ANTIOXIDANTS IN
SPORT
NUTRITION

Edited by
Manfred Lamprecht

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Contents

Preface

CRC Press/Taylor & Francis Group has a long tradition in publishing excellent books in the field of sport nutrition, but up to now no book on redox-active components in sport nutriton has been released. It is an honour that I was asked to edit the first book in this field, and it was exciting for me and my helping team that so many renowned researchers in the field accepted to send a chapter. Many, many thanks to you all!

Originally, I intended to title this book *Redox-Active Components in Sport Nutrition*. But considerations with regard to attracting as many readers as possible led us to title it *Antioxidants in Sport Nutrition*. Although in some respects it is not absolutely appropriate, we perceived the term 'antioxidant' as very suitable as it polarises and fosters discussions. Polarisation and discussion are engines to stimulate research in a specific field.

This book consists of 16 scientifically based chapters with regard to the basic mechanisms of exercise-induced oxidative damage and categorisation of nutritional antioxidants. It covers the antioxidant supply in an athlete's basic nutrition and discusses the controversies of the usefulness or disadvantages of antioxidant supplementation. Many chapters refer to antioxidants and/or bioactives and their effectiveness, and a few chapters cover specific redox-modulating substances and/or supplements. I personally believe it is very interesting for the reader that this book provides chapters that discuss antioxidant supplementation in relation to adaptation and performance as well as the relation between supplementation with redox-modulating compounds and/or supplements and the immune system. Last but not least, two chapters discuss methodological approaches on how to assess oxidative stress and the effectiveness of antioxidant treatment, and two other chapters introduce several biomarkers to estimate the bioefficacy of dietary/supplemental antioxidants in sport.

With this book, sport nutrition scientists and advisors, exercise physiologists, students in related fields as well as coaches, top athletes and recreational athletes will find actual information and practical guidance. Have a good time with it!

Manfred Lamprecht, PhD, PhD
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Editor

Manfred Lamprecht, PhD, PhD, is a medical and exercise scientist at the Medical University of Graz, Austria. He earned a PhD in sport and exercise science at the Karl-Franzens-University of Graz and another PhD in medical science at the Medical University of Graz, Austria.

He holds teaching assignments in the redox biology of exercise and sport nutrition at the Medical University of Graz, Karl-Franzens-University of Graz, and Danube University in Krems, Austria. He is the director of Green Beat—Institute of Nutrient Research and Sport Nutrition, CRO, Graz, Austria.

Dr. Lamprecht is the president of the Austrian Society of Sport Nutrition, head of the sport nutrition experts panel of Styria, Austria, and a board member of the European Nutraceutical Association. He is a member of several advisory boards including the Austrian Nutrition Society and the Austrian Society of Probiotic Medicine.

His fields of research target the redox biology of exercise, exercise-induced intestinal barrier dysfunction and specific sport supplements/bioactives.

Dr. Lamprecht has published numerous papers and served as a referee for more than 20 peer-reviewed, PubMed/Medline listed journals.

He also acts as a consultant for nutraceutical and sport nutrition companies and counsels several top athletes in his specialist fields.

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1 Mechanisms of Oxidative Damage and Their Impact on Contracting Muscle

Chad M. Kerksick and Micah Zuhl

CONTENTS

1.1 INTRODUCTION

An atom or a group of atoms that contains one or more unpaired electron(s) is termed a free radical, which is often a highly reactive and unstable molecule. Two groups of these radical molecules are often classified as reactive oxygen and reactive nitrogen species, respectively. Stabilisation of these radicals requires electron donation from proteins, lipids and DNA which oftentimes leads to degradation and damage to these molecules. Owing to the potential for cellular damage, much controversy was created by initial reports that indicated that physical exercise increased the production of reactive oxygen species (ROS) (Dillard et al. 1978). This initial work did not reveal the specific location, but later work revealed the contracting skeletal muscle to be a prominent source of ROS (Davies et al. 1982). Years later, it was also revealed that contracting muscles also produced nitric oxide (NO), the predominant parent molecule of reactive nitrogen species (Balon and Nadler 1994), and a number of well-constructed review articles since then have confirmed the contribution of skeletal muscle to the production of both

ROS and reactive nitrogen species (Powers and Jackson 2008, Jackson 2009, Powers et al. 2011).

The most abundant biological free radicals are formed when oxygen or nitrogen is incompletely reduced, leading to the production of superoxide ($O_2^{\ast}$) and NO, processes which will be explained in greater detail later in the chapter. The superoxide parent molecule can subsequently be converted into other 'radicals', namely hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH). Removal of ROS is managed by a host of antioxidant systems (e.g. catalase, glutathione/thiol regulation) in the body, and the balance of oxygen species to antioxidants is termed the 'redox state'. As mentioned previously, dysregulation of the redox state results in radical scavenging of key biomolecules such as proteins, lipids (cell membranes are a common target) and DNA, a process which can leave them damaged and unable to function. For these reasons, early theories in the 1980s and 1990s led to the belief that ROS production was mostly a negative consequence of physical exercise. Furthermore, evidence began to mount that a number of clinical situations such as heart disease, amyotrophic lateral sclerosis, irritable bowel disease, diabetes and ageing were a consequence of excessive ROS production and free radical damage (Sies 1985, Powers and Jackson 2008, Jackson 2009, Tsutsui et al. 2011).

Recent perspectives, however, have begun to highlight the fact that both oxygen and nitrogen species exert a key role in the regulation of many intracellular mechanisms and also contribute significantly to various cellular signalling pathways involved with muscle adaptation. For example, several studies and review articles have highlighted the fact that controlled production of both reactive species contribute to mitochondrial biogenesis, angiogenesis, skeletal muscle hypertrophy and proper immune function (Ji et al. 2006, Jackson 2009, Powers et al. 2010, 2011). In this respect, it appears that maintaining a proper balance between radical production and removal is a vital physiological process in the body. The purpose of this chapter is first to briefly explain the main pathways in the human body, which lead to free radical production, and then to highlight the impact of free radical regulation in both cardiac and skeletal muscle tissues. It is these pathways upon which many of the proposed theories for antioxidant regulation occur through manipulation of training, environment, diet or supplementation of the diet with ingredients purported to favourably alter the cellular antioxidant milieu.

1.2   PRIMARY CELLULAR SYSTEMS OF RADICAL GENERATION

1.2.1   Mitochondrial Electron Transport Chain Leaking

The electron transport chain is a four protein complex that uses the reduction potential of molecular oxygen to create an electrical gradient to drive ATP regeneration. Electrons are delivered to complexes I and II by NADH and $FADH_2$, respectively, and the movement of electrons down the chain is controlled by the reduction potential of each successive complex. Molecular oxygen has the highest reduction potential, and is the final electron acceptor in the chain, combining with two protons to create water.

Oxygen consumed by the electron transport chain may undergo one electron reduction, mainly during the corresponding transport of electrons through

components I, II and III. NADH dehydrogenase-coenzyme Q (complex I) accepts electrons from NADH where coenzyme Q is reduced and serves as an electron carrier and transports electrons to cytochrome reductase (complex III). Succinate dehydrogenase-coenzyme Q (complex II) accepts electrons from $FADH_2$ where another coenzyme Q is reduced and transports the electrons to complex III. The cytochrome reductase complex contains cytochrome-b, and -c, along with an iron-containing protein. The cytochromes function as electron-transferring proteins that oxidise coenzyme Q, thus advancing the electrons to cytochrome oxidase (complex IV). Cytochrome oxidase removes the electrons from cytochrome-c, and transfers them to molecular oxygen along with two protons to create water molecules. Evidence suggests that when the reduced form of coenzyme Q (UQH_2) delivers electrons to complex III and is reconverted to the oxidised form (UQ), it passes through a semiquinone anion free radical state ($UQ^{\bullet-}$) (Boss et al. 1998, Becker et al. 1999, Ascensao et al. 2005). Oxygen will accept an electron from the unstable $UQ^{\bullet-}$, and become partially reduced forming a superoxide anion ($O_2^{\bullet-}$). In addition, $O_2^{\bullet-}$ generation has been shown to occur at high levels in complexes I and III (Ide et al. 1999, Drose and Brandt 2012). The inadequate transfer of electrons from complexes I and III requires oxygen reduction mainly through the coenzyme Q redox state as previously reviewed (Muller et al. 2004, Xu et al. 2009, Gomes et al. 2012). It is thought that superoxide generation from complex I migrates towards the mitochondrial matrix, where it is released from complex III and moves into the matrix and inner membrane space (Muller et al. 2004).

In eukaryotic cells, superoxide ($O_2^{\times}$) production is mainly controlled at the site of the mitochondria by a well-equipped antioxidant system (Ascensao et al. 2005). It is important to remember, however, that the contribution of this system is somewhat dependent on the tissue(s) involved, as recent evidence seems to indicate that superoxide production inside the mitochondria of skeletal muscle cells is limited (Powers et al. 2010, 2011). Dismutation of superoxide occurs spontaneously or through catalytic conversion into H_2O_2 using the superoxide dismutase (Cu- or MnSOD) enzyme (Figure 1.1) (Halliwell and Gutteridge 2008). H_2O_2 is considered to be a non-radical and a weak oxidant with a relatively long half-life; a characteristic that sufficiently allows for it to readily diffuse throughout cells and across cell membranes (Halliwell and Gutteridge 2008, Powers et al. 2010). H_2O_2 is further scavenged by the enzymes glutathione peroxidase (GPX) and catalase to produces water (Figure 1.2). Glutathione holds a higher affinity for H_2O_2 than catalase and thus exerts a greater antioxidant effect (Le et al. 1993, Tsutsui et al. 2011). However, when superoxide is produced at high levels, which may occur under conditions of accelerated respiratory chain activity (i.e. exercise), $O_2^{\bullet-}$ levels may exceed the antioxidant capacity of these enzymes. When this occurs, high levels of H_2O_2 are formed and can be further reduced to a hydroxyl radical ($\bullet OH$) either by the Fenton reaction in the presence of iron or the Haber–Weiss reaction (Tsutsui et al. 2011). The hydroxyl radical is the most potent ROS, and is capable of damaging carbohydrates, lipids and DNA (Lipinski 2011).

The production of the superoxide anion alone can lead to molecular damage without the conversion to a hydroxyl radical. In this respect, superoxide dismutase-deficient mice have been shown to develop higher levels of amyloid-β plaque, a major

$$O_2^{\bullet-} \xrightarrow{\text{Superoxide dismutase}} O_2$$

$$O_2 + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2$$
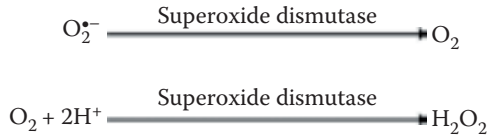
FIGURE 1.1   Superoxide dismutase reaction showing the two-step dismutation of the super-oxide anion to hydrogen peroxide and oxygen.
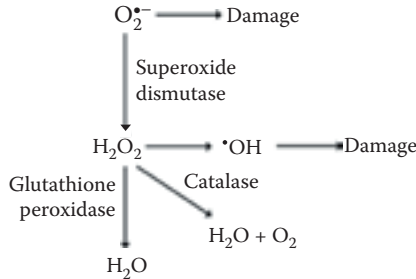
FIGURE 1.2   Glutathione and catalase reactions resulting in the scavenging of H_2O_2.

contributor to Alzheimer's disease (Massaad et al. 2009). Furthermore, superoxide dismutase deficiency can cause severe mitochondrial damage leading to reduced ATP regeneration (Aquilano et al. 2006), a state which could negatively impact performance and overall cellular function. Additional studies indicate that exercise-trained superoxide dismutase knockdown mice develop a form of dilated cardiomyopathy, a maladaptive response to aerobic exercise (Richters et al. 2011). In summary, the elimination of superoxide dismutase leads to excess levels of $O_2^{\bullet-}$ due to the cell's inability to convert the ROS into H_2O_2 and O_2.

In summary, the electron transport chain is the site for mitochondrial respiration resulting in oxygen consumption leading to ATP synthesis. In many eukaryotic cells (but certainly not all), and particularly in basal situations, the electron transport chain is a major site for ROS production, an event that is mostly viewed as physiologically expected. In fact and on a percentage basis, free radical generation in resting conditions is higher than in active or exercising situations. However, when oxygen consumption increases (such as during exercise), an excess of ROS is generated, which may overload the antioxidant enzymes (SOD, GSX, catalase) leading to high levels of oxygen species. An imbalance in the redox state between oxidant production and removal can lead to an enhanced release of superoxides, ultimately resulting in damage to the mitochondria as well as other important cellular structures. The extent of this damage will go on to hinder the ability of the cells to adapt to homeostatic demands, particularly in the face of physical exercise (Riksen et al. 2006, Richters et al. 2011). However, it is important to realise that a balance of ROS production and removal must be struck as evidence exists that too much is detrimental to vibrant cellular function, while a calibrated amount of radical production is needed for optimal cellular function (Powers et al. 2011).

1.2.2 Xanthine Oxidase Pathway

The xanthine oxidase pathway leads to $O_2^{\bullet-}$ production and may also contribute to the production of more potent free radicals such as H_2O_2 and the hydroxyl radical (•OH−). The pathway is triggered under conditions of heavy skeletal muscle contraction, hypoxia or ischaemia. Xanthine oxidase is an enzyme that catalyses the reaction of hypoxanthine to xanthine, uric acid and a superoxide anion. In healthy tissue, xanthine oxidase exists as xanthine dehydrogenase, and is involved in purine metabolism. Under conditions of hypoxia, ischaemia or large bouts of muscular contractions ATP is depleted to ADP and AMP. AMP is further deaminated to IMP by AMP deaminase with additional conversion of AMP into adenosine by 5′ nucleotidase leading to inosine production (Riksen et al. 2006). Purine nucleoside phosphorylase, a key enzymatic step in purine metabolism, converts inosine into hypoxanthine (Canduri et al. 2004) and the large calcium release from muscle contraction triggers a calcium-activated protease, which further changes xanthine dehydrogenase to xanthine oxidase. Activation of xanthine oxidase catalyses the conversion of hypoxanthine into xanthine, which ultimately yields uric acid; superoxide is produced as part of the process as well and is diagrammed in Figure 1.3 (Askew 2002, Sasaki and Joh 2007).

Once the xanthine oxidase pathway is triggered, excess $O_2^{\bullet-}$ production occurs requiring removal through pathways involving MnSOD, glutathione and catalase. As previously reviewed, $O_2^{\bullet-}$ is converted into H_2O_2 with the potential for conversion into the hydroxyl radical. Under hypoxic or ischaemic reperfusion scenarios,
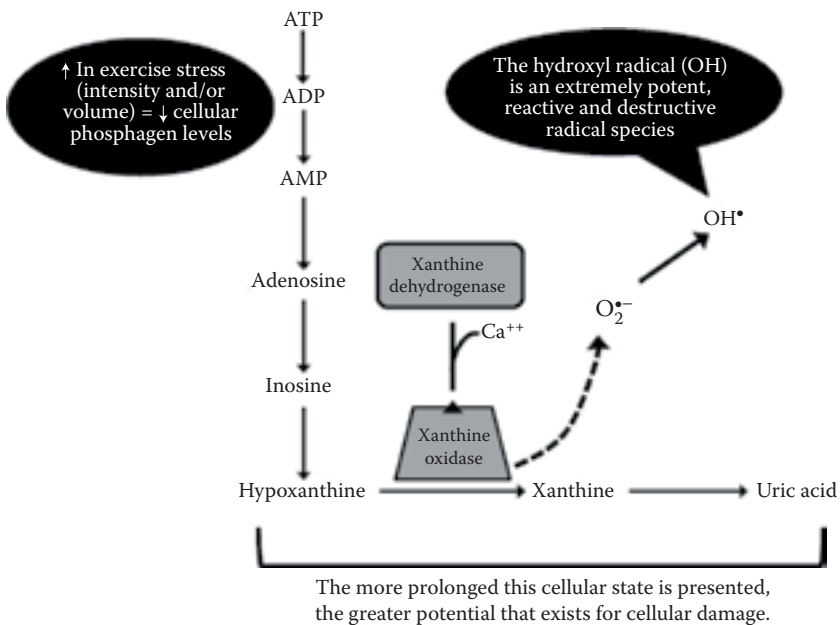
FIGURE 1.3 Xanthine oxidase pathway and production of superoxide and hydroxyl radicals.

xanthine oxidase conversion increases and under these conditions is thought to be a primary contributor to ROS production. In cardiac tissue, pathophysiology of ischaemia–reperfusion leads to tissue damage and eventual heart failure where the xanthine oxidase pathway is involved (Minhas et al. 2006, Burgoyne et al. 2012). In this respect, Minhas et al. (2006) inhibited xanthine oxidase, which ultimately led to a restoration of cardiac function in a rat heart failure model. Furthermore, ROS production from the xanthine oxidase pathway may impair skeletal muscle function, where inhibition results in greater maximal isometric force generation (Ryan et al. 2011).

Superoxide production from the xanthine oxidase pathway, however, might be species dependent and the overall impact of this pathway in human muscle remains to be fully appreciated. Although it is clear that superoxide is produced through this pathway in rat muscle (Gomez-Cabrera et al. 2005), other studies have documented that human skeletal muscle contains much lower levels of xanthine oxidase (Linder et al. 1999, Gomez-Cabrera et al. 2003). As a result, much debate exists as to how much impact the xanthine oxidase pathway contributes to superoxide production in human skeletal muscle.

1.2.3 Nicotinamide Adenine Dinucleotide Phosphate Oxidases Pathway

The nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX 1–5) are a family of enzymes first identified in phagocytes (macrophages and neutrophils) with a primary function of generating ROS as part of the innate immune response to pathogens (Bedard and Krause 2007). The enzyme functions by catalysing electron transfer from NADPH to extracellular molecular oxygen and subsequently producing the superoxide molecule. The production of $O_2^{\bullet-}$ leads to an increase in H_2O_2 levels due to superoxide dismutase activity (see Figure 1.1), which is thought to be the main ROS produced by the NADPH oxidase pathway (Bedard and Krause 2007).

The primary physiological roles of the NADPH oxidase pathway are thought to be host defence and inflammation. During the respiratory burst in neutrophils, the superoxide molecule is converted into H_2O_2 and water. Most of the H_2O_2 produced is converted into hypochlorous acid (HOCl) by myeloperoxidase, a peroxidase enzyme, located in the neutrophil (Klebanoff and Coombs 1992, Hampton et al. 1998). HOCl is a potent oxidant, and destroys pathogens engulfed by the phagocytes. This defence mechanism assists in regulating common bacteria and fungi presented to the body. The inflammatory activity of the NADPH oxidase pathway is the first line of defence against tissue damage or bacterial infiltration. Neutrophils are recruited to the site of infection or damage by chemokines (interleukin-8, tumour necrosis factor-α) released from epithelial cells and macrophages. Once engaged with the pathogen, the respiratory burst begins, causing swelling of the tissue, and formation of green pus (dead neutrophils).

The NADPH oxidase pathway has been identified in other human tissues, including cardiac and skeletal muscle (Bedard and Krause 2007), with this system being responsible for superoxide production at various locations within the myocyte including the sarcoplasmic reticulum, transverse tubules and sarcolemma. In consideration of exercise and sport, limited information is available to fully discuss the

regulation of this system during exercising activity, but several sources of information indicate many potential roles for NADPH oxidase. In this regard, studies indicate that isoforms of NADPH oxidase are found in cardiac tissue (Cheng et al. 2001) and may exert an impact over both the development and protection of cardiac tissue (Li et al. 2006, Satoh et al. 2006, Bedard and Krause 2007, Zhao et al. 2012). The role of NADPH oxidase in skeletal muscle is less clear. Two isoforms of NADPH oxidase have been located near the triad complex of skeletal muscle (Cheng et al. 2001, Hidalgo et al. 2006). This location is consistent with other evidence to suggest that NADPH oxidase activity in skeletal muscle is linked with mechanical stretch and regulation of calcium kinetics (Hidalgo et al. 2006). Finally, endurance exercise has been shown to enhance NADPH oxidase activity in neutrophils, increasing ROS production (Dong et al. 2011). Neutrophil migration into skeletal muscle has been shown to increase after resistance exercise, and may contribute to the inflammatory response (Fielding et al. 1993). Whether or not the NADPH oxidase system contributes to the inflammation is not known, but superoxide levels and muscle damage are reduced when neutrophil migration is prevented (Formigli et al. 1992). Currently, more evidence, particularly on contracting human skeletal muscle, is needed to fully elucidate the impact and function of the NADPH oxidase system.

1.2.4   NITRIC OXIDE PRODUCTION

Much of the focus towards understanding free radical production has centred on the various ROS, a theme which is also displayed throughout this chapter. Production of NO, the primary parent molecule of reactive nitrogen species, may also contribute to free radical production in the electron transport chain as well as through an enzymatic process involving the amino acid L-arginine and one of several NO synthase isoforms (NOS 1–3). As part of the electron transport chain, NO has been shown to bind to cytochrome oxidase (complex IV) of the electron transport chain resulting in inactivation and lowering of oxygen consumption (Cleeter et al. 1994, Poderoso et al. 1996). This promotes a reduced state of the upstream electron carriers (coenzyme-Q or UQH_2) whereby NO then performs the one electron oxidation of UQH_2 leading to superoxide (O_2^x) production (Poderoso et al. 1996, Riobo et al. 2001). Additively, NO can then combine with superoxide (O_2^x) to form peroxynitrite (•ONOO−), one of the more destructive radicals and a powerful oxidising agent (Moylan and Reid 2007, Pacher et al. 2007, Powers et al. 2011).

Peroxynitrite production is associated with various negative cellular outcomes. For example, peroxynitrite is known to damage complex I of the electron transport chain resulting in reduced NADH oxidation and ATP synthesis (Riobo et al. 2001). Other work has illustrated that excess production of peroxynitrite can lead to endothelial and myocardial dysfunction through activation of pro-inflammatory cytokines and DNA damage (Pacher et al. 2007). Finally, increases in peroxynitrite have been shown to be associated with a depletion or modification of cellular thiol groups, two actions that will alter redox signalling and impact numerous cell signalling pathways (Jones 2006). Furthermore, other evidence suggests that formation of peroxynitrite decreases the bioavailability of both superoxide and NO, which will most likely work towards further alteration of cellular signalling activities (Halliwell and

Gutteridge 2008, Powers and Jackson 2008). Of interest, skeletal muscle is known to express two of these isoforms (NOS 1 and NOS 3), while expression of NOS 2 has been reported to occur during inflammatory states (Moylan and Reid 2007). Like some of the oxygen species, NO also has many signalling functions inside the body, particularly those governing the expression and production of nuclear factor-kappa beta (NF-κB) and peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α), PGC-1α and NK-κβ (Powers et al. 2010).

1.3 ROS IN CARDIAC PHYSIOLOGY AND PATHOPHYSIOLOGY

The role as well as the involvement of ROS production in cardiac pathophysiology has been well documented and shown to contribute to contractile dysfunction, cardiomyopathy, arrhythmia, ischaemic reperfusion injury and mitochondrial DNA damage (Burgoyne et al. 2012). In many pathological states, there is a shift in the redox state resulting in an excess production of ROS relative to antioxidant defences (Belch et al. 1991, Hill and Singal 1996). The primary source of excess ROS production is indeed cardiac myocyte mitochondria (ETC), xanthine oxidase and excess NADPH oxidase, along with contribution from neutrophils via respiratory burst actions (Tsutsui et al. 2011). Inside failing hearts when compared with healthy hearts, the electron transport chain produces greater O_2^-, and evidence suggests that complex I is a major site for superoxide production (Sawyer and Colucci 2000, Tsutsui et al. 2011). Furthermore, elevated levels of inflammatory cytokines (e.g. tumour necrosis factor-α) and angiotensin II are common in patients with heart failure, which are linked to increased activity of NADPH oxidase (Tsutsui et al. 2011). In addition, these cellular stimuli also activate p47[phox], which accelerates a cascade of events that ultimately leads to increased production of superoxide via NADPH oxidase (Li et al. 2006). In addition, xanthine oxidase activity is greater in heart failure patients due to the ischaemic/reperfusion cycle, ATP depletion and production of H_2O_2 as well as •OH─ (Cappola et al. 2001). Inhibition of this increased xanthine oxidase activity in a murine heart failure model via allopurinol administration has been shown to improve contractile function (secondary to reduced free radical production) (Cappola et al. 2001).

Recent literature indicates that the mechanism of how excess ROS production causes cardiac dysfunction most likely involves abnormalities in calcium homeostasis (Burgoyne et al. 2012). As indicated previously, ROS production facilitates normal cardiac function through the activation of ryanodine receptor 1 (RyR1) receptors inducing Ca^{2+} release. However, ROS production at high levels can lead to RyR1 malfunction and aberrant calcium release leading to contractile dysfunction, arrhythmia and myopathy (Sag et al. 2011) with further upstream molecular targets being protein kinase A and calcium calmodulin kinase (Sag et al. 2011). Paradoxically, mitochondria are a source of ROS production in the diseased heart, but are also the targets of oxidative damage mainly because much of the ROS produced in the mitochondria do not cross the inner mitochondrial membrane, leaving them trapped (and reactive) in the inner membrane space (Sag et al. 2011). Additionally, mitochondria have their own genomic system for the transcription of mitochondrial DNA, which is disrupted by ROS production and can decrease rates of mitochondrial protein synthesis (Ballinger et al. 2000).

From an exercise perspective, acute fatiguing exercise or several days of exhaustive exercise has been shown to produce ROS in contracting muscle tissue (Fisher-Wellman and Bloomer 2009). During exercise and as expected, both myocardial oxygen consumption and mitochondrial ATP production increase. These acute responses lead to a large increase in electron transport chain activity which opens the door to excessive reduction of molecular oxygen to a superoxide. Indeed, Bo and colleagues have reported an increase in superoxide levels approximately 90 and 120 min after exercise (Bo et al. 2008); the major site of mitochondrial O_2 production is complexes I and II of the ETC (Ji and Mitchell 1994). ROS production causes mitochondrial membrane damage and also disrupts the proton gradient inside this organelle ultimately leading to a reduction in ATP generation (Ji and Mitchell 1994). Training status appears to be a key consideration in this respect as Leichtweis demonstrated a significant reduction in mitochondrial ROS production from aerobically trained rats when compared with lesser trained rats (Leichtweis et al. 1997). In conjunction, trained rats have been shown to have a greater antioxidant response during as well as post exercise (Bo et al. 2008). Whether chronic exercise induced myocardial ROS production leads to cardiomyopathy or dysfunction of a healthy heart is not known. All current research has utilised animal models, but long-term studies have yet to be conducted, particularly in the human species. In summary, balanced ROS production is critical to heart development and also facilitates optimal calcium handling and contractility, but overproduction is unfavourable and in the long run can contribute to the development of heart failure.

1.4   ROS IN SKELETAL MUSCLE

Arguably, the balance of free radicals inside the skeletal muscle is more important, particularly in the context of exercise and sport, than any other tissue. Section 1.3 attempted to highlight some of the key considerations of another important tissue relative to exercise, cardiac muscle, but *in vivo* human work is lacking in this tissue. Fortunately, the available literature in skeletal muscle is more prevalent and recent reviews have highlighted the changes relative to oxidative stress in this tissue. It is now commonly accepted that contracting skeletal muscle is a distinct source of ROS and nitrogen species (Pattwell et al. 2004). Focusing this discussion towards exercise and as mentioned previously, initial reports that exercise increases radical generation led to much controversy and speculation relative to the importance and potential danger of these circumstances (Powers et al. 2010, 2011). Thus, much of the dogma surrounding oxidative stress and exercise 20–30 years ago suggested that oxidative stress was unhealthy and would result in circumstances that negatively impacted on health and performance.

Currently, it is no longer debated that exercise increases oxidative stress throughout the body as this outcome has been illustrated in a number of exercise modes including swimming (Gougoura et al. 2007), prolonged cycling (Michailidis et al. 2007) and eccentric exercise (Nikolaidis et al. 2007, 2008) to name a few. Skeletal muscle readily produces the two parent molecules of both radical species, superoxide ($O_2^{\times}$) and NO. As illustrated in Figure 1.4, superoxide production inside the skeletal muscle occurs in a number of locations: the mitochondria, sarcoplasmic reticulum,
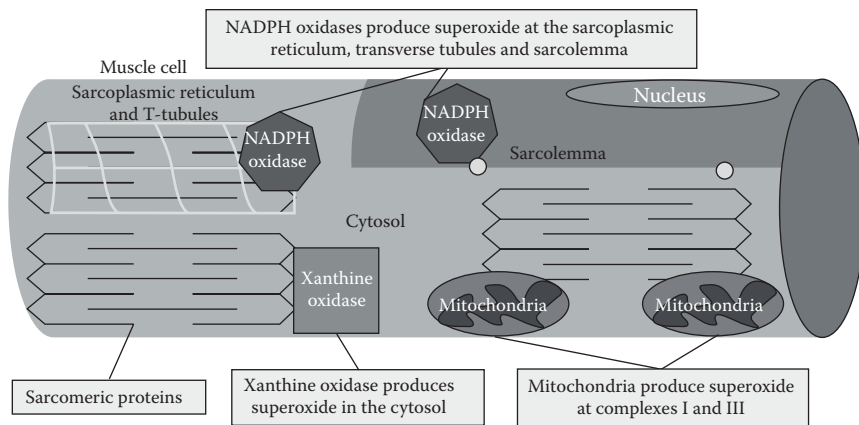
FIGURE 1.4   Illustration of the potential cellular sites for the production of superoxide in muscle fibres. Note that primary sites for cellular superoxide production include mitochondria, NADPH oxidases (located within the sarcoplasmic reticulum, transverse tubules and the sarcolemma), and xanthine oxidase. (From Powers, S.K. et al. 2011. *J Physiol* 589(9): 2129–2138. All rights reserved 2011. The Physiological Society. With permission.)

transverse tubules, sarcolemma and the cytosol (Powers et al. 2011). As highlighted in previous sections, superoxide production inside the mitochondria of the skeletal muscle is focused upon complexes I and III of the electron transport chain (Barja 1999), but recent reports dispute the notion that this location is responsible for an appreciable amount of superoxide production in this tissue. For years, reports that as much as 2–5% of the total oxygen consumed by mitochondria was reduced to superoxide (Boveris and Chance 1973, Loschen et al. 1974) were made and it was thus thought that the more oxygen that was consumed the more radical species were produced. More recent studies using modern-day technology with greater specificity report that only 0.15% of all oxygen consumed by the mitochondria is used to form superoxide (St-Pierre et al. 2002). Moreover, multiple studies also report that more superoxide production may occur during basal respiration than during any form of active respiration (Di Meo and Venditti 2001). In addition to the mitochondria, superoxide is also produced via NADPH oxidases located within the sarcoplasmic reticulum, transverse tubules and sarcolemma (Powers and Jackson 2008), but limited evidence into these mechanisms precludes any further discussion. In addition, xanthine oxidase activity in the skeletal muscle of rat muscle indicates that this mechanism can also be responsible for appreciable superoxide production (Gomez-Cabrera et al. 2005); however, human muscle contains much less of this enzyme making its contribution to superoxide production in human muscle to be negligible (Linder et al. 1999, Gomez-Cabrera et al. 2005). Finally, other sources of superoxide production may be evident at the level of the plasma membrane (e.g. phospholipase A2-dependent), but more research is needed to more fully discuss the impact of these processes (Jackson 2009).

To effectively balance the production of ROS that occur, muscles contain a vast network of endogenous antioxidant enzymes that quench ROS (Ferreira and Reid

2008). In this respect, the sarcoplasm contains CuZn-superoxide dismutase (aka SOD1), catalase and GPX. Moreover, the mitochondrial matrix contains MnSOD (aka SOD2), in addition to GPX. In addition, other less popular thiol-based antioxidant systems exist and include thioredoxin, thioredoxin reductase and peroxiredoxins, although little is known about their regulation and function in skeletal muscle. Multiple non-enzymatic antioxidants exist including lipid-soluble components found nearly exclusively in cellular membranes such as vitamin E, carotenes and ubiquinol. Water-soluble components exist and are ubiquitously distributed across the muscle cell; these include ascorbate (vitamin C), lipoate (lipoic acid), urate and glutathione. In addition, several herbal and botanical species contain various components (e.g. flavonoids, polyphenols, catechins) that are also known to exert antioxidant properties. Of these, glutathione is the most abundant non-protein thiol and also exerts the greatest controls in skeletal muscle over the redox balance of the cell. In this respect, a common marker used in research to represent redox balance is the ratio of oxidised glutathione (GSSG) to reduced glutathione (GSH). For these reasons, glutathione is considered by many to be the most important non-enzymatic antioxidants (Ferreira and Reid 2008).

As highlighted throughout, contracting skeletal muscle contributes radical species from a number of locations, but the notion that these radicals may be needed or may be in some ways beneficial to contracting muscle is an exciting perspective. In this respect, more and more evidence is available to highlight that the balance between the production and quenching of radicals is critically important (Jackson 2009, Powers et al. 2011). On one end of the spectrum, disuse animal models clearly illustrate that this situation is deleterious and results in excessive production of free radicals inside the muscle (Powers et al. 2005, 2007). In a somewhat alarming fashion, other studies show that production of ROS may be a required signal for normal healthy (and desired) remodelling to occur in response to various exercise stimuli (Droge 2002, Jackson 2008). How exactly the muscle tissue and its systemic response identifies and regulates whether the presence of ROS is harmful or helpful is still unknown. While evidence mounts to reveal that factors such as the site of where ROS are produced may differ between an inactive or active state (Powers and Jackson 2008), no clear answer remains. What is known, however, is that multiple cellular signalling pathways throughout the skeletal muscle are impacted and, to some extent, regulation by ROS. Towards this aim, Jackson highlighted and reviewed several cellular signalling pathways that to some extent are impacted by ROS production. As highlighted in Figure 1.5, these pathways represent a wide array of cellular functions including inflammation, proteolysis, hypertrophy, oxidative phenotype and mitochondrial biogenesis (Jackson 2009). More recently, Powers and colleagues elegantly reviewed the evidence surrounding the contribution of two signalling pathways in skeletal muscle known to be heavily influenced by ROS: NF-κB and PGC-1α (Powers et al. 2010, 2011). Briefly, NF-κB is implicated in a large number of cellular processes including inflammation, cell growth, stress responses and apoptosis (Kramer and Goodyear 2007). Evidence in this pathway reveals that ROS and nitrogen species are used by this pathway to transfer signals from inside the cytoplasm to the nucleus ultimately impacting gene expression and eventual phenotypic expression (Droge 2002, Ji et al. 2006). Similarly, another pathway in which the presence and balance of reactive
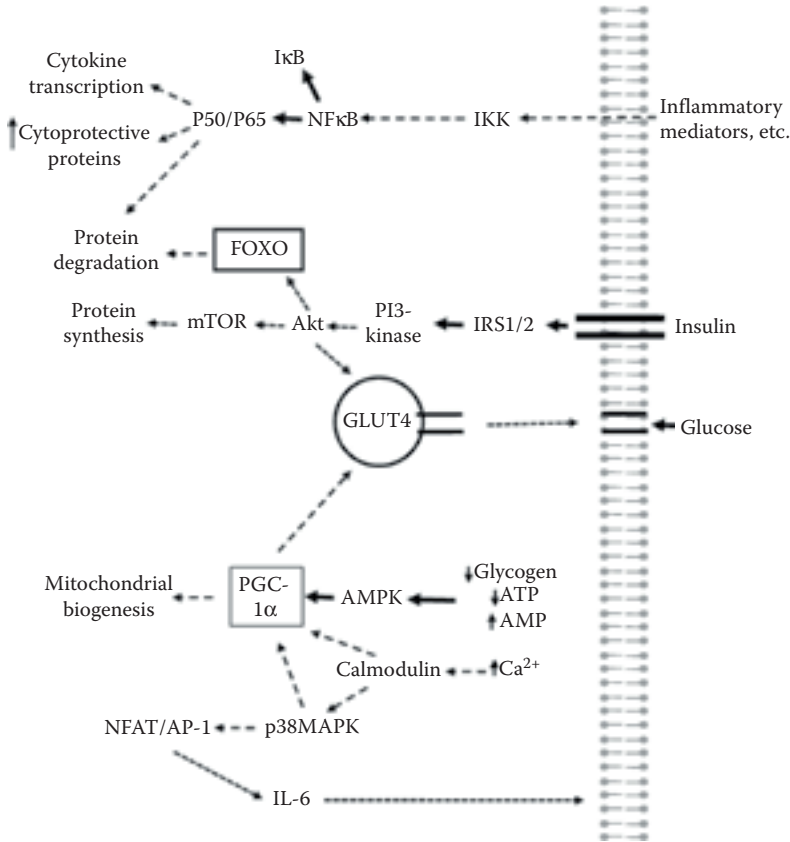
FIGURE 1.5 Candidate signalling pathways in skeletal muscle in which reactive oxygen and/or nitrogen species exert some level of influence over its activity and regulation. (From Jackson, M.J. 2009. *Free Radic Biol Med*. 47(9):1267–1275. With permission.)

oxygen and nitrogen species must be adequately managed involves PGC-1α; a pathway known to control mitochondrial biogenesis and be a primary regulator of an oxidative phenotype inside contracting muscle. As examples, these pathways nicely highlight the key understanding which must be acquired regarding the production and balance of radical species. Additionally, it remains very likely that as more research is completed in this area, the appropriate balance between a pro-oxidant and antioxidant cellular state will also influence the activity and regulation of several other cellular mechanisms inside the contracting muscle.

1.5 CONCLUSION

Exercising a skeletal muscle deals with a complex array of positive and negative signals that must be appropriately balanced and coordinated to achieve optimal health and performance as well as to optimally promote desired adaptations from

exercise training. The impact of free radical species (ROS and reactive nitrogen) in working cells has undergone a vast overhaul in understanding. Gone should be the initial fears of the 1970s where the mere production of radical species was deemed harmful and replacing it should be the need to appreciate and better understand how these factors combine to favourably impact a myriad of cell functions. A number of pathways exist where the parent molecules of both ROS (superoxide) and reactive nitrogen (NO) species are produced inside cells, but most evidence points towards the majority of them being produced throughout the mitochondria, as part of NADPH oxidase activity, and xanthine oxidase involvement. To counteract any excessive production of these radical species and to maintain a favourable state of redox balance, a number of enzymatic and non-enzymatic molecules exist inside cells, in particular muscle cells. Current evidence clearly points towards acquiring a better understanding of what factors result in a cellular environment that results in damage and destruction or regulation and favourable adaptations. In this respect, sound evidence is available to highlight the importance of redox control over pathways involving NF-κB and PGC-1α, and as more interest and research is completed, our understanding of the impact of radical species on other key signalling pathways will also develop. In closing, radical production is expected and should not be viewed as a 'necessary evil', but rather as a potential partner in allowing your body to most favourably adapt and respond to the physical challenges brought upon it.

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2 Nutritional Antioxidants
It Is Time to Categorise

Aalt Bast and Guido R.M.M. Haenen

CONTENTS

2.1 INTRODUCTION

Not many subjects in the field of life sciences have led to so many discussions as antioxidants. Polarised views on antioxidants range from *antioxidants cure any disease* to *antioxidants increase mortality*. Other opposing views are *the more the better* versus *at high doses, antioxidants become pro-oxidant*. In a recent review (Bast and Haenen, 2013), we have addressed 10 misconceptions about antioxidants and analysed this antioxidant controversy. An important factor is that the word antioxidant designates a general common action which does not take into consideration that antioxidants are in fact distinct chemical entities with different modes of action for their specific effects.

Antioxidants should be clearly defined based on their physiological and physicochemical properties. With this in mind, let us categorise and start by asking the question, what is an antioxidant? We will answer this by looking at nutritional antioxidants (i) from a perspective of direct scavengers of reactive oxygen species

(ROS); (ii) with regard to their effect on ROS formation; and (iii) their interplay with other enzymatic and non-enzymatic antioxidants.

2.2   WHAT IS AN ANTIOXIDANT?

2.2.1   REACTION WITH ROS

An antioxidant can be defined in a pragmatic way as 'a compound that in a relatively low concentration, prevents or delays the oxidation of biomolecules'.

Compounds can display an antioxidant action simply by reacting with ROS or preventing the generation of ROS. ROS are generated during the reduction of oxygen and comprise two groups of molecules: (1) free radicals with short biological half-lives, such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals (OH•) and nitric oxide radicals (•NO), and (2) non-radicals, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite (ONOO$^-$) and lipid hydroperoxides (LOOH) (Weseler and Bast, 2010).

Many test tube experiments in which antioxidants were tested for their reactivity towards ROS were thought to be illustrative for the beneficial effect of antioxidants. This was corroborated in cell culture experiments and animal experiments in which imposed oxidative damage could be mitigated by antioxidant treatment. In this way, antioxidant supplementation became synonymous to health. In the ensuing optimism about the health benefit of antioxidants, these compounds were regarded as one group of bioactive molecules and no distinction between the various antioxidants was made. General antioxidant behaviour, namely, reaction with ROS sufficed. Later, it was increasingly recognised that each antioxidant has a unique chemical reactivity. For example, thiols will quite easily react with HOCl (Ching et al., 1994), whereas carotenoids will predominantly quench singlet oxygen (Bast et al., 1988).

The scavenging activity was widely studied. Simple and elegant assays for measuring radical scavenging were developed. Hydroxyl radical scavenging, for example, was measured in a competition assay using a hydroxyl radical generating system. Many compounds could be measured and rate constants of the reaction with hydroxyl radicals could be derived (Halliwell et al., 1987). Later, it was advocated that determination of the hydroxyl radical scavenging by antioxidants was meaningless. It has been argued, and recently with even more emphasis (Forman et al., 2014) that the extremely reactive hydroxyl radical reacts with practically all molecules in a cell with a rate constant approaching the rate of diffusion and that therefore antioxidants can only be active if they react even faster, which is impossible (Bast and Haenen, 2013). However, as we recently debated, site-specific scavenging, that is, by binding to iron and thereby neutralising the radical at the site of formation can provide protection (Bast and Haenen, 2013). Direct antioxidant activity should not be neglected. In fact, we recently established direct attenuation of intracellular oxidative stress by flavanols (Ruijters et al., 2013). Similar findings with other polyphenolic antioxidants were found. New types of intracellular antioxidant mechanisms were proposed. Evidence has been provided that different pools of otherwise inaccessible radicals were scavenged by these polyphenolics at very low concentrations (Lombardo et al., 2013).

Radical scavenging can result in antioxidant scavenging, but the ability to scavenge does not guarantee a physiological relevant activity. Moreover, it has to be realised that each antioxidant has its own radical scavenging profile.

2.2.2   INHIBITION OF SOURCES FOR ROS

ROS are used in several (vital) physiological processes. In fact, many enzymes produce ROS. Antioxidant action might be displayed by inhibiting these ROS-generating enzymes.

2.2.2.1   Xanthine Oxidase

Xanthine oxidase (XO) catalyses the oxidation of hypoxanthine to xanthine and subsequently to uric acid (Figure 2.1). The enzyme can also have xanthine dehydrogenase activity. The dehydrogenase is converted to the oxidase by mild proteolytic modification.

Because XO produces uric acid which may lead to gout, inhibitors of the enzyme like allopurinol are used in the treatment of gout.

Besides uric acid, $O_2^{\bullet-}$ is formed. This is evident in chronic obstructive pulmonary disease (COPD), a lung disease (Ichinose et al., 2003). The reaction of $O_2^{\bullet-}$ with nitric oxide (•NO) may lead to ONOO$^-$ and subsequent tyrosine nitration. Interestingly,
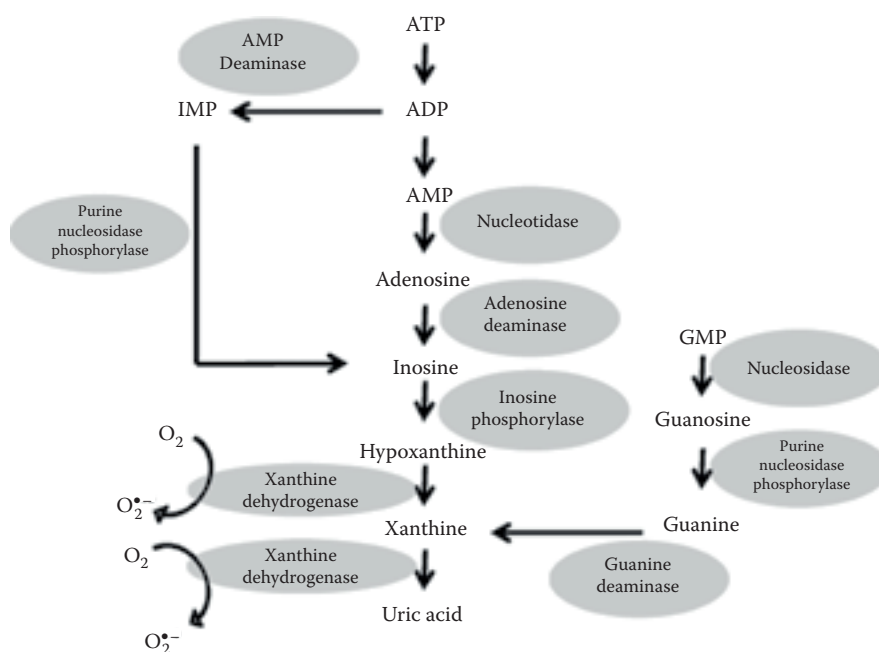
FIGURE 2.1   Xanthine dehydrogenase is transformed into xanthine oxidase by mild proteolysis. Xanthine oxidase catalyses the conversion of hypoxanthine and xanthine into uric acid and $O_2^{\bullet-}$. Note that ATP (and adenosine or guanosine) are precursors for (hypo)xanthine.

allopurinol prevents tyrosine nitration in COPD patients. Eccentric exercise in humans leads to an increased level of XO in human muscle and has been associated with secondary inflammatory processes (Hellsten et al., 1997). Other inhibitors of XO are flavonoids (van Hoorn et al., 2002), which can be found in many plant extracts (e.g. Spanou et al., 2012).

2.2.2.2   NADPH Oxidase

The NADPH oxidases (NOX) were initially assumed to be present only in phagocytes of the innate immune response, where they generate large amounts of $O_2^{\bullet-}$ to kill invading pathogens in the so-called oxidative burst. Upon activation, O_2 is reduced to $O_2^{\bullet-}$ by the transfer of one electron from the reducing equivalent NADH or NADPH. During the last two decades, non-phagocytic NADPH oxidases were described, indicated as the NOX family (Weseler and Bast, 2010). These NOX family NADPH oxidases comprise seven isoforms: NOX1, NOX2, NOX3 (formerly gp91phox), NOX4, NOX5 and dual oxidases 1 and 2 (DUOX1 and DUOX2) (Boots et al., 2009). NOX inhibition is a promising pharmacological concept for treating oxidative stress-related pathologies (Jaquet et al., 2009).

2.2.2.3   Myeloperoxidase

This lysosomal protein is abundantly present in neutrophil granulocytes. It produces HOCl from H_2O_2 and chloride anions (Cl^-). There is a continuous search for safe inhibitors of myeloperoxidase (e.g. Liu et al., 2012). Plant-derived flavonoids have been described as inhibitors of myeloperoxidase (Shiba et al., 2008). Measurement of myeloperoxidase is frequently used as a marker of the accumulation of neutrophils in the inflamed tissue. Fractions of olive oil have been reported to inhibit inflammation as evidenced by this enzyme marker in mice inflammatory models (de la Puerta et al., 2000).

2.2.2.4   Mitochondrial Respiratory Chain

The electron transport chain in mitochondria couples the electron transfer from an electron donor ($NADH^+$ and $FADH_2$) to an electron acceptor (O_2). In this process, an electrochemical proton gradient over the inner membrane of the mitochondrium is generated that is used to synthesise adenosine triphosphate (ATP). This process may lead to partially reduced oxygen radicals. Interestingly, several components of this mitochondrial process have been used in sport nutrition. Oral ATP has been suggested to increase ATP levels. However, it has recently been unequivocally established that ATP is not taken up after oral administration (Arts et al., 2012).

Co-enzyme Q10 is part of the electron transport chain. It is a lipophilic antioxidant. Food supplements are capable of changing the plasma status of Q10. Whether this has a favourable effect on health is still questionable (Bast and Van den Berg, 2011; Ostman et al., 2012).

2.2.2.5   Dysfunctional Enzymes Like eNOS and Cytochrome P450

Some haem containing oxidising enzymes are prone to uncouple (Figure 2.2), that is, in the catalytic oxidation process, oxygen is only partly reduced by the haem iron and leaves the catalytic reaction cycle as such. In this dysfunctional enzymatic catalytic cycle, oxygen reduction is uncoupled from function.

NO synthase   Cytochrome P450

NADPH   NADPH

L-Arginine O$_2$ Uncoupling   O$_2$ RH NADH

At low BH$_4$

With specific
CYP-iso-
enzymes
(CYP2E1)

Uncoupling leads to
superoxide anion radical
generation

L-Citrulline   ROH
                                                              NADP+
•NO

NADP+   NAD+
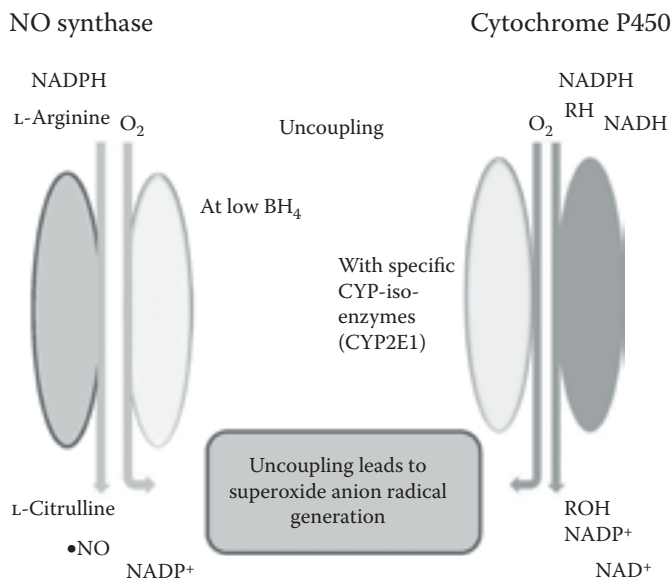
FIGURE 2.2 Function and uncoupling of the haem containing enzymes endothelial NO synthase and cytochrome P450. Uncoupling leads to superoxide radical production. For eNOS, less NO is formed and cytochrome P450 produces less metabolite (ROH).

The flavohaemprotein endothelial nitric oxide synthase (eNOS) oxidises L-arginine to L-citrulline and the vasodilating •NO. A relative shortage in a cofactor like tetrahydrobiopterin (BH$_4$) uncouples eNOS, which generates $O_2^{\bullet-}$ rather than •NO (Vasquez-Vivar et al., 2002).

The haem enzyme cytochrome P450 displays a mono-oxygenase activity in which xeno- or endobiotic substrates receive one extra oxygen atom (Bast, 1986). However, some iso-forms of cytochrome P450, like the ethanol-inducible form cytochrome P450 2E1, readily uncouple. The ROS thus formed may lead to damage of membranes via lipid peroxidation (Bast and Haenen, 1984).

2.2.3 NON-ENZYMATIC AND ENZYMATIC INTERPLAY

From a chemical point of view, an antioxidant is a compound that prevents or delays the oxidation of another compound. Of the two compounds, the one which becomes oxidised functions as an antioxidant for the other (Bast and Haenen, 2013). Dietary antioxidants include vitamins E and C, carotenoids, phenols like hydroxytyrosol and polyphenols like flavonoids.

Antioxidants which are formed during metabolism include glutathione or uric acid.

Upon action of an antioxidant, a hydrogen atom is donated. The resulting free radical of the antioxidant reacts with another free radical of the antioxidant inter- or intracellularly, thus neutralising the reactivity (Figure 2.3).
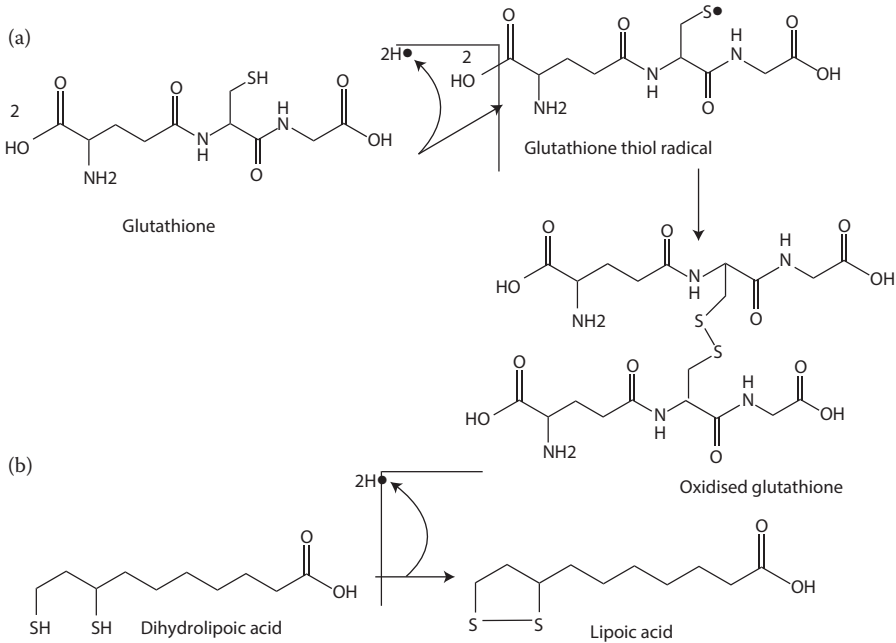
FIGURE 2.3   (a) The tripeptide glutathione acts as a cofactor in various glutathione-dependent antioxidant enzymes. The resulting thiol radical of glutathione reacts with another thiol radical which forms the oxidised form GSSG. (b) A similar reaction can also occur intramolecularly. This is the case with the endogenous compound dihydrolipoic acid. Upon its action as an antioxidant, it gives a thiol radical which can form a disulphide within the molecule. (From Biewenga GP, Haenen GR, Bast A. 1997. *Gen. Pharmacol.* 29: 315–331.)

Other antioxidants form a resonance stabilised free radical after donation of a hydrogen atom. A well-known example in this respect is alpha-tocopherol (vitamin E). Several resonance structures of the alpha-tocopherol free radical render the compound stability (Figure 2.4). Also, the two electron oxidised form of antioxidants give relatively stable products. An example is the flavonoid quercetin which gives relatively stable oxidation products, quercetin quinones (Jacobs et al., 2011).

The quinone of quercetin can be regenerated again to quercetin upon reduction with, for example, vitamin C. Also, the regenation of alpha-tocopherol (vitamin E) from the one electron reduced form, the alpha-tocopherol radical, can take place with vitamin C or glutathione.

In the oxidation of the tocopheryl radical to a tocopherol quinone, one of the rings of the chroman head is opened. In that case, the direct reduction back to α-tocopherol is not possible anymore. *In vivo,* however, other reactions may occur which seems to make reduction of the tocopherol quinone possible (Bast and Haenen, 2002).

Free electrons are not only stabilised within one molecular structure but can also be spread out over various antioxidants that are localised in various cellular

(a)

α-Tocopherol

Semi-quinone radical

Quinone

(b)

Quercetin quinones
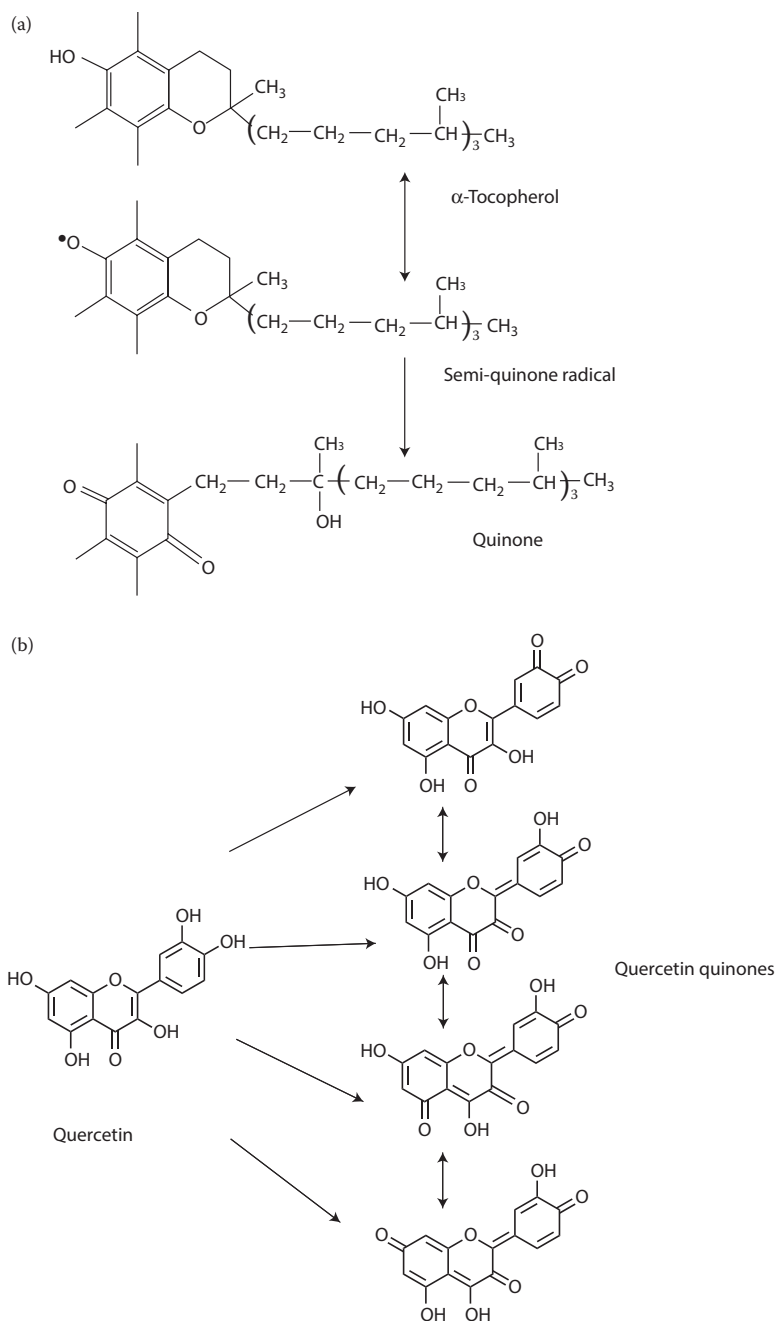
Quercetin

FIGURE 2.4 (a) Alpha-tocopherol and the one electron oxidised alpha-tocopherol free radical (vitamin E free radical) which is stabilised by the various resonance structures. The quinone occurs with a ring opening. (b) The quercetin quinones are the (two-electron) oxidised forms of the flavonoid quercetin. These quinones exist as different tautomers.

compartments (Figure 2.5). Alpha-tocopherol reacts with a lipid peroxyl free radical (LOO•) forming a lipid hydroperoxide (LOOH). Interestingly, some dietary flavonoids can substitute for alpha-tocopherol (van Acker et al., 2000). The relatively stable alpha-tocopherol free radical can react with either vitamin C or with glutathione by which the reduced form of alpha-tocopherol becomes regenerated (Figure 2.5). In this way, antioxidants do not work in isolation but rather form a network in which the free electron disseminates over various molecules and cellular compartments.

It should also be mentioned that enzymatic antioxidant systems participate in this network. LOOH can be reduced to their respective alcohols (LOH) by glutathione peroxidases. Initiating species of lipid peroxidation like H_2O_2 or $O_2^{\bullet-}$ can be metabolised by catalase or glutathione peroxidases (for H_2O_2) or superoxide dismutases (for $O_2^{\bullet-}$) (Bast and Haenen, 2013).

Another interaction is based on the finding that ROS can activate the Nrf2 pathway, thus stimulating the gene expression of enzymatic antioxidants such as catalase, haem oxygenase 1 (Figure 2.6). Oxidised antioxidants have electrophilic properties and can also activate Nrf2. The transcription factor is sometimes called the master switch of cellular antioxidant activity.
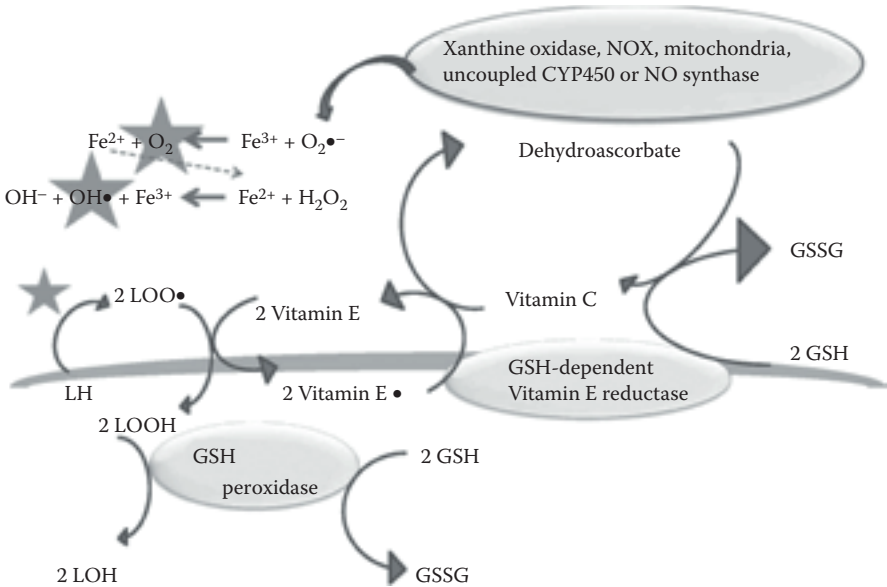
FIGURE 2.5 Illustration of the antioxidant network. It shows that the free electron spreads over various antioxidants which have characteristic physico-chemical features with regard to redox properties and cellular localisation. Regeneration of vitamin E (enzymatically) and vitamin C occurs with glutathione. Various sources for $O_2^{\bullet-}$ are indicated. Reduced iron plays a role in the formation of $OH^{\bullet}$. Both $OH^{\bullet}$ and O_2 are indicated as the trigger and catalyst for lipid peroxidation.
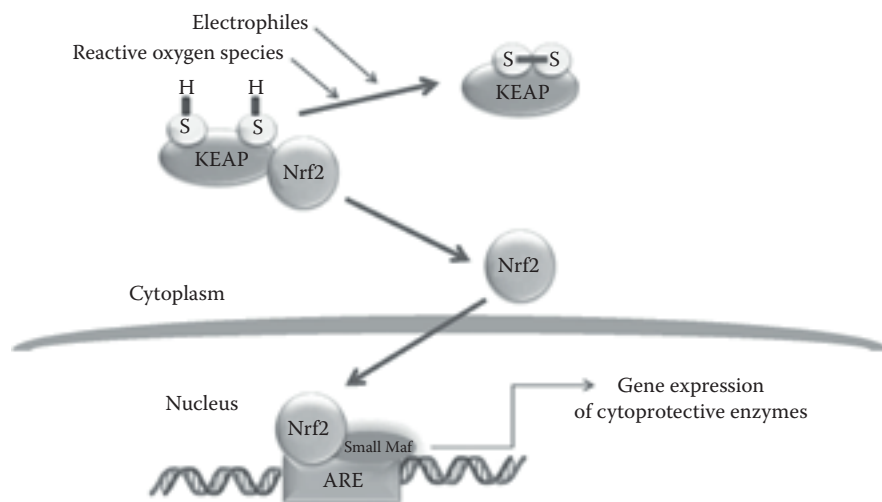
FIGURE 2.6 Reactive oxygen species or electrophiles can oxidise or adduct KEAP1 (Kelch-like ECH-associated protein 1) with leads to activation of the transcription factor Nrf2. Antioxidant metabolites are also known to release Nrf2. Subsequent interaction of Nrf2 with so-called antioxidant responsive elements leads to gene expression of cytoprotective enzymes like glutathione transferases, haem oxygenase or catalase.

2.3 CHANGING VIEWS ON HEALTH EFFECTS OF ANTIOXIDANTS IN TIME

The concept of health has altered in the past 70 years (Editorial, 2009). Interestingly, our view on antioxidants changed at the same pace and along similar lines. Just after the Second World War, the definition of health fitted into the desire of that time (World Health Organization, 1946). A new era began: everything should become better and health was defined as 'a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity'. In other words, with health one strived for perfection: a new world order and compounds that could assist in reaching that goal. Compounds that could do everything: antioxidants. The general notion was that antioxidants could cure many diseases. The reasoning was simple: ROS play a role in the aetiology of many diseases. Therefore, scavenging those harmful species would be beneficial in all those cases. The term 'oxidative stress' was employed to indicate a disbalance between oxidants (ROS) and antioxidants. The general conviction was that restoring the balance would have a beneficial effect. This approach coincided with the attempt to reach a healthy state of perfection. Everything should and could be cured. Gradually, it was realised that antioxidants did not form the Holy Grail and could at best work indirectly (Figure 2.7).

Approximately around the same time, the anti-inflammatory action of antioxidants was advocated (Abou El Hassan et al., 2003, Weseler and Bast, 2012). This was and is also connected with a pleiotropic action. Inflammation can be inhibited in many
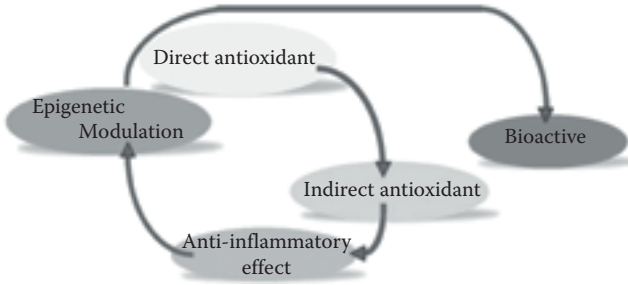
FIGURE 2.7   Changing views on the mode of action of nutritional antioxidants. From 1960 to 1980, nutritional antioxidants were primarily regarded as compounds that directly scavenge free radicals in order to explain their multitude of effects. Ideas gradually shifted towards indirect activity in which compounds act synergistically in network. During the last 15 years, emphasis has been on the anti-inflammatory action of these compounds. In recent years, the long-term effects are explained in terms of epigenetic modulation. Currently, it is suggested to designate nutritional antioxidants not merely as antioxidants, but as 'bioactives', because of their physiological versatile activities.

ways: inhibition of the transcription factor nuclear factor-kappa B (NF-κB) (van den Berg et al., 2001), inhibition of adhesion of neutrophils (Busse et al., 1984) or a beneficial effect on nuclear receptors (Figure 2.8). As shown in Figure 2.8, poly(ADP-ribose)polymerase acts as a co-activator in the action of NF-κB. Interestingly, many nutritional antioxidants are inhibitors of PARP-1. This may partly explain the NF-κB inhibitory activity of many nutritional compounds (Geraets et al., 2007).
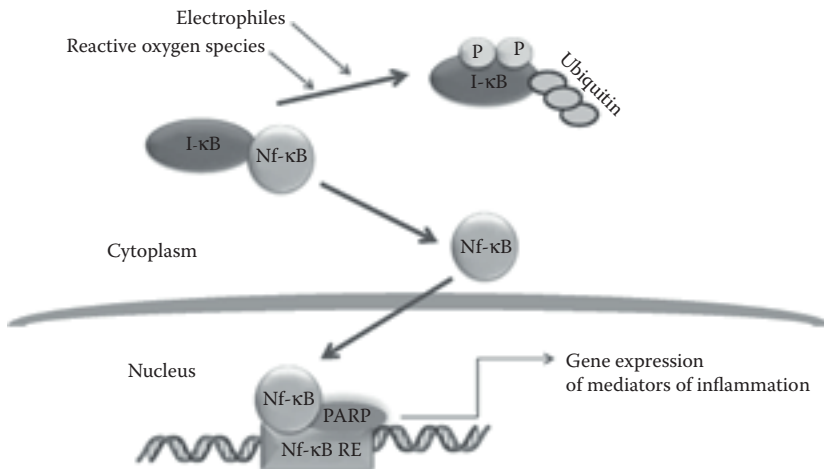
FIGURE 2.8   Activation of the transcription factor NF-κB by reactive oxygen species and electrophiles. Damage to cells activates I-κB kinase, which leads to phosphorylation of the inhibiting factor. Subsequently, ubiquitination occurs and the inhibitor decays by proteosomal activity. NF-κB translocates to the nucleus where it binds. The DNA-repairing enzyme poly(ADP-ribose)polymerase-1 (PARP) is a co-activator. NF-κB leads to gene expression of pro-inflammatory mediators like TNF-α or IL-8.

Many mechanisms are involved. In a recent article in which we investigated the anti-inflammatory action of a well-defined grape seed extract, we defined (Weseler et al., 2011) a series of parameters to show the multitarget subtle effects of the extract. We hypothesise that nutritional 'bioactives' (Figure 2.7) do not act via a single target and with a strong response but rather elicit a multitude of small effects.

2.4 THE ANTIOXIDANT HYDROXYTYROSOL IN EXERCISE

Hydroxytyrosol, or 4-(2-hydroxyethyl)-1,2-benzenediol, is well known as the most powerful antioxidant found in olives. Its chemical structure is depicted in Figure 2.9.

Hydroxytyrosol is held responsible for the various health benefits associated with the consumption of olive oil. Besides the consumption of olives and olive oil, there are other routes leading to hydroxytyrosol intake. During the production of olive oil, a large quantity of wastewater is produced. This wastewater has a higher content of phenolic compounds than olive oil; it contains more than half of the available pool of antioxidants in the olive (~53%). The recovery of phenolic compounds from the wastewater is of major interest, not only from an environmental point of view, but also because these compounds might be useful in the pharmaceutical, food and cosmetic industry. Techniques are being optimised to extract the phenolic compounds (Agalias et al., 2007, Khoufi et al., 2008). Supplements with health-promoting claims are currently being derived from the wastewater (Rietjens et al., 2008), with hydroxytyrosol as the principal ingredient. The content of hydroxytyrosol in some of these extracts is 300–500 times higher than in olive oil. In this way, a waste pollutant is turned into a health-promoting and therefore valuable product. To date, numerous hydroxytyrosol supplements are for sale on the Internet.

2.4.1 RADICAL SCAVENGING PROPERTIES OF HYDROXYTYROSOL

Several studies on the antioxidant properties of hydroxytyrosol have been performed. However, test tube experiments have demonstrated that hydroxytyrosol has superior $ONOO^-$, $OH^\bullet$ and $O_2^{\bullet-}$ scavenging activities, equal to or surpassing that of the reference antioxidant (Figure 2.10). The high potency of hydroxytyrosol for scavenging $ONOO^-$ is in accordance with previous reports (De la Puerta et al., 2001, Deiana et al., 1999). It has been shown that hydroxytyrosol is able to prevent $ONOO^-$-dependent DNA damage and tyrosine nitration (Deiana et al., 1999).

Literature on the activity of hydroxytyrosol as an $O_2^{\bullet-}$ scavenger is contradictory (O'Dowd et al., 2004, Visioli et al., 1998). Test tube experiments unequivocally show that hydroxytyrosol can efficiently scavenge $O_2^{\bullet-}$.
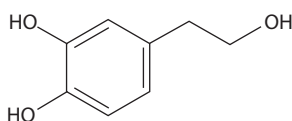
FIGURE 2.9 Chemical structure of hydroxytyrosol.
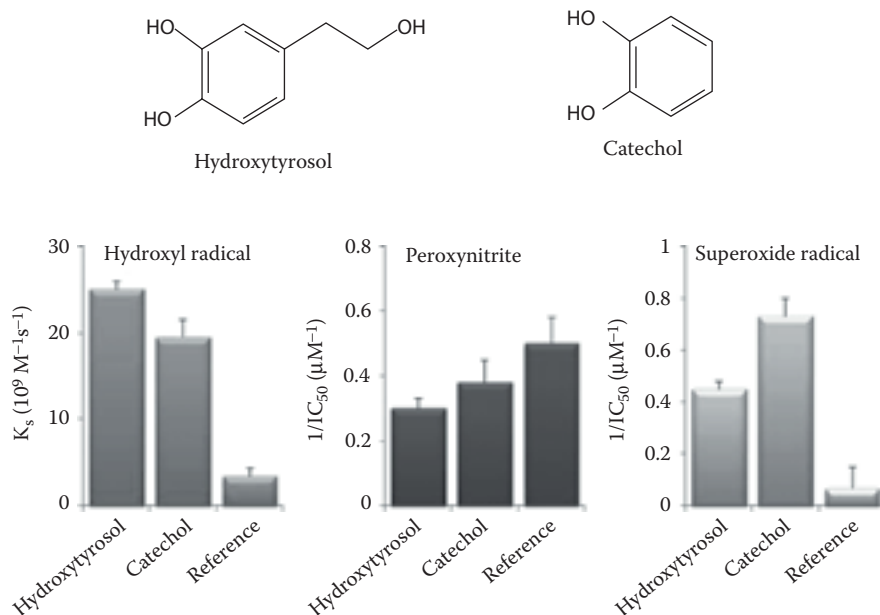
FIGURE 2.10 Antioxidant profiles of hydroxytyrosol and catechol. The OH$^•$, ONOO$^-$ and O$_2^{•-}$ scavenging activities of the compounds were related to the scavenging activities of mannitol (second-order rate constant = $3.4 \cdot 10^9$ M$^{-1} \cdot$s^{-1}), ebselen (IC$_{50}$ = 2.0 μM), and rutin (IC$_{50}$ = 14.8 μM), respectively. The H$_2$O$_2$ and HOCl scavenging activity of the compounds was related to GSH (IC$_{50}$ = 83 μM) and lipoic acid (second-order rate constant = 0.1 mM^{-1} min^{-1}), respectively. None of the phenolic compounds displayed any relevant H$_2$O$_2$ or HOCl scavenging activity.

Previous reports have suggested that hydroxytyrosol is able to scavenge H$_2$O$_2$ (O'Dowd et al., 2004) and HOCl (Visioli et al., 1998). Comparing the activity of hydroxytyrosol to a relevant reference compound clearly shows that hydroxytyrosol hardly possesses any H$_2$O$_2$ or HOCl scavenging activity. The scavenging of the non-radical species H$_2$O$_2$ and HOCl involves a two-electron reaction. In the scavenging of these species, thiols appear to be far superior to phenolic compounds.

Comparing these activities of hydroxytyrosol with those of catechol (1,2-dihydroxyphenyl) shows that the activity of hydroxytyrosol resides in the catechol moiety. As reported previously, the antioxidant potency of the catechol group is superior to that of a resorcinol (1,2-dihydroxyphenyl) or phenol (mono-hydroxyphenyl) group. The relatively high antioxidant activity of catechol can be explained by the high electron-donating effect of one hydroxyl group to the other (Heijnen et al., 2001, 2002).

During the process of radical scavenging, hydroxytyrosol is converted into a radical itself. Like the vitamin E radical, this radical is stabilised due to the formation of several resonance structures of the hydroxytyrosol radical (Figure 2.11). The two electron oxidised form of hydroxytyrosol gives a quinone product. This quinone is readily recycled to hydroxytyrosol in a redox reaction with ascorbate. Alternatively,
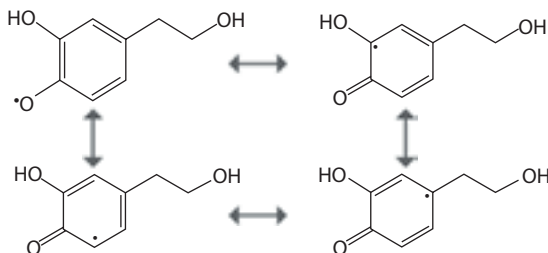
FIGURE 2.11   Resonance structures of the hydroxytyrosol radical.

the quinone can react with GSH in a Michael addition to generate a glutathione conjugate. Through these interactions with either ascorbate or GSH, hydroxytyrosol can pass over the reactivity to the endogenous antioxidant network.

Also, the amphiphilic nature of hydroxytyrosol is of importance; it enables hydroxytyrosol to transfer the reactivity from a lipophilic environment to an aqueous environment. This ability of hydroxytyrosol to transfer radicals from one environment to another is pivotal (Rietjens et al., 2007), especially in the protection of LDL against oxidative damage.

The potent antioxidant activity of hydroxytyrosol is also evidenced by protection against oxidative stress-induced damage to several types of cells, such as intestinal epithelial cells (Manna et al., 1997), erythrocytes (Manna et al., 1999) and hepatocytes (Goya et al., 2007).

2.4.2   ANTIOXIDANT EFFECTS OF HYDROXYTYROSOL *IN VIVO*

For an antioxidant to have beneficial health effects *in vivo*, it is of course essential that it is taken up. Several studies in humans and rats indeed report that the uptake of hydroxytyrosol is good (Visioli et al., 2000b, 2001, Vissers et al., 2002, Tuck et al., 2001, Miro Casas et al., 2003). Plasma concentrations of hydroxytyrosol after consumption of 25 mL of extra virgin olive oil range from 50 to 160 nM (Vissers et al., 2001a, Edgecombe et al., 2000). The consumption of supplements containing a relatively high amount of hydroxytyrosol might lead to an even higher hydroxytyrosol plasma concentration.

Hydroxytyrosol is metabolised in the body by the action of catechol-O methyltransferase on homovanillic alcohol. Ingested oleuropein, one of the other phenolic compounds also present in virgin olive oil, is hydrolysed in the intestine yielding additional hydroxytyrosol. Oleuropein itself hardly reaches the systemic circulation after ingestion (Corona et al., 2006, de la Torre-Carbot et al., 2007).

Visioli et al. (2000c) have shown that hydroxytyrosol was able to inhibit passive smoking-induced oxidative stress in rats, as demonstrated by a reduced urinary excretion of isoprostanes (8-iso-PGF2α). Moreover, a dose-dependent inverse correlation between the rate of 8-iso-PGF2α excretion and increasing amounts of phenolic compounds ingested with olive oil was observed in human volunteers (Visioli et al., 2000a). Furthermore, hydroxytyrosol was able to increase plasma antioxidant capacity in rats (Visioli et al., 2001).

Regarding the protective effect of hydroxytyrosol against the oxidation of LDL, some controversies exist. Several human *in vivo* studies have shown that olive oil phenolic compounds efficiently protect against LDL oxidation (Covas et al., 2006a,b, Fito et al., 2005, Marrugat et al., 2004, Weinbrenner et al., 2004). However, no protective effect of hydroxytyrosol is usually found against LDL oxidation in *ex vivo* experiments (Bonanome et al., 2000, Nicolaiew et al., 1998, Vissers et al., 2001a,b). Additional research on this controversy showed that the lack of effect in the *ex vivo* experiment was actually an artefact. In the *ex vivo* experiment, hydroxytyrosol was given to a volunteer. Subsequently, LDL was isolated by centrifugation. Finally, the isolated LDL was subjected to *in vitro* oxidative stress and the antioxidant effect of the hydroxytyrosol given to the volunteer was tested. It was found that during this isolation of LDL, the hydroxytyrosol was lost from the LDL during the centrifuga-tion step. The apparent loss of hydroxytyrosol from LDL, that occurred during the isolation of LDL, explains why no protective effect *ex vivo* was observed after hydroxytyrosol administration *in vivo* (Rietjens et al., 2007). The final conclusion is that hydroxytyrosol protects LDL against oxidative damage, a claim approved by the European Food Safety Authority (Agostoni et al., 2011).

2.4.3   EFFECT OF HYDROXYTYROSOL IN EXERCISE

During exercise, the production of reactive oxygen and nitrogen species is consider-ably enhanced. In a study with a crossover design in seven untrained healthy men, the effect of supplementation with a hydroxytyrosol-rich olive extract (twice 200 mg) on the glutathione defence system was determined. Details on the exercise are given in the legend of Figure 2.12.

Hydroxytyrosol did not blunt the exercise-induced adaptive response on the glu-tathione system found in erythrocytes and muscle. The exercise-induced increase in erythrocyte GSH concentrations was not affected by hydroxytyrosol supplemen-tation. The erythrocyte GSSG concentration remained during exercise at baseline value, after both hydroxytyrosol and placebo supplementation.

The GSH concentrations in skeletal muscle, that is, in a biopsy from the middle region of the m. vastus lateralis, increased 30 min after exercise. The supplementa-tion of hydroxytyrosol even further increased skeletal muscle GSH levels after exer-cise. Neither exercise nor hydroxytyrosol did affect GSSG concentrations in skeletal muscle (Figure 2.12c). Glutathione reductase activity in skeletal muscle increased after exercise (Figure 2.12b). The increase in glutathione reductase activity induced by exercise was higher after hydroxytyrosol supplementation (Figure 2.12b). The increase in skeletal muscle GST activity by exercise was similar after hydroxytyro-sol and placebo supplementation (Figure 2.12d). This indicates that hydroxytyrosol boosts the exercise-induced adaptive response.

It is of interest to note that the enhancement of the GSH-dependent protection enforces a part of the antioxidant network that was not covered by hydroxytyrosol. In contrast to hydroxytyrosol, GSH is an excellent scavenger of H_2O_2 and HOCl, reactive species not efficiently scavenged by hydroxytyrosol. Also, the interplay between hydroxytyrosol and GSH, as described above, comes into the equation. The complementary profiles of hydroxytyrosol and GSH ensure a protection over a broad

(a)

(b)

(c)

(d)
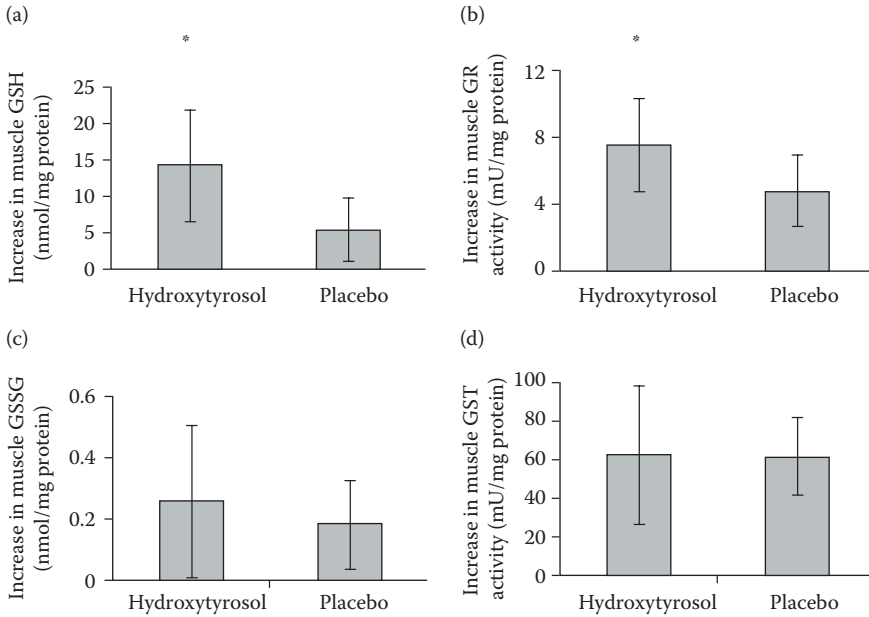
FIGURE 2.12   The effect of hydroxytyrosol on exercise-induced increases in skeletal muscle GSH (a), glutathione reductase (GR) activity (b), GSSG (c) and GST activity (d). Data provided are mean ± SEM. *Significantly different from placebo ($P < 0.10$). The volunteers were asked to participate in the study on two separate occasions. Subjects consumed, in a randomised order, a hydroxytyrosol-rich olive extract or a placebo. The study was blinded for both the volunteers as well as the investigators. The olive extract providing 200 mg hydroxytyrosol per dose was diluted in 150 mL drinking water directly prior to ingestion. The olive extract or the placebo was supplemented twice: on the evening before the test day at 8.00 p.m. and on the test day itself at 8.30 a.m. (t = −30 min). The placebo had no antioxidant capacity. The hydroxytyrosol supplement and the placebo were similar in taste and colour. The subjects arrived at the laboratory at 8.00 a.m., in an overnight fasted state. Then they performed a 5-min low-intensity warm-up using a Stairmaster (Jimsa Benelux BV, Rotterdam, the Netherlands). Thereafter, the resistance exercise session targeted the legs, with eight sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, the Netherlands) and eight sets of 10 repetitions on the leg extension machine (Technogym). The starting workload applied during the resistance exercise session was 75% of the individual 1RM for both the leg press and leg extension with 2-min rest intervals between sets. In total, the exercise regime required approximately 40 min to complete. If subjects could not finish all 10 repetitions at full weight, this was reduced to 65% or 55% of the individual 1RM. Only one of the evaluated volunteers was able to finish the entire protocol at 75%. The average intensity level of the exercise regime was 70%. All subjects were verbally encouraged during the test to complete the entire protocol.

spectrum and prevents the intricate antioxidant network becoming unbalanced. It is concluded that hydroxytyrosol strengthens the endogenous antioxidant defence during a single session of strenuous resistance-type exercise in untrained men.

Hydroxytyrosol supplementation tended to decrease the plasma peak concentration of lactate directly after exercise (Figure 2.13). The area under the plasma
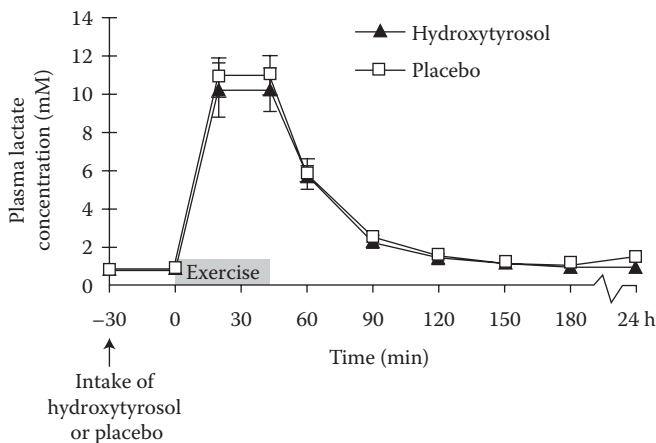
FIGURE 2.13 The effect of hydroxytyrosol on lactate levels during resistance exercise (duration is indicated by the grey bar) and subsequent recovery. Details on the exercise are given in the legend of Figure 2.12. Data provided are mean ± SEM.

concentration time curve (AUC), reflecting the production of lactate, was calculated for each volunteer. The experiment revealed that hydroxytyrosol decreased the AUC of lactate ($P = 0.063$).

Sastre et al. (1992) have reported a positive correlation between oxidative stress, namely the GSSG to GSH ratio, and the lactate to pyruvate ratio during an exhaustive physical exercise. This is in line with the effect of hydroxytyrosol on GSH levels and on lactate. Also, long-term co-supplementation (90 days) with vitamin E, β-carotene and vitamin C reduced the maximal blood lactate concentration during exercise (Schröder et al., 2001). Although the mechanism is unclear, it has been found that antioxidants are able to affect lactate levels during exercise. Nevertheless, additional research is needed to clarify this finding.

In contrast, several studies report on the negative effects of antioxidants. One of the explanations for the controversy is the different population used. Most studies reporting a benefit of antioxidant supplementation in attenuating muscle injury and oxidative stress following resistance exercise have included sedentary, non-resistance trained subjects. In trained subjects, endogenous antioxidant defences may be up-regulated, and therefore these individuals may not benefit greatly from exogenous antioxidant intake in order to reduce muscle injury (Ji, 2008). We selected untrained subjects for the hydroxytyrosol study, based on our speculation that oxidative damage would be greater in a non-trained group, thereby increasing the window of opportunity to detect an effect of hydroxytyrosol supplementation on oxidative damage and antioxidant capacity.

In addition, studies on the use of antioxidants during exercise have used several types of exercise, ranging from long-term endurance exercise to short-term resistance exercise. Aerobic endurance exercise will induce a different flux of radicals than anaerobic resistance exercise does (Pattwell and Jackson, 2004). In designing the study, we speculated that the effect of an acute and high flux of radicals would

be more sensitive to antioxidant supplementation than that of a more prolonged and moderate flux of radicals.

In order to maximise the sensitivity of the study, a single session of strenuous resistance exercise was applied. It has been suggested that the effect of antioxidants depends on the degree of muscle damage (Gomez-Cabrera et al., 2008). During moderate exercise, antioxidants might hamper the beneficial adaptive response. When strenuous exercise is applied, such as in this study, antioxidants are expected to prevent oxidative stress-induced muscle injury (Gomez-Cabrera et al., 2008).

2.5 CONCLUSION

The general overview and the specific example of hydroxytyrosol demonstrate that it is time to categorise. This makes it possible to

- Select the antioxidant with the right profile and multifaceted mode of action that fits the type of oxidative stress involved.
- Translate the antioxidant activity into a realistic health benefit.
- Select the people who are expected to benefit from the antioxidant.

This prevents misconceptions, which, unfortunately, have put dietary antioxidants, food supplements and antioxidant drugs in a bad light. By categorising and making the right choices, we will be able to capitalise upon the myriad of beneficial health effects antioxidants offer and continue to design, develop and disseminate antioxidant strategies.

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3 Antioxidants in Athlete's Basic Nutrition

Considerations towards a Guideline for the Intake of Vitamin C and Vitamin E

Oliver Neubauer and Christina Yfanti

CONTENTS

3.1 INTRODUCTION: ANTIOXIDANTS—A REMAINING HOT TOPIC IN SPORT NUTRITION

Antioxidants in acute physical exercise and exercise training remain a hot topic in sport nutrition, exercise physiology and biology, in general (Jackson, 2008; Margaritis and Rousseau, 2008; Gomez-Cabrera et al., 2012; Nikolaidis et al., 2012). During the past few decades, antioxidants have received attention predominantly as a nutritional strategy for preventing or minimising detrimental effects of reactive oxygen and nitrogen species (RONS), which are generated during and after strenuous exercise (Jackson, 2008, 2009; Powers and Jackson, 2008). Antioxidant supplementation has become a common practice among athletes as a means to

(theoretically) reduce oxidative stress, promote recovery and enhance performance (Peternelj and Coombes, 2011). However, until now, requirements of antioxidant micronutrients and antioxidant compounds for athletes training for and competing in different sport events, including marathon running, triathlon races or team sport events involving repeated sprinting, have not been determined sufficiently (Williams et al., 2006; Margaritis and Rousseau, 2008). Crucially, evidence has been emerging that higher dosages of antioxidants may not necessarily be beneficial in this context, but can also elicit detrimental effects by interfering with performance-enhancing (Gomez-Cabrera et al., 2008) and health-promoting training adaptations (Ristow et al., 2009). As originally postulated in a pioneering study on exercise-induced production of RONS by Davies et al. (1982) in the early 1980s, evidence has been increasing in recent years that RONS are not only damaging agents, but also act as signalling molecules for regulating muscle function (Reid, 2001; Jackson, 2008) and for initiating adaptive responses to exercise (Jackson, 2009; Powers et al., 2010). The recognition that antioxidants could, vice versa, interact with the signalling pathways underlying the responses to acute (and repeated) bouts of exercise has contributed important novel aspects to the continued discussion on antioxidant requirements for athletes.

In view of the recent advances in this field, it is the aim of this report to examine the current knowledge of antioxidants, in particular of vitamins C and E, in the basic nutrition of athletes. While overviews on related topics including basic mechanisms of exercise-induced oxidative stress, redox biology, antioxidant defence systems and a summary of studies on antioxidant supplementation during exercise training are provided, this does not mean that this report is comprehensive. Several issues of the expanding and multidisciplinary field of antioxidants and exercise are covered elsewhere in this book and/or in the literature. Exemplarily, the reader is referred to reviews on oxidative stress (König et al., 2001; Vollaard et al., 2005; Knez et al., 2006; Powers and Jackson, 2008; Nikolaidis et al., 2012), redox-sensitive signalling and muscle function (Reid, 2001; Vollaard et al., 2005; Jackson, 2008; Ji, 2008; Powers and Jackson, 2008; Powers et al., 2010; Radak et al., 2013) and antioxidant supplementation (Williams et al., 2006; Peake et al., 2007; Peternelj and Coombes, 2011) in the context with exercise. Within the scope of the report, we rather aim to address the question regarding requirements of antioxidants, specifically vitamins C and E, during exercise training, draw conclusions and provide practical implications from the recent research.

3.2 OVERVIEW ON BASIC MECHANISMS OF EXERCISE-INDUCED OXIDATIVE STRESS

After more than three decades of research, it is well documented that prolonged, intense exercise and/or exercise involving frequent eccentric/lengthening contractions, especially if unaccustomed, induces the generation of RONS including free radicals [e.g. superoxide ($O_2^{\cdot-}$), nitric oxide ($NO^\cdot$), hydroxyl radical ($OH^\cdot$)] and non-radicals [e.g. hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$)] (Vollaard et al., 2005; Jackson, 2008, 2009; Powers and Jackson, 2008). Potential mechanisms for an exercise-induced RONS generation include the activation of nicotinamide adenine

dinucleotide phosphate–oxidase complexes associated with the sarcoplasmic reticulum and plasma membranes, and variations in perfusion triggering xanthine oxidase activity (Vollaard et al., 2005; Jackson, 2008; Powers and Jackson, 2008). Furthermore, an inadequate electron transfer through the mitochondrial respiratory chain related to the increased oxygen consumption has previously been assumed as a major site for an increased superoxide generation *during* muscle contractions (e.g. reviewed by Powers and Jackson, 2008). However, more recent studies indicate that the RONS generation through an increased mitochondrial oxygen flux during aerobic exercise is rather limited due to internal control mechanisms (Vollaard et al., 2005; Jackson, 2008; Powers and Jackson, 2008). In addition, muscular inflammatory responses characterised by an infiltration of neutrophils and macrophages into exercised skeletal muscle (Stupka et al., 2000), followed by oxidative burst reactions, could contribute to an increased RONS generation until a few days *after* exercise involving muscle damage (Close et al., 2003). Although the phagocytic activity of infiltrated leukocytes appears to be essential for the repair and regeneration of injured muscle tissue, the free-radical-mediated removal of cell debris by phagocytic cells such as neutrophils may elicit secondary tissue damage (Close et al., 2003; Tidball and Villalta, 2010). Each of these potential mechanisms occurs in skeletal muscle tissue, which, as one of the biggest tissues in the human body, is therefore considered the major source for the generation of ROS related to exercise (Powers and Jackson, 2008). However, other tissues have also been discussed as potential sources for an increased exercise-induced RONS generation, including the heart, lungs (Powers and Jackson, 2008) and blood constituents such as leukocytes (Nikolaidis and Jamurtas, 2009), which are mobilised into the circulation and activated as part of the systemic inflammatory response to intense, prolonged exercise (König et al., 2001; Neubauer et al., 2008b, 2013).

Owing to their reactivity, RONS can oxidise and alter the structure and/or function of biomolecules, among which lipids, proteins and DNA are the most vulnerable (and most investigated) cellular targets (Halliwell and Gutteridge, 2007). Depending on the type of stress imposed and how severe the stress is, RONS may accumulate, eventually leading to oxidative damage to these macromolecules and subsequently to an impairment of their physiological functions (Halliwell and Gutteridge, 2007). Progressive oxidative macromolecular damage is evidenced, for example, by disruptions in the cell membrane lipid bilayer, inactivation of membrane-bound proteins, loss of enzyme function, lipoprotein peroxidation and DNA strand breakage (Halliwell and Gutteridge, 2007). Furthermore, it is now recognised that oxidative stress may occur without necessarily resulting in an overall imbalance between pro-oxidants and antioxidants, but rather through a disruption of individual redox-sensitive signalling pathways, some of which, for example, promote proteolytic degradation, inflammation and cell death (Jones, 2006; Jackson, 2009; Powers et al., 2010; Nikolaidis et al., 2012). The chronic exposure to high levels of RONS is associated with the development and/or progression of pathophysiological processes, and implicated in an increasing number of human diseases, such as cardiovascular, metabolic, inflammatory and neurogenerative diseases, cancer as well as muscle atrophy and the ageing process (Vollaard et al., 2005; Halliwell and Gutteridge, 2007; Powers et al., 2010). Exercise-induced oxidative stress has been

discussed to impair performance and muscle force production during exercise (Reid, 2001; Vollaard et al., 2005; Powers and Jackson, 2008), contribute to muscle damage and further promote inflammatory responses after exercise, thereby interfering with recovery (König et al., 2001). Indications for increased oxidative stress have also been reported during periods of overtraining (Palazzetti et al., 2004). Furthermore, some empirical and epidemiological data, paradoxically, suggest that an extraordinary high volume of exercise is associated with an increased risk of developing cardiovascular disease (Lee et al., 1995), potentially due to cumulative oxidative stress as one of the main mechanisms (Knez et al., 2006). On the basis of these data (Lee et al., 1995) and the model of oxidative modifications in atherosclerosis (Stocker and Keaney, 2004), Knez and co-workers hypothesised that the population of ultra-endurance athletes, training for and competing in races with durations of several hours, might be at higher risk of developing atherosclerotic lesions (Knez et al., 2006). To address this issue, one of us together with co-workers recently investigated the time-course of recovery of a broader spectrum of lipid peroxidation and protein oxidation biomarkers in the blood plasma, as well as indices for oxidatively damaged DNA in circulating lymphocytes in response to an Ironman triathlon until 19 days post-race (Neubauer et al., 2008a, 2008c, 2010; Reichhold et al., 2008, 2009). This study indicated that despite a temporary increase in most oxidative stress markers, there is no persistent oxidative stress in response to an acute bout of ultra-endurance exercise, potentially due to training- and exercise-induced changes in the antioxidant defence system (Neubauer et al., 2008a, 2010). Furthermore, recent data of a cross-sectional study showed that physically active, former top-level athletes (who previously participated in endurance sport events and sport games) were characterised by a significantly lower cardiovascular risk profile including a lower oxidative stress status compared with sedentary, former athletes and age-matched, non-athletic individuals (Pihl et al., 2003). Taken together, so far, there is no conclusive evidence that exercise-induced oxidative stress, even in ultra-endurance athletes, elicits any negative impact on health.

3.3 OVERVIEW ON ANTIOXIDANT DEFENCE SYSTEMS, AND THE ROLE OF RONS AS SIGNALLING MOLECULES FOR ENDOGENOUS ANTIOXIDANT DEFENCES

There are several cellular antioxidant defence strategies to counterbalance RONS. These strategies include converting RONS into less active species and preventing the transformation of these less active molecules into ones with higher activity, scavenging RONS and minimising the availability of pro-oxidants (e.g. iron) (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008). The composition of antioxidant defences differs from tissue to tissue and from cell-type to cell-type, but broadly, antioxidant defence systems can be classified into endogenous enzymatic and non-enzymatic antioxidants on the one side, and exogenous, that is, dietary antioxidants on the other (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008). The enzymatic antioxidant defence consists of primary antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), and accessory antioxidant enzymes such as thioredoxin (Halliwell and Gutteridge,

2007; Powers and Jackson, 2008). Examples for non-enzymatic endogenously produced low-molecular weight antioxidants are glutathione, uric acid and bilirubin (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008). Despite some controversial results (e.g. reviewed by Powers and Jackson, 2008), most studies investigating the adaptive responses to exercise-induced RONS generation have shown that both acute (Khassaf et al., 2001, 2003) and regular exercise (Brooks et al., 2008) induces increased activities of antioxidant defence enzymes, in particular SOD, in skeletal muscle (Khassaf et al., 2001, 2003) in mice (McArdle et al., 2004; Brooks et al., 2008) and humans (Khassaf et al., 2001, 2003). The increased SOD activity in the muscle of mice reported in a study of Malcolm Jackson's research group (Brooks et al., 2008) appeared to be primarily due to an increased SOD protein content, reflecting a longer term adaptation to endurance exercise training to counterbalance subsequent redox disturbances and reduce the risk of oxidative damage (Powers and Jackson, 2008). Plasma concentrations of low-molecular mass antioxidants originating from endogenous sources, including bilirubin (Neubauer et al., 2010) and uric acid (Liu et al., 1999; Mastaloudis et al., 2004a; Neubauer et al., 2010), have also been reported to temporarily increase acute bouts after strenuous exercise due to various mechanisms induced during intense exercise (e.g. increased haemolysis and increased purine metabolism) (Liu et al., 1999; Neubauer et al., 2010). Although the exercise-induced changes in these endogenous low-molecular mass antioxidants might not be considered as specific training adaptations, they contribute to enhanced plasma antioxidant defences and, potentially, play a protective role against oxidative damage of blood cell components such as lymphocyte DNA (Neubauer et al., 2010).

Importantly, antioxidant defence systems work in a highly efficient and coordinated manner and are closely related to nutrition. Important low-molecular mass nutritive antioxidants include vitamin C, vitamin E (comprising tocopherols and tocotrienols), carotenoids (e.g. β-carotene) and polyphenols (e.g. flavonoids) (Halliwell and Gutteridge, 2007). Exemplarily, among numerous interactions between antioxidants, the tocopheroxyl radical, which results from the reaction of α-tocopherol with peroxyl radicals, can be 'recycled' to its active vitamin E form by other antioxidants such as vitamin C or glutathione (Traber, 2007). Furthermore, several antioxidant enzymes require trace elements as co-factors for their structural integrity and their functionality. Trace elements with antioxidant function include selenium (required for GPX), iron (CAT), zinc, copper and manganese (all of which are required for different isoforms of SOD). For background information on the biochemistry of these nutritive antioxidants, the reader is referred to the literature (Halliwell and Gutteridge, 2007; Powers and Jackson, 2008). Within the frame of this chapter, the focus is on the vitamins C and E in the context with exercise training, as discussed below.

Of utmost importance for the continued discussion on antioxidants in sport nutrition, it has become an emerging concept that moderate levels of RONS play an important role in the regulation of the muscular contractile function and physiological adaptive responses (Jackson, 2008; Powers and Jackson, 2008; Powers et al., 2010). An increasing number of investigations indicate that RONS generated in response to physiological stimuli such as exercise are a necessary signal to activate redox-sensitive cellular pathways and transcription factors including nuclear factor-κB, activator protein-1 (AP-1), peroxisome proliferator-activated receptor transcription factors and

heat-shock factor (HSF)-1 (Brooks et al., 2008; Jackson, 2008; Radak et al., 2013). In turn, these transcription factors regulate the expression of genes including genes encoding for specific stress and heat shock proteins (HSPs) (Khassaf et al., 2003), genes involved in antioxidant protection (Khassaf et al., 2003; Brooks et al., 2008) and genes associated with mitochondrial biogenesis (Irrcher et al., 2009). The up-regulation of the expression of protective genes/proteins such as HSPs and antioxidant enzymes in response to exercise-induced oxidative stress is associated with an increased protection against subsequent exposure to RONS (McArdle et al., 2004). These seemingly contradictory effects of RONS have been described by implementing the concept of hormesis into this context, a dose–response relationship in which a low dose of a substance is stimulatory or beneficial and a high dose is inhibitory or toxic (Ji et al., 2006; Radak et al., 2013).

3.4 OVERVIEW ON STUDIES ON ANTIOXIDANT SUPPLEMENTATION IN EXERCISE TRAINING

In the following section, we will provide an overview on human studies in this area. The main focus of this overview will be on chronic supplementation (i.e. more than 2 weeks) with vitamins C and E (mainly the α-tocopherol form), either individually or in combination, during exercise training, since these antioxidant vitamins were the most commonly used and more widely examined supplements in these studies. A summary of the studies included in this review is presented in Table 3.1. As mentioned, we do not claim that the list of studies included in the current report is complete, and would like to refer the reader to comprehensive reviews which are already available in this area (Vollaard et al., 2005; Williams et al., 2006; Peake et al., 2007), including a review article of Peternelj and Coombes as one of the most recent (Peternelj and Coombes, 2011). Our approach is rather to exemplarily discuss the findings of a number of key studies and their implications for defining guidelines on the antioxidant intake in athletes. Since it has been suggested that oxidative stress is relative to exercise intensity (Lamprecht et al., 2008), and that in sport with higher oxygen consumption demands, such as marathon- and triathlon-training, higher amounts of RONS are produced, the studies presented are focused on endurance athletes and/or well-trained individuals.

Early studies from the 1970s and 1980s were focused more on the effect of antioxidant supplementation on exercise performance. The rationale behind this effort was based on the fact that the RONS produced during exercise cause muscle damage and fatigue, and consequently decrease performance. It was hypothesised that supplementation with antioxidants would prevent damage or accelerate recovery and, as a result, improve exercise performance. However, the majority of these early studies did not succeed in demonstrating a significant effect of antioxidant supplementation during training. One of the first studies published in JAMA in 1970 (Gey et al., 1970) showed that daily supplementation of 1.000 mg vitamin C during training did not have any effect on endurance performance in well-trained individuals. Some years later, long-term supplementation with vitamin E (α-tocopherol) by competitive swimmers did not show any effect regarding endurance and cardiorespiratory efficiency (Lawrence et al., 1975; Sharman et al., 1976).

TABLE 3.1
Overview on Studies on Antioxidant Supplementation in Exercise Training

Reference	Subjects (Training Status, N, Sex)	Type of Training	Duration of Training (Weeks)	Acute Exercise	Supplementation (Type, Dosage)/ Daily	Duration of Supplementation (Weeks)	Parameters Tested	Results of Supplementation
Gey et al. (1970)	Well-trained, N = 286	Unspecified	12		1000 mg vitamin C	12	Endurance (12 min field test)	No effect
Lawrence et al. (1975)	Trained (swimmers), N = 48	Unspecified	24		900 IU vitamin E	24	Endurance	No effect
Sharman et al. (1976)	Moderately trained (swimmers), N = 27, M/F	Endurance and interval	6		270 IU vitamin E	6	Cardiorespiratory efficiency, motor fitness	No effect
Rokitzki et al. (1994)	Athletes (runners), N = 24	Endurance		Marathon race	400 IU vitamin E, 200 mg vitamin C	4.5	Muscle damage	Beneficial effect
							Lipid peroxidation	No effect
							Antioxidant enzymes	No effect
Zoppi et al. (2006)	Athletes (soccer), N = 10, M	Endurance, strength, anaerobic	12		1000 mg vitamin C, 530 IU vitamin E	12	Antioxidant enzymes	No effect
Mastaloudis et al. (2004a)	Trained (runners), N = 22, M/F	Unspecified	–	50 km ultramarathon	1000 mg vitamin C, 200 IU vitamin E	6	Protein oxidation	No effect
							Lipid peroxidation	Beneficial effect
							Muscle damage	Beneficial effect
							Lipid peroxidation	Beneficial effect
							Inflammation	No effect

TABLE 3.1 (continued)
Overview on Studies on Antioxidant Supplementation in Exercise Training

Reference	Subjects (Training Status, N, Sex)	Type of Training	Duration of Training (Weeks)	Acute Exercise	Supplementation (Type, Dosage)/ Daily	Duration of Supplementation (Weeks)	Parameters Tested	Results of Supplementation
Yfanti et al. (2010)	Trained (cycling), N = 21, M	Endurance (cycling)	12		500 mg vitamin C, 400 IU vitamin E	16	VO_{2max}	No effect
							Lactate	No effect
							Glycogen	No effect
							Metabolic enzymes	No effect
							Antioxidant enzymes	No effect
Yfanti et al. (2012)	Trained (cycling), N = 21, M	Endurance (cycling)	12		500 mg vitamin C, 400 IU vitamin E	16	Lipid peroxidation	Detrimental effect
							Protein oxidation	Detrimental effect
Nieman et al. (2004)	Athletes (triathlon), N = 38, M/F	Endurance	8	Ironman triathlon race	800 IU vitamin E	8	Lipid peroxidation	Detrimental effect
							Inflammation	Detrimental effect
Knez et al. (2007)	Athletes (half and full Ironman triathletes), N = 29, M/F	Endurance	~312	Half-distance and full Ironman triathlon race	560 mg vitamin C, 470 IU vitamin E	~208	Lipid peroxidation	Detrimental effect
							Antioxidant enzymes	No significant effect

Reference	Subjects	Type	Exercise	Supplement	Weeks	Marker	Effect
Lamprecht et al. (2009)	Athletes (cyclists), N = 8, M	Endurance	30 and 60 min cycling test	107 IU vitamin E, 450 mg vitamin C, 36 mg β-carotene, 100 μg selenium	2	Lipid peroxidation	Detrimental effect
Mastaloudis et al. (2004b)	Trained (runners), N = 21, M/F	Unspecified	50 Km ultramarathon	1000 mg vitamin C, 270 IU vitamin E	6	DNA damage	No significant to detrimantal effect
Khassaf et al. (2003)	Untrained, N = 16, M		45 min cycling	500 mg vitamin C	8	Antioxidant enzymes / HSPs / HSP72	No significant effect / Detrimental effect / Detrimental effect
Fischer et al. (2006)	Moderately trained, N=21, M		3 h knee-extensor exercise	500 mg vitamin C, 400 IU vitamin E (α-tocopherol), or 500 mg vitamin C, 292 IU vitamin E (α-tocopherol), 130 IU vitamin E γ-tocopherol	4		
Palazzetti et al. (2004)	Athletes (triathlon), N = 17, M	Endurance	Duathlon test	150 mg selenium, 120 mg vitamin C, 2000 IU vitamin A, 30 IU vitamin E	8	Antioxidant enzymes	Beneficial effect
Gomez-Cabrera et al. (2008)	Human study: Sedentary, N = 14, M	Endurance		1000 mg vitamin C	8	VO$_{2max}$	No effect
	Animal study: N = 18	Endurance		500 mg/kg body weight vitamin C	3–6	Endurance / VO$_{2max}$	Detrimental effect / No effect

TABLE 3.1 (continued)
Overview on Studies on Antioxidant Supplementation in Exercise Training

Reference	Subjects (Training Status, N, Sex)	Type of Training	Duration of Training (Weeks)	Acute Exercise	Supplementation (Type, Dosage)/ Daily	Duration of Supplementation (Weeks)	Parameters Tested	Results of Supplementation
Wray et al. (2009)	Sedentary hypertensive, N = 6, M	Endurance	6		600 mg lipoic acid, 1000 mg vitamin C, 600 IU vitamin E	6	Blood pressure	Detrimental effect
Ristow et al. (2009)	Trained, N = 20, M and Untrained, N = 19, M	Endurance and strength	4		1000 mg vitamin C, 400 IU vitamin E	4	Insulin sensitivity Mitochondrial biogenesis Antioxidant enzymes	Detrimental effect Detrimental effect Detrimental effect
Yfanti et al. (2011)	Trained (cycling), N = 21, M	Endurance (cycling)	12		500 mg vitamin C, 400 IU vitamin E	16	Insulin sensitivity Mitochondrial biogenesis	No effect No effect

Note: 1 IU vitamin E = 0.67 mg *RRR*-α-tocopherol = 1 mg *all rac*-α-tocopheryl acetate.

Abbreviations: M = male; F = female; mg = milligram, IU = international units.

Later studies were focused not only on performance, but also on blood markers and redox status. Rokitzki et al. (1994) reported that administration of combined vitamins C and E for 1 month prior to a marathon race decreased indices of muscle damage after the race while there was no effect on lipid peroxidation. Furthermore, the same combination in soccer players prevented both muscle damage and lipid peroxidation, but it did not affect performance (Zoppi et al., 2006). Mastaloudis et al. (2004a) also tested the effect of the combination of vitamins C and E on lipid peroxidation and inflammation after a 50-km ultra-marathon race. The supplementation prevented lipid peroxidation in response to the ultra-marathon; however, it showed no effect on markers on inflammation which increased dramatically after exercise. One of the authors and co-workers (Yfanti et al., 2010) performed a training study where well-trained individuals consumed the same combination of antioxidant vitamins during 3 months of high-intensity cycling exercise. However, no effect on either cardiovascular or skeletal muscle aerobic adaptations was observed (Yfanti et al., 2010). In contrast, in the same study, higher levels of plasma protein oxidation and lipid peroxidation were measured in the group that consumed the antioxidants compared with placebo, suggesting a pro-oxidative effect of the vitamins (Yfanti et al., 2012). The latter study was not the first one to show such an effect. Some years earlier, Nieman et al. (2004) had found that vitamin E supplementation for 2 months before an Ironman triathlon race promoted lipid peroxidation, assessed by plasma F2-isoprostanes, and inflammation in response to acute ultra-endurance exercise. Furthermore, Knez et al. (2007) examined oxidative stress in half and full Ironman triathletes. They demonstrated that the athletes who were supplemented with vitamins C and E for ca. 1 year had higher levels of lipid peroxidation (assessed by malondialdehyde) after a half-distance- or full-Ironman race, suggesting also a pro-oxidative effect of the supplemented antioxidative vitamins. In addition, in a study by Lamprecht et al. (2009) supplementation for 2 weeks with a vitamin mixture, including vitamin C, vitamin E, β-carotene and selenium, increased plasma malondialdehyde concentration at rest.

However, even after many years of research, it is not possible to draw clear conclusions as a number of studies have not been able to clearly demonstrate excessive damaging effects of exercise with or without antioxidant supplementation. In a large-scale study in Ironman triathletes performed by one of the authors and co-workers, it has been shown that after an ultra-endurance event, DNA-, protein- and lipid peroxidation damage might occur, but that these effects last only transiently (Neubauer et al., 2008a,c; Reichhold et al., 2008, 2009). It is worth noting that the participants in this study were consuming physiological amounts of antioxidants during the course of the study (as described in detail below) (Neubauer et al., 2010). Mastaloudis et al. (2004b) found similar results in runners after an ultra-marathon race, although the study participants were consuming high doses of vitamins C and E. Therefore, taking into account the above published research, it is difficult to support the hypothesis that antioxidant supplementation with vitamins C and E during training is necessary for athletes of ultra-endurance sport, as it seems that it offers minimal or no beneficial effect.

The subject of antioxidant supplementation and exercise training continued to be of high interest. However, the initial view that RONS were, in general, harmful

and that preventing their actions would be beneficial changed over the years. This happened due to some studies showing that RONS produced during exercise play a fundamental role in cellular processes (Irrcher et al., 2009) and that blocking their action would prevent essential cellular processes from taking place.

The more recent human studies investigating the interrelation of antioxidant supplementation and exercise training implemented more sophisticated design, methodologies and techniques and were focused not only on performance, but also on the health aspects of endurance training. Khassaf et al. (2003) examined the effect of vitamin C supplementation during training on antioxidant defence mechanisms and more specifically on SOD and CAT activity, as well as on HSP60 and HSP70. They found that supplementation attenuates the adaptive response to exercise, suggesting a possible negative effect of the supplementation during training. In line with these findings, some years later Fischer et al. (2006) showed that supplementation with vitamins C and E (specifically the γ-tocopherol isoform) inhibits the exercise-induced increase of HSP72 in skeletal muscle as well as in the circulation. The above studies were performed in well-trained individuals and the doses of antioxidants consumed were 5–17 times higher than the recommended dietary allowance (RDA). It is noteworthy that when doses of vitamins C and E close to 100% of the RDA were used during intense training in competitive triathletes, endogenous antioxidant defences were preserved after a duathlon race (Palazzetti et al., 2004). These data suggest that antioxidative requirements of well-trained endurance athletes can be covered by dosages equivalent or close to the RDA, which can be provided by a balanced diet. In addition, it can be suggested that extremely high dosages of antioxidant vitamins that are usually consumed by athletes and individuals engaged to habitual exercise do not offer any additional beneficial effect, but in contrast, they appear in many cases to be harmful.

A number of studies have been published lately which focused on health-related adaptations to endurance training and how antioxidant supplementation interferes in these processes. Although these studies were not performed in well-trained individuals, we believe it is worth mentioning them here as they were the studies that changed the view in the sport world that antioxidant supplements might after all not be required during training. In a study by Gomez-Cabrera et al. (2008), which was performed both in humans and animals, the human results showed that vitamin C supplementation had no effect on maximal oxygen consumption after 8 weeks of endurance training in sedentary men. However, the most striking results were observed in the animal experiment where vitamin C attenuated training-induced mitochondrial biogenesis and endurance capacity in the rodents. A year later, a study was published where a 6-week aerobic exercise training programme with concomitant antioxidant supplementation (vitamins C and E and lipoic acid) was applied in patients with hypertension (Wray et al., 2009). The results showed enhancement of blood pressure and inhibition of exercise-induced flow-mediated vasodilatation in the supplemented group, indicating detrimental effects of the antioxidant supplements (Wray et al., 2009). In the same year, a human study by Ristow et al. (2009) was published that caused a lot of discussion among the researchers dealing with the same subject. In this study, both sedentary and trained individuals trained for 4 weeks while consuming vitamins C and E. The results showed that the antioxidants

inhibited the expected training-induced transcriptional upregulation of genes involved in insulin sensitivity, mitochondrial biogenesis and endogenous antioxidant defence. At the same time that this study was published, one of the current authors and co-workers (Yfanti et al., 2011) were performing an aerobic training study with vitamin C and E supplementation testing some of the same parameters. However, antioxidants during intense cycling exercise had no effect on whole body and skeletal muscle insulin sensitivity or mitochondrial biogenesis in well-trained individuals.

It becomes evident that it is not possible to extract clear conclusions on what is the effect of vitamin C and E supplementation on the adaptive responses to endurance training. The discrepancies among the large number of studies published until now could be attributed to differences in training (i.e. type, duration and intensity), training status of the subjects, supplementation (i.e. type, dosage, duration and timing) as well as the different end points and analytical methods used in each study. Although this particular research area has been extensively studied, additional studies are warranted to obtain more conclusive results on the nutritional antioxidant requirements of professional athletes. Perhaps, more invasive studies should be performed in athletes in order to examine the effect on a molecular level as well as the whole-body level. However, the authors understand that it is difficult to be accepted by professional athletes since such comprehensive investigations interfere with their daily training schedule and recovery.

3.5 IMPLICATIONS FOR ANTIOXIDANT INTAKE IN ATHLETES IN BASIC NUTRITION WITH PARTICULAR FOCUS ON VITAMINS C AND E

On the basis of the data of studies in this area, there is no convincing evidence to recommend antioxidant supplementation during exercise training in addition to the dietary intake of antioxidants. Moreover, on the basis of findings indicating an interference of high doses of supplemental antioxidants with RONS-mediated physiological adaptations to exercise training, caution should be suggested in the use of supplemental antioxidants. Nevertheless, it is very likely that an adequate dietary intake of antioxidants to maintain a physiological antioxidant status is required for athletes undergoing exercise training. In Section 3.5.1, we discuss these antioxidant requirements for athletes by addressing the question regarding antioxidant amounts. Thereby, particular focus is drawn on vitamins C and E, since most of the research in this field has been investigating the effects of these antioxidants in the context with exercise or, vice versa, the potential effects of exercise on the status of vitamins C and E.

3.5.1 Vitamin C

Vitamin C (ascorbic acid or ascorbate) is an essential micronutrient with numerous biological functions, several of which are particularly important to exercise metabolism and exercise immunology (Peake, 2003; Margaritis and Rousseau, 2008). Beyond its role as a potent hydrophilic antioxidant, vitamin C is a cofactor for various metalloenzymes involved in the biosynthesis of collagen, carnitine, neurotransmitter and peptide hormones (Arrigoni and De Tullio, 2002) as well as a regulation

of transcription factors such as AP-1 (Catani et al., 2001), all of which require its properties as a reducing agent (Carr and Frei, 1999; Halliwell and Gutteridge, 2007). Furthermore, ascorbic acid is stored in high concentrations in leukocytes (Levine et al., 1996), and implicated in a number of immune functions such as the (self-) protection of neutrophils from oxidative burst (Gleeson et al., 2004). Considering that these metabolic and immune functions of vitamin C are all related to exercise, it is conceivable that periods of intensified training requiring musculoskeletal growth and repair, as well as an appropriate maintenance of immune function, may increase the requirements of athletes (Peake, 2003; Margaritis and Rousseau, 2008).

Despite some controversies regarding whether changes in the ascorbic acid plasma concentration reflect its actual utilisation by neutralising RONS or other exercise-associated processes, temporary alterations in the ascorbic acid concentration in plasma and leukocytes have been reported following exercise (reviewed by Peake, 2003). Most, but not all (Nieman et al., 2000), of these studies demonstrated an immediate and transient increase of ascorbic acid plasma and/or lymphocyte concentrations after acute bouts of intense endurance (Gleeson et al., 1987; Thompson et al., 2003) and ultra-endurance exercise (Mastaloudis et al., 2004a; Neubauer et al., 2010), perhaps due to a mobilisation of ascorbic acid from the adrenal glands (Gleeson et al., 1987) leukocytes (Viguie et al., 1993) and/or the intake of vitamin C during exercise (Neubauer et al., 2010). Importantly, provided that the investigators monitored time-course dependent recovery responses for a longer period, in several of these studies plasma ascorbic acid concentrations decreased below baseline/pre-exercise values in the days after intense endurance exercise such as a half-marathon (Gleeson et al., 1987) or a marathon (Liu et al., 1999). Hypothetically, the decrease of vitamin C, along with other antioxidants (Neubauer et al., 2010), in the early days of recovery from strenuous, prolonged exercise, in particular if muscle damage and inflammatory responses were induced, may be associated with an increased utilisation of vitamin C due to sustained oxidative stress in the blood (Hessel et al., 2000; Nikolaidis and Jamurtas, 2009) and/or other tissues including skeletal muscle (Peake et al., 2007).

Evidence concerning the chronic effects of exercise training on ascorbic acid concentrations within the plasma or in leukocytes is less conclusive (Peake, 2003). In a recent study (Bergholm et al., 1999), a 3-month training period in marathon runners was accompanied by decreases in all circulating antioxidants measured (including α-tocopherol and β-carotene), except for ascorbic acid, which increased with training. In contrast, in indoor cyclists participating in the Olympic Games (Ferrandez et al., 1996), the ascorbic acid content of lymphocytes and neutrophils decreased immediately prior to the Olympics, concomitantly with increases in serum levels of stress hormones (adrenocorticotropic hormone and β-endorphin), whereas no indications for a suppression of the immune system were found. Importantly, however, antioxidant supplementation at physiological doses (including 120 mg vitamin C) in addition to the dietary antioxidant intake has been shown to preserve the decrease of plasma concentrations of antioxidant vitamins after 4 weeks of overloaded training in well-trained endurance athletes with initially low antioxidant intakes (Palazzetti et al., 2004). The authors of the latter study (Palazzetti et al., 2004) concluded that the amounts of antioxidant vitamins which helped to preserve the status of antioxidant

vitamins can easily be provided by a diversified and well-balanced diet. In agreement with these findings, plasma markers of oxidative stress including F2-isoprostanes were significantly lower in response to 40 min of high-intensity exercise when trained athletes maintained their habitual diet, which was rich in antioxidants, compared with a 2-week diet restricted in antioxidants (Watson et al., 2005).

When attempting to define recommendations for vitamin C, it is important to bear in mind its bioavailability and the well-established dose dependency of vitamin C pharmacokinetics (Lykkesfeldt and Poulsen, 2010). As demonstrated in a recent study by Levine et al. (1996) in healthy volunteers, plasma ascorbic acid concentrations reach a plateau at a vitamin C intake of 200 mg per day, whereas the ascorbic acid contents of neutrophils, monocytes and lymphocytes are saturated at a daily intake of 100 mg. Furthermore, this pharmacokinetic study (Levine et al., 1996) indicated that the optimal bioavailability of vitamin C is close to maximum at an intake of 200 mg vitamin C as a single dose, corresponding to the (sigmoid) dose–response curve reaching a plateau steady-state plasma concentration at the same daily intake (i.e. 200 mg). On the basis of the observation that nearly all of the absorbed vitamin C is excreted at a dose of 500 mg, the investigators concluded that there is no evidence for recommending vitamin C doses above 400 mg (Levine et al., 1996). Regarding the safety and toxicity of vitamin C, Levine et al. (1996) considered safe doses of vitamin C to be less than 1000 mg daily, while most health agencies currently agree on a tolerable upper intake level (UL) of 1000–2000 mg daily (Hathcock et al., 2005; Frei et al., 2012), based on reports that adverse health effects tend to occur above 1000 mg of vitamin C per day (Frei et al., 2012).

Current average population recommendations for vitamin C are 75 mg per day for adult women and 90 mg per day for adult men in the United States (Carr and Frei, 1999), and 100 mg/d for both sexes in the German-speaking countries (DACH, 2000). These recommendations are primarily based on biochemical data such as pharmacokinetics, and estimates of stored tissue ascorbic acid supposed to provide adequate antioxidant protection (Carr and Frei, 1999; DACH, 2000; Lykkesfeldt and Poulsen, 2010). In addition, evidence from epidemiological studies, suggesting that a dietary intake of 90–100 mg vitamin C per day is associated with a reduced risk of cardiovascular disease and cancer, has been taken into account when defining these recommendations (Carr and Frei, 1999; DACH, 2000; Lykkesfeldt and Poulsen, 2010). However, the definition of an optimal vitamin C status remains a matter of debate (Lykkesfeldt and Poulsen, 2010; Frei et al., 2012). Currently, a widely recognised opinion is that the optimum plasma concentration is about the level of saturation, that is, 70 µmol/L (Lykkesfeldt and Poulsen, 2010), which would require a daily intake of about 200 mg vitamin C (Levine et al., 1996). On the basis of the currently available data from human metabolic and pharmacokinetic studies, as well as observational, epidemiologic and randomised placebo-controlled clinical trials, Frei et al. (2012) have proposed 200 mg per day as 'the optimum dietary intake of vitamin C for the majority of the adult population to maximise the vitamin's potential health benefits with the least risk of inadequacy or adverse health effects'.

Concerning the vitamin C status of athletes, a recent cross-sectional study by Rousseau et al. (2004) involving 118 athletes showed that plasma ascorbic acid

concentrations were related to energy expenditure, vitamin C intake and gender. These data (Rousseau et al., 2004) indicated that some athletic individuals (e.g. game-sport athletes) did not meet the dietary intake which is proposed to be optimal, mainly due to poor dietary choices (Margaritis and Rousseau, 2008). However, this study also demonstrated that dietary vitamin C intakes of ≥200 mg per day were achieved, particularly by athletes with higher energy expenditure (e.g. endurance athletes) (Rousseau et al., 2004). In support of this study (Rousseau et al., 2004), our data of 42 participants of an Ironman triathlon suggested that requirements of nutritive antioxidants to maintain an adequate physiological antioxidant status (in reference to current recommendations (DACH, 2000)) to a large, if not the full extent, can be achieved by a diversified and well-balanced diet (Neubauer et al., 2010). The study participants were precisely instructed to avoid antioxidant supplementation at larger doses (e.g. not more than 60 mg of vitamin C daily in the form of supplements) throughout the study period in addition to their normal dietary antioxidant intake, which was primarily consumed with foods such as fruits and vegetables. This implicates that the study participants' dietary vitamin C intake was sufficient to maintain a plasma ascorbic acid concentration of 66.6 ± 13.0 µmol/L following a 6-month training period with a weekly net endurance exercise time of 10.7 ± 2.6 h (values are mean $\pm$ standard deviation), as assessed in resting conditions 2 days before the Ironman race (Neubauer et al., 2010).

Furthermore, it has been postulated that muscle tissue has a high requirement for, and an increased turnover of, vitamin C (Carr et al., 2013), which is most likely following acute bouts of intense, prolonged exercise and periods of intensified exercise training. Most recently, an investigation of Carr et al. (2013) has provided novel data on the bioavailability of vitamin C in human skeletal muscle. This study (Carr et al., 2013) demonstrated that skeletal muscle is very responsive to vitamin C intake and is strongly related to plasma ascorbic acid concentrations, which indicates that muscle tissue apparently is a relatively labile pool and, perhaps, prone to vitamin C depletion with inadequate intake. Of note, on the basis of an analysis of muscle ascorbic acid status relative to quintiles of plasma ascorbic acid concentrations, the investigators proposed that plasma ascorbic acid concentrations of ≥50 µmol/L should be aimed at to optimise the vitamin C status in the skeletal muscle (Carr et al., 2013), which corresponds to current recommendations for the general population (DACH, 2000).

Taken together, high-dosed supplementation with vitamin C during exercise training cannot be recommended, because there is little evidence of benefits. Moreover, there is growing evidence indicating the potential negative outcomes of antioxidant, and, in particular, vitamin C supplementation on health and performance benefits of exercise training (Khassaf et al., 2003; Gomez-Cabrera et al., 2008). The study of Khassaf et al. indicated that chronic vitamin C supplementation attenuated cellular protective adaptations in response to exercise-induced RONS at a dose of 500 mg per day (Khassaf et al., 2003), although no severe harmful health effects might occur at this dose (Hathcock et al., 2005). However, currently available data suggest that a diet containing antioxidant-rich food is capable of both, maintaining a physiological antioxidant status in competitive athletes during heavy training (Palazzetti et al., 2004; Neubauer et al., 2010), and protecting against exercise-induced oxidative stress

(Watson et al., 2005). The question of specific requirements for vitamin C, as for other antioxidants, with exercise training, to date, has not been addressed sufficiently (Margaritis and Rousseau, 2008). However, based on the available data and emerging indications that a plasma concentration of vitamin C of about 70 µmol/L appears to be optimal for health (Lykkesfeldt and Poulsen, 2010), and the vitamin C status of muscle tissue (Carr et al., 2013), a daily intake of about 200 mg of vitamin C to achieve this status (Frei et al., 2012) might serve as an appropriate 'guidance' for athletes. Practical guidelines on how this intake can be achieved are summarised in Table 3.2.

3.5.2   Vitamin E

Vitamin E refers to a group of lipid-soluble compounds including four tocopherols and four tocotrienols (designated as α-, ß-, γ- and δ-). Although all of these naturally occurring vitamin E isomers, as well as the synthetic *all rac*-α-tocopherol, have relatively similar antioxidant activities, α-tocopherol (in its natural form also called *RRR*-α-tocopherol) is the most biologically active vitamin E form. The differences in the potency of the different isomeric vitamin E forms *in vivo* mainly result from the preferential recognition of α-tocopherol by the hepatic α-tocopherol transfer protein, upon which α-tocopherol is transferred to lipoproteins and maintained in the plasma and/or delivered to other tissues (Brigelius-Flohe et al., 2002; Traber, 2007; Traber and Atkinson, 2007). Vitamin E was discovered more than 90 years ago as a micronutrient necessary for foetal development in rats (Evans and Bishop, 1922). While its essential functions are still not completely understood, at least one of the major functions of vitamin E is due to its role as a lipid-soluble antioxidant (Halliwell and Gutteridge, 2007). As a potent scavenger of peroxyl radicals, vitamin E is the primary inhibitor of the free radical-mediated chain reaction of lipid peroxidation in mammals including humans (Halliwell and Gutteridge, 2007). The importance of this function is to maintain the integrity of long-chain polyunsaturated fatty acids in the cell membranes throughout the body, and thus maintain their structure and biological function (Brigelius-Flohe et al., 2002; Traber, 2007; Traber and Atkinson, 2007). On the basis of the oxidation theory (Stocker and Keaney, 2004) and the response-to-injury theory (Ross, 1993), which implicate the involvement of oxidative modifications of low-density lipoproteins and the onset of inflammation in the initiation and progression of atherosclerotic processes, vitamin E is considered to play a key role in the prevention of atherosclerosis and other diseases associated with oxidative stress (Brigelius-Flohe et al., 2002; Roberts et al., 2007). Recently, vitamin E has also been shown to be involved in the regulation of transcription, the release of arachidonic acid (a long-chain polyunsaturated fatty acid and precursor of eicosanoids, which modulates blood vessels and inflammation) and cellular signalling pathways such as protein kinase C signalling (a mechanism regulating cell proliferation and apoptosis) (Brigelius-Flohe et al., 2002; Traber and Atkinson, 2007). However, there is an ongoing debate whether these functions are indeed due to the additional (i.e. non-antioxidant) functioning of vitamin E as a signalling molecule (Brigelius-Flohe et al., 2002) or rather related to its antioxidant and membrane-protecting capabilities (Traber and Atkinson, 2007).

TABLE 3.2
Practical Guidelines for the Intake of Antioxidants in the Athlete's Basic Nutrition

General Guidelines

- The requirements for nutritive antioxidants, in particular vitamins C and E, during exercise training are supposed to be in a range of ca. ≥100–200% of the current recommendations for the general population, dependent on training loads (e.g. intensity, volumes and frequency of the training). The general picture that emerges from the available data is that the antioxidant intake during exercise training to maintain an appropriate physiological antioxidant status in reference to current recommendations can be achieved by consumption of a balanced and well-diversified diet.

- There is no convincing evidence to recommend antioxidant supplementation during exercise training. High dosed antioxidant supplementation can interfere with physiological training adaptations mediated by reactive oxygen/nitrogen species. Therefore, optimizing the nutrition appears to be the wisest option.

- Athletes should be encouraged to consume a diet rich in antioxidants containing a broad variety of fresh fruits, raw and/or steam-cooked vegetables, fruit and vegetable juices, whole grains and nuts. To meet the suggested intake of vitamin E, an increased intake of nuts, margarine, and certain oils including wheat germ oil, sunflower oil corn oil and rapeseed oil is required.

- During periods with a higher training volume and/or a higher training intensity, there might be an increased requirement for antioxidants, which can be achieved through the increased daily energy expenditure and an increased food intake, provided that the nutrient density is appropriate. In particular, an increased consumption of fruit- and vegetable juices can help in such training situations to match increased requirements for energy, carbohydrates and antioxidants.

- Moderately dosed and timely limited antioxidant supplementation and/or the use specialized sports products (e.g. beverages, carbohydrate-rich bars, gels, etc.) fortified with antioxidants may be warranted in specific situations such as during acute bouts of intense endurance exercise lasting several hours and in the early recovery period (within ca. 24 hours) thereafter, or during energy restriction/weight loss programs. However, high dosed antioxidant supplementation, i.e. >>100% of the recommended dietary allowance (RDA)/dietary reference intake (DRI), in addition to the dietary intake of antioxidants, should be avoided.

- Nutritional guidelines for athletic populations need to be fine-tuned through individualized nutritional advice from appropriate professionals, which includes a comprehensive nutritional assessment and blood analysis.

Example How to Achieve an Appropriate Intake of Vitamin C

A daily intake of about 200 mg of vitamin C to is suggested as an appropriate 'guidance' for athletes.

Exemplarily, 228 mg of vitamin C can be provided by a diet including 200 g of an apple (≈24 mg vitamin C) 45 g of a kiwi-fruit (≈20 mg vitamin C), 50 g of a green capsicum (≈39 mg vitamin C), 100 g of a mixture of steam-cooked vegetables (≈45 mg vitamin C) and 200 mL of fresh orange juice (≈100 mg vitamin C).

Example How to Achieve an Appropriate Intake of Vitamin E

A daily intake of about 12–24 mg of vitamin E (or α-tocopherol-equivalents) for female athletes, and 14–30 mg for male athletes is proposed as proxy 'guidance', dependent on the daily energy expenditure and the intake of polyunsaturated fatty acids, both of which are commonly increased in the athletic population compared with the general population.

Exemplarily, 25 mg of vitamin E (or α-tocopherol-equivalents) could be provided by a diet including 200 g of rye bread with oil seed ingredients (≈7 mg α-tocopherol-equivalents), 200 g of muesli (≈6 mg α-tocopherol-equivalents), 25 g of a nut mixture of walnuts, hazelnuts and almonds (≈4 mg α-tocopherol-equivalents), 10 g of rapeseed oil (≈2 mg α-tocopherol-equivalents) and 10 g of sunflower oil (≈6 mg α-tocopherol-equivalents).

Nutritional analyses were performed using Nut.s nutritional software v1.31.33, based on the German/Austrian Food database BLS-Version 3.01.

Given the antioxidant and protective functions of vitamin E, it is not surprising that many investigators in the field have focused on the potential of vitamin E supplementation to counteract exercise-induced oxidative stress and to protect against exercise-induced muscle damage (Jackson et al., 2004; Peternelj and Coombes, 2011). Although there is no consistent evidence for beneficial effects of supplemented vitamin E in the context with exercise, as outlined in our literature overview above, it is well known that the integrity of muscle cells requires an adequate α-tocopherol status (Coombes et al., 2002; Jackson et al., 2004; Margaritis and Rousseau, 2008). When addressing the question regarding requirements for vitamin E during exercise training, however, it is important to note that the assessment of tissue levels of antioxidants, in particular vitamin E, is associated with limitations (Margaritis and Rousseau, 2008; Powers and Jackson, 2008). Exercise training (especially endurance training) is associated with an increase in high-density lipoproteins (Durstine et al., 2001) as main carriers of plasma α-tocopherol, and the cellular vitamin E uptake is related to lipoprotein metabolism (Mardones and Rigotti, 2004). Therefore, it has been hypothesised that certain mechanisms responsible for the incorporation of α-tocopherol into muscle cells could be enhanced due to training (Margaritis and Rousseau, 2008).

In response to acute bouts of endurance (Aguilo et al., 2005) and ultra-endurance exercise (Mastaloudis et al., 2001; Neubauer et al., 2010), increased plasma concentrations of α-tocopherol were observed. This mobilisation of α-tocopherol might be associated with exercise-induced changes in the lipoprotein metabolism, perhaps rather reflecting a shift from tissue stores to plasma circulation rather than a response to the intake of vitamin E during prolonged exercise (Mastaloudis et al., 2001, 2004a). Moreover, similar to plasma concentrations of vitamin C and other nutritive antioxidants, γ-tocopherol decreased temporarily 1 day after an Ironman triathlon, while the decrease in α-tocopherol was not significant (Neubauer et al., 2010). In contrast, Mastaloudis et al. (2004a) reported a prolonged decrease of α-tocopherol (corrected for plasma lipids) from 3 days after a 50-km ultra-marathon run on until 6 days thereafter, regardless of whether the study participants were supplemented with vitamin C and *RRR*-α-tocopheryl acetate or not. In a previous study (Mastaloudis et al., 2001), Mastaloudis and co-workers observed an increased rate of vitamin E turnover (assessed by deuterium-labelled vitamin E) in response to the same ultra-marathon race compared with resting conditions, which the authors attributed not only to an increased lipoprotein turnover, but also to increased oxidative stress. In an attempt to assess the functional status of vitamin E in trained runners, Cases et al. (2006) showed that plasma α-tocopherol concentrations neither increased after a half-marathon run nor after supplementation with an almond-based beverage moderately enriched with vitamins E and C. However, this study indicated that α-tocopherol concentrations in circulating lymphocytes increased in response to the half-marathon in the supplemented and the non-supplemented group, whereas the post-exercise α-tocopherol content in the neutrophils increased in the supplemented group only (Cases et al., 2006). This supports the idea that vitamin E is re-distributed during acute strenuous exercise. Finally, whereas α-tocopherol plasma concentrations decreased in response to a 3-month marathon training period (Bergholm et al., 1999), available data on vitamin E levels in human skeletal muscle

suggest that chronic exercise does not alter vitamin E concentrations in the muscle (Tiidus et al., 1996).

As discussed above, potential limitations have to be considered when interpreting the vitamin E status of athletes based on plasma concentrations (Margaritis and Rousseau, 2008). However, it is notable that the (resting) plasma α-tocopherol concentrations in 42 Ironman triathletes following a 6-month training period and prior to the Ironman race were $22.66 \pm 13.0\,\mu mol/L$ (Neubauer et al., 2010), that is, in a normal physiological range (Traber, 2007), but moderate in reference to the current recommended values for the general population (i.e. $30\,\mu mol/L$) (DACH, 2000). Furthermore, in agreement with a recent cross-sectional study in well-trained athletes (Rousseau et al., 2004), high inter-individual variations among the Ironman triathletes were observed (Neubauer et al., 2010). These data, in addition to observations that the intake of vitamin E among well-trained athletes is often below the recommendations for the general population (Rousseau et al., 2004; Margaritis and Rousseau, 2008), suggest that the vitamin E intake of athletes requires specific attention.

The current dietary reference intake (DRI) for vitamin E (or α-tocopherol-equivalents) is 15 mg per day for both sexes in the United States (Food and Nutrition Board, 2000), and 14–15 mg per day for adult men and 12 mg per day for adult women in the German-speaking countries (DACH, 2000). Owing to insufficient information on more specific functions of vitamin E, these (estimated) guidelines are largely based on the antioxidant activity of vitamin E and its role to protect mono- and polyunsaturated fatty acids from lipid peroxidation (DACH, 2000; Traber, 2001; Brigelius-Flohe et al., 2002). Recent data from prospective, randomised, placebo-controlled clinical trials focused on the potential of vitamin E supplementation in the prevention or modulation of diseases supposedly associated with oxidative stress are inconsistent (reviewed by Brigelius-Flohé et al., 2002). These data convinced neither the Panel of Dietary Antioxidants of the US Food and Nutrition Board nor the German-speaking authorities to recommend an increase of more than 3 mg (Food and Nutrition Board, 2000), or more than 5 mg of vitamin E (or α-tocopherol-equivalents) per day (DACH, 2000) compared with previous recommendations. Furthermore, some of these studies raised health concerns about the long-term safety of high-dosed vitamin E supplementation, and there is an ongoing debate about the toxicity of vitamin E. The US authorities have set the UL at 1000 mg of vitamin E (or α-tocopherol-equivalents) per day (Hathcock et al., 2005). In contrast, authorities in the German-speaking countries have considered potential adverse effects (an increased incidence of bleeding in combination with medication for antiplatelet activity) at doses between 200 and 800 mg of vitamin E (or α-tocopherol-equivalents) for their safety assessment (DACH, 2000). Crucially, in the context with exercise training, detrimental effects have been reported at daily doses of 180 mg of α-tocopherol-equivalents (400 IU vitamin E) in combination with 500 mg vitamin C (Ristow et al., 2009).

Until more specific evidence is available on the vitamin E requirements of exercising individuals and athletes, they should adhere to both the DRIs for the general population and a plasma α-tocopherol concentration of $\geq 30\,\mu mol/L$, which is considered as beneficial in preventing cancer and cardiovascular disease (DACH, 2000).

Importantly and largely in agreement with the DRI (DACH, 2000), the vitamin E intake required during training periods of increased intensity and/or volume might be somewhat above 15 mg per day. As discussed above, the current DRI for vitamin E is based on its potential to protect unsaturated fatty acids, requiring 0.06, 0.04, 0.6, 0.8, 1.0, 1.2 mg of α-tocopherol-equivalents per gram mono-, double-, triple-unsaturated fatty acids, and so on in addition to a basic requirement of 4 mg α-tocopherol-equivalents (DACH, 2000). Considering that typical endurance training requires 500–900 kcal per hour (Jeukendrup, 2008) and that the total energy expenditure (TEE) of endurance athletes can easily increase to ≥4.000 kcal per day, this also increases the DRI for both unsaturated fatty acids and vitamin E. For example, for an athlete with a daily TEE of 4000 kcal, the DRI for monounsaturated fatty acids (i.e. 13% of the TEE) increases to ca. 57.8 g of ω-9-fatty acids, whereas the DRI for the essential polyunsaturated fatty acids (i.e. 2.5% and 0.5% of the TEE for ω-6- and ω-3-fatty acids, respectively) increases to ca. 25.8 g of ω-6- and 5.2 g of ω-3-fatty acids. Provided that athletes achieve these DRIs for unsaturated fatty acids, the DRI for vitamin E concomitantly increases to 24 mg α-tocopherol-equivalents per day. On the basis of these considerations, it is reasonable to suggest a range between 12 and 24 mg of vitamin E (or α-tocopherol-equivalents) per day for female athletes and between 14 and 30 mg for male athletes as proxy 'guidance'.

In view of the discussed detrimental effects of high-dosed vitamin E supplementation, athletes should be encouraged to abstain from supra-physiological doses of vitamin E, but be encouraged to increase their dietary vitamin E intake. The latter requires a substantial increase in the consumption of foods that are rich in fats, such as nuts, margarine and certain oils. This is also supported by the food frequency questionnaire data gained from the study in the Ironman participants, indicating that the plasma α-tocopherol concentration was highest (i.e. 29.6 ± 6.6 µmol/L) in the subject group that reported a daily intake of nuts (Neubauer et al., 2010). Practical guidelines for the vitamin E intake in the athlete's basic nutrition are summarised in Table 3.2.

3.6 GENERAL CONCLUSIONS ON THE INTAKE OF ANTIOXIDANTS IN THE ATHLETE'S BASIC NUTRITION

The general picture that emerges from the available data on antioxidant requirements of athletes is that the antioxidant intake during exercise training to maintain an appropriate physiological antioxidant status in reference to current recommendations can be achieved by consumption of a balanced and well-diversified diet. The potential and importance of dietary sources of antioxidants to achieve these goals has been demonstrated even during very committed endurance training programmes (e.g. training for long-distance triathlon races) (Neubauer et al., 2010). Moderate and timely limited antioxidant supplementation may be warranted in specific situations, for example, during periods of intensified training, during acute bouts of intense endurance exercise lasting several hours and in the early recovery period thereafter or during energy restriction/weight loss programmes. Nevertheless, the ultimate goal in the athlete's basic nutrition is certainly to optimise the nutrition. Crucially, the optimal bioavailability and combined action of multiple phytochemical and

antioxidant compounds derived from fruits, vegetables, whole grains and nuts cannot be replaced by supplementation (DACH, 2000). Furthermore, while phytochemicals such as polyphenols are well recognised for their antioxidant properties, their beneficial physiological effects may be promoted by a multitude of mechanisms (Halliwell, 2009; Hawley et al., 2011).

The current literature is not sufficient to determine definitive recommendations concerning antioxidant requirements for athletes and exercising individuals. However, based on available data, it is feasible to suggest that these requirements, particularly for vitamins C and E, might be in a range of ≥100–200% of the current recommendations for the general population. Whereas an intake of ca. 12–30 mg of vitamin E requires specific attention (see Table 3.2), it is likely that by meeting the 'guidance' of 200 mg vitamin C per day, athletes may also achieve an appropriate intake of other antioxidative nutrients and phytochemicals, since the vitamin C plasma status serves as a proxy or surrogate marker for vegetable and fruit intake (Lykkesfeldt and Poulsen, 2010). Additional research is warranted to define antioxidant requirements during exercise training, which should also take into account nutrigenomic issues (Peternelj and Coombes, 2011). Finally, it is important to note that nutritional guidelines, in particular, for athletes need to be fine-tuned on an individual basis.

DISCLOSURES

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4 Antioxidants in Sport Nutrition
All the Same Effectiveness?

Karl-Heinz Wagner

CONTENTS

4.1 BASIC MECHANISMS OF OXIDATIVE DAMAGE

While oxygen is vital for life of an aerobic organism, the by-products of its metabolism can be harmful to cells. The very small not-to-water-reduced part of oxygen leads to the production of reactive oxygen intermediates, also known as ROS. This is happening ubiquitary but in particular in the working muscle during or after exercise (Alessio et al. 2000; Caillaud et al. 1999; Clarkson and Thompson 2000). ROS includes superoxide ($O_2^{\bullet-}$), nitric oxide (NO$^{\bullet}$) and hydroxyl radicals (HO$^{\bullet}$) and also non-radicals such as singlet oxygen ($^1O_2$) or hydrogen peroxide (H_2O_2). Depending on the type of exercise, a number of potential mechanisms for the generation of ROS within the muscle have been proposed, such as (a) increased formation of $O_2^{\bullet-}$ in the mitochondrial respiratory chain, (b) xanthine oxidase (XO) catalysed degradation of AMP (adenosine monophosphate) during ischaemic muscular work leading to increased production of $O_2^{\bullet-}$, (c) increased ROS formation in the oxidative-burst reaction due to activation of polymorphoneutrophils (PMNs) after exercise-induced muscle damage, (d) loss of calcium homeostasis in stressed muscles, (e) enhanced cytokine production and activation of nuclear factor kappa B (NF-κB), catecholamine

67

autooxidation and many more (König et al. 2007; Niess et al. 1999; Vina et al. 2000). Owing to the unpaired electron in its outer orbit, ROS tend to extract electrons from other molecules to reach a chemically more stable state. However, the generation of ROS is per se not harmful and necessary for the proper functioning of metabolic processes, muscular contraction and immune defence. Muscle antioxidant defence systems are upregulated in response to exercise. NF-κB and mitogen-activated protein kinase are the two major oxidative-stress-sensitive signal transduction pathways that have been shown to activate the gene expression of a number of enzymes and proteins that play important roles in maintenance of intracellular oxidant–antioxidant homeostasis (Ji 2008).

It is only when the body's natural antioxidant defence system is insufficient to detoxify formed ROS that oxidative stress with damage or destruction of cellular macromolecules such as lipids, proteins, nucleic acids and components of the extracellular matrix may occur. Oxidative stress has been associated with decreased physical performance, muscular fatigue, muscle damage and overtraining (König et al. 2001; Margonis et al. 2007; Tiidus 1998). Therefore, it is sometimes suggested that reducing oxidative stress (e.g. by antioxidant supplementation) would improve exercise tolerance and performance. However, to minimise oxidative stress, the organism contains a powerful antioxidant defence system that depends on nutritionally derived antioxidant vitamins such as vitamin E and C, β-carotene, flavonoids, polyphenols as well as endogenous antioxidant (enzyme) compounds, such as glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD), as already described in Chapters 2 and 3 (Clarkson and Thompson 2000; Dekkers et al. 1996). Therefore, oxidative stress during or following exercise can occur only if the exercise-induced generation of ROS is higher than the detoxifying potential of the antioxidant defence systems. Many studies have investigated the effect of physical exercise with respect to the onset and magnitude of oxidative stress and the protective role of antioxidants, however, with various outcomes (König et al. 2001; Peternelj and Coombes 2011; Powers et al. 2004). There is strong support for the assumption that the manifold designs and methods employed to induce and measure oxidative stress are a major cause for conflicting results: Factors such as gender, age, type of exercise (concentric vs. eccentric, maximal vs. submaximal etc.), level and years of training and particularly the exercise-induced local and systemic stress response could account for both differences in ROS generation and the development of antioxidant defence mechanisms. Nevertheless, antioxidants, either endogenously produced or dietary substances that can act as antioxidants, play a major role in the whole network. In the following section antioxidants which are related to physical activity will be described regarding their mode of action and it will be distinguished between various homologues/chemical forms and their antioxidative effectiveness.

4.2 SHORT LOOK ON THE ANTIOXIDANT DEFENCE MECHANISMS

The protective mechanisms against oxidative stress can be divided into two major categories: endogenously produced enzymatic antioxidants that include SOD,

glutathione peroxidase (GPX), CAT, glutaredoxin (GRX) and thioredoxin (TRX). Non-enzymatic antioxidants include nutritionally derived vitamins and provitamins (vitamin E, vitamin C and β-carotene), flavonoids and polyphenols, proteins such as thiols (mainly GSH) and various other low-molecular-weight compounds as ubiquinone, uric acid (UA) and many more (Dekkers et al. 1996; König et al. 2001; Peternelj and Coombes 2011). These substances can either prevent ROS formation or scavenge radical species and convert them into a less active molecule. Furthermore, they avoid the transformation of less active ROS (e.g. $O_2^{\bullet-}$) into more potent forms (e.g. $HO^{\bullet}$), enhance the resistance of sensitive biological targets to ROS attack and assist in the repair of radical-induced damage. The main radical-scavenging functions of the various antioxidants are outlined in Table 4.1. Although most antioxidants are located in specific cellular sites or compartments, they act synergistically and some of them cooperate in the so-called

TABLE 4.1

Enzymatic and Non-enzymatic Antioxidants, Location and Main Function

Enzymatic Antioxidants	Location	Function
SOD	Mitochondria, cytosol	Dismutates superoxide radicals
GPX	Mitochondria, cytosol and cell membrane	Reduces hydrogen peroxide and organic hydroperoxides
CAT	Peroxisomes	Reduces hydrogen peroxide
GRX	Cytosol	Protects and repairs protein and non-protein thiols
TRX	Cytosol	Catalyses the reduction of protein S–S bridges Removes hydrogen peroxide Scavenges free radicals

Non-enzymatic Antioxidants	Location	Function
Vitamin C	Aqueous phase of cells	Scavenges free radicals Recycles vitamin E
Vitamin E, mainly α- and γ-tocopherol	Cell membranes[a]	Breaks lipid peroxidation chain reactions, reduces several ROS to less reactive forms
Carotenoids	Cell membranes[a]	Scavenges free radicals Protects against lipid peroxidation
Glutathion	Ubiquitary non-protein thiol	Scavenges free radicals Removes hydrogen and organic peroxides in a GPX-catalysed reaction Recycles various antioxidants (vitamin C, E)
Flavonoids/ polyphenols	Cell membranes[a]	Scavenges free radicals Metal chelator
Ubiquinones	Cell membranes[a]	Scavenges oxygen radicals and singlet oxygen Recycles vitamin E
UA	Ubiquitary	Scavenges HO radicals

[a] Lipid-rich compartment.

antioxidant chain reaction. This means that, for example, the −SH pool from reduced GSH regenerates vitamins C and E and vitamin C recycles vitamin E. In contrast to other vertebrates, the human organism is not able to synthesise antioxidant vitamins; therefore, non-enzymatic antioxidants such as vitamin E, vitamin C, β-carotene, polyphenols and flavonoids have to be provided by the diet. This implies that the plasma and tissue levels of these non-enzymatic antioxidants are dependent on the quality of foods. In contrast, the enzymatic antioxidants are synthesised within the human organism and several lines of evidence suggest that their production can be upregulated in response to chronic exposure to oxidants (Ji 2008). Although the response may depend on exercise intensity or training duration (Neubauer et al. 2010; Tiidus et al. 1996), most studies have reported an increase in antioxidant enzyme activity following chronic physical exercise (Elosua et al. 2003; Hellsten et al. 1996; Rowiński et al. 2013). This may represent an important mechanism to explain findings from some investigations showing less oxidative stress in trained individuals.

4.3   ANTIOXIDANT SUPPLEMENTATION AND EXERCISE-INDUCED OXIDATIVE STRESS: A CONTROVERSY

Over the past few decades, there have been plenty of exercise studies with measures of oxidative stress as the main outcome when using antioxidant supplementations (see Chapters 7 through 12).

The most common antioxidants used were vitamin E and vitamin C and various antioxidant combinations, also including the latter vitamins. More recently, polyphenols or supplements containing them have been investigated. Not very often, carotenoids, selenium α-lipoic acid or *N*-acetylcysteine have been used. To draw a general conclusion, it can be said that the outcome was inconsistent, from lowering a oxidative stress biomarker to also increasing then (see reviews König et al. 2001; Peternelj and Coombes 2011).

Furthermore, on the population level, many studies have revealed that the 'classical' antioxidants C and E are not only effective in reducing the risk of chronic diseases, but also increasing them slightly (e.g. Abner et al. 2011; Bjelakovic et al. 2007; Gerss and Köpcke 2009). A deeper insight into the network of vitamins C and E can be found particularly in Chapter 3.

4.4   SPECIFIC LOOK ON THE MAIN ANTIOXIDANTS AND THEIR ACTION

Since it is a common practice for athletes to use antioxidants, there is a wide range of vitamins, minerals and different extracts marketed as supplements. However, very often, not in the most active form, overdosed when compared to recommended daily allowances (RDIs), not highly bioavailable and especially as an extract, not even well characterised. Many of the orally taken supplements also have an impact on the antioxidative enzymes or GSH.

4.4.1 GLUTATHIONE

GSH is the most abundant non-protein thiol source in the muscle. Its concentration in the cells is usually in a millimolar range but is having a wide range across organs depending on their radical production. It serves various roles in the cellular defence system by directly scavenging radicals, removing hydrogen and organic peroxides, recycling a variety of other antioxidants such as vitamin E and reducing semi-dehydroascorbate radicals. The various ways of acting and the interaction with exogenic substances show the dependency of the GSH activity on the consumption of substances acting as antioxidants (see review from Powers et al. 2004).

The same is true for SOD, CAT or glutathionperoxidase, which belongs to the consumption of their active micronutrients such as iron, manganese, zinc or selenium.

4.4.2 BILIRUBIN AND UA

Bilirubin and UA represent important endogenous antioxidants mainly found in the plasma. UA serves as a free radical scavenger, can trap peroxyl radicals in aqueous phases and therefore contribute to the plasma antioxidant defence (Wayner et al. 1987). During exercise, energy-rich purine phosphates are used and catabolised, resulting in accumulation of hypoxanthine, xanthine and UA in tissues. The conversion of hypoxanthine into xanthine and UA is associated with the formation of toxic oxygen-free radicals (Sjödin et al. 1990).

Plasma concentrations of the potent hydrophilic antioxidant UA are known to increase during intense exercise (e.g. Neubauer et al. 2008), produced from increased purine metabolism (Liu et al. 1999) and probably also because of impaired renal clearance (Mastaloudis et al. 2004).

Bilirubin has been shown to efficiently scavenge peroxyl radicals and act as a metal-binding species, thus functioning as a selective antioxidant. Similar to UA, bilirubin has also been shown to increase after exercise (Neubauer et al. 2010). Since bilirubin is released into the plasma fluid by destruction of red blood cells, haemolysis which arises during physical activity (e.g. during marathon distance running) can be one explanation.

However, there is now novel information published on hyperbilirubinaemia, showing significant antioxidative effects, protection from non-communicable diseases (NCDs) such as cardiovascular disease (CVD) (Novotny and Vitek 2003) and cancer (Temme et al. 2001; Zucker et al. 2004) as well as a severe impact on lipid metabolism (Bulmer et al. 2013; Wallner et al. 2013). Released bilirubin in the plasma is immediately bound to albumin in blood and transported to the liver. In the liver, unconjugated bilirubin is conjugated to glucuronic acid, consequently gets water soluble and is finally called conjugated bilirubin. A mutation in the gene promoter region of bilirubinuridine–diphosphate–glucuronyl transferase (40–60% impaired glucuronidation) can cause hyperbilirubinaemia, arising in approximately 5–10% of the general population. So far, no link has been drawn to physical activity and adaptation processes in hyperbilirubinaemic subjects, but it is highly expected that a high bilirubin plasma concentration has significant effects on metabolism during physical activity.

4.4.3   Vitamin E: A Group of Tocopherols

Very often, it is ignored that the term vitamin E represents a family of eight natural, structurally related compounds. These compounds contain a chromanol ring with a phytyl side chain, which is saturated for tocopherols and unsaturated for tocotrienols. The α-, β-, γ- and δ-tocopherols and the α-, β-, γ- and δ-tocotrienols differ in the number and the position of methyl groups substituted on the ring.

The forms of vitamin E in most supplements are the synthetic all-rac-α-tocopheryl acetate or all-rac-α-tocopheryl succinate; however, both show not the highest vitamin E activity.

Of all compounds with vitamin E activity, α- and γ-tocopherols are the principal vitamins found in human and animal diets and comprise most of the vitamin E content of tissues.

Interestingly, the intake of γ-tocopherol has been estimated to exceed that of α-tocopherol by a factor of 2–4 in North America (see review Wagner et al. 2004). This is due to the fact that soya bean oil is the predominant vegetable oil in the American diet (76.4%) followed by corn oil and canola oil (both 7%). In Europe, the consumption of the α-form exceeds γ-tocopherol with a ratio of approximately 2:1. However, most of the supplementation studies in exercise science and also on the population level to prevent chronic diseases have been performed with α-tocopherol (Wagner et al. 2004).

The ability to donate the phenolic hydrogen is thus very important for the antioxidant activity of the tocopherols as they scavenge the peroxyl radicals. The lack of the C-5 methyl group decreases the electron density in the phenolic ring, making γ-tocopherol a less potent hydrogen donor than α-tocopherol (Kamal-Eldin and Appelqvist 1996). The bond dissociation energies for the phenolic hydrogens are 75.8 and 79.6 Kcal/mole in the case of α- and γ-tocopherols, respectively (Wright et al. 1997). This makes α-tocopherol a more efficient hydrogen donor and radical scavenger than γ-tocopherol. However, the higher hydrogen-donation ability is a double-edged sword as it makes α-tocopherol participate more readily in side reactions, leading to partial loss of its antioxidant activity. This also contributes to the negative findings of high dose supplementation with various α-tocopherol forms.

4.4.4   Vitamin C

In contrast to tocopherols is vitamin C (ascorbic acid) hydrophilic which acts better in the aqueous environment. It is widely distributed but found in high amounts in leukocytes, adrenal and pituitary glands. Besides its scavenging activity, it is well known to recycle the tocopherol radical, thereby being reduced to the dehydroascorbic acid which can then be regenerated, for example, by GSH.

Some decades ago, the intake of vitamin C was too low; however, nowadays, owing to the availability of fruits and vegetables, the intake has increased and vitamin C is enriched in almost every food, particularly meat and sausages, as antioxidant. Therefore, the vitamin C intake via dietary sources exceeds the recommendations nowadays.

Further, pharmacokinetic data indicate a plasma steady state after a vitamin C intake of 200 mg, whereas the ascorbic acid contents of neutrophils, monocytes and lymphocytes are saturated at a daily intake of 100 mg (Levine et al. 1996). Similar to α-tocopherol, it is also well known that vitamin C turns its antioxidative activity towards pro-oxidative activity after high dose supplementation, particularly in the presence of transition metals such as Fe^{3+}.

Taking the latter into consideration, the total intake of vitamin C should not exceed the recommendations manifold; more details and recommendations are given in this book (Neubauer and Yfanti). Furthermore, recent studies have shown that antioxidative supplements (mainly vitamins C and E) hinder the beneficial cell adaptation to exercise (Gomez-Cabrera et al. 2008, 2012; Ristow et al. 2009).

4.4.5   β-Carotene

Supplementation with carotenoids, particularly β-carotene, should be done with care and with hands on the dose. Particularly for β-carotene, no acceptable daily intake (ADI) is set, since it contributes to an increased risk of cancer (particularly lung cancer) in heavy smokers at an intake of 20 mg/day or higher (Albanes et al. 1996; Omenn et al. 1996; The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994). Carotenoids act along two different main pathways—physical and chemical radical quenching. Physical quenching implies the deactivation of singlet oxygen by energy transfer to the excited oxygen species leading to the carotenoid, yielding a triplet-exciting carotenoid. The energy of this carotenoid is dissipated to recover the ground state; the carotenoid itself remains interactive in the process and is able to undergo more cycles of deactivation. Chemical quenching contributes <0.05% to the total $^1O_2$-quenching by carotenoids, but is responsible for the eventual destruction of the molecule. Besides this quenching ability, carotenoids are able to scavenge peroxyl radicals by chemical interactions. However, mainly, β-carotene acts only at low oxygen partial pressure as antioxidant; hyperoxide conditions, often present during and after physical activity, results in a shift to a pro-oxidant (Wagner and Elmadfa 2003).

4.4.6   Further Antioxidants in Use

From the other antioxidants used, much less data are available, particularly with regard to physical activity.

Flavonoids are a family of secondary active plant constituents which have been associated with antioxidative potential, although very often only in *in vitro* studies. The most prominent ones are flavonones, isoflavonones, flavanones, anthocyanins and catechins. Their biological activities are very broad such as anti-inflammatory, anti-mutagen, anti-tumoral or anti-ischaemic. Most of the observed activities are based on their anti-oxidative potential as a radical scavenger. As polyphenols, they also act as a regenerator of vitamin E radicals or β-carotene. Since they are broadly found in plant products such as black or green tea, grapes and red wine, supplementation must be considered with care. Many of the supplements carrying 'plant extracts' are not well defined and concentrations should be carefully evaluated, if

given at all. Furthermore, they could contain contaminants, which are doing more harm than good and were also responsible for ending the careers of athletes. One other crucial point is their bioavailability which is regularly very low (see reviews Powers et al. 2004; Peternelj and Coombes 2011).

However, recent studies could show the beneficial effects of polyphenols or extracts (grape, beetroot, *Rhodiola rosa* or *Eckonia cava* algae) with regard to oxidative stress in physically active persons, but no effects such as ergogenic acids (e.g. Lafay et al. 2009; Breese et al. 2013; Wylie et al. 2013; Oh et al. 2010). Further, they did not improve muscle force output.

Solely, the antioxidant mechanisms cannot be responsible for the observed effects of polyphenols; therefore, other links are proposed such as the influence on cell-signalling cascades and the interaction with key proteins in these cascades.

Ubiquinones are lipid-soluble quinone derivatives containing an isoprene or farnesyl tail. In humans, predominantly, ubiquinone-10, also called coenzyme-Q, is bioactive. It is found in the diet (soy bean oil, nuts, fish and meat) but also, very often, supplementation is taking place. The antioxidant effects are attributed to their phenolic ring structure, which acts as a radical scavenger, but it is also used to regenerate other primary antioxidants such as tocopherols.

Coenzyme-Q is very popular among athletes, but shows no significant benefit on exercise performance, regardless of age or training status. However, positive effects by Q10 were also shown, such as improved VO_{2max}, faster recovery rate and fatigue recovery (see review from Peternelj and Coombes 2011).

4.5   SUMMARY

The heterogenic role of ROS in living organisms and the beneficial but also deleterious effects of antioxidant supplementation show the complexity of the network and the dependency of various conditions. Since nutritional status, in general, has been improved in the last few decades in many countries worldwide and, at the same time, negative effects of long-term supplementation studies published, the need for high dose supplementation is questionable, particularly for hobby athletes who just fulfil the recommendations for an active person. However, if taking supplements, the chemical form and the concentration must be carefully observed, since the mode of action of many substances can change depending on their concentration, their microenvironment and the network they are acting in. On the basis of the data published, a balanced diet including a large variety of fruits, vegetables, nuts and grain remains the best nutritional approach to maintain optimal antioxidant status.

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5 Well-Known Antioxidants and Newcomers in Sport Nutrition

Coenzyme Q10, Quercetin, Resveratrol, Pterostilbene, Pycnogenol and Astaxanthin

Muaz Belviranlı and Nilsel Okudan

CONTENTS

5.1   INTRODUCTION

Physical exercise induces an increase in production of free radicals and other reactive oxygen species (ROS) (Davies et al. 1982, Borzone et al. 1994, Halliwell and Gutteridge 1999). Current evidence indicates that ROS are the primary reason of exercise-induced disturbances in muscle redox balance. Severe disturbances in redox balance have been shown to promote oxidative injury and muscle fatigue (Reid et al. 1992, O'Neill et al. 1996) and thus impair the exercise performance. There are several potential sources of ROS that can be activated by exercise such as mitochondrial electron transfer chain, in the purine degradation pathway the reaction catalysed by xanthine oxidase, macrophage infiltration and metabolic degradation of catecholamines (Urso and Clarkson 2003, Finaud et al. 2006). The high production of ROS

during exercise is also responsible for muscular damage (Aguiló et al. 2007). On the basis of the above-mentioned information, sportsmen have to improve their antioxidant defence systems to overcome the exercise-induced oxidative damage. Over the past few decades, many attempts have been made to improve antioxidant potential and therefore increase physical performance by improving nutrition, training programmes and other related factors.

An antioxidant is generally defined as any substance that significantly delays or prevents oxidative damage of a target molecule (Halliwell 2007). The antioxidant defence system of the body consists of antioxidant enzymes (superoxide dismutases, catalase and glutathione peroxidase, etc.) and non-enzymatic antioxidants (vitamins A, C and E, coenzyme Q10 (CoQ10) and glutathione, etc.) (Deaton and Marlin 2003). There is a cooperative interaction between endogenous antioxidants and dietary antioxidants; therefore, antioxidant supplementation may improve the muscle fibre's ability to scavenge ROS and protect the exercising muscle against exercise-induced oxidative damage and fatigue. However, antioxidant nutrient deficiency could induce an increased susceptibility to exercise-induced damage and thus leads to impaired exercise performance (Stear et al. 2009). Recently, the problem of whether or not athletes should use antioxidant supplements is an important and highly debated topic. To prevent these hypothetically negative or side effects of physical exercise, supplementation with different types of antioxidants has been used in a great number of studies (Snider et al. 1992, Rokitzki et al. 1994, Reid et al. 1994, Margaritis et al. 1997, Aguiló et al. 2007, Bloomer et al. 2012). In the context of this chapter, information in brief about the well-known and recently used antioxidants such as CoQ10, quercetin, resveratrol, pterostilbene, pycnogenol and astaxanthine is given. The effects of these antioxidants on exercise performance and exercise-induced oxidative stress are also explained.

5.2   COENZYME Q10

CoQ10 (2,3 dimethoxy-5 methyl-6-decaprenyl benzoquinone) is a fat-soluble, vitamin like quinone commonly known as ubiquinone, CoQ and vitamin Q10 (Bonakdar and Guarneri 2005). CoQ10 is an essential cofactor in mitochondrial oxidative phosphorylation, and is necessary for ATP production. It acts as a mobile electron carrier, transferring electrons from complex I to complex III or from complex II to complex III (Molyneux et al. 2008). CoQ10 also appears to increase ATP levels by preventing the loss of the adenine nucleotide pool from cardiac cells (Bonakdar and Guarneri 2005). Additionally, CoQ10 has evidenced activity in preventing lipid peroxidation as an antioxidant, and as an indirect stabiliser of calcium channels to decrease calcium overload (Sugiyama et al. 1980). The function of CoQ10 as an electron shuttle in the electron transport chain has been suggested as a rate limiting step in exercise where energy production is of great importance.

It was generally shown that oral CoQ10 supplementation at different doses led to a marked elevation of CoQ10 levels in various tissues such as skeletal muscle, liver, heart and kidney (Kwong et al. 2002, Kon et al. 2007) and in human plasma (Zuliani et al. 1989, Kaikkonen et al. 1998, Bonetti et al. 2000, Zhou et al. 2005, Kon et al. 2008, Mizuno et al. 2008, Bloomer et al. 2012). Supplementation with CoQ10 could therefore, hypothetically, 'normalise' or even enhance physical performance

by increasing the CoQ10 content in the mitochondria and would potentially enhance the oxidative phosphorylation process (Zhou et al. 2005). Parallel to this, it has been speculated that increased ROS production during physical exercise could decrease the CoQ10 level in muscle tissue and negatively affect physical performance, at least in subjects undertaking strenuous physical training (Karlsson et al. 1996). A positive relationship between exercise capacity and the concentration of CoQ10 in the vastus lateralis muscle was reported in physically active males (Karlsson et al. 1996). Different investigators have consequently tested several related hypotheses regarding CoQ10 and physical performance and oxidative stress and these studies are summarised in Table 5.1.

5.2.1 Effects on Performance

The studies which investigated the potential ergogenic value of CoQ10 have reported mixed results. These studies are categorised according to the type of exercise such as aerobic, anaerobic and muscle injury induced exercises. Although CoQ10 is being suggested to improve exercise capacity, this effect is not supported by empirical data. The previous studies investigating the effects of CoQ10 supplementation on physical performance in humans have found negative effects (Malm et al. 1997), no effect (Braun et al. 1991, Snider et al. 1992, Porter et al. 1995, Weston et al. 1997, Kaikkonen et al. 1998, Nielsen et al. 1999, Svensson et al. 1999, Zhou et al. 2005), positive effects (Cooke et al. 2008, Mizuno et al. 2008, Gökbel et al. 2010), decreased exercise-induced muscular injury in athletes (Kon et al. 2008, Tauler et al. 2008) and positive effects on aerobic and anaerobic threshold and maximal oxygen consumption (VO_{2max}) in cross-country skiers (Ylikoski et al. 1997).

Contradictory results have been found regarding the effects of CoQ10 supplementation on aerobic performance (Laaksonen et al. 1995, Ylikoski et al. 1997, Zhou et al. 2005, Cooke et al. 2008, Bloomer et al. 2012). In placebo-controlled studies, it was shown that CoQ10 supplementation alone (Braun et al. 1991, Weston et al. 1997) or combined with vitamins C and E (Snider et al. 1992, Nielsen et al. 1999) had no significant effect on respiratory capacity and performed work or muscle metabolism. In untrained subjects, Porter et al. (1995) did not find any changes in VO_{2max}, lactate threshold, heart rate and maximal workload during a cycle ergometer test after supplementation with CoQ10 for 2 months. Bonetti et al. (2000) observed that CoQ10 supplementation (100 mg·day^{-1} for 8 weeks) did not affect aerobic power. Similarly, Zhou et al. (2005) demonstrated that CoQ10 supplementation (150 mg·day^{-1} for 4 weeks) did not affect maximal oxygen consumption and ventilatory threshold in healthy sedentary males. In a study by Bloomer et al. (2012), no change in aerobic exercise performance was observed following 4 weeks of CoQ10 supplementation (300 mg·day^{-1}) in physically active subjects. Laaksonen et al. (1995) found that CoQ10 supplementation (120 mg·day^{-1} for 6 weeks) did not affect aerobic performance in trained subjects. In a recent study, Ostman et al. (2012) observed no clear effect on physical capacity, including VO_{2max}, heart rate and lactate threshold after 8 weeks of CoQ10 administration in moderately trained subjects. In contrast to these results, Ylikoski et al. (1997) demonstrated that CoQ10 supplementation (90 mg·day^{-1} for 12 weeks) caused an increase in VO_{2max} in cross-country skiers.

TABLE 5.1
Summary of Published Articles about the Effects of CoQ10 on Exercise Performance and Exercise-Induced Oxidative Stress in Humans

Reference	Subjects	Age (Years)	CoQ10 dosage	Duration	Placebo	Exercise Testing	CoQ10 Levels	Measured Parameters and Effects of CoQ10
Díaz-Castro et al. (2012)	Amateur male athletes	40.5 ± 2.88	30 mg 2 days before, 90 mg previous and 30 mg during the test day	3 days	Yes	50 km running	–	CAT$\uparrow$, TAS$\uparrow$, GPx $\leftrightarrow$, hydroperoxide$\downarrow$, isoprostane$\downarrow$, 8-OH-dG$\downarrow$, IL-6$\leftrightarrow$,TNF-$\alpha\downarrow$
Bloomer et al. (2012)	10 (M) 5 (F) physically active subjects	42.7 ± 10.4	300 mg·day^{-1}	30 days	Yes	GXT and CST	Total CoQ10$\uparrow$ Reduced CoQ10$\uparrow$	GXT, GST, MDA, hydrogen peroxide, lactate, perceived vigour $\leftrightarrow$
Ostman et al. (2012)	23 (M) moderately trained subjects	19–44	90 mg·day^{-1}	8 weeks	Yes	Various exercise capacity tests	–	Exercise capacity, MDA, UA, CK, hypoxanthine $\leftrightarrow$
Gül et al. (2011)	15 (M) sedentary subjects	19.9 ± 0.9	100 mg·day^{-1}	8 weeks	Yes	Repeated WTs	–	MDA$\downarrow$, ADA$\leftrightarrow$, NO $\leftrightarrow$, XO $\leftrightarrow$, SOD $\leftrightarrow$, GPx $\leftrightarrow$, UA $\uparrow$
Gökbel et al. (2010)	15 (M) sedentary subjects	19.9 ± 0.9	100 mg·day^{-1}	8 weeks	Yes	Repeated WTs	–	PP $\leftrightarrow$, MP $\uparrow$, FI $\leftrightarrow$
Cooke et al. (2008)	22 trained and 19 untrained (M and F) subjects	26.1 ± 7.6	200 mg·day^{-1}	14 days	Yes	Isokinetic test WT GXT	CoQ10$\uparrow$	Muscle endurance $\leftrightarrow$, anaerobic capacity $\leftrightarrow$, VO$_{2max}$ $\leftrightarrow$, MDA $\uparrow$, SOD$\downarrow$

Study	Subjects	Age	Dose	Duration		Exercise test	Total CoQ10	Outcomes
Mizuno et al. (2008)	8 (M) 9 (F) physically active subjects	37.5 ± 9.9	100 mg·day⁻¹ 300 mg·day⁻¹	8days	Yes	Fatigue-inducing exercise test on a bicycle ergometer	Total CoQ10 ↑ Total CoQ10 ↑	Physical performance ↑ (with 300 mg/day CoQ10)
Kon et al (2008)	18 (M) elite Kendo athletes	20.1 ± 1.0	300 mg·day⁻¹	20 days	Yes	Muscle injury induced exercise	Total CoQ10 ↑	$CK \downarrow$, $Mb \downarrow$, $LPO \leftrightarrow$
Zhou et al. (2005)	6 (M) physically active subjects	29.7 ± 7.2	150 mg·day⁻¹	2 weeks	Yes	3 submaximal exercise tests	Total CoQ10 ↑	$VO_{2max} \leftrightarrow$, $HR \leftrightarrow$, $RER \leftrightarrow$, $RPE \leftrightarrow$, ventilatory threshold $\leftrightarrow$
Bonetti et al. (2000)	28 (M) cyclist	39.7 ± 6.6	100 mg·day⁻¹	8 weeks	Yes	GXT	Total CoQ10 ↑	$VO_{2peak} \leftrightarrow$, anaerobic threshold $\leftrightarrow$, lactate $\leftrightarrow$, hypoxanthine $\leftrightarrow$, xanthine $\leftrightarrow$, inosine $\leftrightarrow$
Kaikkonen et al. (1998)	37 (M) moderately trained subjects	40.0 ± 7.0	90 mg·day⁻¹	3 weeks	Yes	42 km marathon running	Total CoQ10 ↑	$GSH \leftrightarrow$, $UA \leftrightarrow$, $LDLox \leftrightarrow$, $TRAP \leftrightarrow$
Malm et al. (1997)	18 (M)	25.3 ± 3.4	120 mg·day⁻¹	22 days	Yes	Anaerobic cycling test, VO_{2max}, lactate	–	–
Ylikoski et al. (1997)	25 (M) cross-country skiers	–	90 mg·day⁻¹	6 weeks	Yes	Aerobic threshold	Total CoQ10 ↑	Aerobic threshold ↑, VO_{2max} ↑,
Laaksonen et al. (1995)	11 young, 8 older trained (M)	22–38 60–74	–	–	Yes	Aerobic capacity	Total CoQ10 ↑	$MDA \leftrightarrow$, $VO_{2max} \leftrightarrow$,
Zuliani et al. (1989)	12 (M) sedentary subjects	25.7	100 mg·day⁻¹	1 month	No	VO_{2max}, CK, LA	Total CoQ10 ↑	$CK \leftrightarrow$, $LA \leftrightarrow$

Note: M: male, F: female, GXT: graded exercise test, CST: cycle sprint test, WT: Wingate test, CK: creatine kinase, LA: lactic acid, CAT: catalase, TAS: total antioxidant status, GPx: glutathione peroxidase, 8-OH–dG: 8-hydroxydeoxy guanosine, IL–6: interleukin-6, TNF–α: tumour necrosis factor α, MDA: malondialdehyde, UA: uric acid, ADA: adenosine deaminase, NO: nitric oxide, XO: xanthine oxidase, SOD: superoxide dismutase, Mb: myoglobine, LPO: lipid peroxide, PP: peak power, MP: mean power, FI: fatigue index, HR: heart rate, RER: respiratory exchange ratio, RPE: rate of perceived exertion, LDLox: oxidised low density lipoprotein, TRAP: plasma total antioxidative capacity.

Cooke et al. (2008) showed that both acute (200 mg-60 min before exercise test) and chronic (200 mg·day^{-1} for 14 days) CoQ10 supplementation did not affect time to exhaustion during the aerobic exercise. In contrast, Mortensen (2005) demonstrated that CoQ10 supplementation increased the 6-min walk distance from 269 m to 382 m in patients with chronic heart failure, and they suggested that CoQ10 supplementation increased performance improving the time to exhaustion. Mizuno et al. (2008) found that although 300 mg CoQ10 for 1 week improved physical performance during fatigue-inducing workload trials on a bicycle ergometer, 100 mg of CoQ10 did not affect exercise performance. According to the systemic review of Rosenfeldt et al. (2003), it appears that a modest improvement in the exercise capacity may be observed with CoQ10 supplementation, but this is not a consistent finding.

Studies investigating the effects of CoQ10 supplementation on anaerobic exercise performance (Malm et al. 1997, Cooke et al. 2008, Gökbel et al. 2010, Bloomer et al. 2012) are limited. Malm et al. (1997) demonstrated that 120 mg·day^{-1} for 22 days CoQ10 supplementation had no significant effect on four anaerobic cycling test performances. Similarly, Cooke et al. (2008) showed that CoQ10 supplementation of 200 mg·day^{-1} for 14 days caused no significant change on anaerobic power measured by peak power, mean power and fatigue index compared with placebo. In a study by Bloomer et al. (2012) no change in anaerobic exercise performance was observed following 4 weeks of CoQ10 supplementation. In a recent study (Gökbel et al. 2010), we demonstrated that CoQ10 supplementation 100 mg·day^{-1} for 8 weeks improved the mean power during repeated bouts of the supramaximal exercises. These results were attributed to the contribution of aerobic metabolism and key role of CoQ10 in energy metabolism during the repeated bouts of supramaximal exercises.

Effects of CoQ10 supplementation on exercise-induced muscle injury were investigated in both humans and animals (Shimamura et al. 1991, Kon et al. 2007, Kon et al. 2008). Shimomura et al. (1991) and Kon et al. (2007) reported that intravenous and oral CoQ10 supplementation, respectively, attenuates the rise in markers of muscle damage in rats following downhill running. In addition, Okamoto et al. (1995) provided evidence that CoQ10 protects cultured skeletal muscle cells from electrical stimulation-induced lactate dehydrogenase release. In human subjects, Kon et al. (2008) showed that CoQ10 supplementation prevents exercise-induced increase in creatine kinase (CK) activity and myoglobin levels in kendo athletes after muscle damaging exercise. In contrast, Kaikkonen et al. (1998) claimed that CoQ10 supplementation did not affect CK activity following a marathon run. From these results, it has been stated that CoQ10 supplementation may have the potential to reduce exercise-induced muscular cell damage.

5.2.2 Effects on Exercise-Induced Oxidative Stress

The antioxidant activity of CoQ10 appears only with the reduced form (ubiquinol). The oxidised form (ubiquinone) is readily reduced to ubiquinol enzymatically after dietary uptake (Mohr et al. 1992). CoQ10 inhibits the expression of free radicals from different sources (Sohet et al. 2009, Tsuneki et al. 2007), and therefore it can improve the antioxidant system in the body.

CoQ10 supplementation has been reported to attenuate biomarkers of oxidative stress when measured at rest (Niklowitz et al. 2007, Weber et al. 1994). Weber and his colleagues (1994) reported a decrease in lipid peroxidation in healthy subjects after 2 weeks of CoQ10 treatment (90 mg·day^{-1}).

The effect of CoQ10 supplementation on exercise-induced oxidative stress has been investigated in humans, but the existing data are inconsistent (Braun et al. 1991, Cooke et al. 2008, Gül et al. 2011, Bloomer et al. 2012, Díaz-Castro et al. 2012, Ostman et al. 2012). Laaksonen et al. (1995) found that neither the CoQ10 supplementation nor the exercise affected serum malondialdehyde (MDA) concentration in endurance-trained athletes. Similarly, Cooke et al. (2008) showed that acute CoQ10 supplementation did not affect serum MDA levels and SOD activities during and following exercise. In a study by Bloomer et al. (2012), it has been indicated that, in physically active men and women, 30 days of CoQ10 supplementation (300 mg·day^{-1}) did not affect resting or exercise-induced measures of oxidative stress. In a study by Ostman et al. (2012), it has been demonstrated that supplementation of 90 mg·day^{-1} CoQ10 for 8 weeks in moderately trained men did not affect oxidative stress indices. In contrast, in a study by Gül et al. (2011), we showed that supplementation of 100 mg·day^{-1} CoQ10 for 8 weeks decreased oxidative stress indices immediately after the repeated bouts of supramaximal exercises in sedentary men. In a recent study (Díaz-Castro et al. 2012), it has been reported that CoQ10 supplementation during high-intensity exercise is efficient in reducing the degree of oxidative stress (decrease membrane hydroperoxides, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and isoprostanes), which would lead to the maintenance of cell integrity. Also in the same study, CoQ10 administration modulated inflammatory signalling associated with exercise by preventing over-expression of tumour necrosis factor (TNF)-α after the exercise.

The effect of CoQ10 administration on exercise-induced oxidative stress has been also investigated in rats, but the existing data are also inconsistent (Faff and Frankiewicz-Jóźko, 1997, Kon et al. 2007, Okudan et al. 2012). Faff and Frankiewicz-Jóźko (1997) demonstrated that 4 weeks of oral CoQ10 supplementation in a daily dose of 10 mg·kg^{-1} body mass markedly suppresses exercise-induced lipid peroxidation in the liver, heart and gastrocnemius muscle. In a recent study (Okudan et al. 2012), we showed that 6 weeks of intraperitoneal CoQ10 injection in a daily dose of 10 mg·kg^{-1} body mass and exercise training significantly inhibits exhaustive exercise-induced lipid peroxidation and DNA damage, but did not affect glutathione levels and SOD activity in the heart tissue of rats. As a result of the study, we concluded that CoQ10 supplementation and exercise training have interactive effects on lipid peroxidation and DNA damage. In contrast, Kon et al. (2007) reported that 4 weeks of oral CoQ10 supplementation in a daily dose of 300 mg·kg^{-1} body mass ameliorates exercise-induced oxidative damage in skeletal muscle, but not in the liver after the muscle damaging exercise.

CoQ10 is a relatively large hydrophobic molecule (Kaikkonen et al. 2002). Therefore, absorption of CoQ10 into tissues is often slow and limited. Additionally, ingestion of CoQ10 at fast-melt or in capsule form could affect its plasma availability. It has been suggested that 'fast-melt' CoQ10 formulations enhanced the absorption kinetics into the bloodstream (Joshi et al. 2003) and the increased bioavailability may enhance greater uptake into the muscle. The difference among the results in

both human and animal studies is most likely dependent on the type, dosage and time frame of treatment of the antioxidant(s), the tissue sampled, the exercise protocol used to induce oxidative stress, the time of measurement, the assays used and the test subjects recruited (i.e. trained versus untrained, old versus young and healthy versus diseased), among other variables (Bloomer 2008).

5.3 QUERCETIN

Quercetin (3,4,5,7-pentahydroxylflavone) is a natural bioactive flavonoid found in a wide variety of natural foods, such as nuts, grapes, apples, berries, onions, broccoli and black tea (Boots et al. 2008, Kelly 2011). *In vitro* and animal studies indicate that quercetin has many biological effects such as antioxidant, antiinflammatory, anticarcinogenic, antiviral, psychostimulant, cardioprotective, neuroprotective, antipathogenic, immune regulatory and increasing mitochondrial biogenesis (Davis et al. 2009a). Antioxidant properties of quercetin are attributed to its chemical structure, particularly the presence and location of the hydroxyl (-OH) substitutions.

The beneficial effects of quercetin largely depend on its bioavailability after oral administration. Although initial reports indicated that bioavailability of quercetin was limited, recent evidence suggests that quercetin can be detected in plasma within 15–30 min of ingestion of a 250 or 500 mg quercetin chew preparation, reaching a peak concentration at approximately 120–180 min, returning to baseline levels at 24 h in humans (Boots et al. 2008, Davis et al. 2009a). Quercetin also has been shown to reach and accumulate in various tissues such as the colon, kidney, liver, lung, muscle and brain, though the tissue distribution has not yet been studied in humans (de Boer et al. 2005, Harwood et al. 2007).

Quercetin supplementation studies in athletes have focused on the potential effects of exercise-induced inflammation, oxidative stress, immune dysfunction and exercise performance (Nieman et al. 2012). The available evidence for a beneficial effect of quercetin on exercise performance, while encouraging, is limited by the lack of sophisticated clinical trials. The first human exercise study investigating quercetin supplementation was published in 2006 (MacRae and Mefferd 2006), with many more published in the past few years and continuing to be published. When athletes are studied, most of the researches have failed to find an ergogenic effect (Quindry et al. 2008, Utter et al. 2009), in contrast to that of a study of elite cyclists, who exhibited an improvement of their aerobic performance (MacRae and Mefferd 2006). MacRae and Mefferd (2006) indicated that administration of quercetin (1200 mg) for 6 weeks resulted in performance improvement in cyclists. Davis et al. (2010) examined the effects of 7 days of quercetin (1000 mg) supplementation on both VO_{2max} and time to fatigue on a bicycle ergometer in healthy untrained men and women. Increases in both VO_{2max} (3.9%) and time to fatigue (13.2%) were found.

In a recently published meta-analysis (Pelletier et al. 2013), it has been demonstrated that quercetin supplementation improves endurance performance by $0.74 \pm 1.04\%$ compared with placebo. However, no relationship was found between quercetin duration and percentage changes in endurance performance between

groups. In this meta-analysis, it was demonstrated that quercetin confers an increase in performance which is much less than this efficacy threshold, thereby indicating that it is unlikely to confer any ergogenic value, at least within the length of supplementation used and quercetin doses provided by the actual studies. The authors concluded that quercetin is unlikely to improve performance, independent of the training state. Athletes may hope to benefit from use of a sport nutrition supplement during out of doors, real-world exercise conditions, if it produces an effect under laboratory-controlled exercise conditions that is 1.3–1.6% (Hopkins et al. 1999, Hopkins and Hewson 2001) greater than the effect of the placebo.

In a study by Dumke et al. (2009), no effect of quercetin supplementation (1000 mg·day^{-1}) was observed on cycling time trial performance in elite cyclists. Quindry et al. (2008) reported that quercetin supplementation (1000 mg·day^{-1} for 3 weeks) had no effect on race performance at the Western States 100-mile race. A single, very high dose of quercetin (2 g) was also shown not to increase exercise performance in moderately fit military personnel during exercise in the heat (Cheuvront et al. 2009). Supplementation with quercetin and vitamin C for 8 weeks did not improve exercise performance but reduced muscle damage and body fat percent in athletes (Askari et al. 2012). Sharp et al. (2012) demonstrated that supplementation with quercetin (1000 mg·day^{-1} for 9 days) did not improve aerobic capacity, aerobic performance, steady state load carriage exercise and change the metabolic or perceptual responses to exercise. In a recent study, Casuso et al. (2013) suggested that quercetin supplementation showed no effect on VO$_{2peak}$, speed at VO$_{2peak}$ or endurance time to exhaustion after 6 weeks of quercetin supplementation.

Some studies (MacRae and Mefferd 2006, Davis et al. 2010, Nieman et al. 2010) reported an improvement in exercise performance in humans after ingestion of quercetin, whereas most others failed to find statistically significant benefits in exercise capacity (Cureton et al. 2009, Utter et al. 2009, Bigelman et al. 2010, Ganio et al. 2010, Sharp et al. 2012, Askari et al. 2013).

The most important novel effect of quercetin related to a possible benefit on endurance performance comes from two recent *in vitro* and rodent studies (Rasbach and Schnellmann 2008, Davis et al. 2009b) that show a benefit on mitochondrial function. Davis et al. (2009b) found that quercetin feedings (12.5 and 25 mg·kg^{-1}day^{-1}) for 7 days improve running time to fatigue by stimulation of mitochondrial biogenesis, including peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and sirtuin 1 (SIRT1) gene expression, mitochondrial DNA (mtDNA) and cytochrome c enzyme concentration in both the brain and soleus muscle of rats. However, this effect has not yet been observed in humans. A study investigating markers of mitochondrial biogenesis in humans after quercetin administration (1000 mg·day^{-1} for 2 weeks) observed trends towards increased markers of mitochondrial biogenesis such as cytochrome c oxidase and muscle mtDNA but failed to reach statistical significance (Nieman et al. 2010).

Possible reasons for the inconsistent findings among these studies may include the range of subject fitness levels, differences in plasma quercetin concentration obtained via the various supplementation protocol/supplement types and differences in research design.

In accordance with anti-inflammatory properties, it has been shown that quercetin modulates intracellular signalling pathways, including the inflammatory signalling cascade, by inhibiting activation proinflammatory transcription factor and nuclear factor-kappa B (NF-κB) (Harwood et al. 2007). Strenuous exercise is capable of damaging muscle and initiating an inflammatory response. Nieman et al. (2007a,b,c) examined the effect of quercetin upon inflammation after three consecutive days of cycling and following an ultralong endurance run. Except for an attenuation of interleukin (IL)-8 and IL-10 mRNA in blood leukocytes following the cycling bouts, quercetin failed to attenuate any of the measured markers of muscle damage, inflammation, increases in plasma cytokines and alterations in muscle cytokine mRNA expression. Recently, Overman et al. (2011) reported that quercetin decreased expression of inflammatory cytokine TNF-α, interferon-γ, IL-6 and IL-1β transcripts in cultured human macrophages, which are known to be contributors to secondary muscle damage. O'Fallon et al. (2012), McAnulty et al. (2008) and Abbey and Rankin (2011) demonstrated no effect of quercetin supplementation on the markers of muscle damage and no effect of quercetin or eccentric exercise on biological markers of systemic inflammation (IL-6 and C-reactive protein) in untrained and trained individuals. Konrad et al. (2011) reported that ingestion of a quercetin-based supplement (1000 mg) 15 min before the 2 hours of treadmill run did not attenuate exercise-induced inflammation or immune changes or improve performance. In a study, quercetin feedings reduced self-reported symptoms of upper respiratory tract infection (URTI) following 3 days of exhaustive exercise (Nieman et al. 2007c). In this study, highly trained cyclists ingesting 1000 mg·day^{-1} of quercetin during a 3-week period experienced a significantly lower incidence of URTI during the 2-week period following the 3 days of intensified training. However, there was no beneficial effect of quercetin on any of the immune components measured, including natural killer (NK) cell lytic activity, polymorphonuclear respiratory burst or phytohaemagglutinin-stimulated lymphocyte proliferation, despite the reduced incidence of URTI symptoms that were observed after quercetin feedings. However, in a similar study, Henson et al. (2008) reported no benefit on illness rates following the Western States Endurance Run. Davis et al. (2008) reported that quercetin supplementation (12.5 mg·kg^{-1}·day^{-1}) for 7 days reduces susceptibility to influenza infection following stressful exercise in rats. No effects of quercetin were found on leukocyte subset counts, granulocyte respiratory burst activity and salivary immunoglobulin A following quercetin supplementation for 3 weeks before and 2 weeks after the Western States Endurance Run (Henson et al. 2008).

Another interesting property of quercetin which may enhance mental and physical performance is its caffeine-like psychostimulant effect. Psychostimulants, like caffeine, can delay fatigue during endurance exercise, because of their ability to block adenosine receptors in the brain, which results in an increase in dopamine activity (Davis et al. 2003). A psychostimulant effect of quercetin has also been reported *in vitro* (Alexander 2006) in a manner similar to that of caffeine (Ferré 2008), but this effect was not found in human subjects (Cheuvront et al. 2009).

5.4 RESVERATROL

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenolic flavonoid (Baur and Sinclair 2006). It is freely available in food supplements and is found in the seeds and skins of grapes, red wine, mulberries, peanuts and rhubarb (Baur and Sinclair 2006, Nieman et al. 2012). Many *in vivo* and *in vitro* studies (Brisdelli et al. 2009, Ventura-Clapier 2012) have provided evidence for neuroprotective, antiatherogenic, antithrombotic, antihypercholesterolemic, antiinflammatory, antioxidant, proangiogenic, vasorelaxing and anticancer effects of resveratrol. Interestingly, it has also been shown that resveratrol increases skeletal muscle mitochondrial biogenesis and fatty acid oxidation in many tissues as well as exercise performance in mice (Dolinsky et al. 2012).

Pharmacokinetic studies indicate that resveratrol has a poor bioavailability. Resveratrol, even at the high dosage of 750 mg (kg·body weight^{-1}) per day for 13 weeks by the oral route, has been shown to have no adverse effects (Edwards et al. 2011). Pharmacological studies also suggest that therapeutic doses of resveratrol are non-toxic, easily absorbed and well tolerated by humans. A dose of 150 mg·kg^{-1}·day^{-1} has been used in the study of Dolinsky et al. (2012), while other studies have shown that lower doses of 20 mg.kg^{-1}.day^{-1} proved to be efficient in preventing cardiac dysfunction (Rimbaud et al. 2011) and pulmonary hypertension (Csiszar et al. 2009) and also in vasoprotection (Ungvari et al. 2007).

Interest in resveratrol in sport medicine arose after animal studies assessed endurance performance of mice and found a dose-dependent increase in exercise tolerance, improved motor skills and increased number and activity of mitochondria in muscle cells. Both exercise and resveratrol are thought to trigger biochemical cascades, leading to improved mitochondrial function and energy metabolism. Indeed, it has been shown that resveratrol enhances mitochondrial biogenesis and induces adenosine 5′ monophosphate-activated protein kinase (AMPK) in the skeletal muscle of mice (Baur and Sinclair 2006, Lagouge et al. 2006). However, when SIRT1 was knocked out, these effects were absent (Price et al. 2012). Resveratrol as a food supplement in sport medicine has not received much attention especially in human studies, despite some basic scientific evidence that this substance could have multiple indications related to high-performance sport (Nieman et al. 2012).

Resveratrol has been also touted as an exercise mimetic effect through its activation of SIRT1 and AMPK (Hart et al. 2013). To support this hypothesis, it has been demonstrated that resveratrol supplementation increases the exercise performance in aged mice (Murase et al. 2009) and mice fed by a Western diet (Lagouge et al. 2006) in the absence of exercise training, suggesting that resveratrol can stimulate pathways similar to exercise. Ryan et al. (2010) demonstrated that 10 days of resveratrol supplementation also diminishes the basal levels of oxidative stress associated with ageing. Functional measurements of maximal isometric force and rate of fatigue were unaffected by resveratrol supplementation in aged animals. Mice treated with resveratrol demonstrated elevations in AMPK activation and PGC-1α expression, along with increases in mitochondria in animals fed by a high fat diet (Baur and Sinclair 2006). Additionally, enhanced SIRT1 activity like exercise

training decreases plasma glucose levels, improves insulin sensitivity, increases mitochondrial number and function, decreases adiposity, improves exercise tolerance and potentially lowers body weight (Elliott and Jirousek 2008). The induction of PGC-1α and activation of AMPK are commonly observed following both exercise and resveratrol administration (Ruderman and Prentki 2004, Baur and Sinclair 2006, Lagouge et al. 2006, Zang et al. 2006).

Menzies et al. (2013) demonstrated that SIRT1 protein is responsible for the partial maintenance of basal mitochondrial content and function, in addition to lowering mitochondrial ROS generation and improving fatigue in skeletal muscle. They also showed that resveratrol can activate both AMPK and p38 in temporally distinct stages, which could promote post-translational changes in PGC-1α, thereby altering its activity (Jäger and Nguyen-Duong 1999). These studies (Jäger and Nguyen-Duong 2007, Menzies et al. 2013) also demonstrated that high doses of resveratrol were necessary for AMPK-mediated activation of SIRT1. The resveratrol-induced improvement in energy metabolism is at least partly mediated by specific signal transduction pathways and resveratrol seems mediated by enhanced mitochondrial biogenesis with the activation of the AMPK-SIRT1-PGC-1α pathway (Ventura-Clapier 2012).

Resveratrol administration seems to induce a higher aerobic capacity in mice, as shown by the increased running time and oxygen consumption in muscle fibres (Menzies et al. 2013). Similarly, Hart et al. (2013) suggested that resveratrol supplementation enhanced the effects of exercise on endurance capacity, and this was shown in rats which already had a high level of aerobic endurance. These findings suggest that resveratrol could be used as a performance enhancer (Baur and Sinclair 2006, Lagouge et al. 2006). Dolinsky et al. (2012) demonstrated that a combination of resveratrol and exercise training increased time to exhaustion compared to exercise training. The authors suggested that resveratrol optimises fatty acid metabolism, which may contribute to the increased contractile force response of skeletal muscles.

5.5 PTEROSTILBENE

Pterostilbene (*trans*-3,5-dimethoxy-4-hydroxystilbene) is a stilbenoid chemically similar to resveratrol and is found in grapes, wine and berries (Rimando et al. 2004). Pterostilbene is generated by plants in response to microbial infestation or exposure to ultraviolet light (Langcake 1981). Pterostilbene is closely related structurally to resveratrol (a naturally occurring dimethylether analogue of resveratrol) and shows many of the same characteristics, as well as its own unique therapeutic potential (Rimando et al. 2002).

Pterostilbene might show higher biological activity compared with resveratrol, because substitution of a hydroxy with a metoxy group increases the transport into cells and increases the metabolic stability of the molecule. Therefore, pterostilbene is not as quickly glucuronidated and sulphated as resveratrol.

Pterostilbene is known to have many pharmacological benefits for the prevention and treatment of a wide variety of diseases, including cancer (McCormack and McFadden 2012), dyslipidaemia (Rimando et al. 2005), diabetes (Amarnath Satheesh and Pari 2006), cardiovascular degeneration (Amarnath Satheesh and Pari 2008) and pain (Hougee et al. 2005). Antioxidant and antiinflamatory effects of

pterostilbene are also demonstrated (Roupe et al. 2006, Perečko et al. 2010, Hsu et al. 2013). Pterostilbene possesses strong, dose-dependent antioxidant effects (Rimando et al. 2002, Amorati et al. 2004). The antioxidant activity of pterostilbene was first demonstrated *in vitro* by its inhibition of methyl linoleate oxidation (Roupe et al. 2006). It inhibits the production of hydroxyl radicals (Perečko et al. 2010). In terms of the antiinflamatory effect of pterostilbene, Hsu et al. (2013) demonstrated that pterostilbene downregulates inflammatory TNF-α, IL-6, cyclooxygenase-2, inducible nitric oxide synthase, IL-1β, monocyte chemotactic protein-1, C-reactive protein and plasminogen activator inhibitor-1 expression by inhibiting the activation of NF-κB.

According to our knowledge, to date no study has investigated the effects of pterostilbene supplementation on exercise performance, exercise-induced oxidative stress and inflammatory response in both sedentary and trained individuals. On the basis of the current studies, pterostilbene may improve athletic performance by activating and supporting both antioxidant and antiinflamatory cascades in untrained and trained subjects. However, detailed animal and human studies are needed in this subject.

5.6 PYCNOGENOL

Pycnogenol (also referred to as picnogel or pycnogel) is the registered trade name for a natural extract from the bark of a French maritime pine (Pinus Pinaster). It is a standardised extract composed of a mixture of flavonoids, mainly phenolic acids, catechin, taxifolin and procyanidins, and each component exerting a unique biological effect (Packer et al. 1999). Recommended doses of pycnogenol range widely and depend on the treatment aim. For example, to combat chronic venous insufficiency, recommended doses range from 150 to 360 mg·day^{-1}, whereas others have recommended approximately 75–90 mg·day^{-1} to prevent oxidative tissue damage. In a majority of clinical trials, the duration of supplementation is generally 2–3 months. Side effects of pycnogenol supplementation are minimal (Gleeson et al. 2012). Studies indicate that pycnogenol components are highly bioavailable. Interestingly, pycnogenol displays greater biologic effects as a mixture than its purified components do individually, indicating that the components interact synergistically (Packer et al. 1999).

Pycnogenol supplementation has been reported to have a wide range of health benefits, including improved cognitive function, endothelial function, blood pressure regulation and venous insufficiency (Maimoona et al. 2011, Gleeson et al. 2012). Pycnogenol also acts as an antiinflammatory and antioxidant agent (Packer et al. 1999, Devaraj et al. 2002, Williamson and Manach 2005). The antioxidant effect of pycnogenol is attributed to the high procyanadin content (Grimm et al. 2004). Pycnogenol has also been reported to have cardiovascular benefits, such as a vasorelaxant activity, angiotensin-converting enzyme inhibiting activity and the ability to enhance the microcirculation by increasing capillary permeability (Packer et al. 1999).

There are a limited number of studies in the current literature about the effects of pycnogenol on exercise performance, exercise-induced oxidative stress and inflammatory response. In a previous study (Pavlovic 1999), examining the effect of

pycnogenol on endurance performance demonstrated a significant increase in endurance performance in recreationally trained athletes. Mach et al. (2010) demonstrated that pycnogenol-rich antioxidant cocktail improves time to fatigue by increasing the serum NAD^+ levels. In a recent study, Bentley et al. (2012) showed that an acute single dose of pycnogenol supplement is able to improve endurance performance in trained athletes. Additionally, Vinciguerra et al. (2006) demonstrated that pycnogenol ingestion reduces the number of events in subjects with cramps and muscular pain without causing negative effects. However, additional experiments are required to confirm these results, to examine the optimal timing and dose amount of this supplement, as well as to establish the physiological mechanisms that explain the increased time to exhaustion during intense endurance exercise.

5.7   ASTAXANTHIN

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a natural compound (one of the xantophyll carotenoids) found in algae, fish and birds (Aoi et al. 2008). Astaxanthin has been shown to be one of the most effective antioxidants against lipid peroxidation and oxidative stress in *in vivo* and *in vitro* systems (Chan et al. 2009, Tripathi and Jena 2009, Choi et al. 2011). It has potential health-promoting effects in the prevention and treatment of various diseases such as cancer, chronic inflammatory diseases, diabetes, cardiovascular and neurodegenerative diseases (Yuan et al. 2011). Astaxanthin also has immunomodulating, antiinflammatory actions (Park et al. 2010) and stimulates fat oxidation (Aoi et al. 2008, Res et al. 2013). *In vitro* studies (Kurashige et al. 1990) have demonstrated that astaxanthin is a several fold more active free radical scavenger than β-carotene and α-tocopherol.

In mammals, astaxanthin accumulates in muscle, liver and kidney tissues after oral administration and dietary astaxanthin attenuates muscle damage and inhibits peroxidation of DNA and lipids due to prolonged exercise (Aoi et al. 2003). Prolonged astaxanthin supplementation has been reported to improve both swimming and running time to exhaustion in mice (Aoi et al. 2003, Ikeuchi et al. 2006). Keisuke et al. (2002) observed that 4 weeks of astaxanthin supplementation showed performance enhancing effects by reducing the lactic acid build-up following 1200 m of running. Earnest et al. (2011) reported a significant 5% improvement in 20 km time trial performance following 4 weeks of astaxanthin supplementation (4 mg·day^{-1}) in seven trained cyclists. In contrast to these reports, Res et al. (2013) demonstrated that astaxanthin supplementation did not improve exercise performance in endurance trained cyclists.

One possible explanation of the performance enhancing effect of astaxanthin is to increase the fat oxidation. In several studies (Ikeuchi et al. 2006, Aoi et al. 2008) using a mouse model, 4–5 weeks of astaxanthin supplementation (6–30 mg·kg body weight^{-1}) has been reported to improve fat utilisation during exercise and subsequently increase swimming and treadmill running time to exhaustion. The observed increase in fat oxidation was attributed to a greater capacity for fatty acyl-CoA uptake into the mitochondria via an improvement in carnitine palmityol transferase 1 (CPT1) function. CPT1 is located on the mitochondrial membrane and is regarded as the rate limiting enzyme of fatty acid metabolism (McGarry and Brown 1997). Astaxanthin supplementation may improve CPT1 function by inhibiting the

accumulation of damaging ROS on the mitochondrial membrane (Naguib 2000, Mortensen et al. 2001). Astaxanthin also inhibited the elevation of plasma lactate and reduced muscle glycogen catabolism during exercise, which supports the lipolytic effect of astaxanthin (Aoi et al. 2008). In a recent study, Res et al. (2013) demonstrated that 4 weeks of astaxanthin supplementation (20 mg·d^{-1}) increases plasma astaxanthin levels, but this did not augment fat oxidation rates at rest and/or during submaximal exercise.

In accordance with antioxidant activity, 12 weeks of astaxanthin supplementation has been demonstrated to improve total antioxidant capacity and decrease MDA levels in sedentary, obese subjects (Choi et al. 2011) and lower levels of lipid peroxidation in healthy untrained males (Karppi et al. 2007). In a recent study, Res et al. (2013) observed the apparent absence of any antioxidant properties of astaxanthin in endurance trained athletes and they attributed this situation to the duration of the supplementation period. In a recent study, Baralic et al. (2013) demonstrated that astaxanthin supplementation had a beneficial effect on paraoxonase activity towards paraoxon and diazoxon, as well as total sulphydryl content in young soccer players. They suggest that astaxanthin might be of special interest for the athletes who are more susceptible to oxidative stress, providing additional support for enzymatic and non-enzymatic endogenous antioxidant defence systems in order to attenuate increases in ROS production. In a recent study, Park et al. (2010) demonstrated that dietary astaxanthin decreased biomarkers of oxidative DNA damage (8-OHdG) in young healthy females.

Studies (Jyonouchi et al. 1994, Chew et al. 1999, Bennedsen et al. 1999, Park et al. 2010) investigating the immunomodulatory effects of astaxanthin have demonstrated that astaxanthin stimulates immune response in both animals and humans. Dietary astaxanthin enhanced both cell-mediated and humoral immune responses in young healthy females (Park et al. 2010). The immune markers significantly enhanced after astaxanthin supplementation such as T-cell and B-cell mitogen-induced lymphocyte proliferation, NK cell cytotoxic activity, INF-γ and IL-6 production and leukocyte function antigen-1 expression (Park et al. 2010). Astaxanthin increased cytotoxic T-lymphocyte activity in mice (Jyonouchi et al. 2000) and inhibited stress-induced suppression of NK cell activity (Kurihara et al. 2002).

There are contradictory findings about the effects of astaxanthin on exercise-induced damage. Aoi et al. (2003) suggested that astaxanthin can attenuate aerobic exercise-induced damage in mouse skeletal and heart muscle, including the associated neutrophil infiltration that may potentiate further injury. However, Bloomer et al. (2005) demonstrated no benefit for astaxanthin on eccentric exercise-induced muscle damage. Interestingly, astaxanthin is currently being used by some athletes as a natural sun blocking agent. It has been reported that astaxanthin supplementation protects against UVA-skin damage by providing a photo-protective effect of the dermal layer (Camera et al. 2009, Suganuma et al. 2010, Hama et al. 2012).

5.8 CONCLUSION

In conclusion, antioxidant supplements are widely used in many sport fields, even though some of them are probably ineffective. There is insufficient evidence to recommend antioxidant supplements for exercising individuals who consume the

recommended amounts of dietary antioxidants through food because of the contradictory findings. During the antioxidant supplementation, exercising individuals consider that not only type, but also dosage and duration of the supplement is important for effective prevention. However, further studies are needed to clarify the interactive effects of exercise training and antioxidant supplementation.

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6 Polyphenols in Sport
Facts or Fads?

Francesco Visioli

CONTENTS

6.1 INTRODUCTION

Every professional sport athlete and nearly every amateur frequently comes across some sort of advertisement, lay press article, TV advertisements and so forth touting the salubrious activities of polyphenols. The near totality of such pieces of 'information' underscores the need to add antioxidants to the athlete's diet, pointing to polyphenol-rich food or supplements as a valuable source of free radical scavengers. From a mere scientific viewpoint, the reality is—currently—very different.

In this chapter, I will review the (scant) available evidence on the use of polyphenols as antioxidants in sport and try to discuss what the road ahead should look like.

6.2 WHY SHOULD WE INGEST ANTIOXIDANTS?

As also outlined in other chapters of this book, physical exercise induces oxidative stress (Nikolaidis et al. 2012b). Whether mild or severe, oxidative stress might provoke damage to cellular macrocomponents, namely lipids, protein, sugar and DNA (Nikolaidis et al. 2012b). Therefore, the hypothesis is being formulated that the ingestion of antioxidants would prevent such damage and, in addition, augments performance (Gomez-Cabrera et al. 2012).

Indeed, increased oxidative damage can be detected after physical exercise (see ad hoc chapters of this book) and—theoretically—this would translate into increased risk of degenerative diseases, where oxidative stress plays a role (Visioli and Davalos 2011, Visioli et al. 2011).

The problem here is that we are faced with several methodological limitations. The first and most important one is the correct assessment of oxidative damage (Halliwell 2012). To start with, there is—as of today—no proper and accurate way to measure free radical production. Free radicals are too short-lived to be measured *in vivo* (especially in humans), and therefore we try to fight an enemy that we cannot even see.

6.3 ANTIOXIDANTS ARE GOOD FOR OUR HEALTH, RIGHT?

Well, yes, in appropriate amounts that are very difficult to define. Please note that if we look at the results of clinical trials with antioxidants, the near totality of them showed null (Vivekananthan et al. 2003) or even harmful (Bjelakovic et al. 2007) effects. In other words, we do not have sufficient experimental evidence to suggest the intake of pharma-nutrition preparations based on antioxidants. In fact, the converse might be true and we probably should discourage their use (Nikolaidis et al. 2012a, Ristow et al. 2009, Bjelakovic et al. 2007). However, epidemiological studies are quite clear: higher intakes of antioxidants (vitamins, but also polyphenols) are associated with better prognosis (Visioli and Davalos 2011, Visioli et al. 2011).

Why this apparent conundrum? Probably, the foremost explanation is that we confuse *in vitro* with *in vivo* antioxidant activities. Whereas the former are fairly easy to assess and usually require simplified systems, the latter are nearly impossible to accurately establish. Moreover, *in vitro* activities do not take into account bioavailability issues: with the progress of our knowledge of polyphenol metabolism, we now know that these molecules undergo extensive first-pass metabolism and reach plasma and target organs in minute amounts (Visioli et al. 2011). Indeed, cellular concentrations of exogenous antioxidants probably do not add much weight to the antioxidant array (enzymes, but also glutathione, etc.) that we are endowed with. We should stress that human cells already contain several layers of antioxidants, some of which are enzymatic in nature, for example, superoxide dismutase and catalase. Intracellular antioxidants often reach millimolar concentrations, whereas polyphenols' circulating concentrations normally do not exceed the low micromolar range (Lotito and Frei 2006).

6.4 WHAT ARE POLYPHENOLS?

Although an extensive discussion of polyphenols' chemistry is beyond the scope of this chapter (appropriate reviews can be found elsewhere), readers might benefit from a quick overview of these nutritionally important molecules. Polyphenols are the product of plants' secondary metabolism. These secondary metabolites are not required for plant development and growth, but they are essential to plant communication and defence (Iriti and Faoro 2009). Among their most important biological roles, polyphenols are involved in the interaction with pathogens, herbivores and other plants; they protect plants from ultraviolet radiation and oxidants, repel or poison predators and, finally, attract beneficial insects or microbes and are indispensable to pollination (Winkel-Shirley 2002). Indeed, according to plant biologists,

stimulation of plant active defences, that is, secondary metabolic pathways, is a very important goal in crop production. Since agriculture was developed in 10,000 BC, humans have been modifying plant secondary metabolite profiles. By selecting fruit, flower and vegetable colours, farmers involuntarily elected higher anthocyanin content, whereas in selecting for scents they modified volatile phenolics. From a biosynthetic viewpoint, the enzyme phenylalanine ammonia lyase catalyses the deamination of phenylalanine to cinnamic acid, the precursor of the polyphenols (Iriti and Faoro 2004, 2009) (Figure 6.1). These include simple phenylpropanoids, flavonoids, stilbenes and tannins, which contain a second aromatic ring originated from three molecules of malonyl CoA (Figure 6.1).

It is important to underscore that, in addition to focused breeding, several molecular techniques can be employed to enhance the polyphenolic content of plants (Iriti and Faoro 2009).
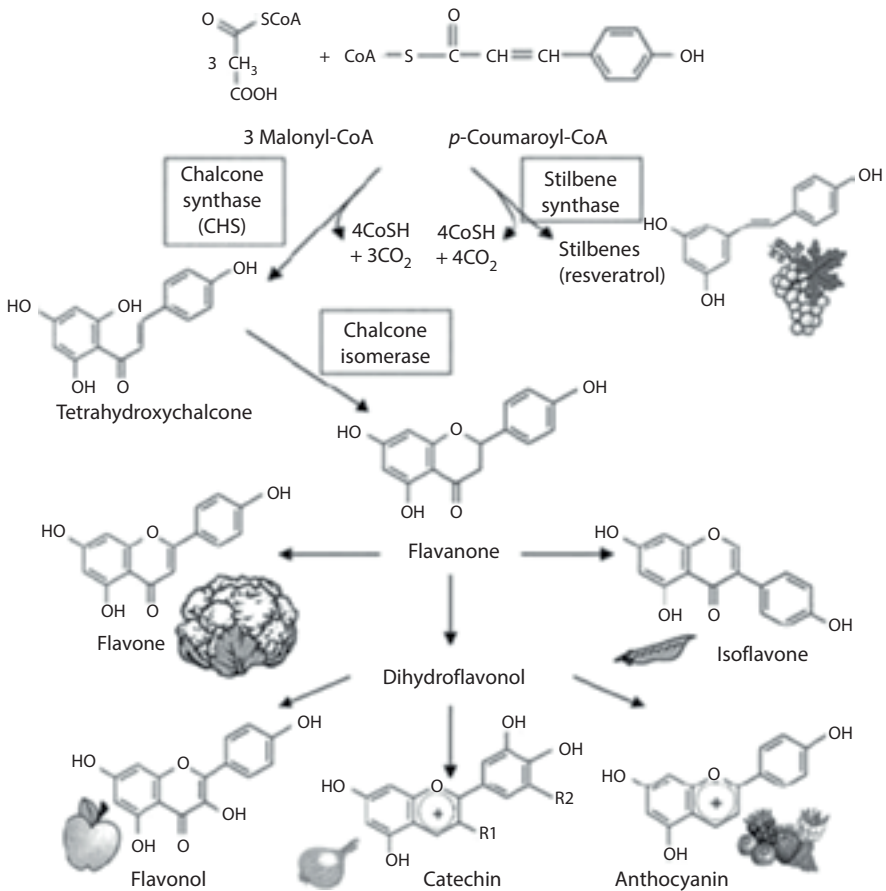
FIGURE 6.1   Major polyphenol biosynthetic pathways in plants. (Adapted from Iriti, M., and F. Faoro. 2004. *Curr Topics Nutr Res* 2:47–65.)

6.5   POLYPHENOLS: MUCH MORE THAN ANTIOXIDANTS (IN FACT, THEY PROBABLY ARE WEAK ANTIOXIDANTS ONCE INGESTED)

It is now thought more likely that some phytochemicals, including polyphenols, are processed by the body as xenobiotics. They stimulate stress-related cell-signalling pathways (Figure 6.2) that result in increased expression of genes encoding cytoprotective proteins. Nrf2 (NF-E2-related factor 2) is a transcription factor which binds to the antioxidant response element (ARE) in cells and thus regulates enzymes involved in antioxidant functions or detoxification (e.g. thioredoxin reductase-1 and glutathione peroxidases) (Shay et al. 2012). Polyphenols might increase gene transcription of Nrf2 mediated by such response elements (Chiva-Blanch and Visioli 2012). This provides grounds for the theory of hormesis, that is, when mild stress triggers defence mechanisms. In the case of polyphenols, it indicates that they could most likely have an indirect antioxidant action (Figure 6.2).

One human example of these effects has been reported by Visioli et al. (2009), who published a study in which 98 Chinese/Malay subjects ingested an olive preparation which was high in phenolics. After 1 h, no difference in plasma antioxidant capacity was observed, but a significant increase in total plasma glutathione concentration was measured. The authors postulated that the observed effects of the olive phenols on glutathione levels might be governed by the ARE-mediated increase in Phase II enzyme expression.
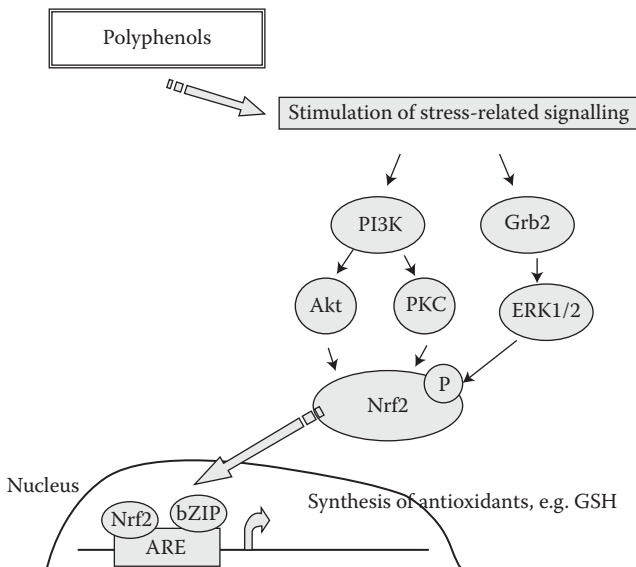
FIGURE 6.2   Polyphenols activate cellular stress-related signalling pathways, which lead to the mobilisation on nrf2 and the activation of the nuclear antioxidant response element (ARE). The ARE stimulates the production of Phase II enzymes and of antioxidants, namely glutathione (GSH). Therefore, polyphenols, rather than being direct antioxidants, act as xenobiotics and stimulate the hermetic cellular response that leads to higher endogenous antioxidant production.

6.6 POLYPHENOLS IN SPORT: A REVIEW OF THE CURRENT EVIDENCE

As mentioned, the current hype on 'antioxidants and health' also involves polyphenols of natural origin. A marketing mix of real science, consumer demand, appeal and targeted advertisement is encouraging sport researchers to undertake experiments to assess the antioxidant virtues of raw mixtures or individual compounds in physical exercise.

The first thing to be said is that the near totality of studies aims at demonstrating some antioxidant action, even though when other antioxidant vitamins have been tested the outcomes have been really disappointing (see above). Indeed, if some conclusion can be drawn, it is that provision of antioxidant molecules to athletes or amateurs actually decreases performance and increases muscular damage (Childs et al. 2001, Ristow et al. 2009). Of note, these studies did not conduct performance diagnostics (only biochemical measurements); therefore, any inference on the actual effects of antioxidant supplementation on performance (either way) is premature (Nikolaidis et al. 2012a).

As of today, the vast majority of studies on polyphenols and physical exercise concerned green tea catechins. As an example, Jowko et al. tested the activities of green tea catechins in healthy individual (Jowko et al. 2011) and soccer players (Jowko et al. 2012). They report very modest protection from oxidative damage in the former and no effect in the latter. In addition, the question should be asked of whether provision of catechins increased or decreased performance, in addition to their putative cellular antioxidant activities. Kerksick et al. also tested a combination of epigallocatechin-gallate and *N*-acetylcysteine in healthy volunteers who performed eccentric exercise bouts (Kerksick et al. 2013). Again, no effect of this intervention was recorded.

In summary, despite the active search for 'natural', polyphenol-rich extracts that might enhance physical performance and decrease oxidative damage because they are antioxidants, the information we currently have is very limited and actually suggests the converse (Gomez-Cabrera et al. 2012).

6.7 CONCLUSIONS

The concept that physical activity induces oxidative stress which needs to be counteracted by increased intakes of antioxidants is—apparently—well anchored in professionals and amateurs. Though the first part might be true (intense exercise does induce oxidative stress in muscles and, probably, other tissues), provision of antioxidant vitamins at high doses apparently does more harm than good. In this respect, we should also move beyond the belief that polyphenols (many of which are indeed potent *in vitro* antioxidants) exert remarkable antioxidants activities in humans. As mentioned, these molecules are heavily metabolised and their true contribution to the cellular antioxidant array is most likely minimal. However, polyphenols stimulate the stress-related biochemical pathways that in the long run afford protection from subsequent insults. In short, it is time we break away from the equation polyphenols = antioxidants, because these molecules are endowed with several other activities (Visioli et al. 2011).

As of 2014, we should discuss 'polyphenols in sport' within the framework of hormesis and adaptive response (including effects on immunity). Even though hormesis is quite difficult to precisely define and quantify, emerging evidence strongly suggests that 'controlled damage' such as that induced by physical exercise stimulates resilience and, in turn, provides better health (Gaman et al. 2011, Calabrese et al. 2012, Ristow and Schmeisser 2011).

In conclusion, physical exercise and sport are essential to a healthy lifestyle despite the fact that they induce oxidative stress (or maybe even 'because' of that) (Nikolaidis et al. 2012b, Gomes et al. 2012). The current hype on polyphenols as antioxidant agents that would prevent muscular damage and enhance performance does not rest on solid ground and many more years of ad hoc studies are required to clarify their precise role in sport.

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7 Supplemental Antioxidants and Adaptation to Physical Training

Micah Gross and Oliver Baum

CONTENTS

7.1   INTRODUCTION

Free radicals are commonly thought of as perpetrators of cell damage, ageing and even cancer, while antioxidants are seen as the defence against these threats. As awareness about the harmful effects of free radicals has increased, so has consciousness regarding the importance of dietary antioxidants. As a result, many health-conscious people turn to nutritional supplements containing vitamins and other antioxidants. Particular interest in antioxidant supplements has arisen among athletes and people who train regularly. Indeed, antioxidants are among the most common sport supplements used by amateur and professional athletes (Krumbach et al. 1999; Margaritis and Rousseau 2008; Sobal and Marquart 1994). A quick survey of the labels on most energy bars and recovery drinks would seem to erase any doubt that athletes have an extraordinary need for antioxidants. However, it has not been conclusively shown that this is the case, and it remains debatable whether large amounts of supplementary antioxidants are sensible at all for athletes in training (Padilla and Mickleborough 2007).

Indeed, there is a growing body of evidence that the appearance of free radicals in skeletal muscle, besides having certain negative effects, also fulfils important physiological functions in cells, and that the right balance between antioxidants and free radicals is necessary for the desired physiological adaptations (Gomez-Cabrera
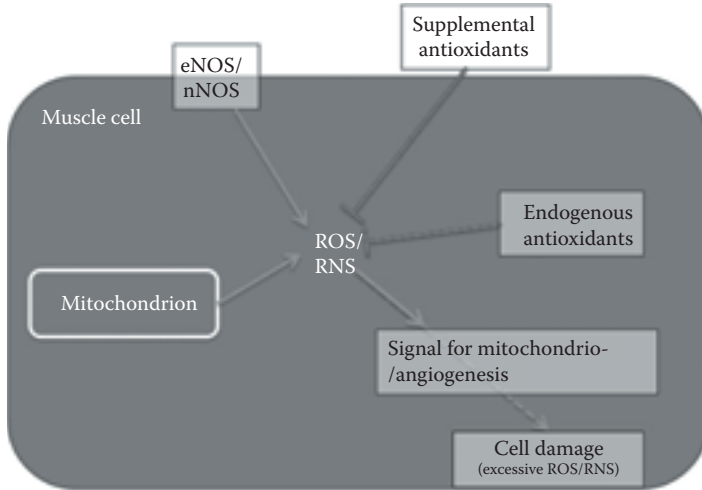
FIGURE 7.1 Overview of the interaction between free radicals (ROS/RNS), antioxidants and their effects.

et al. 2008; Ji 2008). Thus, it becomes necessary to assess the prudence of antioxidant supplementation, particularly among athletes in training (Gross et al. 2011).

In this chapter, we briefly address the production of free radicals at rest as well as during and in response to exercise or training. Then we reflect upon the negative and positive effects free radicals can have on skeletal muscles, highlighting the signalling functions of free radicals in the process of physiological adaptation to training. Finally, the influence of supplemental antioxidants on free radical biology and training adaptations is discussed. An overview of the situation discussed in this chapter is presented in Figure 7.1.

7.2 FREE RADICAL PRODUCTION IN SKELETAL MUSCLE

Free radicals are a heterogeneous group of molecules that are characterised by a free valence electron in their outer atom orbital. Owing to this unpaired electron, free radicals may rapidly react with other molecules. Accordingly, their biological half-life time is very short in biological solutions, including cellular surroundings.

In skeletal muscle fibres, three main free radicals are found: nitric oxide (NO$^\bullet$) as the main representative of reactive nitrogen species (RNS) as well as superoxide anion (O$_2^{-\bullet}$) and hydroxyl radicals ($^\bullet$OH) as the most important reactive oxygen species (ROS). Hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) also belong to the group of ROS without being free radicals.

The gaseous NO$^\bullet$ is generated by NADPH-dependent oxidation of L-arginine by the catalytic activity of intracellular NO synthases (NOS). Skeletal muscle contains high concentrations of neuronal NOS (nNOS) anchored at the sarcolemma. Additionally, NO$^\bullet$ may be generated in skeletal muscle by endothelial NOS (eNOS),

which is expressed in endothelial cells of arterial and venous vascular sections and capillaries.

$O_2^{2-\bullet}$ represents the top of the ROS cascade, which is mainly generated by the activity of membrane-associated NADPH oxidase and as a by-product by the respiratory chain complexes I and III (for review, see Jackson et al. 2007; Powers et al. 2011) in the mitochondria. Superoxide is highly toxic, as it inactivates enzymes that contain iron–sulphur clusters as a prosthetic group (Valko et al. 2007). Therefore, the rapid detoxification of superoxide is of physiological relevance. The reaction of $O_2^{2-\bullet}$ with NO, which yields $ONOO^-$, represents one possible mode of elimination. This reaction is approximately 3 times more efficient at scavenging superoxide than is that catalysed by the superoxide dismutase (SOD) system (Pattwell et al. 2004).

Various enzymes generate significant levels of H_2O_2 expressed in skeletal muscle fibres (for review, see Jackson et al. 2007). The most important are the three forms of SOD: the sarcoplasm SOD-1 and the mitochondrial SOD-2 require copper and zinc on their active side, while the manganese-dependent SOD-3 is located in the extracellular fluid.

Far less is known about the biology of $^\bullet OH$. Presumably, in a microsomal compartment in the presence of Fe^{2+}, this ROS is generated non-enzymatically from H_2O_2 and very rapidly scavenged by endogenous antioxidants.

Studies using nNOS-knockout mice and nNOS-overexpressing transgenic mice have previously shown that an alteration of the nNOS expression levels in skeletal muscle is accompanied by a change in the availability of $O_2^{2-\bullet}$ and, subsequently, H_2O_2 (SOD-1 dependent) and/or $ONOO^-$ (Da Silva-Azevedo et al. 2009; Sakellariou et al. 2011). These studies suggest that levels of $NO^\bullet$ and ROS may influence each other, indicating that free radical metabolism is highly regulated and balanced in muscle tissue.

7.3   NEGATIVE EFFECTS OF FREE RADICALS

High concentrations of free radicals, in particular those of rapidly released ROS, lead to a strong oxidative stress in cells, for example, in neutrophils and monocytes of the immune system but also in skeletal muscle. This is useful for defending against microbiological pathogens, and while the abrupt increase in oxidants helps eliminate infiltrating cells, it also harms the defending cells, which are not protected against this burst of oxidants. Even low levels of ROS might induce cell damage by chemical inactivation of important molecules such as deoxyribonucleic acid (DNA) by base damage and single-strand breaks, unsaturated fatty acids by lipid peroxidation or amino acids and cofactors in proteins by oxidation.

Free radicals may also bring the cellular homeostasis out of balance indirectly. The formation of $ONOO^-$ reduces the levels of bioactive $NO^\bullet$, which is required to dilate terminal arterioles, feed arteries and resistance arteries. In addition, $O_2^{2-\bullet}$ and $ONOO^-$ also lead to apoptosis or inflammatory responses via activation of redox-sensitive signalling cascades. Accordingly, imbalance of the ROS metabolism is associated with cardiovascular diseases, stroke and heart attack.

7.4 SIGNALLING FUNCTIONS OF FREE RADICALS

Although free radicals have traditionally been considered mainly as a threat to cell stability and health, results from several recent studies present the framework for a functional role of free radicals, such as $NO^\bullet$ and $O_2^{2-\bullet}$ as well as H_2O_2, as important cell-signalling molecules. Endogenous oxidant defence is up-regulated by negative feedback from ROS, especially $O_2^{2-\bullet}$ (Gomez-Cabrera et al. 2008), but free radicals also play an important role in stimulating physiological adaptation, especially those related to endurance training. With endurance exercise, increased flux of oxygen through mitochondria causes acute increases in $O_2^{2-\bullet}$ production; exercise-associated depletion of substrates, leading to a drop in glutathione reductase activity, and hyperthermia, which promotes mitochondrial uncoupling and loss of respiratory control, can also contribute to additional free radical production during exercise. Further, transient hypoxia during anaerobic exercise leading to acidosis, as well as reperfusion of hypoxic muscle, can increase oxidative stress (Kanter 1998). Finally, the mechanical stress of exercise can itself increase free radical formation (Symons 1988). These are all phenomena which occur due to training and help provide the stimulus for physiological adaptation.

Further, free radicals may act in the extracellular space; for example, $NO^\bullet$ induces vasodilation in feeding and resistance arteries (Clifford and Hellsten 2004), leading to increased blood-flow velocity (Kayar et al. 1992). The resulting increase in shear stress in the microvasculature of muscle fibres is an important stimulus for angiogenesis in muscle (Baum et al. 2004). ROS also affects the metabolism of carbohydrates and lipids by regulation of the mRNA expression levels of key enzymes (Hoppeler et al. 2011), and there is increasing evidence suggesting that they play an important role in modulating redox-sensitive signalling pathways on the way to further muscular adaptations (Jackson 2009). Also, ROS helps to stimulate the up-regulation of peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 alpha), a master regulatory gene of mitochondrial and vascular adaptation, via the $5'$ adenosine monophosphate-activated protein kinase (AMPK) pathway (Hoppeler et al. 2011).

The most intensively characterised signal transduction pathway triggered by $NO^\bullet$ comprises the activation of soluble guanyl cyclase which, via its synthesis product cyclic guanosine monophosphate (cGMP), subsequently increases the activity of the cGMP-dependent protein kinase G (PKG). However, $NO^\bullet$ might also covalently bind to amino acids within the primary structure of other signalling enzymes. These post-translational modifications, for example, cysteine S-nitrosylation (reversible) and tyrosine nitration (irreversible), enable the initiation of subsequent triggering of cellular cascades (Godecke et al. 2008; Stamler et al. 2008). In the skeletal muscle of humans, $NO^\bullet$ production has been identified to directly regulate the transcription rate of stress genes after exercise (Steensberg et al. 2007).

Adaptations of the free radical metabolism following training may be dependent on changes to cellular thiol:disulphide ratios, or redox potentials, caused by free radicals (Jackson 2009) or the transient appearance of $O_2^{2-\bullet}$ (Gomez-Cabrera et al. 2008), as these appear to stimulate the up-regulation of certain important transcription factors within this pathway. ROS levels after aerobic endurance training have

been associated with muscle and heart hypertrophy, angiogenesis and glucose transport ability (Gibala 2009; Ji 2007). Furthermore, they seem to provide signals for the expression of genes related to metabolism, as will be addressed below.

Free radicals may also have acute positive effects on contractility and improvement of performance. In low concentrations, they help maintain muscle force production (Jackson 2009; Powers and Jackson 2008). Further, during the oxidative burst of phagocytosis, macrophages release $O_2^{2-\bullet}$, H_2O_2 and $NO^\bullet$ as part of the clearing out of damaged or dead cell material, which helps to speed up the repair process (Valko et al. 2006).

7.5   EFFECTS OF SUPPLEMENTAL ANTIOXIDANTS ON TRAINING ADAPTATIONS

The negative effects discussed above, which can be exhibited by RNS and ROS, supposedly provide the basis for the beneficial effect of supplemental antioxidants such as vitamins C and E or β-carotene (non-enzymatic antioxidants, which are not synthesised in humans and must be obtained exogenously). These substances are able to scavenge various free radicals by proton donation (Sies and Stahl 1995).

There have been some benefits of vitamin supplements in certain populations or situations. It has been shown that vitamin C can help strengthen immune defence (Kreider et al. 2004; Valko et al. 2006) while vitamin E could enhance energy balance at high altitude (Simon-Schnass and Pabst 1988). Further, the two function together to protect against lipid peroxidation. Vitamin A is particularly well suited for scavenging $O_2^{2-\bullet}$, $^\bullet OH$ and peroxyl radicals such as $ONOO^-$ (Valko et al. 2004).

However, evidence from clinical studies does not support a protective effect of vitamins C and E or β-carotene against DNA damage or cancer (Valko et al. 2004) or against cardiovascular disease (Myung et al. 2013). Further, it should be noted that under certain circumstances, these antioxidants may become pro-oxidative agents. β-carotene, in the presence of increased partial pressure of oxygen, can be converted into a peroxyl radical, and vitamin C can form DNA-damaging genotoxins from lipid hydroperoxides in the presence of transition metal ions (Valko et al. 2004).

Further, questions have been raised about the efficacy of high doses of supplemental dietary antioxidants such as vitamins C and E during endurance training. In some cases, counteracting ROS accumulation via acute antioxidant supplementation can positively affect athletic performance. For example, pharmacologically boosting the capacity to convert H_2O_2 into water protects against ROS-induced fatigue or enhances time to exhaustion (Medved et al. 2004; Reid 2008). However, most such studies employ intravenous infusions instead of common oral supplements. In general, evidence does not support the belief that vitamins and antioxidants are ergogenic or contribute to enhanced training effectiveness. The consensus is that supplemental vitamins C and E and ubiquinone are not ergogenic in normally nourished athletes at low altitude and vitamins C and E and β-carotene do not prevent training-induced muscle damage in humans (Williams 2004).

Moreover, several publications suggest that these may actually be counterproductive (Gomez-Cabrera et al. 2005, 2008; Ji 2008; Ristow et al. 2009; Wray et al.

2009), since it is supposed that radicals such as ROS and NO• play an important signalling role for metabolism, mitochondriogenesis and angiogenesis, and artificial suppression with supplemental antioxidants may weaken these signals. For example, one response to the elevated oxidative stress associated with exercise is increased oxidant defence via up-regulation of antioxidant enzymes such as SOD and glutathione peroxidase (GPx). However, antioxidant supplementation discourages such adaptations by interfering with the radical-mediated signal (Gomez-Cabrera et al. 2005, 2008). The importance of this consequence may not be obvious if one assumes a surrogate protective effect from exogenous antioxidants; however, endogenous mechanisms could be more important when radical production is particularly high. Accordingly, significantly greater oxidative damage has been observed following half and full ironman triathlons in athletes who took antioxidant supplements than in those who did not (2007).

The challenges faced by energy production systems during training stimulate enhanced capacity through the likes of increased mitochondrial volume density and capillarisation of muscle fibres, and improved provision and utilisation of the substrate. Here too, placebo-controlled studies with animals and humans provide evidence for interference of orally supplemented antioxidants, including vitamin C on exercise-induced signalling and dependent events such as expression of the mitochondrial enzyme cytochrome C, which is representative of mitochondrial volume (Gomez-Cabrera et al. 2008), and improvements to insulin sensitivity (Ristow et al. 2009). Elsewhere, in humans involved in an endurance-training programme, acute supplementation of vitamins C and E seemed to prevent exercise-induced vasodilation (Wray et al. 2009), which, in addition to causing acute hypertension, could also blunt the blood-flow-induced stimulus for angiogenesis. Moreover, angiogenesis is prevented if NO• release from eNOS is blocked (Baum et al. 2004; Hudlicka et al. 2000).

As discussed above, PGC-1 alpha is an established inducer of mitochondriogenesis (Handschin and Spiegelman 2008) and the expression of the gene for PGC-1α increases with training. However, this response is suppressed or eliminated by the administration of supplemental antioxidants in mice (Meier et al. 2013), rats (Gomez-Cabrera et al. 2008) and humans (Ristow et al. 2009), which suggests that PGC-1 alpha mRNA expression is favoured by ROS and that the up-regulation of PGC-1 alpha, in particular, in response to endurance exercise is very sensitive to the redox state of the skeletal muscle. This being the case, mitochondrial proteins may nonetheless increase with training combined with antioxidant supplementation, despite interference of antioxidants with PGC-1a mRNA expression, as has been observed in humans and rats (Gomez-Cabrera et al. 2008; Irrcher et al. 2009; Powers et al. 2011). Possibly, sustained activation of PGC-1 alpha at the post-translational level (phosphorylation, acetylation) (Powers et al. 2011) could prolong the half-life time of the PGC-1 alpha protein to compensate for the lack of mRNA induction.

The capacity of skeletal muscles for glycolysis and lipid oxygenation is increased in response to endurance exercise, which is accompanied by a higher storage potential for energy substrates, in particular carbohydrates (glycogen) and lipids (triacylglycerine). These endurance exercise-related changes in skeletal muscle structure are evoked by characteristic alterations in the transcriptional rates of various key

metabolic genes (Hoppeler et al. 2011). To verify the hypothesis that the administration of antioxidants influences the expression patterns of genes of the metabolism in skeletal muscle of mice during treadmill training and in sedentary animals, we have recently quantified the mRNA levels of eight marker genes that are involved in the oxidation or exercise-dependent storage of carbohydrate and lipids, and which are known to be up-regulated in response to endurance exercise (Meier et al. 2013). As anticipated, the mRNA for each of the eight enzymes was expressed at higher levels in the tibialis anterior of trained mice than in sedentary mice. This outcome underlined the general shift of the carbohydrate and lipid metabolism in skeletal muscle towards oxidation and storage in response to endurance exercise.

While the mRNA levels of five of the eight enzymes (G6PDH, GYG, MCAD, CD36 and FABP-3) were not affected by supplemental antioxidants either in sedentary animals or with training, the concentration of HK-II, GLUT-4 and SREBF-1c mRNA was higher in the sedentary mice receiving supplemental antioxidants than in those receiving a placebo, suggesting that antioxidant supplementation at least partially mimicked the effect of endurance training on the transcription of these two genes (Figure 7.2). However, although antioxidant supplementation or endurance training each increased the expression of GLUT-4 (an important protein involved in glucose transport across the sarcolemma) to a similar degree when administered in isolation, there was no additional effect of combining the two (Meier et al. 2013). This pattern was similar for HK-II, which catalyses the phosphorylation of glucose within glycolysis (Printz et al. 1997), although in this case the effect of antioxidant supplementation alone produced the largest increase in expression (Meier et al. 2013). It has also been shown, however, that administration of vitamins C and E in isolation had no impact on GLUT-4 and HK-II expression in human skeletal muscle (Yfanti et al. 2011). However, expression of SREBF-1c, a pivotal transcription factor in biosynthesis of fatty acids (Shimano 2001), increased significantly with antioxidant supplementation or exercise alone, and to the largest degree when the two were combined (Meier et al. 2013).

While most studies addressing the question of whether excessive radical scavenging can actually reduce selected training stimuli by suppressing the radical-dependent signal for adaptation have been conducted at the molecular level, effects on training-induced changes in aerobic and endurance capacity have been measured in some cases. For example, daily vitamin C supplementation has been shown to greatly inhibit the peripheral adaptations to training (i.e. capillarisation and mitochondrial genesis) and mean improvement in maximal oxygen uptake has been shown to be greater (approximately double) in humans and in rats that received a placebo than in those that received vitamin C ($1 \text{ g} \cdot \text{d}^{-1}$ in humans). In this study, improvement of endurance capacity (measured only in rats) was sevenfold greater (Gomez-Cabrera et al. 2008), supposedly because this parameter is more specifically related to peripheral adaptations.

Another instance of antioxidant supplementation interfering with training is when muscle injury occurs, such as after intense, unaccustomed and especially eccentric exercise. Vitamins C and E have been shown to delay healing and recovery of strength, and increase oxidative stress after such muscle-damaging exercise (Beaton et al. 2002; Childs et al. 2001; Close et al. 2006; Teixeira et al. 2009).
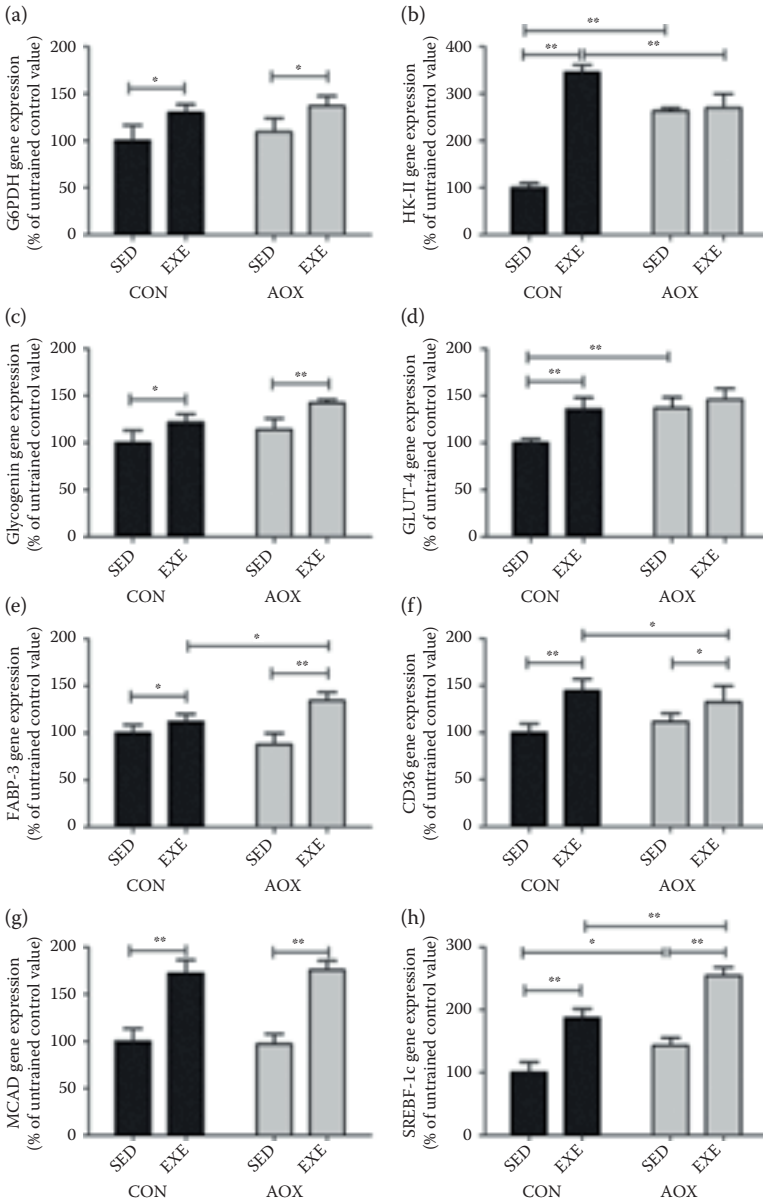
FIGURE 7.2 mRNA expression levels of eight marker genes of the carbohydrate (a through d) and the lipid (e through h) metabolism determined by real-time PCR (polymerase chain reaction). Values represent means_standard deviation from six mice for each group. Asterisks refer to values of treated mice significantly differing (*$p \leq 0.05$ and **$p \leq 0.01$) from values of mice not administered with antioxidants (AOX) and/or remaining untrained (CON), respectively. (Adapted from Meier, P., M. Renga, H. Hoppeler and O. Baum. 2013. *Cell Biochem Funct* 31: 51–9.)

7.6 CONCLUSIONS

The experimental findings described above are intriguing from the standpoint of athletes and coaches who wish to improve performance capacity through training. Could it be that many are unknowingly counteracting training effectiveness through banal practices such as consuming an antioxidant-rich recovery drink after an endurance training session or taking a daily multivitamin? The issue is far from resolved. If and to what extent supplemental antioxidants are beneficial or detrimental to well-nourished athletes in training is a complex question. The answer lies, in part, in the type of training and the associated training goals.

Clearly, there are certain situations where supplementation is probably advantageous; these include high-altitude training periods, since radical production is intensified and endogenous defence weakened in hypoxia (Pialoux et al. 2006, 2009a,b), or around important competitions, where only (possible) benefits remain relevant. And of course, in the case of a diagnosed deficiency, supplementation is recommended, although this is seldom the case in healthy endurance athletes eating a balanced diet (Knez et al. 2007; Margaritis and Rousseau 2008).

In any case, it is clear considering the positive and negative effects of free radicals, and that the right balance between these and antioxidants is necessary for health and optimal training effectiveness. For the time being, identifying the optimal balance remains elusive, making the area of free radicals, antioxidants and training, especially in athletes, a ripe one for further research. Specifically, questions regarding the effects of dose (if possible, based on individual needs), timing (acutely in relation to training sessions or chronically in relation to training cycles) and setting (e.g. hypoxia) of antioxidant supplementation on RNS/ROS signalling during endurance training wait to be answered. These circumstances could be decisive in determining whether supplementation is largely beneficial or detrimental to training effectiveness. Meanwhile, few practical recommendations can be made, other than to realise that, at least for endurance athletes, antioxidant supplementation is not a case of 'the more, the better'.

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8 Green Tea Catechins and Sport Performance

Ewa Jówko

CONTENTS

8.1 INTRODUCTION

Green tea is brewed from the unfermented dried leaves of the plant *Camellia sinensis*. The predominant constituents of green tea are polyphenols belonging to the family of catechins, mainly (–)-epigalocatechin gallate (EGCG), with lesser amounts of catechin (C), epicatechin (EC), epigalocatechin (EGC) and epicatechin gallate (ECG). In addition, caffeine, theanine, theaflavins and phenolic acids such as gallic acid are present in smaller quantities (Cooper et al., 2005). A typical brewed green tea beverage (250 mL) contains 50–100 mg of catechins and 30–40 mg of caffeine. However, the concentration of bioactive compounds of green tea can vary widely according to preparation methods, that is, brewing time or water temperature (Rains et al., 2011). Therefore, standardised green tea extract (GTE) has been developed for research to provide uniform levels of green tea catechins (GTCs).

In recent years, many health benefits of consuming green tea have been reported, including the prevention of diseases associated with free radicals and reactive oxygen species, such as cancer, or cardiovascular and neurodegenerative diseases. In addition to the antioxidant properties of the catechins, their anti-diabetic, anti-bacterial, anti-inflammatory and anti-obesity activities also have been reported (Zaveri, 2006). The health benefits of green tea are mainly attributed to its anti-oxidant properties, including the ability of catechins to scavenge reactive oxygen species or chelate with metal ions (Kashima, 1999). In addition to antioxidant effects, GTCs have been purported to influence several molecular targets in signal transduction pathways associated with cell death and survival (Murase et al., 2002). However, it is not known so far whether these effects on molecular endpoints in signal transduction pathways are

downstream events of the modulation of pro-oxidant/antioxidant balance in cells or if they result from direct action of the catechins on molecular targets, independent of antioxidant properties (Zaveri, 2006).

This chapter highlights the recent research on the efficacy and mechanisms of action of GTCs on body weight, fat metabolism and oxidative stress parameters, with particular interest in their application in healthy, physically active and trained individuals.

8.2 EFFECTS OF GTCs ON BODY WEIGHT

The majority of evidence associated with green tea consumption and health benefits come from epidemiological studies. In one of these studies in Taiwan (Wu et al., 2003), a lower percentage of total body fat, smaller waist circumference and decreased waist-to-hip ratio were observed in subjects with average habitual tea consumption of 434 mL/day for more than 10 years, compared to non-habitual tea drinkers. In accordance with the above are the results of experiments in rodent models, which demonstrated the reducing effects of tea catechins on dietary fat-induced weight gain and body fat mass (Hasegawa et al., 2003; Chen et al., 2009). The results of human intervention studies are inconsistent, however; they indicate that the presence of caffeine in green tea supplements appears to be necessary to see an effect of GTCs on weight loss (Manore, 2012). Phung et al. (2010) performed meta-analysis of 15 randomised clinical trials to characterise the relation between GTCs with or without caffeine and changes in anthropometric variables. In a majority of these reviewed studies, a habitual normal diet was maintained. The authors concluded that GTCs alone (caffeine-free) do not seem to alter positively anthropometric measurements. However, ingestion of GTCs with caffeine (about 12 weeks, total catechins 583–714 mg/day and caffeine ≤114 mg/day) is associated with a reduction in body weight (average −1.38 kg) or other anthropometric parameters (BMI and waist circumference) as compared to caffeine-matched controls (Phung et al., 2010).

It seems that ethnicity (Asian vs. Caucasian) may be a potential moderator of weight loss magnitude after green tea ingestion (Hursel et al., 2009). As reported in more recent reviews regarding studies with overweight or obese adults with duration 12–13 weeks (Jurgens et al., 2012), the mean weight loss in six studies conducted outside Japan was −0.04 kg, whereas in eight Japanese studies the weight loss ranged from −0.2 kg to −3.5 kg in favour of green tea preparations over controls. Indeed, in a 12-week clinical study in Taiwan (Tsai et al., 2009), performed on a group of 120 healthy overweight and obese persons, the average weight loss in individuals who ingested green tea amounted to 6.8 kg (vs. 0.8 kg in the control), and the average loss in body fat mass amounted to 7.6% in green tea consumers (vs. 0.5% decrease in the control). Smaller effects were obtained by Zhang et al. (2012) in a population of 118 Chinese adults with a high proportion of abdominal visceral fat. Those authors investigated the effects of ingestion of catechin-enriched green tea beverage for 12 weeks (609.3 mg catechins and 68.7 mg caffeine daily) or a control beverage (86.2 mg catechins and 40.4 mg caffeine daily), without any intervention of diet, on anthropometric parameters. After 12 weeks, a significant reduction in visceral fat, as well as body weight (−1.0 kg) and body fat (−0.7 kg) were observed by ingestion of catechin-enriched green tea, whereas no significant changes were seen in the control group (Zhang et al., 2012).

It must be emphasised that a number of factors, apart from ethnicity, may affect the results of GTC supplementation, such as the characteristics of the studied population (children, healthy adults or adults with overweight, obesity, hyperlipidemia or diabetes mellitus), doses and types of tea catechins used, and timing of tea catechin ingestion relative to meals (Phung et al., 2010). Additionally, it has been mentioned that the magnitude of habitual caffeine intake can also influence study results (Thielecke and Boschmann, 2009). Two studies examined the effects of GTCs on body weight maintenance after a period of weight loss in overweight and moderately obese male and female subjects. In a study by Kovacs et al. (2004), a 4-week weight loss period (with a very low caloric diet) was followed by a 13-week weight maintenance period in which the subjects (overweight or moderately obese) consumed their habitual diet and received green tea extract (total catechins 573 mg/day, of which 323 mg was EGCG, and caffeine 104 mg/day) or a placebo. No significant differences in body weight regain were observed between the green tea and placebo groups; however, in the green tea treatment, habitual high caffeine consumption (>300 mg/day) was associated with a higher weight regain compared with habitual low caffeine consumption (Kovacs et al., 2004). In another study by these authors (Westerterp-Plantenga et al., 2005), a green tea–caffeine mixture (270 mg EGCG + 150 mg caffeine per day) gave further significant reductions a in both body weight and body fat during a period of weight maintenance following a weight loss period, but only in low-level caffeine consumers, whereas no effect was seen in high-level caffeine consumers. These results confirm the hypothesis that high habitual caffeine intake may mask the beneficial effects of GTCs on weight loss and weight maintenance. In line with the above are also the results obtained by Diepvens et al. (2006), who investigated the effect of GTEs with added caffeine (1207 mg catechins + 237 mg caffeine a day) along with low-energy diet on body composition in moderate caffeine users (200–400 mg caffeine/day). In that study, no significant differences in rate of weight loss were observed between the placebo and GTE groups. Finally, it has been suggested that the effect of green tea preparation on weight loss or weight maintenance can be influenced by protein content in the diet. In a study by Hursel and Westerterp-Plantenga (2009), a green tea–caffeine mixture (270 mg EGCG + 150 mg caffeine/day, ingested in three doses before the meals) or a placebo were added to a high- or low-protein diet (four groups studied), during a 13-week period of weight maintenance after a weight loss period (4-week) in moderately obese subjects. Both green the tea–caffeine mixture and the high-protein diet improved weight maintenance independently through thermogenesis, fat oxidation and sparing fat-free mass. However, no synergistic effect between the green tea–caffeine mixture and the high-protein diet was found, most likely because of the formation of complexes and, in turn, a reduction in absorption (Hursel and Westerterp-Plantenga, 2009).

Few studies added GTCs to diet and exercise in a weight loss program. Meanwhile, theoretically, the administration of GTCs could have an additive effect on weight loss, above and beyond that caused by exercise alone. Shimotoyodome et al. (2005) reported that, in mice fed a high-fat diet, the combination of dietary GTE and regular exercise (treadmill running) had a greater effect on body weight gain and fat utilization than regular exercise alone. Maki et al. (2009) compared the effects of a high-GTC-beverage (625 mg/d GTC + 39 mg caffeine) versus a control beverage

(39 mg caffeine) on body composition and fat distribution in 132 overweight and obese adults during a 12-week weight loss programme (moderate-intensity exercise, ≥180 min/week). Although the percentage changes in fat mass did not differ between the GTC and control groups, greater percentage reductions in both total abdominal fat area (−7.7% vs. −0.3%) and subcutaneous abdominal fat area (−6.2% vs. 0.8%) were observed after GTC versus control beverage administration. In the latest double-blind and placebo-controlled study (Cardoso et al., 2013), a combination of green tea ingestion with resistance training (8 weeks) was examined in overweight and obese women. Green tea combined with resistance training decreased body fat as well as increased lean body mass and muscle strength, as compared to resistance training alone. However, in overweight and obese women undergoing regular endurance exercise (a 12-week programme: walking or running 3 times a week for 45 min at 75% HR_{max}), no additional changes in both body composition and abdominal fat, above those caused by the exercise, were observed after supplementation with EGCG alone (300 mg/day) (Hill et al., 2007). This observation confirms that GTCs without concomitant caffeine ingestion do not exert their weight-loss effect, and it may also indicate that the effect of GTCs might be due to the combination but not catechin alone (Phung et al., 2010).

In the available literature, as described above, most studies involved overweight and obese populations to evaluate the effects of green tea ingestion on weight loss. Very few reports concern the use of this supplement in weight loss programmes for athletes. Weight loss in athletes is motivated by a desire to optimise performance by improving the power–weight ratio, or for aesthetic reasons in leanness sport (Garthe et al., 2011). In sport divided by weight categories (e.g. combat sport such as wrestling, boxing, taekwondo and judo), the rationale for achieving rapid weight loss is to compete at the lowest possible weight class. Weight regulation for elite combat sport athletes is considered an important component of the athletes' mental preparation for competition (Pettersson et al., 2012). To achieve a rapid weight reduction, athletes use a variety of methods, such as reduced liquid ingestion, use of saunas, blouses and plastic suits, reduced energy intake, fasting one day prior to the weigh-in, reduced carbohydrate and fat intake or more aggressive actions such as vomiting and diuretics (Franchini et al., 2012). Weight regulation practices have been proven to negatively affect health parameters, including nutritional and hormonal status, immune function or psychological status, the latter related to dehydration or hypoglycemia (Pettersson et al., 2012). Despite the well-documented negative consequences of rapid weight loss on health status, the prevalence of aggressive and harmful procedures for rapid weight reduction is very high in most combat sport (Franchini et al., 2012).

It has been recommended that, on average, weight loss goals should be approximately 0.5–0.9 kg per week and should not exceed 1.5% of body weight loss per week. A higher rate of weight loss indicates dehydration or other restrictive or unsafe behaviours that can negatively affect performance and health (Turocy et al., 2011). To achieve a weight loss of 0.5–1.0 kg/week, an energy deficit corresponding to 500–1000 kcal/day is needed. However, reducing daily energy intake by 1000 kcal can compromise recovery and impair training adaptation in athletes, especially with an already low energy intake (American College of Sports Medicine, 2009). Garthe et al. (2011) compared the effects of 5–6% body weight loss at slow and fast rates

(0.7% and 1.4% weekly, respectively) on changes in body composition and strength- and power-related performance in elite athletes. They found that the slower weight-loss intervention had more positive effects on lean body mass and performance than the faster weight-loss intervention (Garthe et al., 2011).

Only one study investigated the effect of tea catechins combined with caffeine on body weight and composition in athletes (Bajerska et al., 2010). In this study, wrestlers ingested GTE (509.9 mg EGCG and 36.9 mg caffeine daily), oolong tea extract (OTE: 172 mg EGCG and 138.2 mg caffeine daily) or a placebo (cellulose) for 6 weeks. The study was conducted during a pre-season conditioning programme, when the athletes were on regular diet (55% calories from carbohydrates, 25% from lipids and 15% from proteins). As compared to baseline levels, significant weight loss was observed after ingestion of both GTE (0.6 kg or 0.8%) and OTE (1.4 kg or 1.6%), but not with the placebo. Body weight reduction was accompanied by absolute fat loss (GTE: 1.3 kg, 8.6%; OTE: 1.9 kg, 8.0%). Surprisingly, only in the OTE group was absolute fat loss significant after 6 weeks, as compared to the baseline. However, changes in relative fat mass (% body weight) during 6 weeks were significantly different in both intervention groups, as compared to the placebo (−1.5%, −1.7% and +0.4% for GTE, OTE and placebo, respectively). Although, in the above-mentioned study by Bajerska et al. (2010), no differences between GTE and OTE were observed regarding all parameters, more marked changes in the OTE group were probably due to the higher caffeine content.

Favourable effects of GTCs on body weight and body fat have been shown in a number of intervention trials with a dose of GTCs from 270 mg to 1200 mg/day (Rains et al., 2011). So far, however, an optimal dose of GTC has not yet been established (da Silva Pinto, 2013). Moreover, there is lack of data about the possible side effects, especially of high doses of GTC. Although GTE at a dose of 3.0 g/day (Freese et al., 1999) and EGCG in pure form at a dose of 800 mg (Chow et al., 2003) have been considered to be safe for humans, excessive amounts of GTC used for weight reduction may cause hepatic toxicity (Yang et al., 2011).

Taken together, ingestion of green tea extract rich in catechins and caffeine may be a successful and safe strategy for athletes to gradually lose weight, especially when combined with moderate energy restriction and increased energy expenditure. Additionally, supplementation with green tea catechin-enriched caffeine can improve weight maintenance after a period of weight loss. However, more studies are needed to confirm these effects and evaluate the dose and content of tea catechins and caffeine required to achieve expected results. Before evaluating precise recommendations to athletes, habitual caffeine use should be taken into account because there are convincing data that high habitual caffeine intake can blunt the beneficial effects of green tea supplements on body weight loss. Therefore, for high-level caffeine consumers (≥300 mg/day), a higher dose of tea catechins may be needed to obtain desirable results. Moreover, based on meta-analysis of intervention studies, it seems that the effects of a GTC–caffeine mixture may be more pronounced in Asian than Caucasian subjects. Finally, green tea extract may be an alternative strategy for intake of caffeine alone in high doses, because no change in heart rate or blood pressure was reported after green tea extract ingestion. In contrast, ingestion of high doses of caffeine (400 mg) has been related to side effects such as elevated

systolic and diastolic blood pressure, anxiety, palpitation, headache and dizziness (Westerterp-Plantenga, 2010).

8.3 MECHANISMS OF ACTION OF GTCs ON WEIGHT LOSS

Both green tea catechins and caffeine are considered the two active substances contained in GTE that are responsible for weight loss. Several biological mechanisms of action by which green tea can help reduce body weight have been proposed, including decreased fat absorption, reduced adipocyte lipogenesis or increased thermogenesis and fat oxidation (Wolfram et al., 2006). As far back as in the 1970s (Borchardt and Huber, 1975), an *in vitro* study showed an inhibitory effect of GTCs on catechol-*o*-methyl-transferase (COMT), the enzyme that degrades norepinephrine. Among GTCs, EGCG and ECG have been found to be the most potent COMT inhibitors (Chen et al., 2005). As is well known, norepinephrine, being a neurotransmitter of the sympathetic nervous system, plays an important role in the control of thermogenesis and fat oxidation. Thus, catechins ingested by inhibiting COMT may cause an increase and a more prolonged effect of norepinephrine on thermogenesis and fat metabolism. (The inhibition of COMT by catechins allows norepinephrine to exert a prolonged influence on thermogenesis and fat metabolism.) In turn, caffeine can also exert a thermogenic effect, but through a different pathway: by inhibiting phosphodiesterases and prolonging the life of cyclic adenosine monophosphate (cAMP) in the cell (Shixian et al., 2006). Consequently, increased cAMP stimulates the sympathetic nerve system and activates hormone-sensitive lipase, which promotes lipolysis (Westerterp-Plantenga, 2010). It has been suggested that caffeine and GTCs may act additively or even synergistically to prolong the sympathetic stimulation of thermogenesis (Phung et al., 2010; Manore, 2012). As indicated by the meta-analysis of six short-term (24-h) studies (Hursel et al., 2011), both the catechin–caffeine mixture and caffeine alone have a significant effect on energy expenditure, in a dose-dependent manner. The dose–response effect on energy expenditure occurred with a mean increase of 0.53 kJ/mg for catechins and 0.44 kJ/mg for caffeine. However, as compared to a placebo, a stimulating effect on fat oxidation was observed only after the catechin–caffeine mixture ingestion, whereas no effect was seen after ingestion of caffeine alone (Hursel et al., 2011). The greater effect of caffeine-containing GTE than that of an equivalent amount of caffeine on the metabolic rate was found in a study by Dulloo et al. (1999). In that study, sedentary healthy young men, whose mean BMI was 25.1 ± 1.2 kg/m^2, spent 24 h in a respiratory chamber three times, consuming on separate occasions: GTE (150 mg caffeine and 375 mg catechins provided daily, of which 270 mg was EGCG; ingested in three divided doses with the main meals), caffeine alone (150 mg/day) and the placebo. Compared to the placebo, a significant increase in 24-h energy expenditure (4%) and a significant decrease in 24-h respiratory quotient (RQ, from 0.88 to 0.85) were observed during treatment with GTE, whereas no significant changes in these parameters were found during treatment with caffeine alone or placebo (Dulloo et al., 1999). As a lack of differences between treatments in urinary nitrogen excretion (thus in the protein oxidation rate) was observed in a study by Dulloo et al. (1999), a lower RQ (ratio of expired CO_2 to inspired O_2) indicated higher fat oxidation because more O_2 is required in

relationship to expired CO_2 during fat burning (the RQ of carbohydrate oxidation is 1:1 and fat is 0.7). The effect of increasing energy expenditure by 4% over 24 h increases the contribution of the thermogenesis component to the total energy expenditure (Dulloo et al., 1999). Typically, thermogenesis contributes 8–10% of the daily energy expenditure, so green tea extract ingestion could increase thermogenesis by 35–43% (75–100 kcal). Although it seems small for a single day, the long-term effects are significant (Bell and Goodrick, 2002).

A significant increase in total 24 h urinary norepinephrine excretion observed in a study by Dulloo et al. (1999) after GTE ingestion is consistent with the inhibiting effect of green tea on COMT and confirms the stimulatory effect of GTCs on sympathetically mediated thermogenesis and fat oxidation. It has been suggested that more positive results of studies with Asian subjects than studies with Caucasian subjects may be caused by differences in COMT enzyme activity between ethnic groups (Westerterp-Plantenga, 2010; Hursel et al., 2011). There is evidence that this difference is due to the COMT gene polymorphism, since Asian populations have a higher frequency of the thermostable, high-activity enzyme, COMT^H allele (*Val/Val* polymorphism) opposite to the higher frequency of the thermolabile, low activity enzyme, COMT^L allele (*Met/Met* polymorphism) in Caucasian populations (Palmatier et al., 1999).

8.4 GTCs AND SPORT PERFORMANCE

8.4.1 Effect of GTCs on Exercise Metabolism

As is well known, total energy expenditure is composed of three factors: resting metabolic rate, thermic effect of feeding (diet-induced thermogenesis) and thermic effect of physical activity (Shixian et al., 2006). In a study by Dulloo et al. (1999), the increase in daily energy expenditure during green tea treatment was due to a cumulative increase in postprandial thermogenesis in the diurnal period. However, in a more recent study (Lonac et al., 2011), no acute effect of EGCG alone (in total, seven capsules per 135 mg EGCG were consumed over 48 h; three capsules/day, and the final capsule was ingested 2 h before measurement) on a resting metabolic rate and thermic effect of feeding was observed in healthy young people with a normal BMI (average 24.6 ± 1.2 kg/m^2). It has also been purported that the effect of GTE could be greater under conditions of elevated sympathetic tone and norepinephrine release (i.e. higher activity of COMT) during higher activity levels (Dulloo et al., 1999). In fact, in healthy normal-weight, active men (with a mean BMI 23.9 ± 0.8), ingestion of GTE in a 24-h period before a 30-min cycling exercise at 60% VO_{2max} (total 890 mg polyphenols, in which 366 mg was EGCG, no caffeine) caused a 17% increase in both whole body fat oxidation and the contribution of fat oxidation to total energy expenditure, as compared to placebo (Venables et al., 2008). In another study (Ichinose et al., 2011), the hypothesis that endurance training in combination with GTE supplementation would further accelerate whole-body fat utilisation during exercise, compared with training alone, was investigated. To test it, healthy men were undergoing 10-week endurance training (cycling for 60 min/day at 60% VO_{2max}, 3 days/week). In that time, they ingested GTE (delivering daily 572.8 mg total catechins, of which 100.5 mg was EGCG, and 76.7 mg caffeine) or

placebo + caffeine (77.6 mg/day). Before and after training, respiratory gas exchange was measured during 90 min exercise at 55% VO_{2max}. As a result of training combined with GTE supplementation, a significant decrease in the respiratory exchange ratio (RER; post-training: 0.816 vs. pre-training: 0.844) during exercise was seen, indicating an increase in the proportion of whole body fat utilisation during exercise. Despite interaction between moderate-intensity training and GTE supplementation, no change in RER was observed after training alone (Ichinose et al., 2011). Moreover, in this study, increased fat oxidation during exercise in the GTE group only, without any change in the caffeine-matched placebo group, indicates that tea catechins were responsible mainly for enhancement of fat utilisation.

It has been suggested that increased fat oxidation during aerobic exercise could have a glycogen-sparing effect and consequently result in an improved endurance capacity (Jeukendrup et al., 1998). The results of animal studies (Murase et al., 2005, 2006) may confirm this hypothesis. In mice fed GTE for 10 weeks, lower RQ and a higher rate of fat oxidation (as determined by indirect calorimetry) were observed during swimming exercise to exhaustion, as compared to control mice (Murase et al., 2005). In addition, feeding with GTE increased β-oxidation activity in skeletal muscles and free fatty acid concentration in plasma, as well as decreased plasma lactate concentration. An increase in lipid utilisation as a result of GTE ingestion contributed to improvement in endurance capacity, since prolonged swimming time to exhaustion was observed in animals fed GTE as compared to controls (by 8–24%). Furthermore, the effect of GTE on endurance performance was dose-dependent, and the catechin responsible for observed effects of GTE is, at least partially, EGCG (although the effects of EGCG appeared weak compared with those of GTE). Similarly, in the next study of this research group (Murase et al., 2006), a 10-week intake of catechin-rich GTE in mice, together with habitual exercise, improved running endurance (an increase in running time to exhaustion by 30%, as compared to control mice), and these effects were caused by increasing whole body lipid utilisation and sparing muscle glycogen content.

However, some human studies did not support the stimulating effect of GTE supplementation on fat oxidation during exercise (Eichenberger et al., 2009; Dean et al., 2009; Hodgson et al., 2013). In a double-blind crossover study by Eichenberger et al. (2009), healthy endurance-trained men ingested, for 21 days, GTE (159 mg catechins/day, of which 68 mg/day was EGCG, and only 28 mg caffeine/day) or placebo (corn starch). At the end of the respective supplementation period (both GTE and placebo), a submaximal cycling exercise (for 2 h at 50% W_{max}) was performed. GTE supplementation did not influence fat metabolism during exercise, as indicated by plasma fatty acids, 3-β-hydroxybutyrate and triacylglycerol, or RER and energy expenditure (Eichenberger et al., 2009). It must be mentioned, however, that the dose of catechins used in this study was relatively low (159 mg catechins, which approximately corresponds to the content in two cups of green tea brew) in comparison to other studies; in these, the dose of catechins reached the equivalent of six to seven cups of green tea beverage (Ichinose et al., 2011) and even more—up to 10 cups (Venables et al., 2008). In another double-blind crossover study (Dean et al., 2009), male cyclists performed exercise (60 min of steady-state cycling at 60% VO_{2max}, immediately followed by a self-paced 40-km cycling time trial) three times: after EGCG (270 mg/day), after placebo

and placebo + caffeine (3 mg/kg/day) ingestion over a 6-day period, and 1 h before exercise testing. The study found little benefit of EGCG on fat oxidation or cycling performance, unlike caffeine which enhanced cycling performance. Therefore, the positive effect of green tea on thermogenesis and fat oxidation may be attributed to an interaction between GTCs and caffeine on sympathetic activity rather than individual catechin components (Dean et al., 2009). In a more recent study (Hodgson et al., 2013) in healthy physically active men, the metabolic responses following 7-day GTE supplementation (1200 mg catechins + 240 mg caffeine/day consumed in two doses: before breakfast and dinner) at rest and during moderate-intensity exercise (60-min cycling at 56% VO_{2max}) were examined using gas and liquid chromatography-mass spectrometry. As indicated by metabolic profiling, GTE enhanced lipolysis and fat oxidation when compared to placebo, but only under resting conditions, whereas no effect of GTE was seen during exercise. Because the metabolic effects observed during exercise were largely attributed to acute ingestion of the last dose of green tea (for 2 h before exercise), the authors concluded that a single dose of GTE used may not be potent enough to stimulate further metabolism above that already upregulated by exercise. Moreover, the supplementation period in these two studies (Dean et al., 2009; Hodgson et al., 2013) seems to be too short (6–7 day) to exert an adaptive stimulating effect of GTE on exercise fat metabolism.

Unexpectedly, in the study by Hodgson et al. (2013), no increase in plasma norepinephrine was seen after GTE supplementation, not only during exercise, but particularly at rest when a stimulating effect of green tea on fat metabolism was observed. It questions COMT inhibition as a putative mechanism of action of GTE *in vivo*, and suggests that green tea could stimulate lipolysis via non-adrenergic mechanisms, including upregulation of lipid-metabolizing enzymes by attenuating nuclear factor-κB (NF- κB) activity (Hursel and Westerterp-Plantenga, 2010). It is generally assumed that NF-κB is a transcription factor regulating the expression of several genes which are important in such cellular responses as inflammation or growth (Yang et al., 2001). NF-κB has been cited as a typical redox-regulated factor and ROS have been claimed to be principal regulators of NF-κB activation in many situations (Powers and Jackson, 2008). For example, after hepatic warm ischaemia and reperfusion in rats, an increase in free radical formation was observed, with concomitant NF-κB activation and elevated pro-inflammatory cytokine production (Zhong et al., 2002). However, GTE prevented ischaemia–reperfusion injury to the liver. This protective effect of GTE was associated with decreased free radical formation, as well as inhibited NF-κB activation and, in turn, blunted cytokine production. Taking into account the pro-oxidant stimulation of NF-κB activation, and the fact that a number of diverse antioxidants are inhibitors of this signalling pathway, it is likely that GTCs inhibit NF-κB activation by scavenging free radicals (Zhong et al., 2002). Moreover, evidence indicates that the stimulating effect of GTCs on fat oxidation may be mediated by peroxisome proliferator-activated receptors. In high fat-fed mice treated with dietary EGCG for 16 weeks, increased expression of peroxisome proliferator-activated receptor α was observed in the skeletal muscles, as compared to high fat-fed controls (Sae-Tan et al., 2011). Therefore, GTCs, as antioxidants, can block the activation of NF-κB, which is no longer able to inhibit the peroxisome

proliferator-activated receptor and the latter upregulates lipid-metabolising enzymes involved in fat oxidation (Hursel and Westerterp-Plantenga, 2010).

By contrast with animal studies (Murase et al., 2005, 2006), no improvements in endurance performance were observed after GTC consumption in all the above-mentioned human studies (Eichenberger et al., 2009; Dean et al., 2009; Ichinose et al., 2011), even if augmented fat oxidation was observed (Ichinose et al., 2011). So far, only one study in humans has reported an increase in sport performance after GTC ingestion (Richards et al., 2010). In this study, 19 healthy adults performed incremental cycling exercise until volitional fatigue twice, after placebo and EGCG consumption (7 capsules for 135 mg EGCG, in total 945 mg EGCG; 3 capsules/day were ingested over 48 h prior to exercise, and the last capsule was taken 2 h before exercise testing). Short-term ingestion of EGCG increased significantly VO_{2max} (this increase was observed in 14 out of 19 subjects); however, other physiological parameters (maximal values of RER, stroke volume, cardiac output, work rate and heart rate) were not affected by EGCG consumption (Richards et al., 2010). The authors tried to explain the mechanisms responsible for the increase in VO_{2max} after EGCG consumption. They speculated that EGCG may increase arterial–venous oxygen difference in skeletal muscles, since maximal cardiac output was not augmented as a result of EGCG ingestion. However, it was not supported with direct empirical data. Alternatively, uncoupling of mitochondrial respiration from ATP production by EGCG was suggested (Richards et al., 2010). Indeed, increased mRNA levels of different uncoupling proteins, which are considered to be implicated in thermogenesis and energy metabolism, were found in animal studies after green tea catechin supplementation (Nomura et al., 2008; Lee et al., 2009). As a result of EGCG ingestion, increased mRNA expression of uncoupling protein 2 in mouse liver was reported, whereas RQ during night was decreased, supporting a decreased lipogenesis and increased fat oxidation (Klaus et al., 2005). However, taking into account that no change in RER was found after EGCG ingestion in a study by Richards et al. (2010), it does not seem that the uncoupling mechanism could be responsible for the increase in VO_{2max} after consumption of EGCG. Similarly, it is unlikely that attenuation of norepinephrine degradation via inhibition of COMT could be the mechanism of EGCG action in the above-mentioned study, since not only RER but also maximal heart rate and stroke volume were not affected by EGCG ingestion. Finally, the possibility that tea catechins may impact VO_{2max} through its antioxidant properties was discussed (Richards et al., 2010). It has been mentioned that ROS bioactivity/oxidative stress can modulate cardiac responsiveness to β-adrenergic receptor stimulation by the sympathoadrenal system (Mak and Newton, 2001). In accordance with the above, the intracoronary infusion of vitamin C as antioxidant augmented the inotropic response to an exogenous β-receptor agonist dobutamine in humans (Mak and Newton, 2001). Similarly, the thermogenic responsiveness to non-selective β-adrenergic receptor stimulation was augmented after intravenous ascorbic acid administration (Bell et al., 2006). However, this effect was observed in sedentary but not in habitually aerobic exercising healthy adults. Thus, it does not seem that in endurance-trained persons, antioxidant administration could offer additional benefits over these caused by training alone since, as confirmed in a study by Bell et al. (2006), regular aerobic exercise alone increases thermogenic

responsiveness to β-adrenergic receptor stimulation. Although there is no information about the training status of subjects in the above-mentioned study, the mean value of VO_{2max} points rather to a low level. Therefore, it remains open whether GTC supplementation could increase VO_{2max} in trained athletes, especially via the above-suggested mechanisms.

8.4.2   GTCs and Exercise-Induced Oxidative Stress

The results of controlled interventional studies indicate that regular consumption of green tea (at least 0.6–1.5 L/day) may increase total antioxidant potential in plasma, reduce lipid/protein peroxidation and protect against DNA damage in healthy subjects (Ellinger et al., 2011). However, it seems that green tea ingestion may be effective when antioxidative/oxidative balance is impaired. Thus, these beneficial effects of green tea consumption occur more likely in subjects exposed to increased oxidative challenge, that is, cigarette smoke, benzene exposure and exhaustive exercise (Ellinger et al., 2011).

It is fairly well accepted that intense exercise, both aerobic and anaerobic, can induce an oxidative stress, a condition in which pro-oxidant production (including free radicals and other reactive oxygen and nitrogen species) overwhelms antioxidant defences. As a result, increased oxidative modifications of proteins, nucleic acid and lipids have been reported (Bloomer et al., 2007). Growing evidence indicates that reactive oxygen species can contribute to both the initiation and the progression of muscle fibre injury, which may lead to decreased muscle contractile ability and force production and, as a consequence, to impaired muscle performance (Bloomer, 2007). From the above point of view, much attention has been focused on the supporting endogenous antioxidant defence system by antioxidant supplementation as a strategy to reduce oxidative stress and muscle damage, as well as to improve exercise performance (Urso and Clarkson, 2003; Nikolaidis et al., 2012).

The effects of green tea ingestion on exercise-induced oxidative stress were studied for the first time in rats (Alessio et al., 2002). After green tea ingestion for 6.5 weeks, rats performed an acute running exercise. Green tea ingestion increased post-exercise levels of total antioxidants in plasma, as well as prevented exercise-induced lipid peroxidation in kidneys. Human studies confirmed a protective effect of GTCs against oxidative damage caused by exercise (Panza et al., 2008; Jówko et al., 2011). In a study by Panza et al. (2008), healthy men, involved in recreational weight training, performed intense resistance exercise twice: after 7-day ingestion of water and then after 7-day green tea intake (daily 600 mL brew, total phenol concentration 771 μg/mL). The exercise performed after control treatment increased both plasma creatine kinase activity (an indicator of muscle damage) and xanthine oxidase activity (the main source of free radicals in the ischaemia–reperfusion conditions), without any change in plasma lipid hydroperoxide levels (indicators of lipid peroxidation). However, as a result of green tea ingestion, increased antioxidant potential of plasma (i.e. increased concentrations of reduced glutathione, total polyphenols and ferric reducing ability of plasma) and decreased plasma lipid hydroperoxide (both at rest and post-exercise) were observed. Moreover, green tea intake prevented a post-exercise rise in plasma creatine kinase and xanthine oxidase activities (Panza et al., 2008). In

our study (Jówko et al., 2011), physical education students were subjected to a 4-week strength training (focused on strength endurance development), in combination with placebo or GTE supplementation (daily 640 mg polyphenols, 500 mg of which were catechins). Moreover, the students completed an intense muscular endurance test (one set of bench press and back squat to exhaustion, at 60% one repetition maximum) twice: in the pre- and post-treatment periods. Resting values of both the total plasma polyphenols and the total antioxidant potential of plasma were elevated after GTE supplementation, as compared to placebo. Furthermore, GTE ingestion prevented the increase in plasma creatine kinase activity induced by the muscular endurance test, as well as the increase in plasma hydroxyperoxides caused by the 4-week strength training. These results show that GTE supplementation can protect against oxidative damages induced by both single intense strength exercise and prolonged strength training, at least in previously untrained subjects (Jówko et al., 2011). However, in a more recent study (Jówko et al., 2012), no changes in oxidative stress parameters were seen after acute ingestion of GTE in professional soccer players. In that study, the athletes performed an intense strength exercise (3 sets of bench press and back squat to exhaustion, at 60% one-repetition maximum) 1.5 h following ingestion of GTE in a single dose (640 mg polyphenols). While total plasma polyphenols increased slightly after GTE ingestion, the total antioxidant potential of a plasma was unaffected by acute GTE intake. Furthermore, ingestion of a single dose of GTE did not affect both plasma lipid peroxidation levels and plasma creatine kinase activity. Thus, it is likely that the acute ingestion of GTCs may be insufficient to modify the antioxidant potential in plasma and a longer period of GTE supplementation is necessary to diminish the oxidative damages induced by intense exercise (Jówko et al., 2012). In our latest study (in press), we investigated the effect of GTE ingestion on blood markers of oxidative stress in sprint-trained athletes during their preparatory period. In this placebo-controlled crossover study, the athletes performed repeated a sprint test (4×15 s on a cycloergometer, separated by 1-min rest intervals) three times: at baseline, after a 4-week treatment with a placebo and following a 4-week treatment with GTE. Supplementation with GTE increased the total antioxidant potential of plasma at rest and prevented oxidative stress induced by the high-intensity repeated sprint test; but, conversely, no protection from exercise-induced muscle damage was found in sprinters as a result of GTE ingestion (unpublished). These results are in contrast to previous findings in non-athletes (Panza et al., 2008; Jówko et al., 2011) or endurance trained cyclists (Eichenberger et al., 2009), in whom a decrease in plasma creatine kinase activity was observed after GTC ingestion.

Finally, it does not seem that GTE supplementation could improve physical performance through attenuation of oxidative stress, since no changes in exercise parameters were noted after GTE intake in all the above-mentioned studies. On the contrary, there is growing evidence that decreasing free radical production through the use of excessive amounts of antioxidants could inhibit the signalling induced by reactive species, which are necessary to specific cellular adaptations to exercise (Gomez-Cabrera et al., 2005, 2008a). Indeed, there is clear evidence that redox-sensitive transcription factor NF-κB is activated with exercise leading to the upregulation of gene expression of antioxidant enzymes. Furthermore, this adaptation was abolished when production of ROS was prevented by allopurinol, which is known as

an inhibitor of xanthine oxidase (Gomez-Cabrera et al., 2005). Although in our studies (Jówko et al., 2011; and unpublished data) GTE intake did not affect superoxide dismutase activity, it cannot be excluded that inhibition of NF-κB activation by high doses of GTCs could prevent a training-induced adaptive increase of other antioxidant enzyme activities. From the above point of view, the need for antioxidant supplementation for athletes on a well-balanced diet should be re-evaluated. However, in the case of a low intake of antioxidant nutrients, an antioxidant-rich diet appears to be a prudent recommendation (Panza et al., 2008). It may be a matter of concern for some athletes practising weight control sport and those who, for some reason, do not eat a well-balanced diet (Williams, 2004).

In addition, it appears that only moderate regular exercise can attenuate oxidative stress via mild generation of ROS, inducing hormesis-like effect, manifesting upregulation of antioxidant enzymes, repair enzymes and enzymes responsible for degradation of potentially harmful damaged molecules (Goto et al., 2007; Gomez-Cabrera et al., 2008a). These adaptive responses protect the body against more severe stresses in future (Ji et al., 2010). On the other hand, excessive generation of ROS by exhaustive exercise may be harmful to unprepared cells (Goto et al., 2007). Based on these data, antioxidant supplementation can be recommended to limit the effects of oxidative stress in athletes involved in heavy training (Williams 2004) or before competition (Gomez-Cabrera et al., 2008b). In a study by Margonis et al. (2007), severe resistance training decreased antioxidant potential in plasma and increased lipid peroxidation products in plasma and urine, and these changes were highly correlated with performance drop and training volume increase. Although in our study (unpublished) GTE supplementation did not attenuate exercise-induced muscle damage in sprinters during their preparatory period, it cannot be excluded, however, that these effects could be observed in terms of a more intense period of training. Thus, further studies are needed to evaluate whether chronic GTE supplementation may be beneficial in athletes during their competition period.

8.5 CONCLUSION

Consumption of GTCs has been shown to increase fat oxidation and energy expenditure, particularly if combined with caffeine. This effect was seen in both sedentary and physically active individuals during exercise. Thermogenic properties of green tea seem to be beyond that explained by its caffeine content. The mechanisms by which GTCs may stimulate fat oxidation and energy expenditure include, among others, inhibition of COMT and prolonged stimulation of the sympathetic nervous system by norepinephrine. Moreover, GTCs as antioxidants may block the activation of the oxidative stress-sensitive transcription factor NFκB and, in turn, activate peroxisome proliferator activated receptors that are important transcription factors for lipid metabolism. The above-mentioned mechanisms of action may explain the positive effect of green tea extract with caffeine on weight loss and on weight maintenance, which were found in a population of overweight and moderately obese individuals. However, these effects can be influenced by different modulators like ethnicity or habitual caffeine intake. Moreover, more studies are needed to evaluate the efficiency of GTC–caffeine mixture on body weight and body composition in

non-obese individuals, with potential implications for body weight control in athletes. In addition, the optimal dose of GTCs, as well as side effects, especially of high doses of GTCs, has not yet been established so far.

As evidenced in animal studies, increased fat oxidation during aerobic exercise as a result of GTC ingestion could have a glycogen-sparing effect, which can result in an improvement of endurance capacity. However, these observations cannot be confirmed by human studies. Moreover, chronic but not acute ingestion of GTCs can increase the antioxidant potential in plasma and alleviate both oxidative stress and muscular damage induced by exhaustive exercise. However, taking into account that excessive intake of antioxidant supplements may hinder training adaptations, further studies with GTE supplementation are needed in regard to trained athletes, especially in the heavy training period. It is difficult to answer the question regarding whether athletes need antioxidant supplements to counter increases in oxidative stress from exercise. It is still unknown at what level of oxidative stress the potential benefits will outweigh the risks. Thus, more research is necessary to address these issues and to provide guidelines for recommendations to athletes.

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9 Acute and Chronic Effects of Antioxidant Supplementation on Exercise Performance

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CONTENTS

9.1 INTRODUCTION

Reactive oxygen and nitrogen species (RONS), also known as free radicals, are continually produced within the body as part of normal oxidative metabolism (Finaud et al. 2006; Powers et al. 2011). These molecules act as intracellular messengers and are necessary for proper physiological function (Dröge 2002; Niess 2005). However, high concentrations of RONS can be toxic and cause significant oxidative damage to the cellular structure of lipids, protein and DNA (Halliwell and Gutteridge 1999; Powers et al. 2004). The concentration of RONS within the body is controlled by an extensive antioxidant system, which works to scavenge free radicals (Halliwell and Gutteridge 1999; Powers et al. 2004). Antioxidants (AOX) are present in both the intra- and extra-cellular matrix forming a complex defence system to protect cells and tissue against oxidative damage (Powers and Lennon 1999). The antioxidant

141

defence system is commonly divided into enzymatic (endogenous) and non-enzymatic (exogenous) AOX.

Seifried et al. (2007) define AOX as a group of compounds characterised by their ability to be oxidised in place of other compounds present. The main enzymatic AOX include superoxide dismutase, catalase and glutathione peroxidase. Vitamin A, vitamin C, vitamin E, thiols, flavonoids and ubiquinones represent the main non-enzymatic AOX, which can be obtained in a conventional diet and with supplementation. This has led to the suggestion that AOX supplementation may result in an acute improvement in skeletal muscle contractile performance (MacRae and Mefferd 2006; Oh et al. 2010). This hypothesis is based on the finding that the rapid elevation of oxidant concentration during exercise may be a contributory factor to muscle fatigue (Reid et al. 1994; Gomez-Cabrera et al. 2008). However, other reports have stated that AOX supplementation in combination with exercise training may blunt exercise-induced biochemical adaptations to exercise (Reid 2001; Watson et al. 2005). This chapter provides a comprehensive overview of the acute exercise responses and longer term adaptations to the common antioxidant supplements.

Prolonged exercise training induces marked changes in physiological function and skeletal muscle contractile performance (Kubukeli et al. 2002; Laursen and Jenkins 2002). The oxidant–antioxidant balance during exercise has been shown to greatly influence muscular contraction (Clarkson and Thompson 2000) and adaptation to physical training (Palazzetti et al. 2003). It has been proposed that optimising skeletal muscle oxidant concentration by consuming antioxidant substances (acutely) results in a greater force production and power output during prolonged high-intensity endurance exercise (Poulsen et al. 1996; Alessio et al. 2000). However, studies have also shown that physiological adaptations to exercise may be blunted when oxidant production is suppressed by AOX (Palazzetti et al. 2003; Watson et al. 2005). Therefore, consideration should be made of the research concerning both acute supplementation with AOX and exercise performance and also more chronic consumption and the possible consequences. The type of AOX substance/content is also important when evaluating the potential use and physiological significance of the different AOX supplements.

9.2   REACTIVE OXYGEN SPECIES, EXERCISE PERFORMANCE AND FATIGUE

An elevated concentration of oxidants within the skeletal muscle may cause oxidative damage to the mitochondria and muscle contractile proteins, interfering with the excitation–contraction coupling process (Vollaard et al. 2005; Powers et al. 2011). However, *in vitro* studies have also shown that myofibril contraction is inhibited when RONS production is suppressed, prompting the suggestion that optimal force production requires the maintenance of a moderate level of oxidants within the muscle during exercise (Reid 2001; Gomez-Cabrera et al. 2008). Based on these findings, an inverted-U shaped model of cellular redox balance and contractile function has been proposed as shown in Figure 9.1 (Reid 2001).

It is well established that RONS production is increased following most exercise types, durations and intensities in a dose-dependent manner (Ashton et al. 1998;
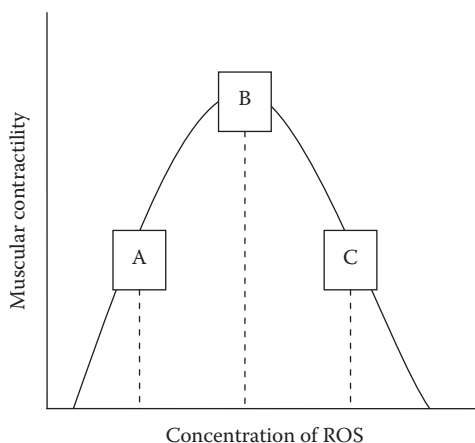
FIGURE 9.1 Optimal redox state for skeletal muscle contraction. A is the basal level of ROS within the muscle, B is the optimal level of ROS for contractile function and C is the excessive amount of ROS inhibiting muscle function and inducing fatigue.

Alessio et al. 2000; Aguiló et al. 2005; Watson et al. 2005; Finaud et al. 2006). In order to prevent the occurrence of severe oxidative damage during exercise, the body must continually re-enforce its antioxidant protection. Physiological adaptation to oxidative stress relies on the process of redox signalling (Dröge 2002). When an increased production of RONS occurs, redox signalling is used to induce protective mechanisms, predominately the up-regulation of antioxidant responses, to restore the redox homeostatic balance (Dröge 2002). During exercise, the redox balance is disturbed due to the exercise-induced increase in free radical production (Ashton et al. 1998). This initiates a redox signalling cascade, which leads to an increase in antioxidant enzyme expression and facilitates the mobilisation of exogenous AOX (Atalay et al. 1996; Miyazaki et al. 2001; Aguiló et al. 2003; Groussard et al. 2003). Allowing the body to adapt to the oxidative stress through improving the antioxidant defence system enables the re-establishment of redox homeostasis.

The balance between oxidant production and antioxidant removal is vital to the regulation of cellular functions (Banerjee et al. 2003). Low concentrations of free radicals are necessary for proper regulation of cellular function, but higher concentrations can lead to cellular damage and oxidative stress (Halliwell and Gutteridge 1999; Lachance et al. 2001; Reid 2001).

If the antioxidant response is insufficient or free radical production is chronically increased, the body may not be able to return to the previous level of redox homeostasis (Dröge 2002). Instead, the oxidant/antioxidant balance will find a new point of equilibrium, which will have a substantially increased concentration of free radicals. The new higher equilibrium point may affect the redox signalling pathways, resulting in altered patterns of gene expression and an inability to adapt to further oxidative stress (Dröge 2002).

A pro-oxidant shift in redox homeostasis has been observed in several pathological diseases such as diabetes, cancer, rheumatoid arthritis and ageing (Clarkson and

Thompson 2000; Gomez-Cabrera et al. 2008). It is likely that intensified periods of physical training with minimal recovery may also perturb redox homeostasis, inducing a state of chronic oxidative stress (Itoh et al. 2000; Santos-Silva et al. 2001; Palazzetti et al. 2003). Chronic oxidative stress may then inhibit physiological adaptations to exercise and contribute to negative exercise physiological states including the development of overreaching and, in severe cases, overtraining (Palazzetti et al. 2003). It is therefore important to control the production of free radicals within the body in order to maintain redox homeostasis and normal physiological function.

Under conditions of physiological stress such as during intense exercise, free radical production increases dramatically, altering the redox state of the muscle and possibly inhibiting muscle contractile function (Reid et al. 1994; Powers et al. 2011). The resulting consequence would be an increased onset of muscle fatigue and reduction in exercise performance. However, it has been postulated that the consumption of AOX during exercise may assist in the maintenance of an optimal level of RONS within the skeletal muscle. Antioxidant substances may help neutralise free radicals and thereby prolong skeletal muscle integrity and prevent a decline in performance (Morillas-Ruiz et al. 2005; Oh et al. 2010).

9.3   ANTIOXIDANT SUPPLEMENTATION AND EXERCISE ADAPTATION

9.3.1   Chronic Adaptation to Exercise Training and Antioxidant Supplementation

The theory of training highlights the importance of imposing a certain amount of stress upon the body in order to stimulate physiological adaptations. Exercise is a significant stressor to the body, which initiates adaptive processes in biological systems to accommodate the increase in physical work demand. The outcome of regular exercise training is largely known including physiological adaptations such as improved cardiovascular function and skeletal muscle respiratory capacity as well as improved functional performance (Bigard et al. 1996; Billat et al. 1999).

It has become increasingly evident that RONS act as intra-cellular messengers to stimulate changes in cell function and regulate gene expression (Pattwell and Jackson 2004). Nuclear factor kappa-B (NF-κB) is one of the most commonly investigated redox sensitive transcription factors. Some authors believe that activation of NF-κB may be an important regulator of adaptation to exercise training (Ji et al. 2004; Ho et al. 2005; Cuevas et al. 2005). This hypothesis is based on several factors including the NF-κB induced up-regulation of antioxidant enzymes to maintain red-ox homeostasis (Zhou et al. 2001), the inflammatory response associated with NF-κB on skeletal muscle (Ortega et al. 1999) and the reduced binding capacity of NF-κB during fatiguing exercise (Durham et al. 2004).

Exercise-induced increases in RONS production are likely to stimulate the red-ox sensitive NF-κB, resulting in the up-regulation of antioxidant gene expression (Hollander et al. 2001; Ho et al. 2005). An example of this has been shown by Hollander et al. (2001) where a significant increase in the binding capacity of NF-κB and subsequent up-regulation of the antioxidant enzyme superoxide dismutase was

observed in response to a single bout of exercise in rat skeletal muscle. Moreover, in a study by Gomez-Cabrera et al. (2005), inhibition of xanthine oxidase-mediated RONS production by the antioxidant allopurinol significantly blunted the exercise-induced increase in NF-κB and prevented increases in antioxidant enzymes from occurring.

These studies show the importance of NF-κB activation to up-regulate antioxidant protection following exercise (Ji et al. 2004; Fernández et al. 2005). Individuals who regularly participate in physical activity display an augmented endogenous antioxidant enzyme capacity (Brites et al. 1999) and an increased tolerance to exercise-induced oxidative stress (Brites et al. 1999; Pittaluga et al. 2006). Without a dynamic and adaptable antioxidant defence system, the body would not be able to cope with increasing amounts of oxidant production and a state of chronic oxidative stress may occur (Dröge 2002). Consequently, it may be detrimental to the endogenous antioxidant defence system if the exercise-induced redox signalling processes are blunted by exogenous AOX supplementation. It is hypothesised that chronic antioxidant supplementation may blunt training adaptations and be harmful to exercise performance (Reid 2001; Watson et al. 2005). This is supported by a study where triathletes who regularly used antioxidant supplements incurred a greater amount of oxidative stress following an Ironman race than those who did not use antioxidant supplements (Knez et al. 2007). However, not all investigations have shown that AOX supplementation impedes exercise-induced activation of redox sensitive signalling pathways (Petersen et al. 2012). It has also been demonstrated that quercetin supplementation (1000 mg/day) promotes skeletal muscle mRNA expression of genes involved in mitochondrial biogenesis in 26 previously untrained males during a 2-week physical training period (Nieman et al. 2010). These studies highlight the difference in AOX supplementation strategies that have been used in combination with exercise training and also that it may be more beneficial to only increase antioxidant consumption during periods of elevated training stress. It is also likely that a dose-response effect could be evident, where the amount of AOX required to optimise oxidant content in the skeletal muscle is relative to the type and amount of exercise undertaken. This is yet to be fully investigated and should be a focus of future antioxidant research.

9.3.2 Acute Exercise Responses to Antioxidant Supplementation

The majority of studies have focused on the use of AOX to minimise exercise-induced oxidative stress, muscle damage and inflammation (Tauler et al. 2006; Gomez-Cabrera et al. 2005; Mastaloudis et al. 2004; Thompson et al. 2004; Morillas-Ruiz et al. 2005). While these data are relevant for the potential efficiency of AOX supplementation as a strategy for optimising adaptations to exercise training, there is less research available on the potential (acute) ergogenic effect of AOX on muscle contractile performance during exercise.

During exercise/muscle contraction antioxidant stores may become depleted, leaving the body susceptible to oxidative damage (Powers et al. 2004; Morillas-Ruiz et al. 2005). Numerous studies have investigated the effects of antioxidant supplementation on exercise-induced oxidative stress with equivocal results. This is most likely due to the wide variety of exercise/supplementation protocols including the length of supplementation period and type of AOX and the exercise task used by

each research group. Many investigations have tested one particular AOX substance, yet it appears that a combination of different AOX could be more effective at combating RONS than a single substance (Tauler et al. 2006; MacRae and Mefferd 2006; Bloomer et al. 2006). This reflects the complex nature of the antioxidant defence system, whereby each antioxidant is most efficient at quenching a certain type of radical species.

Medved et al. (2004) reported a 26.3% increase in cycling time to fatigue at 90% $\dot{V}O_2$ peak when N-acetylcysteine (NAC) was intravenously infused in eight endurance trained men. Kingsley et al. (2005) observed that supplementation with 750 mg·day^{-1} phosphatidylserine for 10 days had a tendency to improve time to exhaustion in a shuttle running test. These studies support the idea that a single AOX supplement can improve exercise performance. An investigation by MacRae and Mefferd (2006) reported that the addition of the flavonoid quercetin to a liquid antioxidant supplement (green tea extract, vitamin C, vitamin E, caffeine, niacin, taurine and vitamin B groups) significantly enhanced the antioxidant effect of the supplement and resulted in a 3.1% performance improvement during a 30 km cycle time trial. Hence, it is possible that a combination of AOX compounds may induce larger effects on exercise performance. However, in contrast, other investigations have not shown any improvement in exercise performance after a period of AOX supplementation. For instance, no increase in time to exhaustion in moderately fit men was found by Romano-Ely et al. (2006) when the subjects consumed a carbohydrate–protein–antioxidant drink containing vitamins E and C.

The majority of studies examining the effects of AOX supplementation have investigated the effects on endurance exercise performance. Resistance or weight training is known to induce considerable increases in RONS (Bloomer 2007). There are very few studies that have examined the effects of AOX supplementation (short or long term) on resistance exercise performance. In one study, Lafay et al. (2009), using a double-blind crossover design, investigated the influence of 4 week supplementation (400 mg/day) of a grape extract rich in polyphenols or a placebo on jumping force capacity. After 4 weeks of polyphenol supplementation, there was a 19% improvement in work capacity during 45 s of bodyweight squat jumps (Lafay et al. 2009). Similarly, 30 days of supplementing with a vitamin C and E combination helped attenuate a decline in maximal force production, peak concentric torque and total work performed during 300 consecutive isometric contractions (Shafat et al. 2004). Despite the equivocal findings, these results suggest that the acute consumption of AOX during exercise may assist in the maintenance of an optimal level of RONS within the skeletal muscle and lead to improvements in both endurance and resistance training performance.

9.4 SPECIFIC TYPES OF ANTIOXIDANT COMPOUNDS AND EXERCISE

9.4.1 Vitamins E, C and Carotenoids

The vitamin E family consists of at least eight structural isomers, with α-tocopherol displaying the most potent antioxidant properties (Burton et al. 1982). Vitamin E is a phenolic compound and is capable of hydrogen donation, which can convert

superoxide and hydroxyl radicals to more stable forms (Burton et al. 1982; Powers et al. 2004). Vitamin E is lipid soluble and is found in lipid-rich structures such as the sarcoplasmic reticulum and cellular membrane where it is capable of scavenging free radicals produced from the mitochondria (Ji 1995).

Vitamin C (ascorbic acid) is water soluble and is present in the cytoplasm of the cell. Vitamin C can scavenge for superoxide and hydroxyl radicals and also help to recycle vitamin E back to its reduced state. Vitamin C also functions as a pro-oxidant by reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) (Aust et al. 1985; Powers et al. 2004; Yu 1994). Carotenoids are similar to vitamin C in that they are capable of antioxidant and pro-oxidant functioning. Both types are also lipid-soluble AOX mostly located in cellular membranes and are effective in preventing lipid oxidation by quenching free radicals such as singlet oxygen (Yu 1994; Powers et al. 2004).

The majority of studies have attempted to examine the longer term effects of vitamin E or C supplementation on exercise performance and antioxidant capacity (Kanter et al. 1993; Mastaloudis et al. 2004). Vitamin C and E supplementation for 6 weeks has been shown to decrease lipid peroxidation (F-2-isoprostanes) after endurance exercise (Mastaloudis et al. 2004). Similarly, 600 mg of vitamin E supplementation for 6 weeks also decreased lipid peroxidation (MDA) after 30 min of exercise (Kanter et al. 1993). There is evidence to suggest that the ergogenic effect of vitamins A, C and E during exercise is minimal as 500 mg vitamin C supplementation per day for 1 month did not improve maximal aerobic capacity ($\dot{V}O_{2max}$) in healthy young males. Furthermore, vitamin E supplementation (900 IU/day) for 6 months did not improve swimming performance over the study period (Bell et al. 2005; Lawrence et al. 1975). An antioxidant cocktail mixture including vitamins A, C and E and orally consumed for 6 weeks had no ergogenic properties during a 30 km cycling time trial (TT) in well-trained male cyclists (MacRae and Mefferd 2006). These results suggest that elevated ingestion of these vitamins did not optimise oxidant content within the skeletal muscle or have any effect on muscle contractile function and therefore did not improve exercise performance.

9.4.2 GLUTATHIONE AND N-ACETYLCYSTEINE SUPPLEMENTATION

Glutathione (GSH) is the most abundant low-molecular weight thiol present in muscle cells and the intracellular levels of GSH are normally around 0.5 mM (Yu 1994). Glutathione can react with free radicals, such as the hydroxyl radical and carbon radicals, by donating a hydrogen atom and is needed by the body to remove both hydrogen and organic peroxidases such as lipid peroxide (Powers et al. 2004).

N-Acetylcysteine is a reduced thiol donor with antioxidant properties that support glutathione synthesis. Importantly, it is the acetylated derivative of cysteine which is the rate limiting step in glutathione synthesis (Sen 2001; Ferreira and Reid 2008).

Studies using isolated muscle preparations have shown a reduction in fatigue in NAC-treated rabbit diaphragm muscles and NAC infusion has also been shown to improve the functioning of skeletal muscle (Reid et al. 1994). Similarly, in a double-blind crossover study, transcutaneous electrical stimulation was employed to stimulate the tibialis anterior muscle for 30 min in healthy young males with and without NAC infusions (150 mg/kg). The NAC infusion trial resulted in an improved fatigue

resistance of, on average, 15% during the trial. However, as transcutaneous stimulation is not a normal physiological process, the practical application of these findings on exercise performance is limited (Ferreira and Reid 2008; Reid et al. 1994). More recent work involving endurance-trained participants has demonstrated that intravenous infusion of NAC at 125 mg·kg^{-1} h^{-1} prior to exercise (20 min) and then during the exercise protocol at a rate of 25 mg·kg^{-1} h^{-1} improved endurance cycling time to fatigue (Medved et al. 2004; McKenna et al. 2006). Participants cycled for 45 min at a workload that corresponded to 71% peak $\dot{V}O_{2max}$; they then cycled to fatigue at a workload of 92% $\dot{V}O_{2max}$. During the NAC trials, time to fatigue at 92% $\dot{V}O_{2max}$ increased by 23.8% and 26.3%, respectively, during two separate studies (Medved et al. 2004; McKenna et al. 2006). While these results show a positive effect of NAC on exercise performance, the effect of NAC via methods other than infusion needs to be established. Oral NAC supplementation of 1800 mg per day for 4 days increased knee extensor endurance during sub-maximal knee extensions and an oral solution containing 150 mg kg^{-1} of NAC improved the endurance of handgrip repetitions during sub-maximal handgrip contractions (Koechlin et al. 2004; Matuszczak et al. 2005). In a recent study, Slattery et al. (2014) have shown that an oral dose of NAC (1200 mg/day for 9 days) improved repeat sprint performance during a cycle ergometer race simulation. Not all studies have shown that NAC improves exercise performance. NAC infusion did not have any effect on total work production during three short intensive cycling bouts (45 s/bout) in healthy male subjects and it did not improve time to fatigue at 130% $\dot{V}O_{2max}$ (Medved et al. 2003). The authors suggested that NAC supplementation did not improve performance because of the high force requirements of the exercise task. The available studies suggest that NAC delays fatigue in moderate intensity exercise, but NAC appears to have few ergogenic properties during severe high-intensity exercise tasks (Ferreira and Reid 2008).

9.4.3 FLAVONOIDS

Flavonoids are commonly found in edible plants and are diphenylpropanes which include family members such as flavones, isoflavones and anthocyanins (Cao et al. 1997). Polyphenolic flavonoids are capable of scavenging superoxide, hydroxyl and peroxyl free radicals and are therefore potent AOX (Powers et al. 2004).

Quercetin is an example of one such polyphenolic flavonoid substance that has been shown to improve exercise performance (MacRae and Mefferd 2006; Davis et al. 2010). In a recent meta-analysis containing data from 11 studies on human participants, quercetin was reported to have a small but significant ergogenic effect on endurance performance (Kressler 2011). Indeed, Davis et al. (2010) utilised a placebo- controlled crossover design to investigate the effects of a 7-day quercetin supplementation protocol (500 mg twice per day) on time to fatigue at 75% $\dot{V}O_{2max}$ during a cycle ergometer trial in healthy male and female participants. Following quercetin supplementation, time to fatigue increased by 13% (105 min compared to 93 min) when compared to the placebo trial (Davis et al. 2010). A beneficial effect of quercetin on exercise was also observed by Nieman et al. (2010) using a double-blind crossover design in male participants supplemented with 1 g/day of quercetin for 2 weeks. The participants walked on a treadmill for 60 min at an intensity of 60%

$\dot{V}O_{2max}$ and then had 12 min to cover as much distance as possible. Quercetin supplementation significantly increased the distance covered in the 12 min distance trial by ~3% compared to the placebo trial. There was also a 3% improvement in 30 km time trial performance in trained male cyclists after 6 weeks of quercetin supplementation (MacRae and Mefferd 2006). Nonetheless, these improvements in performance with quercetin supplementation have not been consistently reported (Ganio et al. 2010). Exercise performance has been shown to improve following supplementation with other flavonoid antioxidant substances. In a recent study, ecklonia cava polyphenol (ECP) was shown to improve time to fatigue during a running trial (Oh et al. 2010). Using a double-blind crossover design, healthy male subjects consumed either a liquid placebo solution or a liquid ECP solution containing 60 mg of ECP 30 min before exercise. The acute polyphenol supplementation with ECP significantly increased time to fatigue by around 2.5 min (Oh et al. 2010). These results may be either due to ECP's ability to scavenge excessive RONS or to the ability of polyphenol flavonoids to exert a vasodilatory effect which may promote blood flow to the working muscles (Pietta 2000; Oh et al. 2010).

Pycnogenol (PYC) is a commercially available pine bark extract containing oligomeric proanthocyanidins (OPC), which has been shown to be associated with a variety of clinical therapeutic benefits (Slayback and Watson 2002; D'Andrea 2010). However, the effects of PYC supplementation on exercise performance have received little scientific investigation. In one study by Pavlovic (1999), PYC supplementation was evaluated using a double-blind crossover design incorporating athletic males who were supplemented with 200 mg of either a placebo or PYC per day for a month. The exercise test was a time to fatigue protocol at 85% $\dot{V}O_{2max}$ and there was a significant 21% increase in this parameter after a month of PYC supplementation.

In a more recent investigation, Clifford et al. (2013) found that orally consuming 120 mg of PYC with 600 mg of bioflavonoids (PYC-B) had a positive influence on cycling performance. Using a double-blind crossover design, trained cyclists and triathletes supplemented with either a placebo or the PYC-B supplement for 3 days prior to a 20 km time trial. The PYC-B supplement enabled participants to significantly enhance their power output in the final 5 km of the trial and improve their time by 3.8 s in the final 1 km.

Pycnogenol is the active ingredient of a new commercially available supplement 'cocktail'. The acute effects of this supplement have been investigated in both endurance and resistance exercise modes (Bentley et al. 2012; Mach et al. 2010). In a double-blind crossover design study, the effects of a single dose of this supplement on time to fatigue in an endurance cycle exercise in trained and untrained subjects were investigated. The subjects consumed 150 mL of liquid placebo or 150 mL of the supplement containing 360 mg of PYC prior to exercise. The subjects cycled at workloads of 50% and 70% peak power output for 4 min per stage; then time to fatigue was assessed at 95% peak power output, and PYC supplementation significantly increased time to fatigue in both trained and untrained cyclists by approximately 17% (Mach et al. 2010). This was subsequently confirmed by another separate investigation in trained cyclists (Bentley et al. 2012). These studies indicate that, depending upon the supplement type and dosing period, PYC has potential acute ergogenic effects, which are in contrast with those of other AOX compounds.

9.5   SUMMARY

In summary, RONS or free radicals are required at low concentrations for many important physiological functions. However, the dramatic increase in RONS during severe exercise can damage cell membranes and interfere with excitation contraction coupling, having deleterious effects on skeletal muscle performance. An increase in RONS disturbs redox balance within the muscle, prompting red-ox signalling to up-regulate the use of AOX, particularly exogenous AOX. It has therefore been suggested that antioxidant supplements may be able to assist exercise performance by reducing the excessive exercise-induced oxidative stress response. However, other important chronic physiological adaptations to exercise may be blunted if skeletal muscle oxidant concentration is too low. Nonetheless, as skeletal muscle requires a moderate oxidant concentration to optimise force production, it has been suggested that acute doses opposed to chronic consumption of AOX may be more beneficial to exercise performance. Numerous antioxidant supplements have been studied for their ergogenic potential including vitamins A, C and E, n-acetylcysteine and various flavonoids. While not all studies have found positive results on exercise performance or adaptations, many investigations have demonstrated that antioxidant supplementation, particularly when combined as a 'cocktail', can have an ergogenic effect on both high intensity endurance and resistance exercise performance.

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10 Evaluation of Quercetin as a Countermeasure to Exercise-Induced Physiological Stress

Manuela Konrad and David C. Nieman

CONTENTS

10.1 INTRODUCTION

Polyphenols are a large class of colourful, plant-based, phenolic organic compounds (USDA 2007). They are enriched in certain vegetables, fruits, seeds and beverages (e.g. tea and wine) and are regarded as semi-essential nutrients in humans.

Flavonoids, a polyphenolic subgroup, provide many of the colours in fruits and vegetables (Nieman et al. 2010a). As a natural antioxidant, flavonoids constitute significant components of the human diet and exhibit a diverse array of biological effects (Kandaswami and Middleton 1994, Korkina and Afanas'ev 1997, Li et al. 2000, Middleton et al. 2000).

Flavonoids compromise a large group of plant metabolites, 6000 of which have been identified to date (Erdman et al. 2007) and can then be divided into six subgroups. One of these six groups is flavonols, which it contains the abundant and diffuse flavonoid quercetin (Nieman et al. 2010a). Food-based sources of quercetin include tea, onions, apples, peppers, blueberries and dark green vegetables (Chun et al. 2007, USDA 2007).

The intake of these compounds improves an individual's health and decreases their risk of cardiovascular disease (Korkina and Afanas'ev 1997, Kim et al. 2004, Scalbert et al. 2005).

10.2   ABSORPTION, BIOAVAILABILITY AND METABOLISM OF QUERCETIN

The first investigations on the pharmacokinetics of quercetin in humans were published in 1975 (Gugler et al. 1975), and the amount of research in this area has increased enormously.

The estimated absorption of quercetin glucoside, the naturally occurring form of quercetin, ranges from 3% to 17% in healthy individuals receiving 100 mg. The relatively low bioavailability of quercetin may be attributed to its low absorption, extensive metabolism and/or rapid elimination. Quercetin absorption is affected by differences in its glycosylation, the food matrix from which it is consumed, and the coadministration of dietary components such as fibre. Recent data indicate that the bioavailability of quercetin increases with the co-ingestion of fat (Guo et al. 2013).

The average terminal half-life of quercetin is 3.5 h. The total recovery of C-quercetin in urine, faeces and exhaled air is highly variable, depending on the individual (Moon et al. 2008). These results are consistent with those of other authors who have measured quercetin absorption and appearance in plasma after ingesting the pure quercetin aglycone as well as various glycoside forms contained in foods such as shallots, onions and apples (Egert et al. 2008). Additional literature indicates that isoquercetin (glycosylated quercetin) is more completely absorbed than is quercetin in the aglycone form, and that the simultaneous ingestion of quercetin with vitamin C, folate and additional flavonoids improves bioavailability (Manach et al. 2005, Harwood et al. 2007).

Quercetin accumulates in the outer and aerial tissues (skin and leaves) because biosynthesis is stimulated by exposure to sunlight. Human subjects can absorb significant amounts of quercetin from food or supplements, and elimination is quite slow, with a reported half-life ranging from 11 to 28 h (Conquer et al. 1998, Manach et al. 2005).

A more recent study showed that flavonol intake is about 13 mg/day for U.S. adults, and quercetin represents three-quarters of this amount. The estimated flavonoid intake ranges from 50 to 800 mg/day, both depending on the consumption of fruits and vegetables and the intake of tea (Chun et al. 2007). In Spain, however, the average daily intake of flavonoids is significantly higher than in the United States and was measured at 313 mg/day based on sources like tea, citrus fruits and juice, beers and ales, wines, melon, apples, onions, berries and bananas (Zamora-Ros et al. 2010).

10.3   QUERCETIN AND SAFETY

Quercetin has GRAS status (generally recognised as safe) according to criteria established by the U.S. Food and Drug Administration (Davis et al. 2009a). Not only is quercetin accepted as safe, but the European Food Safety Authority has

published a number of health claims finding that quercetin has beneficial physiological effects in the protection of DNA, proteins and lipids from oxidative damage (EFSA 2011).

In studies on both animals and humans, quercetin supplementation is regarded as medically safe and has not been found to cause any adverse symptoms or harmful physiological effects (Harwood et al. 2007, Henson et al. 2008, Utesch et al. 2008, Davis et al. 2009a, Knab et al. 2011).

Long-term feeding of quercetin in rats leads to accumulation in several organs including the lungs, testes, kidneys, heart, liver, thymus and muscle (de Boer et al. 2005). It was not possible to replicate this finding, however, in pigs, where quercetin was found only in organs involved in its metabolism and excretion, namely the small intestine, kidneys and liver (Bieger et al. 2008).

These data question the degree to which quercetin is incorporated into human tissues including the lung, heart and muscle. Further research is needed using tissue biopsies and radiolabelled procedures (Nieman 2010).

10.4   ROLE OF SUPPLEMENTATION TO COUNTER OXIDATIVE STRESS

During strenuous exercise, there is a dramatic increase in oxygen uptake in various organs, particularly the skeletal muscle. The resting body is equipped with both enzymatic and non-enzymatic antioxidant reserves (Morillas-Ruiz et al. 2006). Cells continuously produce free radicals and reactive oxygen species (ROS) as part of metabolic processes. These free radicals develop an antioxidant defence system consisting of enzymes such as catalase, superoxide dismutase, glutathione peroxidase and numerous non-enzymatic antioxidants, including vitamins A, E and C, glutathione, ubiquinone and flavonoids. Exercise, though generally recognised as healthy and advantageous, can also produce an imbalance between ROS and antioxidants, which is referred to as oxidative stress. Physical activity increases the generation of free radicals in several ways and as oxidative phosphorylation increases in response to exercise, there will be a concomitant increase in free radicals. Catecholamines released during exercise can lead to free radical production. Other sources of free radicals increase with exercise-induced prostanoid metabolism, xanthine oxidase, NAD(P)H oxidase and several secondary sources, such as the release of radicals by macrophages recruited to repair damaged tissue (Jackson 2000).

In order to cope with the excess of free radicals produced upon oxidative stress, the human body has developed mechanisms for maintaining redox homeostasis. These protective mechanisms include scavenging or detoxifying ROS, blocking ROS production, sequestering transition metals, as well as enzymatic and non-enzymatic antioxidant defences produced in the body, that is, endogenous (Hayes and McLellan 1999, Masella et al. 2005) and others supplied within the diet, namely, exogenous ones.

Among them, dietary polyphenols have been widely studied for their strong antioxidant capacities and cell regulatory properties (Hollman et al. 1997, Hartman et al. 2006, Landete 2012).

Athletes use antioxidant supplementation as a means to counteract the oxidative stress of exercise. Whether or not strenuous exercise does, in fact, increase the need for additional antioxidants in the diet is not clear. If the increase in free radicals is greater than the ability to neutralise them, the radicals will attack cellular components, especially lipids. The attack on lipids initiates a chain reaction called lipid peroxidation, which leads to the generation of more radicals and ROS that can harm other cellular components. The body appears to be able to withstand a limited increase in free radicals and, in fact, data suggest that an increase in ROS is necessary for muscle adaptation to occur (Jackson 1999, Urso and Clarkson 2003).

As previously discussed, intensive and sustained exercise can create an imbalance between ROS and antioxidant defences, leading to oxidative stress that causes lipid peroxidation and protein oxidation (Nieman et al. 2003, Mastaloudis et al. 2006).

Although pathways between oxidative stress during heavy exertion and immune dysfunction have been described, data support is widely lacking (Nieman et al. 2003). Moreover, the proposed benefits of antioxidant supplementation in attenuating both oxidative stress and exercise-induced immune dysfunction remain unsubstantiated (Petersen et al. 2001, Nieman et al. 2003, Mastaloudis et al. 2006).

Taking into account all of the results of human studies, antioxidant supplementation to counter both oxidative stress and immune dysfunction in endurance athletes during heavy exertion cannot be recommended. The majority of investigations have failed to show that the ingestion of antioxidants such as vitamins E and C has meaningful effects on exercise-induced inflammation and muscle damage, and immune perturbations (Nieman 2008).

Quercetin is a powerful *in vitro* antioxidant and free radical scavenger (Loke et al. 2008). Quercetin (aglycone form) has several phenolic OH groups that protect against free radical damage via radical scavenging activity (Santos and Mira 2004). However, low bioavailability and metabolic transformation (i.e. conjugation of absorbed quercetin with glucoronic, sulphur and methyl groups) reduce the likelihood of *in vivo* scavenging activity (Loke et al. 2008). The majority of human studies conclude that supplementation with quercetin in its aglycone form does not exert antioxidant effects even in daily doses of up to 1000 mg over 12 weeks (Boyle et al. 2000, Egert et al. 2008, Knab et al. 2011).

McAnulty et al. (2008), for example, reported no effect of quercetin supplements in countering exercise-induced oxidative stress or other indicators of physiologic stress. Forty athletes were recruited and randomly given either quercetin or a placebo. Subjects consumed either 1000 mg of quercetin or the placebo every day for 3 weeks before and during 3 days of cycling at 57% work maximum for 3 h. The findings from this study indicate that quercetin supplementation increased the circulating plasma values of quercetin; however, the increase in plasma quercetin metabolites did not affect oxidative stress, inflammation or plasma antioxidant capacity (McAnulty et al. 2008).

Quindry et al. (2008) tested quercetin as an antioxidant countermeasure during the 160-km Western States Endurance Run, where oxidative stress is high due to the length and effort involved. In the double-blind study, 63 subjects received either 1000 mg of quercetin or a placebo. Biomarkers of plasma antioxidant status were not influenced by quercetin after three weeks ingestion (Quindry et al. 2008).

Abbey and Rankin (2011) also failed to reduce oxidative stress via the inhibition of the enzyme xanthine oxidase after 1 week of supplementation with 1000 mg of quercetin in a repeated sprint performance (Abbey and Rankin 2011).

Quercetin's anti-inflammatory and anti-oxidative effects may be augmented by the co-ingestion of N-3 polyunsaturated fatty acids (Camuesco et al. 2006, Mostafavi-Pour et al. 2008) and epigallocatechin 3-gallate (EGCG) (Ivanov et al. 2008). For example, the concurrent administration of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) with quercetin resulted in a synergistic anti-inflammatory effect in rats with inflammatory intestinal disorders (Camuesco et al. 2006). *In vitro* data indicate that quercetin exhibits antiviral activity only when protected against oxidative degradation by ascorbate (Vrijsen et al. 1988). Another potential weakness of these early human studies was the supplementation regimen. Quercetin supplements were ingested 10–24 h prior to doing exercise, a time period that may have been too long, given the half-life of quercetin (Manach et al. 2005, Moon et al. 2008).

Subjects of both sexes in the general population ($n = 1002$) were recruited and had a quercetin intake of 500 mg or 1000 mg/day or were on placebo, respectively, over 12 weeks' time. As a consequence, the plasma quercetin level was significantly increased, but there was no influence on several markers of oxidative stress and antioxidant capacity (Shanely et al. 2010). In their serum, total cholesterol was measured between the 500 mg group and the placebo group, and there was a small decrease in high-density lipoprotein cholesterol levels in the 1000 mg group. Both quercetin groups experienced a small decrease in serum creatine and an increase in the glomerular filtration rate. Interestingly, the drop in mean arterial blood pressure occurred through a combined but nonsignificant reduction in both systolic and diastolic blood pressure for both quercetin groups compared with the placebo group (Knab et al. 2011).

In the elderly, those with chronic diseases and obese subjects, oxidative stress is also increased (Voss and Siems 2006). Because plant extracts work, as has been observed in the context of attenuation exercise-induced oxidative stress and inflammation (Hurst et al. 2010, Nieman et al. 2010a), there is also increasing interest in applying this to the attenuation of obesity-induced inflammation, oxidative stress and immune dysfunction. It is also shown in healthy overweight male subjects with a mildly elevated level of inflammation marker that after a supplementation of different food components, modulating inflammation, oxidation was possible and the metabolism status was altered, all detected by a nutrigenomic approach (Bakker et al. 2010).

Quercetin is a powerful antioxidant and free radical scavenger, as demonstrated by *in vitro* studies (Rice-Evans et al. 1996, Hou et al. 2004, Loke et al. 2008, Duenas et al. 2010).

Loke et al. (2008) showed in an *in vitro* study that free quercetin provides greater protection from oxidative stress than its conjugated metabolites found in the plasma. There seems to be better evidence to support the intake of foods rich in antioxidants within the diet than there is for supplementation due to the consequence of increased uric acid levels (Lotito and Frei 2006).

Taken together, the results and benefits of pure high-dose quercetin as an antioxidant are very limited, and in contrast, using quercetin as part of an antioxidant-cocktail has been found to be much more efficient and can be generally recommended.

10.5   QUERCETIN AS AN IMMUNE BOOSTER

It is well known from the studies cited below that after the heavy exertion of a marathon, cytokines (interleukin (IL)-6, IL-10, IL-1ra and IL-8) increased strongly in response to the race and remained elevated 1.5 h later. The pattern of change did not differ significantly in terms of either sex or age (Nieman et al. 2001, Suzuki et al. 2002, Croisier et al. 1999). This means that intensive exercise elicits a depression of several aspects of acquired immune function. This depression is temporary and cell numbers and functions usually return to pre-exercise values within 24 h. During prolonged periods of intensified training in elite athletes and if recovery between exercise sessions is insufficient, this temporary decrease in cell function can become a chronic depression of acquired immunity (Walsh et al. 2011b).

In contrast to moderate physical activity, prolonged and intense exercise also causes an increased risk of certain types of infection, for example, an upper respiratory tract infection (URTI) (Nieman 2007). Overtraining or training with insufficient recovery can further exacerbate these effects (Gleeson 2007).

To counter exercise-induced inflammation, muscle damage and soreness, the intake of non-steroidal anti-inflammatory drugs (NSAID) like ibuprofen is widespread among athletes, especially if they engage in ultra-distance sport. Ibuprofen in the context of heavy exertion is ineffective, however, and has been found to be potentially harmful (Nieman et al. 2006). Users compared with non-users experienced the same amount of muscle damage and soreness and, additionally, a small leakage of colon bacteria into the circulation that promoted even more inflammation. Ibuprofen use was associated with 25–88% higher plasma levels of seven cytokines, and significant elevations in blood neutrophil counts and serum CRP, urine F2-isoprostanes, alanine and aspartate aminotransferase, and blood urea nitrogen (Nieman 2009). In addition, the level of oxidative stress rose and the side effects of mild kidney dysfunction were observed (McAnulty et al. 2007). Athletes strongly believe that NSAID help them and only a better substitute would make them discontinue its use (Nieman 2012).

There are several studies in humans investigating the correlation of quercetin and its immunomodulatory effects. Quercetin does indeed reduce illness after intensive exercise. Again, under double-blind conditions, Nieman et al. (2007) showed that a supplement of 1000 mg of quercetin alone 3 weeks before, during and two weeks after a 3-day period of 3 h of cycling in the winter resulted in a markedly lower incidence of URTI in well-trained subjects in the two weeks after the intensified training, but had no effect on exercise-induced immune dysfunction, inflammation and oxidative stress (Nieman et al. 2007).

The literature is supportive of the antipathogenic capacities of quercetin when it is cultured with target cells and a broad spectrum of pathogens including URTI-related rhinoviruses, adenoviruses and coronaviruses. Quercetin blocks viral patterns at an early stage through several mechanisms, including inhibition of proteases by molecular docking, binding of viral capsid proteins and suppression of virulence enzymes such as DNA gyrase and cellular lipase (Chiang et al. 2003, Chen et al. 2006).

The impact of the co-ingestion of two or more flavonoids increases its bioavailability and the outcomes on immunity: Nieman et al. (2009) tested after 2 weeks of

supplementation in trained athletes, before and after a period of heavy exertion, with or without 120 mg of EGCG, 400 mg of isoquercetin and 400 mg of EPA-DHA. It resulted in significantly reduced post-exercise measures for both inflammation and oxidative stress, with a chronic augmentation of granulocyte oxidative burst activity (Nieman et al. 2009).

When taken together, the results of a high-dose mixture of flavonoids, including quercetin, showed a successful reduction in the illness rates of exercise-stressed athletes as well as a chronic augmentation of their innate immune function (Nieman 2010).

10.6   DURATION AND AMOUNT OF SUPPLEMENTATION

There are different approaches to determining the appropriate duration of flavonoid supplementation to achieve any positive effect. Powers et al. (2010) contend that the appropriate duration of antioxidant supplementation is a key issue in investigations on exercise performance.

There have been many attempts in human trials to investigate the impact of quercetin on different outcomes; the longest was as many as 60 days between exercise and supplementation, but the majority varied between seven and 21 days (Nieman et al. 2010a), and in the context of polyphenols even a 7-day supplementation of quercetin is regarded as a 'short-term' supplementation (Davis et al. 2009a). Most investigations using flavonoid-rich products or extracts in athletic settings have utilised supplementation periods of at least 7 days prior to heavy exertion, and report varying levels of success in attenuating inflammation and oxidative stress (McAnulty et al. 2005, Chang et al. 2010, Nieman et al. 2010a, Trombold et al. 2010, Goldfarb et al. 2011).

Quercetin supplementation covers periods ranging from 2 to 6 weeks in untrained and trained subjects and has been linked to an inconsistent influence on exercise performance (Nieman et al. 2010a,b).

Few studies used an acute dose of a flavonoid-rich product or extract prior to exercise (Wiswedel et al. 2004, Morillas-Ruiz et al. 2006, Lyall et al. 2009, Davison et al. 2012), and the results were not in favour of acute supplementation *in vivo* (Konrad et al. 2011, Davison et al. 2012). Short-term (48 h) supplementation with EGCG was related to a small but significant increase in maximal exercise performance (Richards et al. 2010), but in general acute supplementation has limited support, which suggests that a longer loading period is necessary (Konrad et al. 2011).

As has been shown, the range of the duration of any supplementation varies a great deal and there are few studies measuring short-term effects. Taken together, there are no consistent recommendations regarding the timing of the supplement. There is little evidence to support any effects of an acute dose of polyphenols on performance and immunological changes in contrast to NSAIDs, which do not need a loading period prior to heavy exertion (Nieman 2012).

10.7   NECESSITY OF SUPPLEMENTATION

Recent evidence suggests that athletes, more so than non-athletes, seem to require increased antioxidants in order to reduce exercise-induced oxidative damage.

Some question whether or not exercise-induced oxidative stress and inflammation due to the higher turnover of oxygen radicals imposed by high-intensity training and competition should be attenuated in athletes, as antioxidant supplementation blocks many of the beneficial effects of exercise on metabolism; however, transiently increased levels of oxidative stress reflect a potentially health-promoting process at least with regard to the prevention of insulin resistance and type 2 diabetes mellitus (Ristow et al. 2009). Yfanti et al. (2010) did not show any effect on training adaptions with a focus on performance after a 12-week supplementation with a mix of antioxidants in highly trained athletes (Yfanti et al. 2010).

It is well known that certain fruits and vegetables can help prevent or treat chronic human diseases and that a broad spectrum of bioactive food can be more effective than any single-component synthetic drug (Raskin et al. 2002, Lila 2007). These natural components accumulate simultaneously in a plant and show a multiple defensive strategy for the human consumer (Lila 2007).

Data are limited and the method and regimens vary widely, but the main conclusion is that flavonoid–nutrient mixtures or any extracts of fruits, vegetables and tea consumed acutely or chronically before exercise diminish post-exercise oxidative stress, inflammation and delayed onset muscle soreness. To avoid the risk of exceeding intake, the most effective way is to consume a varied diet focused on fruit, vegetables and whole grain (Nieman et al. 2010a).

10.8 EFFECTS OF QUERCETIN ON PERFORMANCE

Active skeletal muscle mitochondrial density has been shown to be increased by as much as 20–100% through cardiorespiratory endurance exercise, depending on the level of intensity (Hoppeler and Fluck 2003). This occurrence is mediated by the rise in intracellular calcium levels when muscles contract and includes the harmonised expression of mitochondrial and nuclear genes including the transcriptional coactivator peroxisome proliferator-activated receptor γ-coactivator-1 (PGC-1α) (Diaz and Moraes 2008).

Researchers who used animal models found that certain adaptions in muscle phenotype provoked by exercise can be replicated partly by energy restriction, genetic manipulation, drug treatment and with some forms of plant polyphenols including soya isoflavone derivatives, resveratrol and EGCG (Lagouge et al. 2006, Civitarese et al. 2007, Narkar et al. 2008, Rasbach and Schnellmann 2008). The indications suggest that supplementation with quercetin will also induce a rise in mitochondrial biogenesis and endurance performance in mice (Davis et al. 2009b). The study using 'Imprinting Control Region' male mice revealed an increase in soleus muscle PGC-1α (~100%) and SIRT1 (~200%) messenger RNA (mRNA), cytochrome C concentration (18–32%) and treadmill running time until fatigue (~37%) after a 7-day period of quercetin ingestion of 12.5 and 25 mg/kg. The soleus muscle mitochondrial DNA (mtDNA) of the mouse had roughly doubled after a week of the 25 mg/kg quercetin dose; however, this was not so with the 12.5 mg/kg dose. All of the mice were separately housed in regular cages and did not receive any training by means of forced treadmill running. As a second experiment, mice that were administered

quercetin and provided access to running wheels augmented their running distance after 6 days by 35% when compared with the placebo group (Davis et al. 2009b).

There are multiple studies investigating the effects of quercetin supplementation on performance variables in humans as well, but the results are inconsistent.

After a 2-week supplementation with quercetin (1000 mg/day) vs. placebo in untrained male subjects who provided a blood and muscle biopsy, quercetin was associated with a small but significant improvement in a 12-min time trial (15% treadmill with a self-selected speed) and modest but insignificant increases in the relative copy number of mtDNA and mRNA levels of four genes related to mitochondrial biogenesis (Nieman et al. 2010b).

Another study in untrained volunteers did show a modest improvement of their VO_{2max} (3.9% vs. placebo; $p < 0.05$) along with a substantial (13.2%) increase in ride time to fatigue ($p < 0.05$) after a 1-week supplementation with quercetin (1000 mg·d^{-1}) compared with the placebo (Davis et al. 2010).

One study of 11 elite cyclists reported a 1.7% 30-km time trial performance enhancement compared with the placebo group following six weeks of quercetin supplementation mixed with green tea leaf extract and antioxidant vitamins (MacRae and Mefferd 2006).

Another study with trained cyclists showed differing results: 39 trained cyclists were randomised to a placebo or to quercetin (mixed with EGCG), took the supplements for 2 weeks and cycled on 3 consecutive days for 3 h/day. Subjects from both groups were able to maintain a mean power output of $56.9 \pm 0.6\%$ $W_{max.}$ The total time trial duration did not differ between groups, and there was no difference in mRNA expression for genes related to skeletal muscle mitochondrial biogenesis (Nieman et al. 2009).

Similarly, a study of 40 trained cyclists randomly given 1000 mg/day of quercetin or a placebo for 3 weeks failed to show any group differences in measures of cycling efficiency or skeletal muscle mRNA expression for PGC-1α or SIRT1 (Dumke et al. 2009).

There was also no effect in a study wherein 39 trained cyclists took 1000 mg/day of quercetin supplements compared with a placebo in terms of mRNA expression for mitochondrial biogenesis or cycling time trial performance when engaging in 5-, 110- and 20-km time trials at the end of three 3-h cycling bouts (Nieman et al. 2009).

The number of quercetin-induced changes in muscle PGC-1α and SIRT1 mRNA, and endurance performance in mice (Davis et al. 2009b) was found to be significantly higher than in untrained human subjects ($p < 0.05$) (Nieman et al. 2010b). There could be numerous potential reasons for this, one being the applicability of mouse model findings to humans in quercetin and flavonoid-based research. Supplementation issues that must also be considered include the length of supplementation, the type of supplementation and amount of quercetin ingested.

There is good evidence to support the hypothesis that quercetin may be able to increase endurance exercise capacity, which comes primarily from *in vitro* and *in vivo* studies in rodents that show that quercetin has a combination of biological properties known to affect both physical and mental performance and the ability to increase mitochondrial biogenesis in both the muscle and brain of mice. After 7

days of quercetin supplementation, the mice underwent a change in mitochondrial biogenesis resulting in an increase in both maximal endurance capacity and voluntary wheel-running activity (Davis et al. 2009b). After a short period of supplementation (7–8.5 days) with 1000 mg/day of quercetin alone, there was no significant effect on performance during an aerobically demanding exercise (Abbey and Rankin 2011, Sharp et al. 2012), but that might be due to the short supplementation time (McAnulty et al. 2005).

In a meta-analysis on quercetin and its ergogenic effects, data on 254 subjects did show a small but significant benefit. The mean VO^2_{max} ranged among studies from 41 to 64 mL/kg/min, had a median treatment duration of 14 days and a median dosage of 1083 mg/day. Despite variability among studies, quercetin provides a small but significant benefit in physiological measures of human endurance exercise capacity (Kressler et al. 2011).

Interestingly, quercetin may also enhance mental and physical performance with its caffeine-like psycho-stimulant effects. Many studies have shown that psycho-stimulants like caffeine can delay fatigue during endurance exercise, at least partly due to their ability to block adenosine receptors in the brain, and as a consequence the dopamine activity increases (Davis et al. 2003).

Several flavonoids also exhibit adenosine A_1 receptor antagonist activity *in vitro*. Quercetin had the highest affinity for this receptor, which is similar to caffeine, of all the tested flavonoids (Alexander 2006).

In general, polyphenols appear to be beneficial for athletic performance; however, the exact mechanisms are unknown, as each class is likely to have a different physiological effect (Powers et al. 2004, Braakhuis et al. 2011). The improvement of physical performance in athletes, however, might be due to quercetin's antioxidant properties (Cureton et al. 2009). Future quercetin performance studies should have a longer supplementation period with quercetin consumed in combination with other nutrients, flavonoids and adjuvants like EGCG, luteolin, tiliroside and isoquercetin. Multiple performance measures should also be utilised and these should be selected to appropriately measure the desired outcome (Nieman 2010).

10.9 SUMMARY

The popularity of endurance and ultra-endurance events is increasing and, as a result, there is an increased interest in improving health and, of course, performance in athletes, as heavy exertion takes a toll on their immune systems. For this reason, many athletes take NSAID to boost their immune system and to help them to deal with the physical pain that results from such heavy exertion. On the downside, however, the utility of NSAIDs is very limited, as they come with the possibility of severe side effects and may be even harmful or counterproductive (Nieman et al. 2006).

Quercetin is a widespread flavonoid and is predominant in tea, onions, apples, peppers, blueberries and dark green vegetables (Nieman et al. 2010a). It is generally regarded as safe (Davis et al. 2009a) and is of increasing interest based on its broad range of biological effects in athletes as well as in the general population. The results of these effects are not consistent, however, and the outcomes need to be carefully evaluated as they are dependent on the type of subject and their level of fitness.

With regard to the immune system, there is a smaller incidence of URTI after supplementation with quercetin alone; however, inflammation markers are not significantly influenced after heavy exercise *in vivo* (Nieman et al. 2007). On the contrary, quercetin co-ingested with isoquercetin, EGCG, EPA and DHA does reduce post-exercise measures for both inflammation and oxidative stress with a chronic augmentation of granulocyte oxidative burst activity (Nieman et al. 2009). The outcomes of oxidative stress and quercetin supplementation are not entirely clear: there is *in vitro* evidence, but *in vivo* the supplementation fails or has little impact.

Overall, the intake of quercetin appears to provide a small but significant benefit for athletes in terms of physical as well as mental performance (Kressler et al. 2011). There is support in recommending the ingestion of quercetin, especially if it is administered together with green tea extract (EGCG) and fish oil, which is reinforced by *in vitro* studies that report strong anti-inflammatory, anti-oxidative and antipathogenic effects (Walsh et al. 2011b). The potential synergism between the initiation of exercise training and quercetin supplementation should be studied to determine whether untrained subjects achieve amplified performance outcomes.

Taken together, we know definitively that a flavonoid cocktail is much more efficient than a high dose of one single component. In the majority of the literature, we find references to the benefits of prolonged supplementation with quercetin; with either a mix of several components or only a single dose, only very limited utility has been reported.

The future challenge will be to find the mixture of flavonoids that deliver optimal benefits and especially to establish a recommendation for their protracted intake; this could be within a carbohydrate drink, for example, and would have more of an effect than only ingesting a large dose of a single molecule (Walsh et al. 2011a).

The research in this area is continuing in order to determine the proper outcome measures, dosing regimen and adjuvants that may amplify any perceived bioactive effects of quercetin *in vivo* (Nieman et al. 2012).

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11 Inflammation and Immune Function

Can Antioxidants Help the Endurance Athlete?

Lisa J. Elkington, Maree Gleeson, David B. Pyne, Robin Callister and Lisa G. Wood

CONTENTS

11.1 INTRODUCTION

Endurance athletes and coaches often pose these questions: How do we maintain good health and optimise performance? Are there nutritional strategies that promote good health during periods of heavy exercise training and preparation for competition? Are diet or supplement intakes of antioxidants beneficial, and if so, which is the best strategy? Researchers and practitioners are also interested in these issues and there has been concerted effort in laboratory and field-based studies to identify the clinical and performance effects of supplementation, and the underlying physiological mechanisms.

This review examines some of the important aspects of antioxidant supplementation in endurance athletes including increases in free radical production and subsequent oxidative stress created by high endurance training loads, the impact of endurance training and oxidative stress on immune function, the impact of improving

antioxidant status on factors affecting performance, recovery and adaptation, and whether the source of supplementation is best obtained directly from dietary sources or nutraceutical supplements. Two special circumstances, ultra-endurance events and altitude training, which may invoke specific oxidative stress, are also examined.

11.2 ENDURANCE TRAINING AND OXIDATIVE STRESS

Endurance athletes, such as those competing in the individual sport of running, cycling, swimming and triathlon, undertake many hours of aerobic exercise training each week. Endurance training relies on oxygen use in skeletal muscle to provide the energy for these activities. The oxidative nature of this training may increase the production of free radicals, which are highly reactive, and antioxidant defences are necessary to protect cells from free radical damage. This potential to damage cells is described as oxidative stress and may result in an inflammatory response from the immune system to protect host tissues.

There is a substantial body of evidence that high intensity or prolonged duration endurance-training loads stimulate increased free radical production and oxidative stress (Watson et al. 2005). Endurance training yields an increased production of reactive oxygen species (ROS) (Powers and Jackson 2008) and reactive nitrogen species (Reid 2001; Powers and Jackson 2008). Superoxide and nitric oxide are the ROS most commonly produced in cells (Powers and Jackson 2008). Although oxidative stress may result in an inflammatory response, it is also possible that free radicals play an important physiological role in training adaptations. There has been considerable debate on whether excessive antioxidant intake may reduce training related adaptations (Gross et al. 2011). Achieving an appropriate balance between pro-oxidants and antioxidants may be a challenge for many endurance athletes (Atalay et al. 2006; McGinley et al. 2009).

Regular physical activity can also reduce oxidative stress and inflammation, and improve immune function (McTiernan 2008; Shanely et al. 2011). The volume, intensity and nature of the exercise activity influence this relationship. Although high-intensity endurance training can increase antioxidant enzyme activity, as well as reduce markers for exercise-induced oxidative stress (Miyazaki et al. 2001), very high training loads are associated with an acute reduction in antioxidant capacity and an increase in markers of oxidative stress (Neubauer et al. 2008). This effect has also been demonstrated in athletes competing in ultra-endurance events, including ultra-marathons and ironman triathlons (Knez et al. 2007; Neubauer et al. 2008; Turner et al. 2011). Clearly, athletes have to balance training loads to avoid a heightened risk of fatigue, illness or injury.

11.3 IMPORTANCE OF ANTIOXIDANTS

Antioxidants protect the body from oxidative stress, thereby preventing damage to a wide range of cell structures including lipids, proteins and DNA (Martin 2008). In the body, antioxidants are usually categorised as either endogenous or exogenous. The main endogenous antioxidants include superoxide dismutase, catalase and glutathione peroxidase enzymes and glutathione. Exogenous antioxidants are

obtained from the diet and include, but are not limited to, vitamin E (tocopherols and tocotrienols), vitamin C (ascorbic acid), coenzyme q10 and carotenoids. These substances exert their effects in different biological ways, some by converting the free radicals into less reactive substances, some by protein binding to minimise availability, and others by acting as free radical scavengers (Knez et al. 2007; Powers and Jackson 2008).

Endurance training in preparation for competition places substantial acute and chronic demands on physiological, metabolic and energetic processes. Meeting the nutrient demands can be a challenge for athletes. Competition for key nutrients between the energetic and immune systems during prolonged exercise training is one explanation for the heightened risk of illness in some athletes. There are many options available to athletes wanting to increase their antioxidant intake via either dietary sources or supplements. Antioxidant supplements are increasingly promoted in the general and sporting communities with many claims relating to improved energy availability, faster recovery from exercise, and improved cardio-vascular and immune health. Supplement use is common among endurance athletes with daily consumption rates of up to 90% reported in college athletes in the USA (Frioland et al. 2004).

11.4   ENDURANCE EXERCISE AND SYMPTOMS OF RESPIRATORY ILLNESS

Upper respiratory symptoms are one of the most common reasons for an elite athlete to present for medical review (Robinson and Milne 2002) and there is an established link between training load and risk of respiratory illness (Walsh et al. 2011). Some athletes experience frequent episodes of upper respiratory illness. These symptoms are consistent with an inflammatory response and until recently were assumed to be the result of upper respiratory infection. However, this is not always true and the aeti-ology of the airway inflammation in endurance athletes is varied (Spence et al. 2007) including infection, localised inflammation, allergy and poorly managed asthma.

Although moderate amounts of exercise are typically protective, high volumes of training can increase the risk of respiratory symptoms compared with inactive or moderately active individuals (Nieman 1994). Bouts of endurance training at high intensity, high volume or both can yield transient changes in immune cell activity, which may be responsible for a clinically significant period of increased susceptibil-ity to infection. The risk of upper respiratory tract illness is thought to be highest during periods of overreaching or overtraining and around competition. A period of increased vulnerability, the so-called 'window of immunosuppression' after exer-cise, is based on data showing that immune perturbations can last up to 72 h after competition or a hard training session (Nieman 2007).

The changes in immune activity in the hours after intense physical exertion can be briefly summarised as follows: an acute neutrophilia and lymphopenia, a decrease in natural killer cell activity and T-cell function, a decrease in salivary IgA, and an increase in pro-inflammatory cytokines and chemokines (Nieman 2007). These changes in cellular and soluble components of the immune system have been well described. The biological regulators of these immune responses are thought to

include catecholamines, cortisol, blood flow, body temperature and dehydration (Nieman 2007).

An underlying infectious cause is not always well established for the upper respiratory symptoms experienced by athletes. The notion that inflammation not associated with infection plays a significant role in many clinical presentations is well established. In a study examining the aetiology of upper respiratory symptoms in elite athletes, bacterial infections accounted for only 5% of presentations (Reid et al. 2004), with other inflammatory causes accounting for 30–40% of upper respiratory symptoms. In support of this finding, viral aetiology was identified in only 30% of athletes with illness with similar pathogens to the general community (Spence et al. 2007). Epstein–Barr virus reactivation was deemed responsible for 22% of athletes with recurrent symptoms (Reid et al. 2004). Other causes of upper respiratory illness in athletes include asthma, allergy and unresolved non-respiratory infections and autoimmune disease (Spence et al. 2007).

Athletes may also be at increased risk of airway dysfunction as a consequence of the substantial mechanical stresses on the airways, dehydration and exposure to agents capable of inducing airway injury (pollutants, irritants, allergens). These effects are largely a consequence of the large and prolonged movements of air associated with endurance training. Oxidative stress has been identified as a major factor in pollutant-induced bronchospasm but only a few studies have investigated the role of these agents in eliciting respiratory symptoms in athletes (Chimenti et al. 2009).

11.5 ANTIOXIDANTS AND RESPIRATORY ILLNESS

Antioxidant supplementation has the potential to be a useful nutritional strategy for athletes at risk of respiratory illness. Athletes on a high-antioxidant diet, or who consume antioxidant supplements, may have increased protection against both training- and pollution-induced respiratory illness; however, studies investigating these proposals are lacking. Antioxidants are known to play a role in modifying inflammation of the airways outside the athletic community. A study of asthmatic individuals in the general community examined the role of a high antioxidant diet versus low antioxidant diet (Wood et al. 2012). In this study, the low antioxidant diet led to a worsening of two commonly used measures of asthma severity (percentage of predicted forced expiratory volume in one second, and percentage of predicted forced vital capacity, increased the concentration of the inflammatory marker C-reactive protein in serum, and reduced the time to acute asthma exacerbation compared with those on the high antioxidant diet (Wood et al. 2012). Importantly, this study also demonstrated the benefit of whole-food antioxidant intakes. Increasing the intake of whole foods rich in antioxidants may be beneficial due to the synergistic effect of multiple nutrients consumed in combination. It is also possible that other, as yet unidentified, compounds present in fruit and vegetables may contribute to the beneficial effects of antioxidant-rich foods on airway inflammation.

Another study examining the effects of increasing lycopene concentrations in participants with asthma reported a substantial reduction in airway inflammation as measured by a lower concentration of sputum neutrophils in these participants. The increase in lycopene concentrations was achieved by consumption of either tomato

extract or a lycopene-equivalent dose of tomato juice for a short period of only 7 days (Wood et al. 2008). Given the evidence that inflammation is implicated in a significant number of upper respiratory symptoms reported by athletes, and a substantial reduction in airway inflammation is associated with increased consumption of dietary antioxidants in non-athletic subjects, supplementing the diet with foods that are antioxidant rich may offer similar protection to athletes. Further experimental work in athletic groups is needed to confirm this hypothesis.

11.6 EFFECTS OF INFLAMMATION ON PERFORMANCE, RECOVERY AND ADAPTATION

There is only limited evidence that respiratory inflammation including infections is associated with reduced sport performance. In elite swimmers, an episode of respiratory symptoms prior to international competitions was associated with a decrement in performance (Pyne et al. 2005). Endurance events such as a marathon, an iron-man competition or triathlon can induce muscle damage and an acute inflammatory response—however, there is also an associated increase in anti-inflammatory cytokines (Suzuki et al. 2006). The balance between inflammatory and anti-inflammatory effects depends on a variety of factors. The increase in ROS produced in skeletal muscle during physical activity is dependent on the intensity and duration of the task being performed as well as the antioxidant capacity. Although low activity levels of ROS appear to improve contractility (*in vitro*), high levels are likely to impair function.

11.7 ANTIOXIDANT STUDIES IN ATHLETES

Several studies have attempted to attenuate the inflammatory effects of exercise using antioxidant-rich supplements. A reduction in creatinine kinase and urinary 8-hydroxy-guanosine has been reported following pre-season supplementation with a blend of antioxidants and amino acids in collegiate soccer players (Arent et al. 2010). Although no performance benefit was demonstrated in the players, this could imply a possible recovery benefit. Acute supplementation of trained cyclists 4 h prior to an exercise trial with a pine bark extract, Pyconogenol®, increased time to exhaustion, maximal oxygen uptake and economy (Bentley et al. 2012). Another study which supports the theory of increased antioxidant capacity examined the impact of cherry juice supplementation compared with an energy-matched placebo on knee extension maximum voluntary contractions (Bowtell et al. 2010). Cherry juice supplementation substantially improved post-exercise recovery of isometric strength compared with placebo. Although this study is not specific to endurance athletes, the outcomes support the concept that improved antioxidant availability can delay time to fatigue and promote muscle recovery that potentially improves performance (Bowtell et al. 2010). Blueberry consumption prior to prolonged exercise (2.5 h of running) resulted in higher post-exercise NK cell counts and an increased concentration of anti-inflammatory cytokines compared with a control group (McAnulty et al. 2011).

Quercetin is one of the few antioxidant supplements that has been examined in a number of studies and demonstrated a consistent performance benefit; however, the

studies have been conducted mostly using untrained subjects. In a study of running performance in a 12-min treadmill test, a substantial improvement was observed in untrained subjects (Nieman et al. 2010). In another study, maximal oxygen uptake and cycle time to fatigue were increased following 7 days of quercetin supplementation compared to placebo (Davis et al. 2010), again in untrained subjects. It is unclear whether this benefit would be seen in highly trained athletes.

A topic of much discussion in sport nutrition is whether the use of supplements may compromise natural physiological processes. Some researchers contend that antioxidant supplementation may interfere with cellular signalling function of ROS, and therefore prevent the adaptations that are necessary for performance improvements (Gross et al. 2011). An alternate view is that dietary supplements simply augment natural antioxidant capacities in the face of very high demands associated with endurance training and the fear of physiological interference is overstated. Further studies are needed to resolve this controversy.

11.8   DIETARY VERSUS SUPPLEMENT SOURCES OF ANTIOXIDANTS

A perennial question for athletes is whether they can obtain adequate antioxidant intakes from normal dietary sources or nutritional supplements are needed. Evidence that supplementation with any one antioxidant is sufficient to prevent oxidative damage from free radicals produced during exercise, or prevent immune disturbances or respiratory inflammation associated with exercise, is inconclusive (Nieman 2008). Evidence to resolve this issue in athletes is lacking. However, in individuals with asthma, the view has emerged that whole foods or multi-formulation supplements that contain more than one antioxidant may be more effective in enhancing antioxidant capacity (Wood et al. 2012).

A Mediterranean diet appears to protect against oxidative stress in the general population. The ATTICA study, a large epidemiological study of 3000 residents of urban and rural areas surrounding Athens in Greece, reported significant associations between adherence to the Mediterranean diet (rich in fruit, vegetables, legumes, whole grains, fish, nuts and low-fat dairy products) and health benefits (Kontogianni et al. 2012). Better adherence to this diet was associated with higher total antioxidant capacity and reduced levels of oxidised low-density lipoprotein (LDL)-cholesterol. The reduction in LDL-cholesterol is thought to account for its protective effect on cardiovascular health. Furthermore, this study demonstrated an association between the Mediterranean diet and reductions in markers of inflammation and coagulation.

While a high antioxidant diet is associated with a reduction in airway inflammation and markers of disease severity in asthma and chronic airway disease patients (Wood et al. 2012), there is some doubt as to the benefit and even safety of supplementation with a high-dose single antioxidant. For example, studies with isolated vitamin E supplementation in the form of α-tocopherol have, somewhat counterintuitively, increased markers of oxidative stress compared with placebo during the Triathlon World Championships (Nieman et al. 2004).

A number of food-based antioxidant supplements have shown some promise in improving outcomes during exercise, including quercetin, blueberries and even cherry juice (Nieman et al. 2007, 2010; Bowtell et al. 2010; Davis et al. 2010; McAnulty

et al. 2011). Multi-nutrient supplementation could be a safer choice compared with very high doses of individual antioxidants, or nutrients providing increased antioxidant defence, with fewer risks of potential harm (Atalay et al. 2006). It seems prudent to recommend a diet rich in natural antioxidants including generous quantities of a variety of fruits and vegetables.

11.9 ULTRA-ENDURANCE EVENTS

Ultra-endurance events are one area of endurance exercise and sport that warrants specific consideration for dietary antioxidant supplementation. These events attract substantial numbers of non-elite recreational competitors as well as elite endurance athletes. The most well known of these extreme events is the ironman triathlon incorporating a 4-km swim, 180-km bike and a full 42-km marathon (Knez et al. 2007; Turner et al. 2011). A study examining the impact of full and half ironman triathlons on markers of oxidative stress reported that the ultra-endurance athletes had lower levels at rest compared with relatively inactive controls (Knez et al. 2007), but elevations post-competition indicating a marked inflammatory response. These athletes had relatively higher concentrations of erythrocyte antioxidant enzymes at rest but reductions in these enzymes post-race indicating a depletion of antioxidant defence mechanisms. Markers of oxidative stress may remain elevated for several days after a prolonged bout of physical exercise. Neubauer et al. (2008) demonstrated increases in a variety of markers of oxidative stress after an ironman triathlon event; these markers took 5 days to return to baseline after the event (Neubauer et al. 2008).

Athletes taking antioxidant supplements can have greater elevations in markers of oxidative stress after half or full iron man triathlon events than age-matched relatively inactive control subjects (Knez et al. 2007). Similarly, vitamin E (α-tocopherol) supplementation 2 months prior to an ironman event produced greater elevations in post-race markers of oxidative stress compared with placebo (Nieman et al. 2004). In another study, examining the impact of ultra-marathon swimming on oxidative stress (Kabasakalis et al. 2011), there was no significant difference observed between pre- and post-race markers of oxidative stress, possibly due to the low intensity nature of this activity compared with that examined in other sport which are at a higher average percentage of VO_2 max. Another study examining oxidative stress markers in response to training efforts in swimmers found that despite higher resting levels of oxidative stress compared with inactive controls, a juice-based flavonoid supplement pre- and post-training failed to reduce post-exercise oxidative stress (Knab et al. 2013).

11.10 ALTITUDE TRAINING

The impact of training at altitude warrants special consideration, as altitude exposure can increase the production of oxidative stress independent of the intensity or volume of exercise undertaken (Bakonyi and Radak 2004; Pialoux et al. 2009a,b). It, therefore, seems logical that improving antioxidant supply during this period of increased oxidative stress would benefit health and possibly exercise performance. Studies have been conducted using the 'live high, train low' method of altitude

exposure. Endurance training with intermittent resting hypoxia resulted in a decrease in resting plasma antioxidant levels, with little change in the control group without the hypoxic exposure (Pialoux et al. 2009b). The resting hypoxia group also had a greater increase in post-training markers of oxidative stress. It appears that training with the added hypoxia yields an increase in the production of free radicals that depletes the body's antioxidant capacity. The increased antioxidant intake during this time may assist in maintaining antioxidant levels. The depletion in the hypoxia group had not returned to baseline levels after 2 weeks of recovery (Pialoux et al. 2009a), indicating a more sustained impact on antioxidant levels. Another study reported only a trivial difference in markers of oxidative stress in the supplemented group following 2 weeks of moderate intensity exercise at high altitude (Subudhi et al. 2004). In this study, the concentration of markers of oxidative stress did not change following prolonged submaximal (55% VO_2 max) cycling. The increased oxidative stress produced by altitude exposure may play an important role in adaptation, and dampening this effect with antioxidant supplementation may theoretically impair adaptation.

11.11   SUMMARY

Antioxidants can diminish the potential oxidative stress produced by high volume and intensity endurance training. However, it is not entirely clear whether an increased oxidative stress caused by training is actually harmful to the athlete. The degree that an increase in free radical production during high training loads regulates signalling required for training adaptations warrants further investigation. While these issues are being resolved, athletes should seek advice on antioxidant supplementation from their health care practitioner(s) who should be assessing individual requirements in terms of underlying health, dietary intakes and training loads.

There is some evidence that increased dietary antioxidants modify the disease pattern in illnesses with an inflammatory aetiology. It is likely that diets that increase fruit and/or vegetable intake (and therefore high in dietary antioxidants) have a number of unknown beneficial biological actions that cannot currently be identified or measured. More research is needed to determine whether dietary interventions that benefit disease groups in the general community, such as those with asthma, transfer directly to hardworking but otherwise healthy endurance athletes.

Mixed diets high in antioxidants may be safer than antioxidant supplementation and possibly confer greater benefits. Higher antioxidant intakes may help maintain a normal pro-oxidant/antioxidant balance. Endurance athletes who undertake very high levels of training, either living and/or training at moderate to high altitudes, or who participate in ultra-endurance competitions, may benefit from antioxidant supplementation.

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12 Influence of Mixed Fruit and Vegetable Concentrates on Redox Homeostasis and Immune System of Exercising People

Manfred Lamprecht, Georg Obermayer and Werner Seebauer

CONTENTS

12.1   INTRODUCTION

Sufficient and regular consumption of fruits and vegetables (FV) is commonly regarded as an essential nutritional, preventive activity to maintain health. A lot of scientific publications demonstrate that adequate consumption of plant foods is associated with a decreased risk of chronic degenerative diseases, such as coronary heart disease, stroke, diabetes or certain types of cancer (Bazzano et al., 2008; Dauchet et al., 2005, 2006; Iqbal et al., 2008; Joshipura et al., 1999; Ness and Powles, 1997; Nikolić et al., 2008; Nöthlings et al., 2008; Pomerleau et al., 2006; Steinmetz and Potter, 1996; Wright et al., 2008; Yamaji et al., 2008). The risk-reducing effects are attributed to bioactive components including phytochemicals, phytonutrients and vitamins, minerals and fibre (Brown et al., 1999; Dragsted et al., 2004; Herrera et al., 2009; Lampe, 1999).

Around the world, various Public Health Nutrition strategies such as '5 a day' are applied to encourage people to increase consumption of FV. However, these have met with limited success: nutrition reports and surveys reveal that people consume about 300 g of FV per day (Billson et al., 1999; Casagrande et al., 2007; Elmadfa et al., 2008; German Ministry for Nutrition, Agriculture and Consumer Protection, 2011; Naska et al., 2000), far less than the recommended 400 g up to 650 g per day (Danish Veterinary and Food Administration, 1998; United States Department of Agriculture, 2010; WHO, Food and Agriculture Organization of the United Nations, 2003).

Many people rarely achieve the recommended intake of FV due to several reasons such as taste preferences, convenience, availability, difficult coordination with their working world, ignorance and so on. FV consumption before exercise training can also lead to digestive discomfort during exercise due to the high fructose and fibre content (Ivy and Portman, 2004; Lamprecht and Smekal, 2004). Inadequate FV intake in a person's daily diet can lead to underconsumption of bioactive compounds. This situation provides a rationale for offering concentrated FV nutrition, especially for exercising people.

A well-balanced mixture of phytonutrients, vitamins, minerals and other bioactives from a variety of FV may lead to additive and synergistic interactions in human metabolism that result in health benefits (Liu, 2003; Oude Griep et al., 2010). Hence, to bring as many as possible of these FV bioactives together in one supplement might be superior to supplements containing only vitamins, phytochemicals, juice or powder from just one or a few fruits and/or vegetables.

This chapter refers to studies that used supplementation with mixed FV concentrates in relation to exercising people and their redox and immune system, independent of the training status. We address three questions:

- Can supplementation with mixed FV concentrates modulate redox homeostasis in exercising people?
- Can supplementation with mixed FV concentrates modulate the immune system of exercising people?
- How can sport nutrition advisors decide whether or not to supplement with mixed FV concentrates?

A search of Medline/PubMed and the Cochrane library returned six original articles—with only one FV concentrate (Juice Plus + ®, NSA LLC, Collierville, TN, USA)—that pertain to exercising people and their redox and immune systems.

12.2 CAN SUPPLEMENTATION WITH MIXED FRUIT AND VEGETABLE CONCENTRATES MODULATE REDOX HOMEOSTASIS IN EXERCISING PEOPLE?

Increased metabolism due to exercise training results in enhanced demands for energy, protein, carbohydrate, water, essential fatty acids and also micronutrients such as vitamins, phytonutrients and minerals. A deficit of micronutrients with redox functions can result in imbalanced redox biology in favour of accumulation of reactive oxygen and nitrogen species (RONS) and disturbed redox signalling and control. This situation is called oxidative stress, which results in molecular, cell and tissue damage (Chevion et al., 2003; Peternelj and Coombes, 2011; Petibois and Déléris, 2005; Urso and Clarkson, 2003). In physical exercise, an overwhelming production of RONS can occur which leads to increased inflammatory processes, decreased immunity, increased susceptibility to injury and prolonged recovery (Fischer et al., 2004; Kuipers, 1994; Peters et al., 1993; Saxton et al., 1994).

Scientific literature is scarce regarding exercising people with respect to supplementation with mixed FV concentrates, although a recent systematic review included research on healthy subjects, both trained and untrained (Esfahani et al., 2011). This review revealed that daily consumption of the commercially available encapsulated mixed fruit and vegetable concentrates increased serum concentrations of major antioxidant vitamins. Esfahani et al. (2011) also reported reduced concentrations of oxidative stress and inflammatory markers and promising health advantages on markers of immunity and endothelial function. They noted a diversity of studies with respect to design, study population and the variability in the measured outcomes and assays utilised.

12.2.1 Randomised Controlled Trials That Investigated Fruit and Vegetable Supplementation on Redox Homeostasis in Exercising People

Bloomer et al. (2006) reported that use of the three-blend fruit, vegetable and berry (FVB) form of this supplement resulted in reduced exercise-induced increase of plasma protein carbonyl concentrations—a marker of RONS induced protein oxidation—compared with placebo after 30 min treadmill running at 80% of VO_{2max}. Trained subjects had consumed the nutraceutical continuously for 2 weeks with their meals.

This group also conducted a gender comparison of exercise-induced oxidative stress (Goldfarb et al., 2007). They found that trained women had higher resting antioxidant levels (reduced glutathione, vitamin E) than their trained male counterparts. With FVB supplementation, plasma vitamin E differences disappeared. Markers of oxidative stress (protein carbonyls, oxidised glutathione, malondialdehyde) increased

similarly in both genders in response to exercise of similar intensity and duration. The FVB supplementation attenuated the reduced glutathione (GSH) decrease, and the oxidised glutathione and protein carbonyls increased compared with the placebo group—with no gender differences. Also, plasma vitamin C increased with mixed FV supplementation compared with placebo. They concluded that 2 weeks of supplementation with the FVB concentrate can attenuate exercise-induced oxidative stress equally in both genders.

In a randomised, double-blinded, placebo-controlled trial in a cohort of trained men (Lamprecht et al., 2007), all non-smokers, we also demonstrated that daily supplementation for 28 weeks with the FVB capsules (with meals) reduced carbonyl proteins (CP). It is noteworthy that this group clearly demonstrated a non-adequate intake of FV: instead of >5 portions/day, they only consumed 2 portions/day.

With the same cohort of trained men, we also conducted endurance tests with distinct intensities: at 70% of VO_{2max} and at 80% of VO_{2max} (Lamprecht et al., 2009b). The 70% intensity was adjusted about 10% below the anaerobic threshold, and the second slightly above. At each intensity level, we tested both a placebo group and the active group, which received the encapsulated mixed FVB concentrate. We found that post-exercise CP concentrations increased significantly at 80% VO_{2max} intensity but not at 70%; only in the placebo group this phenomenon occurred at all measured time points (Table 12.1). Towards the end of the study (28 weeks), when individual stress profile was increased by 45% more hours on duty per week, CP concentrations approached 1 nmol/mg protein after 80% VO_{2max} intensity in the placebo group. Referring to our own laboratory data obtained from athletes during the past few years, CP concentrations close to 1 nmol/mg protein (based on our applied method, described in Lamprecht et al., 2009b) are related to increased events of common cold and inflammation (unpublished data).

In another randomised, double-blinded, placebo-controlled study, we investigated a cohort of obese women and assessed the effects of 8-week FBV supplementation

TABLE 12.1

Differences between Long-Term FVB Supplementation and Placebo in Post-Exercise CP Concentrations of Stressed, Non-Smoking, Trained Men

Weeks with FVB/Placebo	FVB 70% VO_{2max}	FVB 80% VO_{2max}	Placebo 70% VO_{2max}	Placebo 80% VO_{2max}
0, baseline	=	↑	=	↑
4 weeks	=	=	=	↑
16 weeks	=	=	=	↑
28 weeks	=	=	=	↑ (~1 nmol/mg)

Source: Modified from Lamprecht, M., Oettl, K., Schwaberger, G., Hofmann, P., and Greilberger, J. F. 2009. Protein modification responds to exercise intensity and antioxidant supplementation. *Medicine and Science in Sports and Exercise*, 41(1), 155–163.

and a single bout of controlled walking exercise on CP, tumour necrosis factor (TNF)-alpha, total oxidation status (TOS) and oxidised LDL (oxLDL) (Lamprecht et al., 2013). Evaluation of the women's diet also revealed that they did not consume enough FV, just two portions per day. Following 8 weeks of supplementation compared with placebo, the FBV group had a significant reduction in CP, oxLDL, TOS and TNF-alpha. It is noteworthy that low-grade inflammation marker TNF-alpha was above the normal range at baseline and decreased to a physiological range after 8 weeks of mixed FVB supplementation. Thirty minutes of walking exercise at 70% of VO_{2max} did not show any significant influence on the measured biomarkers indicating that this kind of exercise does not induce oxidative stress and inflammation in obese women. Furthermore, this study demonstrates that obese middle-aged women with non-adequate FV intake can benefit from mixed FVB supplementation in respect of redox-balancing and attenuation of low-grade inflammation.

Additionally, it is important to mention that FVB supplementation showed neither pro-oxidant effects nor changes of antioxidant enzymes in our studies (Lamprecht et al., 2007, 2009b). This is in contradiction to some exercise studies, which have reported increased lipid peroxidation and decreased plasma glutathione peroxidase (GPx) in trained men after antioxidant supplementation (Knez et al., 2007; Lamprecht et al., 2009a; Nieman et al., 2004). A decrease of antioxidant enzyme GPx could cause a weakening of the body's antioxidant system. This means that supplementation with antioxidants does not provide a net benefit when internal antioxidant systems are regulated down in parallel. Obviously, this down-regulation does not occur with adequate supplementation with mixed FVB concentrates, even after long-term supplementation of 7 months (Lamprecht et al., 2007).

Under the focus of resistance exercise, there is some evidence that antioxidant supplementation could offer protection from exercise-induced muscular and oxidative damage, inflammation, muscle force loss and fatigue (Bloomer et al., 2004; Bryer and Goldfarb, 2006; Nakhostin-Roohi et al., 2008; Palazzetti et al., 2004; Silva et al., 2010). If so, this would accelerate recovery, especially from resistance training, and consequently lead to increased strength performance. However, a number of studies suggest that antioxidant supplementation might promote muscle damage and hinder recovery (Avery et al., 2003; Childs et al., 2001; Close et al., 2006; Teixeira et al., 2009). These conflicting data are due to the diversity of study protocols with different methods, subjects, surrogate endpoints, outcome measures, products and so on.

Recently, Goldfarb et al. (2007) showed that 4-week supplementation with the mixed FVB juice concentrate (with meals) leads to significantly lesser increases in CP, MDA and oxidised glutathione (GSSG) after eccentric exercise in young people (18–35 years). Although the authors conducted diet analyses their paper did not reveal information about the subject's fruit and vegetable intake. We might speculate that this was not different from other age-matched cohorts and thus too low. However, they found no differences between supplementation and placebo in the context of functional changes related to pain and muscle damage between their non-resistance trained study subjects.

To summarise, these six described publications reveal that

- Increased resting CP and TOS values at baseline are reduced significantly after FV supplementation, in groups with non-adequate FV intake
- Increased resting values of low-grade inflammation marker TNF-alpha in obese women: FVB supplementation can decrease it significantly
- After 8 weeks, FV supplementation can decrease oxLDL concentrations significantly in obese women with very low FV consumption
- With strenuous endurance exercise or eccentric exercise, the increase in CP, MDA and GSSG can be avoided by FVB supplementation
- FVB supplementation can attenuate exercise-induced GSH decrease
- Both genders respond similarly to the FVB supplementation
- Activity of erythrocyte antioxidant enzymes seems not to be affected

12.2.2   Fruit and Vegetable Supplementation and Adaptation to Physical Exercise

Several studies postulate that antioxidant supplements could hinder the beneficial cell adaptations to exercise via RONS-induced signal transduction. Some studies (Fischer et al., 2006; Gomez-Cabrera et al., 2008; Khassaf et al., 2003) showed that antioxidant supplementation induced decreased activation of protein kinases, followed by blunted DNA binding of transcription factors. This mechanism results in reduced gene expression of antioxidant enzymes. Gómez-Cabrera et al. (2003) observed that antioxidant supplementation inhibited up-regulation of antioxidant enzymes GPx and superoxide dismutase (SOD) in animal muscles. Gomez-Cabrera's findings are in contrast to our findings with mixed FVB supplementation: we did not observe changes in human's erythrocyte GPx and SOD activities after 8, 16 and 24 weeks of supplementation. However, in dietary counselling for athletes, it is very important to be aware that chronic antioxidant supplementation could blunt exercise-induced redox signalling and adaptation. The application of antioxidant supplements has to be balanced with the priority of the individual goal: poal that has to be fevoured; adaptation or redox/immune stabilisation (see Section 12.3.4)? In this regard, further research on FV supplementation and adaptive mechanisms to exercise is needed to clear this discussion.

Interestingly, recent studies with polyphenol containing supplements including quercetin, *Rhodiola rosea* or beetroot juice revealed performance enhancing effects in trained cyclists or rowers (Bailey et al., 2009; MacRae and Mefferd, 2006; Skarpanska-Stejnborn et al., 2009). Nieman et al. (2010) demonstrated that supplementation with quercetin (1000 mg/day) promotes skeletal muscle mRNA expression of genes involved in mitochondrial biogenesis in 26 previously untrained males during a 2-week physical training period. There is emerging evidence that the antioxidant potential of phenolic compounds is unlikely to be the sole mechanism responsible for the biological effects. Interaction with various key proteins in cell signal transduction cascades is described (Vauzour et al., 2010). FVB supplements can consist of a lot of phenolic compounds, which is an interesting aspect for future exercise research. For more information on antioxidant supplementation and exercise adaptation, see Chapters 7 to 9 in this book.

12.3 CAN SUPPLEMENTATION WITH MIXED FRUIT AND VEGETABLE CONCENTRATES MODULATE THE IMMUNE SYSTEM OF EXERCISING PEOPLE?

12.3.1 RECOMMENDATIONS AND PRACTICAL CONSIDERATIONS FOR FRUIT AND VEGETABLE INTAKE

To stabilise the immune system, increased consumption of FV is prudent. However, there is no generally accepted recommendation of FV consumption for exercising people or athletes, although some institutions and authors recommend up to 13 portions a day, in proportion to energy expenditure (Casagrande and Gary-Webb, 2010).

The standard recommendation to consume five portions of FV per day is already difficult to achieve for the general population (The European Food Information Council (EUFIC, 2012). For exercising people, in many cases, consuming a high volume of plant foods is not realistic. With reference to national nutrition campaigns in Austria (Austrian Ministry of Health, 2011) and Germany (German Ministry for Nutrition, Agriculture and Consumer Protection and Ministry of Health, 2011), one portion of uncooked vegetables and fruits weighs 100–200 g. One portion of cooked vegetables weighs 200–300 g. In total, this leads to a minimum weight of 500 g/day and an average weight of 750 g of FV per day. Very often, sporty people and, of course, top athletes conduct training regimens with long and extensive training units several times a week or even daily. On these days, consuming more than three portions or 400 g of fruits and/or vegetables, with meals/snacks before or within training sessions, might cause digestive issues, especially due to the high content of fibre in FV. Therefore, from the scientific and practical point of view, it makes sense to search for alternatives that can—at least in part—compensate inadequate consumption of plant foods in the athlete's basic diet.

Mixed FV juice concentrates have been on the market since 1993. They focus primarily on a normal population which feels it does not eat enough plant foods on a regular basis. In recent years many sporty people and athletes have also adopted these products to circumnavigate the detrimental digestive effects of high FV intake, while getting some of the beneficial effects.

12.3.2 FRUIT AND VEGETABLE SUPPLEMENTATION, IMMUNITY AND RELATED STUDIES

In a randomised, double-blinded, placebo-controlled trial in a cohort of trained men (Lamprecht et al., 2007), all non-smokers, we demonstrated that daily supplementation for 28 weeks with the FVB capsules (with meals) affected the subjects' immunity expressed via a reduced frequency of common cold, sore throat and fever. This effect was even more pronounced when the subjects' duty became more stressful towards the end of the study, due to more hours of work and circadian imbalance. Compared with the placebo, we also showed a significant decrease in the low-grade inflammation marker TNF-alpha after 16 and 28 weeks. Interestingly, this also occurred in the stressful time towards the end of the study period. No adverse effects of supplementation were observed. However, it is again necessary to note that the

investigated group only consumed two portions of FV per day, far below the recommendations. We believe that this was the main reason why the supplementation with the mixed FVB concentrate could demonstrate these clear beneficial results.

In a randomised, double-blinded, placebo-controlled trial, Nantz et al. (2006) investigated 59 healthy law students who consumed either the two-blend form of the encapsulated FV concentrate or the placebo for 11 weeks. They measured $\alpha\beta$- and $\gamma\delta$-T cells, cytokines, lymphocyte DNA damage, antioxidant status and levels of carotenoids and vitamin C. A log of illnesses and symptoms was also maintained. The FV group tended to have fewer total symptoms than the placebo group. After 11 weeks, there was a 30% increase in circulating $\gamma\delta$-T cells and a 40% reduction in DNA damage in lymphocytes in the FV group relative to the placebo group. The plasma antioxidant status improved and plasma levels of vitamin C, β-carotene, lycopene and lutein increased significantly from the baseline in the FV group. Interferon-γ from lymphocytes was reduced 70% in the FV supplemented group. This study demonstrated that the FV supplementation resulted in an increased plasma nutrients and antioxidant capacity, a reduction in DNA strand breaks and an increase in circulating $\gamma\delta$-T cells.

In a study conducted by the Charite' University Medical Centre, Berlin, Germany, Roll et al. (2010) observed the preventive effect of this dietary FV supplement on common cold symptoms. In this randomised, double-blinded, placebo-controlled trial, healthcare professionals (mainly nursing staff aged 18–65 years, 80% females) from a university hospital in Germany were randomised to the FV supplement or placebo daily for 8 months, including a 2-month run-in period. The number of days with moderate or severe common cold symptoms within 6 months was assessed by diary self-reports. They included 529 subjects in this study. The analyses revealed that the intake of the encapsulated mixed FV concentrate was associated with a 20% reduction of moderate or severe common cold symptom days in these healthcare professionals, particularly those exposed to patient contact.

Although the last two described studies were not exercise-related and did not provide information about how many portions of FV were consumed per day, we assessed the results as interesting as they support the immune supporting effects of FV supplementation.

12.3.3   Dosage of Mixed Fruit and Vegetable Supplementation

High-dose supplementation with certain antioxidants can exert pro-oxidant effects, displace other important antioxidants or interfere with essential defence mechanisms, such as apoptosis, damage to essential lipids and competition for absorption of other essential compounds (Soni et al., 2010). In an exercise study, we demonstrated that supplementation with higher concentrations of vitamin E and β-carotene increased lipid peroxidation and decreased GPx in plasma. Several studies on clinical endpoints revealed that supplementation with vitamin E and/or beta-carotene can increase the risk of mortality, incidence and recurrence of cancers, and increased the risk for cardiovascular diseases (ATBC Cancer Prevention Study Group, 1994; Bairati et al., 2006; Belch et al., 2008; Cook et al., 2007; Lonn et al., 2005; Miller et al., 2005; Omenn et al., 1996). These studies suggest that >400 IU/day of vitamin

E and ≥20 mg of β-carotene/day are rather detrimental and support the occurrence of the described outcomes.

Pro-oxidant and antioxidant roles have also been found for bioactive phytochemicals, such as flavonoids, naturally occurring phenolic compounds derived from plants. Flavonoids have been reported to inhibit lipid peroxidation, chelate metal ions, inactivate lipoxygenase, autoxidise and form high-reactive RONS, act in signal transduction and so on (Soni et al., 2010). Consumption of >20 mg of polyphenols per serving reduces iron absorption and could be an issue for populations with marginal iron status (Corcoran et al., 2012). Despite these data, it is commonly believed that in Western populations consuming adequate haem iron and ascorbic acid (which enhances non-haem iron absorption), the risk of developing anaemia due to dietary flavonoid intake is low.

The sole mixed FV concentrate (Juice Plus + , NSA LLC, Collierville, TN, USA) from which publications with randomised controlled trials can be found on PubMed/Medline contains different vitamins such as E, C and β-carotene as well as a variety of phytochemicals. From the composition of the product labelled on the packaging (<10 mg β-carotene/day and <100 IU/day of vitamin E) and the body of papers reporting beneficial clinical outcomes, we conclude that the daily recommended dosage for intake of this FV(B) supplement does not reach dosages of certain antioxidants as described above for detrimental outcomes, not even when applied in the long term. In addition, the investigator handbook of this product reveals that only 13 adverse events (like minor gastrointestinal complaints and hive-like rash) were reported in more than 25 clinical studies with almost 2000 subjects, which is far less than 1%.

Juice Plus+ is a low-dosed supplement which provides a variety of bioactives, including vitamins and phytochemicals. We speculate that this fact might contribute to the observed beneficial clinical outcomes.

To summarise, data from studies with FV and FVB supplementation indicate that people's immunity can benefit when dietary consumption of FV is low and an increased psycho-physiological stress profile occurs.

Overdosing with the sole FV or the FVB concentrate reported on PubMed/Medline is not realistic as long as consumers contain themselves on dosage recommendations of 4–6 capsules/day.

Nevertheless, further research on FV(B) supplementation in relation to immunity and exercise—especially mechanistic studies and randomised clinical trials—is warranted to corroborate the existing data (Table 12.2).

12.3.4   Inevitable: Immunity and Performance Are Housed under the Same Roof

Stabilised immunity has the largest impact on physical exercise performance. In competition periods with high intensities and (too) short recovery periods, stressful general conditions and a higher risk to suffer oxidative stress, inflammation and imbalanced immunity occur. During such periods, stabilisation of immunity has priority and a possible non-favourable effect on adaptation is secondary. There is already evidence that FV supplementation has beneficial effects on oxidative stress, inflammation and immunity in trained and untrained people (Bloomer et al., 2006;

TABLE 12.2
Summary of Sport Studies with Mixed FV or FVB Supplements

Dosage of Supplementation	Subjects' Characteristics	Time Points of Sample Collection	Design Methodology	Results Outcome	Author (Year)
Fruit, vegetable and berry juice powder conc.: 2×3 capsules a day for 2 weeks; 400 IU vitamin E + 1000 mg vitamin C for 2 weeks	Trained men and women: 25 men, 23 women; 18–30 years; Non-smokers	Blood collection at baseline, after 2 weeks of FVB supplement, after 1 week washout; also before exercise and immediately post exercise	Double-blind, randomised, placebo-controlled, 3 parallel groups: 2 supplemented groups and 1 placebo group; controlled diet before exercise tests; 30 min running exercise at 80% VO_{2max} before and after 2 weeks supplementation/placebo and after 1 week washout	Reduced exercise-induced increase of plasma protein carbonyls in FVB supplemented groups; no differences in protein carbonyls between supplemented groups; no impact of supplementation on MDA and 8-OHdG	Bloomer et al. (2006)
Fruit, vegetable and berry juice powder conc.: 2×3 capsules a day for 28 weeks	41 trained men; 30–40 years; Non-smokers	Blood collection at baseline, 4, 16 and 28 weeks of supplement or placebo; resting values	Double-blind, randomised, placebo-controlled, 2 parallel groups: supplemented and placebo group; standardised nutrition	Plasma CP lower in the supplemented group; differences between groups more distinct with increased stress profile; tendency to fewer duty days lost due to illness ($p = 0.068$) in supplemented group	Lamprecht et al. (2007)
Fruit, vegetable and berry juice powder conc.: 2×3 capsules a day for 2 weeks; 400 IU vitamin E + 1000 mg vitamin C for 2 weeks	Trained men and women: 25 men, 23 women; 18–30 years; Non-smokers	Blood collection at baseline, after 2 weeks of FVB supplement, after 1 week washout; also before exercise and immediately post exercise	Double-blind, randomised, placebo-controlled, 3 parallel groups: 2 supplemented groups and 1 placebo group; controlled diet before exercise tests; 30 min running exercise at 80% VO_{2max} before and after 2 weeks supplement/placebo and after 1 week washout; gender comparison of exercise-induced oxidative stress	Women have higher resting antioxidant levels than men; markers of oxidative stress increased similarly in both genders in response to exercise of similar intensity and duration; 2 weeks of antioxidant supplement can attenuate exercise-induced oxidative stress equally in both genders	Goldfarb et al. (2007)

Supplement	Subjects	Protocol	Sampling	Results	Reference
Fruit, vegetable and berry juice powder conc.: 2 × 3 capsules a day for 28 weeks	41 trained men; 30–40 years; Non-smokers	Double-blind, randomised, placebo-controlled, 2 parallel groups: supplemented and placebo group; standardised nutrition; 40 min cycle ergometer exercise at 70% and 80% of VO_{2max} at baseline, 4, 16 and 28 weeks	Blood collection at baseline, 4, 16 and 28 weeks of supplement or placebo; also before exercise and immediately, 30 min and 30 h post exercise	Increase in CP in the placebo group after 80% VO_{2max} exercise; post-exercise CP differences more distinct with increased stress profile; no influence of supplementation on antioxidant enzyme activities and redox state of albumin	Lamprecht et al. (2009)
Fruit, vegetable and berry juice powder conc.: 2 × 3 capsules a day for 28 days + 4 days post exercise	44 non-resistance trained subjects; no gender specification; 18–35 years; non-smokers	Double-blind, randomised, placebo-controlled, 2 parallel groups: supplemented and placebo group; documented diet before exercise tests; 4 sets of eccentric elbow flexion with non-dominant arm	Blood collections and muscle function tests before exercise and immediately, 2, 6, 24, 48 and 72-h post exercise	Muscle soreness, range of motion and maximal isometric force not influenced by treatment; protein carbonyls, MDA and glutathione ratio increased post exercise in the placebo group, whereas there were no changes in the supplemented group; no influence of treatment on lipid peroxides	Goldfarb et al. (2011)
Fruit, vegetable and berry juice powder conc.: 2 × 3 capsules a day for 8 weeks	42 obese, pre-menopausal, untrained women, 36–46 years, non-smokers	Double-blind, randomised, placebo-controlled, 2 parallel groups: supplemented and placebo group; standardised nutrition; 30 min walking exercise at 70% of VO_{2max} at baseline and 8 weeks	Blood collection at baseline and 8 weeks of supplement or placebo; also before and immediately post exercise	Reduction in CP, oxLDL, TOS and TNF-alpha; FVB supplementation decreased markers of oxidation and inflammation; improved skin microcirculation due to FVB supplementation and the model of exercise	Lamprecht et al. (2013)

Lamprecht et al., 2007, 2009; Roll et al., 2010). These effects seem to increase when basic nutrition is lacking in plant food consumption (Lamprecht et al., 2007).

Gómez-Cabrera et al. (2003) demonstrated the beneficial effect of restricted RONS production in competitive periods via cyclists taking part in the Tour de France: when given allopurinol (a xanthine oxidase inhibitor and antioxidant), they had lower increases in the activity of creatine kinase and aspartate aminotransferase.

Once an athlete becomes sick, full recovery from the common cold in top endurance sport takes 6–8 weeks to reach top form again. In cross-country skiing, for example, this would mean a lost season if such a disease event occurred around Christmas time. As long as the athlete can keep his health, he/she stays competitive. Moreover, in competition periods, athletes usually are not focused on performance improvement. The time period in which the highest level of fitness is reached has to be programmed prospectively and is already achieved when the competition season begins.

Consequently, it is useful to base one's decision regarding FV supplementation on the primary goal: adaptation to a programmed exercise stress or stabilisation of immunity and health? In preparation and development periods, supplementation with antioxidants containing FV supplements might be counterproductive due to hindered RONS-induced signal transduction. In competitive periods, the supplementation is beneficial to stabilise immunity, if adequate testing on the product demonstrated these immune stabilising effects. However, other domains contribute to a pro or con decision as described in Section 12.4.

12.4   HOW CAN SPORT NUTRITION ADVISORS DECIDE WHETHER OR NOT TO SUPPLEMENT WITH MIXED FV CONCENTRATES?

To decide pro or con supplementation with mixed FV concentrates, at least five domains have to be considered. In a person's life, at the time point of counselling, each domain has a certain manifestation: favourable or non-favourable. Table 12.3 depicts the five influencing domains with examples for pro and contra supplementation, respectively. We suggest terming these domains 'diet', 'biochemical checkup', 'exercise/competition regimen', 'basic conditions' and 'product quality':

1. *The person's diet: food and fluid intake*: A 7-day food record is necessary to estimate the basic diet and also daily plant food intake. A non-favourable characteristic of this domain indicates supplementation with FVB concentrates, for example, individual digestive situation or certain aversions/preferences do not allow dietary intake of 5 portions/day or more.
2. *Biochemical check-up*: Analysis of oxidative stress and inflammatory parameters in the blood and urine as well as a standard blood chemistry panel. With respect to the athlete's clinical history, age, gender and genetics, it is useful to conduct a panel of several parameters at rest and post-exercise. FVB supplementation is a tool to combat excess oxidative stress or inflammation.

TABLE 12.3

Overview and Examples for Non-Favourable or Favourable Manifestations of Each Influencing Domain

Favourable Conditions for Health → Con Supplement	Non-Favourable Conditions for Health → Pro Supplement
Diet:	Diet:
• Consumption of 5 or more portions of FV per day	• Consumption <5 portions of FV per day
• Balanced mixed basic diet	• Diets with undersupply of micronutrients, especially antioxidants (e.g. severe weight loss practice and unbalanced diet)
• Periods with normal energy uptake (especially depending on the exercise regimen)	• Periods of high energy uptake (most likely determined by exercise regimen) and consumption of less than 6–13 portions of FV per day
• Consumption of >5 portions of FV in periods of high energy expenditure	
• Consumption of other food supplements or specific functional food	• Consumption of other food supplements or specific functional food
Biochemistry and anamnesis:	Biochemistry and anamnesis:
• Oxidative stress, inflammatory parameters or antioxidants in blood and/or urine within appropriate ranges before and post exercise	• Oxidative stress + inflammatory parameters + antioxidants in blood and/or urine out of appropriate ranges before and post exercise
• Appropriate concentrations of parameters of the standard blood chemistry panel	• Inappropriate standard blood chemistry panel (e.g. imbalance in lipid metabolism)
• Beneficial genetic constitution	• Unfavourable genetic constitution
• Inconspicuous anamnesis	• Unfavourable anamnesis
Exercise/competition regimen:	Exercise/competition regimen:
• Low intensity and duration	• Strenuous competition period
• Low frequency, adequate recovery periods	• Rehabilitative or weight management training
• Periods with strenuous training units to achieve optimised performance with adequate time for recovery/super compensation	• Periods with high intensity and frequency combined with inevitable short recovery periods and high energy expenditure
Basic conditions:	Basic conditions:
• Favourable and relaxed psycho-physiological situation	• Significantly increased psycho-physiological stress profile
• Good environment, facilities, equipment for training, favourable humidity and so on	• Inappropriate facilities, environment and humidity, stay in high elevations, pollutants and so on
FV product:	FV product:
• No scientific evidence for bioefficacy of the product (no testing on the product)	• Documented scientific evidence for bioefficacy of the product (data/publications from tests on the product)
• High concentrations of antioxidant vitamins added to the product	• Good quality: safety and risk assessment certificates available
• No safety and risk assessment certificates	

3. *Exercise/competition regimen*: In certain time periods, stabilisation of immunity and avoidance of inflammatory processes are of higher priority than adaptation. During this time, FVB supplementation is indicated. During macrocycles primarily focused on adaptation, it might be indicated to reduce or even avoid supplementation with mixed FVB concentrates.

4. *Underlying 'basic conditions' in an athlete's training period*: Training in hot and humid weather, stay in higher regions, cold and dry climate, polluted air, unfavourable training facilities and so on. Also, stressful daily life, mentally/emotionally disturbed everyday life, job, family and so on fall into this domain and contribute to decide pro or con FVB supplementation.

5. *Quality and evidence of the FVB supplement/product itself*: If FVB supplementation is indicated, the product has to provide scientific evidence of bioefficacy and must fulfil all safety criteria. Well-described methodology, evaluated dosage, timing and investigated target groups are key factors necessary to decide to choose a product. Possible adverse effects and recommendations to avoid digestive problems should be published to give an opportunity for quick adjustment.

Each domain should be evaluated for advantageousness on the athlete's health and performance as well as the 'interaction' between domains. In the event that one or more certain domains reveal non-favourable characteristics/manifestations, the likelihood of FVB supplementation increases (except for the FV product, see Table 12.3 and Figure 12.1).

If FVB supplementation is indicated, the product should be added to one or more meals, depending on the regimen being used from published scientific studies with that product.

The decision pro or con regarding FVB supplementation has to be reevaluated continuously. On the basis of the subject's genetic profile, absorption rates, bioavailability, pharmacokinetics, bioactivity, etc. might be different among people and could lead to a different response, even to supplementation regimens with scientific evidence for efficacy. Thus, after 3–16 weeks, depending on the training macro-cycle and/or period of the specific type of sport, frequently conducted counselling sessions with check-ups comprising all influencing domains reflect the most professional handling with this issue.

Note: Supplements are no replacement for dietary fibre! Even if FVB supplementation is indicated, these supplements cannot substitute for the recommended daily fibre intake of 20–35 g (Marlett et al., 2002). The uptake of dietary plant foods should increase to at least 5 portions/day, especially in less strenuous training periods, as long as this amount and frequency of intake does not result in digestive problems.

12.5   CONCLUDING REMARKS

The main base to stabilise redox homeostasis and immunity is a mixed, balanced diet. Supplementation with mixed FVB concentrates is indicated if health stabilisation and avoidance of oxidative stress and inflammation have priority.
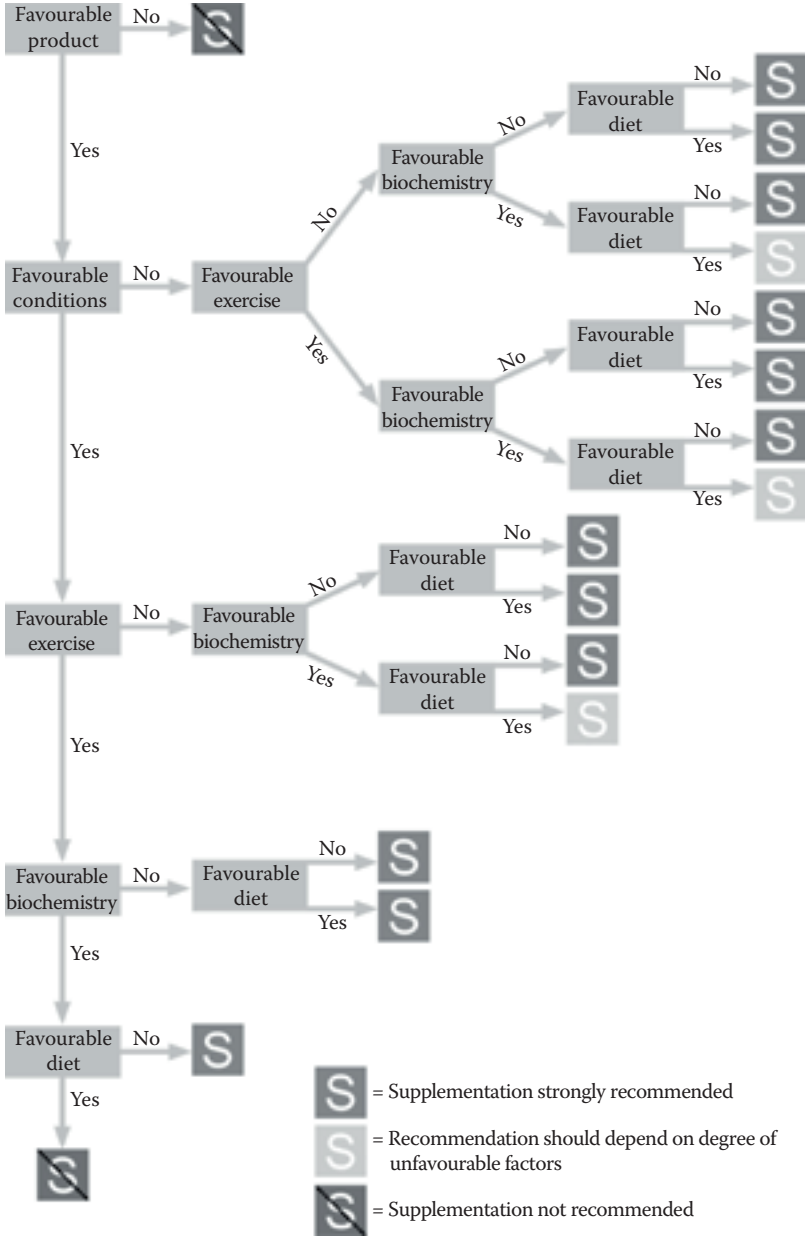
FIGURE 12.1 A 'decision path' to ease decision pro or contra FV supplementation to athletes' and sporty people's diet. First, the product should be evaluated in respect of scientific evidence of its bioefficacy as well as safety and tolerance. In the event this is favourable, nutrition and biochemistry constitute the most important factors in favour of supplementation. If either shows deficits (non-favourable characteristics), a supplementation should be strongly considered. If both are inconspicuous, the exercise regimen and basic conditions should be assessed. In the event that all conditions are near optimal, supplementation is not indicated.

It is not realistic to provide general advice pro or con regarding supplementation with mixed FVB concentrates. Pro or con and also amount and dosage of supplementation underlie individual evaluation of each influencing domain in every single person. The manifestation(s) of the five domains, diet, biochemical analyses and clinical history, exercise training, basic conditions and the product itself, are crucial for decision-making. Thus, in practice, a counsellor's decision pro or con FVB supplementation should be based on a systematic decision-making procedure. A 'decision tree' as provided in this chapter might guide in finding the right solution.

In future, the number of exercise studies with these promising nutraceuticals should increase. The results from research in this field should be combined with practical observations and documentary reports to achieve sustainable health and performance of sporty people.

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13 Methodological Considerations When Evaluating the Effectiveness of Dietary/Supplemental Antioxidants in Sport

Martin Burtscher, Dominik Pesta, Dietmar Fuchs, Maximilian Ledochowski and Hannes Gatterer

CONTENTS

13.1 INTRODUCTION

In recent years, there has been an increasing practical, clinical and scientific interest in the effects of free radicals and antioxidants related to sporting activities and exercise performance (Nikolaidis et al. 2012a,b; Peternelj and Coombes 2011; Powers et al. 2011; Reid 2008; Stear et al. 2009). Most of these reviews agree that oxidative stress represents a fundamental biological response to exercise stimuli. The generation of reactive oxygen species (ROS) increases with inclining oxygen utilisation during endurance exercises and is potentially associated with the risk of damage to muscles and other tissues. The key question is how effectively athletes can defend against the increased free radicals resulting from exercise. Do athletes need to take extra antioxidants? Studies provide arguments for and against the use of antioxidants but also emphasise that additional research will be required until a final judgement can be made (Peternelj and Coombes 2011; Powers et al. 2011; Stear et al. 2009). Obviously, large discrepancies exist between scientific evidence and the promotion by manufacturers and distributors of antioxidants. The promises of manufacturers and distributors are often based on anecdotal reports, not well-controlled observational studies or even on theoretical assumptions or speculations. It is readily understandable that the expectations of athletes encourage business interests. However, the main goal of scientific studies is to find the truth. To accomplish this goal, appropriate study design and sound statistical methods are of utmost importance.

13.2 HOW TO OBTAIN THE TRUTH

Subjects taking nutritional supplements such as antioxidants expect 'truly' beneficial effects. Such an expectation may sometimes provoke substantial placebo effects which are often misinterpreted as true effects. Most of the consumers, however, will not really experience benefits. Thus, a rigorous scientific and practical verification of true effects is paramount. Based on the definition of John Dewey, a student of the pragmatic philosophers Sanders Pierce and William James, truth should be coherent with other knowledge, should be repeatable, and lead to correct predictions (Spector 2009). To meet these requirements, rigorous scientific methods have to be used, finally enabling the prediction of effects, for example, those of supplemental antioxidants.

13.3 TYPES OF STUDIES AND THEIR CONCLUSIVENESS AND LEVEL OF EVIDENCE

There are three types of research or studies advancing scientific knowledge: descriptive, explanatory and predictive research.

13.3.1 Descriptive Studies

Descriptive studies, also referred to as observational studies, collect information on existing relationships, for example, between health status or level of performance and the regular intake of antioxidants. They include not only cross-sectional and longitudinal (cohort) studies but also studies on single cases and case series. Whereas cross-sectional studies look on existing associations at a single time point or time interval within a defined population of interest, longitudinal studies follow the individuals of such a population over time. These studies do not change the environment or conditions but simply describe 'what is' or 'what was'. The findings obtained from these studies provide associations between outcome variables but do not allow causality to be established. Thus, after this first step of research, explanatory and predictive studies are necessary to assess causality.

13.3.2 Explanatory Studies

Explanatory studies try to answer 'how' and 'why' an intervention works. For instance, when descriptive research found associations between the intake of antioxidants and high levels of performance, it will be of interest to show 'how' and 'why' antioxidants can improve performance, that is, to establish cause and effect relationships. Interventions in explanatory studies are strictly defined and controlled. Randomised controlled trials (RCT) are considered as simple and powerful tools in clinical research. In this type of study, participants are randomly assigned to the intervention group, for example, receiving supplemental antioxidants, or to the control group, for example, receiving placebo. To avoid biases (systematic errors), it is highly desirable that researchers performing outcome measurements as well as study participants are 'blinded', that is, they do not know whether somebody has been allocated to the intervention or the control group.

13.3.3 Predictive Research

Predictive research forecasts intervention effects based on the knowledge gathered by descriptive and experimental studies. For example, supplemental antioxidants, which have been demonstrated to be beneficial in descriptive studies and cause and effect relationship, have also been shown in explanatory studies and are consumed by a defined population, for example, athletes. Ideally, beneficial effects of the intervention should be predictable in a large proportion of the subjects studied.

13.4 EVIDENCE-BASED MEDICINE OR EVIDENCE-BASED PRACTICE

The most important reasons for prasticing evidence-based medicine (EBM) or evidence-based practice (EBP) are to improve quality of care by the use of practices that really work, and the elimination of those that do not work or are even harmful (Gray and Pinson 2003). According to Sackett et al. (1996), EBM means using the best evidence in making decisions about the care of the individual patient based on

TABLE 13.1

Levels of Evidence

1 a: Systematic reviews (with homogeneity) of randomised controlled trials (RCTs)

1 b: Individual RCTs (with narrow confidence interval)

1 c: All or no RCTs

2 a: Systematic reviews (with homogeneity) of cohort studies

2 b: Individual cohort study or low quality RCTs (e.g. <80% follow-up)

2 c: 'Outcomes' research; ecological studies

3 a: Systematic review (with homogeneity) of case–control studies

3 b: Individual case–control study

4 a: Case series (and poor quality cohort and case–control studies)

4 b: Expert opinion without explicit critical appraisal, or based on physiology, bench research or 'first
   principles'

Source: Levels of Evidence, Centre for Evidence-Based Medicine, Oxford, 2013.

the best available evidence from systematic research integrating individual clinical expertise and patient values. The same is true with regard to medical care and support for athletes. The higher the level of evidence (Table 13.1), the better the quality of research and the more likely that the predicted effects will actually occur.

13.5   GENERAL ASPECTS AND CRITICAL VIEW REGARDING EFFECTS OF ANTIOXIDANT SUPPLEMENTATION ON HEALTH BENEFITS AND FAVOURABLE ADAPTATIONS IN RESPONSE TO PHYSICAL ACTIVITY

When planning studies of antioxidant effects on exercise performance, a comprehensive knowledge of integrated biochemical and physiological mechanisms of exercise metabolism, free radicals and antioxidants is essential. The following section intends to provide some general thoughts and will focus on potentially negative effects of antioxidants. These adverse effects are discussed in more detail in other chapters of this book.

The dietary vitamin and supplement industry is one of the world's fastest growing markets, with 32 billion dollars in revenue for nutritional supplements alone (www. forbes.com). Although it is generally accepted that the prevalence of vitamin deficiency is low in industrialised countries and physically active individuals do not show increased requirements of vitamins and minerals, food supplements are increasingly used by the general population and athletes. There is increasing evidence that antioxidants may not only lack health benefits but may even prevent health-promoting effects of physical exercise in humans (Ristow et al. 2009). Bjelakovic et al. (2007) looked at data from 67 studies on antioxidant supplementation and they concluded that β-carotene, vitamin A and vitamin E supplementation seemed to increase the risk of death. When a 6-week aerobic exercise training programme was carried out by patients with hypertension, supplementation of antioxidants (vitamins C and E

and lipoic acid) maintained the hypertensive status of subjects and the inhibition of exercise-induced flow-mediated vasodilatation (Wray et al. 2009). Although a possible explanation (free radical acting as vasodilators) is put forward, the authors do not provide experimental evidence for the mechanism of their finding. Also, though the training period is rather short (6 weeks), the authors fail to include a time control group to account for a time effect. The authors apply spin trapping and electron paramagnetic resonance spectroscopy (EPR) to assess plasma-free radical concentration, which is considered the gold standard method. However, they used a different training modality (graded maximal cycling) than was used for the exercise training programme itself (knee-extensor exercise). Secondary, stress-related effects due to a difference in exercise modality for training and testing cannot therefore be excluded. Ristow et al. (2009) found a blunted induction of gene expression and an increase in insulin sensitivity in a vitamin C and E supplemented group undergoing about 60 min of circuit-type training 5 times a week for 4 weeks. By contrast, only the non-supplemented group increased the ROS-dependent transcription factors and insulin-mediated glucose disposal in response to the exercise training. Thiobarbituric acid-reactive substances (TBARS) levels were significantly increased in response to exercise in the non-supplemented group but not in the group taking the antioxidants. Ristow et al. (2009) emphasises the importance of oxidative stress to induce the above-mentioned changes. Plasma levels of vitamins C and E were not determined in the participants and, surprisingly, Ristow et al.'s report (2009) increased mRNA levels of PGC-1α, PGC-1ß, PPARγ as well as superoxide dismutase (SOD) 1 and 2 only in the non-supplemented group. Along these lines, it is worth mentioning that euglycemic–hyperinsulinemic clamps and muscle biopsies were performed 1 week after completion of training. It has been shown that increases in the mRNA levels of the above-mentioned transcription factors in response to exercise are transient in nature and return to baseline levels about 24 h after an exercise bout (Pilegaard et al. 2003). The same is true for training-induced increases in insulin-stimulated glucose disposal which quickly decays 3–4 days after cessation of training, being undetectable after 7 days (Oshida et al. 1991; Burstein et al. 1985). Thus, owing to some methodological flaws, the findings by Ristow et al. (2009) are difficult to comprehend. Further, the number of participants to be included to observe a true effect was not planned, which reduces the validity of the study. The authors state that 'supplementation with antioxidants may preclude these health-promoting effects of exercise in humans'. This conclusion is not comprehensive, given the fact that hundreds of different antioxidants interplay to regulate the pro-oxidative–antioxidative balance in the body. Therefore, this statement cannot be extrapolated to antioxidants in general by using only two specific antioxidants.

Similarly, Yfanti et al. (2011) studied the effect of vitamin C and E supplementation on insulin sensitivity in response to endurance training. The training consisted of a mixture between interval and continuous cycling ~80 min, 5 times a week for 12 weeks. The authors report similar increases in insulin-stimulated glucose uptake in both groups of about 15% as well as VO_{2max} of about 18% in both groups. They concluded that supplementation with vitamins C and E for 12 weeks of cycling exercise training had no effect on performance or insulin-stimulated glucose uptake. Unfortunately, no markers of oxidative stress were reported in this study providing

no information on the effectiveness of antioxidant supplementation on levels of oxidative stress.

It is interesting that both Ristow et al. (2009) and Yfanti et al. (2011) use a similar regime and yet the outcome of their studies is distinct. A closer look, however, reveals a few differences between the two studies: Yfanti et al. (2011) provide 500 mg vitamin C and 400 IU vitamin E daily for 16 weeks (4 weeks lead-in phase), whereas in the study of Ristow et al. (2009), participants took double the amount of vitamin C (1000 mg) and the same dose of vitamin E (400 IU) daily for a total time of only 4 weeks. In the Ristow study, the supplement dosages of vitamin C and vitamin E for the participant's age group are therefore 10 and 17 times higher, respectively, than the recommended dietary allowances. Also, the total time training is very different, being 4 weeks, 5 times a week (20 sessions) in the Ristow study and 12 weeks, 5 times a week (60 sessions) for the Yfanti study. Thus, more research is needed to clarify whether these parameters can explain the observed differences between the two studies. Furthermore, it is likely that methodological issues may partly account for the inconsistent results when comparing these two studies (see above).

ROS seem to play a role in disease prevention and the extension of the lifespan. ROS are actually 'required' for the extension of the lifespan, by a mechanism that is called mitohormesis (Radak et al. 2008; Ristow 2012). Antioxidants that interfere with ROS formation seem to prevent this effect (Gomez-Cabrera et al. 2012). This finding is discussed controversially (Higashida et al. 2011), but the vast majority of experimental evidence clearly advises against supplementation of antioxidative vitamins. Additionally, there is also growing evidence that antioxidative vitamins may play a role in the development of obesity (Mangge et al., 2013).

The number of overweight and obese individuals has reached epidemic proportions with serious social and psychological dimensions. For this development, lifestyle changes with increased consumption of more energy-dense but nutrient-poor foods with high levels of sugar and saturated fats, combined with reduced levels of physical activity, are considered to be most important. Reduced caloric intake in combination with regular physical activity is certainly the most effective way of preventing the development of obesity.

Recently, it was claimed that antioxidant supplements diminish the health-promoting effects of exercise in patients with type 2 diabetes (Ristow et al. 2009). As the most important mode of action, antioxidants neutralise ROS and thus counteract physiological responses to exercise-induced ROS. However, this relationship could be of much broader relevance and some other possible consequences could be extrapolated that are more generally relevant (Theodorou et al. 2011).

The increased mitochondrial formation of ROS induced by exercise (Powers and Jackson 2008; Kyparos et al. 2009) is central to oxidation of carbon fuels. It is also well documented that physical exercise and sport activities elicit a significant activation of inflammatory responses and create an oxidising milieu which involves the release of specific immune products like cytokines and neopterin and their serum-soluble receptors, sIL-2R, sTNF-R55 and sCD23 (Tilz et al. 1993). The pro-inflammatory cytokine cascade is activated by signal transduction elements such as nuclear factor-kappaB (NF-κB), which is inducible by ROS and critical for establishing a pro-inflammatory response (Schreck et al. 1991). Antioxidant compounds are able

to dampen this pro-inflammatory response. As a consequence, ROS production as well as the molecular mediators of endogenous ROS defence, such as superoxide dismutase and glutathione peroxidise, are diminished in response to physical exercise (Ristow et al. 2009). However, whether this finding justifies their conclusion that antioxidant supplements prevent the health-promoting effects of physical exercise is probably too far-reaching and questionable, because the induction of antioxidant mediators cannot be regarded as the one and only benefit of sport and physical exercise.

Regular physical activity may be considered the most important condition counteracting metabolic and cardiovascular diseases (Szostak and Laurant 2011). There is ample evidence that physical activity is an independent and important factor for the assessment of cardiovascular risk and that increasing levels of physical fitness protect against elevations in most risk factors in subjects with and without cardiovascular diseases. Although, in general, there seems to be a reduction in biomarkers of oxidative stress with antioxidant intake, the physiological meaning of this is not clear and the significance for health implications is still poorly understood. The ergogenic potential of antioxidants is still debated; however, a meta-analysis of 68 randomised antioxidant supplement trials revealed that antioxidant intake does not improve overall health but may in fact increase mortality (Bjelakovic et al. 2007). Further studies are required before final conclusions can be drawn.

13.6 STUDIES OF ANTIOXIDANTS AND EXERCISE PERFORMANCE: WHAT IS THE SUPPORTIVE EVIDENCE?

This section is not intended to be comprehensive but tries to provide some representative examples underpinning the necessity of careful study planning. For an extensive review, the reader may refer to Peternelj and Coombes (2011).

Many athletes take vitamins and antioxidants with the expectation of increasing exercise performance (Huang et al. 2006). Athletes seem to use supplements more frequently than the general population, with a prevalence of about 46% in athletes and an increasing prevalence with higher levels of professionalism (Sobal and Marquart 1994). Therefore, it has long been of interest to athletes and coaches as well as to scientists whether the support of the endogenous antioxidant defence systems by additional supplementation of antioxidants is an effective strategy to reduce oxidative stress and ROS production and hence to possibly improve exercise performance. However, there is evidence that ROS are important signalling molecules that act on pathways essential to and beneficial for exercise-induced adaptations (Burgoyne et al. 2007; Powers et al. 2010) with regard to tissues such as skeletal muscle. As early as 1971, it was shown that vitamin E supplementation (400 IU/day for 6 weeks) caused unfavourable effects on endurance performance in swimmers (Sharman et al. 1971), and more recent studies also suggest negative effects of antioxidant supplementation such as blunted adaptation to exercise stimuli or promotion of exercise-induced oxidative stress (Malm et al. 1996, 1997; Avery et al. 2003; Nieman et al. 2004).

The number of antioxidants presented in the scientific literature and on the market is countless, including substances such as colostrum, caffeine, selenium, carotenoids

such as β-carotene, lycopene, lutein, zeaxanthin, coenzyme Q and many others. It is worth mentioning that most of these substances have not undergone proper evaluation and that there is insufficient scientific evidence regarding the ergogenic effects and long-term safety. In this section, we will therefore focus on studies assessing the connection between antioxidant supplementation and performance using either vitamin E or C as they are among the most powerful and well-characterised antioxidants (Beyer 1994). Vitamin E is one of the most important nutritional antioxidants accounting for membrane stability and fluidity by preventing lipid peroxidation (Jiang et al. 2011). Vitamin C contributes to the prevention of lipid peroxidation in interstitial fluid and plasma (Bradshaw et al. 2011). Different studies report blunted adaptations to the endurance exercise. Gomez-Cabrera et al. (2008) found a significant increase of ~186% in endurance performance in rats subjected to running for 6 weeks, 5 days/week on an animal treadmill. Endurance performance was significantly blunted (increase ~26%) in rats that received a daily dose of vitamin C (0.24 mg/cm^2 body surface area). The authors found that vitamin C supplementation had a significantly negative effect on mitochondrial biogenesis. VO_{2max}, however, was not negatively affected by antioxidant supplementation along the lines of an excess capacity of muscle mitochondria over O_2 delivery by the circulation (Boushel et al. 2011). Similar results were obtained in the same study with men training for 8 weeks (Gomez-Cabrera et al. 2008). Subjects receiving an oral dose of 1 g vitamin C per day increased VO_{2max} by 11% compared to a 22% increase in the non-supplemented group after 8 weeks of training. The results were not significant, probably due to a small sample size of only five people in the supplemented group. Also, no mechanistic pathway analysis from muscle tissue obtained from biopsies was performed in the human group due to ethical restraints. Although it is, in general, commendable to include a human and an animal trial in one study, direct comparisons between humans and rats cannot be made and translations of results are not always straightforward. It is worth mentioning that inbred rats are nearly genetically identical compared to human subjects, who are inherently genetically diverse. Sample size calculations based on findings from homogeneous, nearly identical animals would therefore lead to an underestimation of subjects in human studies. This study represents a typical example where findings from an animal study were not confirmed in the human study branch possibly due to a type 2 error. The use of an adequate sample size is a prerequisite to obtaining meaningful results.

In another human study by Khassaf et al. (2003) the negative effects of ascorbic acid supplementation on the adaptive responses of endogenous antioxidant enzymes and stress proteins were demonstrated. The authors found increased baseline activities of SOD, catalase (CAT) and HSP60 on vitamin C supplementation, which could be an indication of a pro-oxidant effect of vitamin C (Carr and Frei 1999). However, it still remains unclear whether this up-regulation of defence mechanisms is beneficial or deleterious to the tissue. It is also not clear whether suppression of the expression of these proteins following stress to skeletal muscle will be beneficial to skeletal muscle viability over the longer term. Further studies are necessary to elaborate on these questions. Furthermore, it has been shown that supplementation with ascorbic acid does not preserve muscle function but hinders the recovery process, thereby being detrimental to future performance (Close et al. 2006).

Asha Devi et al. (2003a, 2003b) studied the effect of vitamin E supplementation on swimming performance in rats of different age groups (4, 8, 12 and 22 months of age). The authors report significant differences in endurance capacity between supplemented and control rats over all age groups studied with vitamin E-supplemented rats showing higher endurance capacity. Further, supplementation with vitamin E resulted in a beneficial plasma lipid profile. The findings of Asha Devi et al. suggest an ergogenic effect of vitamin E supplementation on exercise performance. However, not all animal studies have shown performance increases following antioxidant administration. As an example, rats supplemented with vitamin E failed to improve treadmill time to exhaustion in two studies (Mehlhorn et al. 1989; De Oliveira et al. 2003). Exercise per se lead to an up-regulation of CAT and SOD activity in all age groups except the old group. Old animals only showed increased SOD activity when supplemented with vitamin E. This might indicate that supplementation is not necessary during youth. Malondialdehyde (MDA) content was lower in supplemented rats compared to the non-supplemented control group. Interestingly, the authors report reduced tissue vitamin E levels in the control group concluding that the antioxidant is utilised during exercise to scavenge free radicals. Importantly, this is again just true for the animals from the old group. As for the aforementioned studies also in this study, the sample size is rather low and it remains elusive whether these findings are transferable to humans. Whereas a number of studies report an ergogenic effect of antioxidants, the majority of studies found no changes or even negative outcomes. For a review, see Peternelj and Coombes (2011).

Intake of dietary supplements is very prevalent among the community of professional athletes (Huang et al. 2006). It is well established that a number of commercially available dietary supplements can be cross-contaminated with prohibited substances found on the Prohibited List of the World Anti-Doping Agency (Geyer et al. 2008). The intake of dietary supplements is therefore not only connected with a high risk of inadvertent doping but can also jeopardise the health of an athlete. Athletes should therefore use caution when considering supplementation with antioxidants, especially at higher doses.

In one particular case, the use of antioxidants for athletes may be justified. Dietary recall studies show that some athletes do not consume a balanced diet containing enough fruits and vegetables, and therefore these athletes potentially lack antioxidants (Farajian et al. 2004). In this case, it may be necessary to supplement with antioxidants in reasonable dosages to achieve physiological levels of antioxidants. Apart from that, athletes are rather encouraged to focus on a healthy, energetically adequate diet that is rich in antioxidant-containing foods (such as whole grains, fruits, vegetables, nuts and seeds). A balanced diet is the best nutritional approach to optimise a person's antioxidant status.

13.7   TYPICAL FLAWS AND PITFALLS OF STUDIES

The studies mentioned above show inconclusive results regarding the effects of antioxidants on health benefits and exercise performance. One limitation of these studies is the training status of the subjects. Participants are mostly untrained or moderately trained subjects with a VO_{2max} in the range of around 45–60 mL/kg/min. Besides

a limited number of recreationally active individuals who take supplements, it is mostly highly trained or even professional athletes who use antioxidants. It appears that there is a considerable difference with regard to oxidative stress between a single bout and regular physical activity. Repeated bouts of physical activity such as are experienced by athletes induce an up-regulation of the endogenous antioxidant defence system to minimise oxidative damage (Radak et al. 2008), whereas a single bout of exercise seems to be insufficient to induce these adaptations (Ji 2008). It is therefore not surprising that a number of markers of oxidative stress, for example, protein carbonyls (PC) and MDA appear to be unaffected in trained subjects in response to normal or even strenuous exercise (Farney et al. 2012; Bloomer et al. 2006) or if at all, seem to be more affected by a high fat diet than by exercise (McCarthy et al. 2013). These data suggest that oxidative stress is not elevated in exercise-trained subjects. Further, most of the studies only include an insufficient number of subjects and can therefore be considered as underpowered. Most studies do not provide sample size or power calculations in order for the reader to comprehend the likelihood that the findings are real. An important addition in every study would be to characterise the endogenous redox state of the subjects at baseline to characterise their defence system. It can be speculated that the endogenous defence system is better developed in well-trained than in untrained people, which again has an influence on the outcome in these different populations. Factors such as training status, age and sex have not been extensively studied and may have a considerable impact on oxidative stress response to exercise. Lastly, the site of occurrence between different organs and timing of the maximal effect of oxidative stress in response to an exercise bout have to be considered.

For future directions, it will be important for researchers to incorporate other substances than vitamins C, E and coenzyme Q10 based on the large number of available antioxidants that warrant systematic testing. The fact that these three substances were mainly studied makes generalisations difficult.

Regarding study design, a crossover design would be a clean way to study the effects of antioxidants. Also, established animal models of increased or decreased oxidative stress (e.g. the superoxide dismutase 2 nullizygous mouse as a model for increased oxidative stress and the transgenic mouse over-expressing mitochondrial catalase such as the mCAT mouse model for decreased oxidative stress) may prove very useful as positive or negative controls. It is, however, important to mention that findings from animal experiments cannot be automatically translated to humans.

Plasma volume decreases due to exercise-induced dehydration. This fact might be responsible for an overestimation of post-exercise markers of oxidative stress in studies that did not account for the changes in plasma volume.

A general problem of *in vivo* detection of antioxidants in humans lies in their short-lived nature. Although antioxidants have been detected *ex vivo* (Ashton et al. 1998), the transition to their behaviour *in vivo* is therefore often not necessarily scientifically sound. Free radicals exhibit a high reactivity and a relatively short half life (e.g. 10^{-5} s for superoxide radicals and 10^{-9} s for hydroxyl radical) (Fisher-Wellman and Bloomer 2009). They are therefore difficult to measure directly. A direct measurement method and gold standard of free radical assessment is electron spin resonance

spectroscopy using spin traps (Ashton et al. 1998). Although these methods provide accurate information their application is costly and requires specialist personnel. That is why the majority of researchers make use of indirect methods that have been developed to assess biomarkers of oxidative stress. These markers are always selective and never thorough, and studies using a variety of markers do not always provide consistent results (Vollaard et al. 2005). As a general rule, it can be stated that the more markers that are assessed the better. Investigating only one marker does not give a representative view of the oxidative stress status of the subject. One extensively used marker is TBARS, a reagent commonly used to assay MDA, which is an end product of lipid peroxidation. As there are other cellular sources of MDA, the TBARS assay is not entirely representative for lipid peroxidation (Trevisan et al. 2001). The fact that TBARS is not specific for MDA is often ignored (Bird and Draper 1984). Methodological shortcomings such as TBARS production during the assay (Vollaard et al. 2005) have to be taken into account and different markers of oxidative stress (e.g. isoprostane, lipid hydroperoxide, conjugated dienes and pentane) have to be considered. To account for methodological problems of assessing biomarkers of oxidative stress, it is therefore paramount to include a non-exercising control group as the baseline readout. Furthermore, information on exact procedures of assays is often neglected but should be included in every publication to comprehend the outcome. Similar methodological constraints apply when assessing levels of the popular antioxidant marker glutathione with its reduced (GSH) and oxidised (GSSG) fractions.

13.8   APPROPRIATE STUDY DESIGN AND METHODS TO ASSESS THE EFFECTS OF ANTIOXIDANTS ON EXERCISE PERFORMANCE

Proper planning of the study is essential. A simple checklist is provided in Table 13.2.

TABLE 13.2
Checklist to Support Study Planning

Which question is the researcher trying to answer?

What is already known on the topic of interest? Literature review?

What is the population of interest? Normal population? Athletes? Age? Gender?

Aim of the study and hypothesis?

Type of intervention or exposure? Product? Appropriate dosage? Duration of intake?

Appropriate study design? Randomised? Controlled? Blinded? Counterbalanced?

Primary and secondary outcome measures?

Standardised tests? Reliability and validity of applied tests?

What is the appropriate sample size? Statistical power?

What are the appropriate statistical analyses?

Ethical considerations? Informed consent? Approval by an ethics committee?

13.8.1   Rationale, Study Aim and Hypothesis

Numerous studies have investigated the effects of antioxidant supplementation on exercise performance, each having strengths but to some extent also weaknesses (Simon-Schnass and Pabst 1988; Reid et al. 1994; Matuszczak et al. 2005; Subudhi et al. 2006; Matzi et al. 2007; Gatterer et al. 2013). The rationale to study such effects is the hypothesis that oxidative stress possibly contributes to muscle fatigue and might impair exercise performance (Reid 2001). Supposed mechanisms are impairments of muscle and cellular function due to the modifications or damages of contractile proteins, suppression of the calcium sensitivity of myofilaments, alterations of mitochondrial function and its degradation (Callahan et al. 2001; Ott et al. 2007; Murphy et al. 2008). Consequently, eliminating oxidative stress is assumed to improve performance.

When investigating within this field, knowledge of the specific literature is of utmost importance. This enables gaps to be established in the research and is valuable in formulating the study rationale and the study aims. Importantly, any interventional study greatly benefits from a prospective hypothesis and a study design that makes it possible to accept or reject the hypothesis within the primary end point (Preiser et al. 2002). Within the field of nutritional studies, a double-blinded, randomised and placebo-controlled study design is favoured.

13.8.2   The Study Participant

When selecting participants it is important to recognise that various characteristics of the participants might influence the outcome of the investigation. These include anthropometric and demographic parameters (e.g. body composition, age and sex distribution), type of practised sport (e.g. cycling vs. running), fitness level (e.g. recreationally active individuals vs. elite athletes), training periodisation (e.g. off-season vs. in-season) and baseline oxidative stress and defence variables (e.g. participants with high vs. low levels of oxidative stress at baseline). Thus, selection depends on the study aims and hypotheses. Moreover, chronic and/or acute diseases of the participants and behavioural factors, including smoking, caffeine and alcohol consumption, drug use and special diet, need to be controlled and might, according to the study goal, be considered as exclusion criteria. It has to be mentioned that studying healthy trained subjects having a balanced oxidative and antioxidative status already at the start of the investigation might challenge the detection of effects of a supplementation (Ellinger et al. 2011).

13.8.3   Randomisation and Blinding

After the selection of participants, a random assignment to different groups (intervention or placebo) is advisable. Stratification for fitness level, oxidative status, age and sex results in homogeneous groups, and allows documentation of possible intervention effects. It is important that participants as well as investigators involved in data processing are, at best, blinded to the intervention. Needless to say, participants should receive antioxidants or placebos that are identical in taste and appearance,

and only investigators not involved in data processing are allowed to manage group allocation and supplement distribution. This procedure guarantees that placebo effects and investigator biases can be excluded.

13.8.4 Sample Size and Statistical Power

To obtain appropriate results, the use of an adequate sample size is a prerequisite. Established statistical procedures will help to choose proper sample sizes. It is not enough to reject the null hypothesis only because of statistical significance, but also because of practical importance and clinical relevance. Further, type I and type II errors have to be considered. A type I error (corresponding to a significance level of usually 0.05) is an incorrect rejection of the true null hypothesis. A type II or beta error is an error not rejecting a false null hypothesis. The statistical power (1-beta) is the probability that a test will reject the null hypothesis, although it is false. Thus, a beta error of 0.1 corresponds to a power of 90%, meaning a 90% probability of rejecting an actually false null hypothesis. A statistical power of at least 80% is necessary for a test to detect an effect if it actually exists.

13.8.5 Main Outcome Measures

The effectiveness of the supplementation is evaluated by measuring exercise performance and oxidative status before and after the intervention. Tests need to fit the aims of the study. With respect to exercise performance, laboratory or field tests are applicable. Within the laboratory setting, incremental exercise tests, time-to-fatigue tests or time trial tests in combination with gas analyses, lactate diagnostics and so on may provide valuable information on the physiological responses and the mechanisms leading to performance changes. Field tests are considered to resemble competition situations more closely but might miss explanatory measures. It has to be added that effects of antioxidant administration on performance may critically depend on the type of exercise testing (Reid et al. 1994). With respect to the measured oxidative stress and defence parameters, a large number of markers are investigated. Selecting parameters supposedly involved in muscle and cellular function might yield additional information. Most frequently, hydrogen peroxide, total and oxidised glutathione, 8-hydroxydeoxyguanosine, MDA, protein carbonyl content, 4-hydroxynonenal and nitrotyrosine were investigated (Subudhi et al. 2006; Nikolaidis et al. 2012a; Gatterer et al. 2013). Moreover, activity levels of CAT, CuZnSOD, MnSOD, glutathione peroxidase and plasma concentrations of α-tocopherol and β-carotene were also used to reflect antioxidative status (Subudhi et al. 2006; Nikolaidis et al. 2012a). It has to be recognised that oxidative stress markers measured in blood plasma might not reflect the conditions within the working muscles (Subudhi et al. 2006).

13.8.6 Considerations during the Experiment

The study period (i.e. long-term vs. short-term supplementation) needs to be in accordance with the aims of the study. During the supplementation period, two different procedures can be followed. First, the training practice of participants is kept

unaltered, with groups performing approximately the same training load. Second, participants of both groups perform the same training load which is different from their usual training regime, for example, high altitude training or high intensity training. Such procedures allow investigation of the effects of the supplementation on specific training adaptations. In any case, a training logbook helps to document training activities. Additionally, it is advisable to maintain normal nutritional habits throughout the study and to document any changes. Participants suffering from acute infections or injuries during the intervention period preferentially have to be excluded from the study, since such conditions might impact outcomes (Schwarz 1996; Tiidus 1998).

13.8.7　Use of Appropriate Statistical Tests

Nutritional studies typically compare the efficacy of a new preparation with another and/or a placebo. Besides a pure description, it is important to know whether observed differences between groups (preparations) are just random or are really true. Inferential statistical tests commonly used in the nutrition profession have been recently summarised by Saracino et al. (2013) and these authors also report how to choose them.

13.9　CONCLUSION

Making decisions about medical care and support of athletes, including the use of dietary/supplemental antioxidants in sport, should be based on the best available evidence from systematic research. However, large discrepancies exist between scientific evidence and the promotion by manufacturers and distributors of antioxidants. When evaluating the effectiveness of dietary/supplemental antioxidants, comprehensive knowledge of integrated biochemical and physiological mechanisms of exercise metabolism, free radicals and antioxidants is as necessary as the consideration of stringent methodological standards.

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14 Common Questions and Tentative Answers on How to Assess Oxidative Stress after Antioxidant Supplementation and Exercise

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CONTENTS

14.1 INTRODUCTION

Redox biology has been one of the most rapidly developed fields of biology and one of the most popular in the mass media. Free radicals (reactive species in the present review) have been linked to many different biological processes, such as cell signalling (Forman et al. 2010), enzyme activity (Stubbe and Van Der Donk 1998), synthesis of antibiotic substances (Lesniak et al. 2005) and pathophysiology of diseases (Valko et al. 2007). From the results of thorough investigations conducted in the past three decades, it is now clear that acute exercise induces oxidative stress, whereas chronic exercise enhances the endogenous antioxidant mechanisms (Camiletti-Moirón et al. 2013; Theodorou et al. 2011). Along with the progress of the exercise redox biology, the *in vitro* molecular and biochemical properties of many nutrient compounds possessing redox properties (i.e. pro-oxidants and mostly antioxidants) have also been revealed. However, despite the long-standing research efforts, it is still uncertain whether and how the exogenous administered antioxidants affect redox homeostasis *in vivo* and physical performance (Bell et al. 2013; Braakhuis 2012; Nikolaidis 2012c; Peternelj and Coombes 2011; Powers et al. 2010).

Why did it prove to be difficult to reveal the effects of antioxidant supplementation on oxidative stress and human physiology? We believe that the main reason is the methodological uniqueness of each study, particularly regarding the research strategy that investigators adopt on issues relevant to redox biology. Taking into account that redox biology of exercise is a relatively new field, research is driven more on intuition and less on sound methodological evidence. Thus, it is desirable to develop and achieve some agreement on key influencing factors, which investigators should take into account when designing studies in the area of redox biology. Therefore, the aim of this chapter is to provide a methodological framework and broad directions on setting up appropriate experimental set-ups. In particular, we have introduced and tentatively answered eight questions, which a researcher may come across when designing experiments in the redox biology of exercise. It is emphasised, particularly considering the inherent complexity of redox biochemistry, that the following answers are based on the current knowledge; therefore, they can always be amended or disproved by new evidence and should not be accepted as the final answers.

14.2 QUESTION 1: WHICH REDOX BIOMARKERS TO MEASURE?

'Oxidative stress' remains until today a term not clearly defined. In this chapter, when referring to oxidative stress we mean 'any increase in the level of reactive species and/or oxidant biomarkers', which is consistent with the interpretation of oxidative stress in most studies (Nikolaidis et al. 2012b). In addition, any shift (increase or decrease) in the level of reactive species, oxidant biomarkers, antioxidants and/or redox-active molecules will be referred to as an 'alteration in redox homeostasis' (Nikolaidis et al. 2012b).

In most studies, redox biomarkers are traditionally considered as some end or intermediary products of a chemical reaction between a reactive species and a biomolecule. However, other molecules, for example, hydrogen peroxide (H_2O_2), vitamin C and others, can also be considered as redox biomarkers since they can affect redox
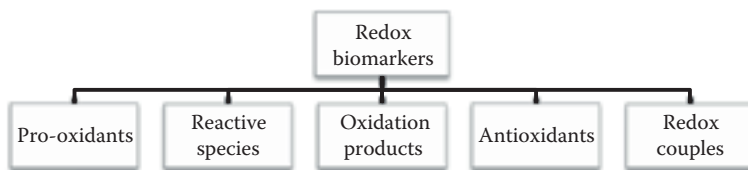
FIGURE 14.1   Classification of redox biomarkers.

homeostasis. Therefore, we broadly categorised redox biomarkers as pro-oxidants, reactive species, oxidation products, antioxidants and redox couples (Figure 14.1). Only a small portion of the available redox biomarkers in each category is presented. The selection was based on three factors: (i) the frequency with which they are currently used in research, (ii) the ability to be measured without requiring specialised equipment and (iii) the reliability and validity of their measurement.

14.2.1   PRO-OXIDANTS

The chemical compounds that promote the oxidation of biomolecules can be defined as pro-oxidants (e.g. iron and copper ions). This is mediated either by generating reactive species or by inhibition of antioxidant mechanisms. Hydrogen peroxide is probably the most well-studied pro-oxidant. Although it is actually a reactive species, we have included it in the category of pro-oxidants because it is a weak oxidising agent and poorly reactive [not able to oxidise lipids, DNA and most proteins (Halliwell and Gutteridge 2007)]. Besides, one of the supposedly major functions of H_2O_2 is to act as a pro-oxidant when reacting with ferrous iron (Fe^{2+}) or tyrosine leading to the generation of highly reactive species, such as hydroxyl radical ($^\bullet OH$) and tyrosyl radical.

Except for the H_2O_2, Fe^{2+} (the other reactant of the Fenton reaction) is another representative member of the pro-oxidant family. Ferrous iron is present in several human cells (e.g. haemoglobin, macrophages, myoglobin, liver, intestinal lumen) and has the ability to donate and accept electrons with relative ease (Pantopoulos et al. 2012). An interesting fact is that many chemical compounds that traditionally belong to antioxidants, under certain circumstances, can act as pro-oxidants as well (Villanueva and Kross 2012). Such compounds are vitamin C (Bradshaw et al. 2003), vitamin E (Tafazoli et al. 2005) and uric acid (Sautin and Johnson 2008). For example, although uric acid accounts for most of the antioxidant ability in plasma, it becomes a pro-oxidant at high concentration, thus leading to oxidation of LDL and liposomes by peroxynitrite as well as oxidation of LDL in the presence of copper ions (Cu^+ and Cu^{++}) and lipid hydroperoxides (Sautin and Johnson 2008).

14.2.2   REACTIVE SPECIES

The term 'reactive species' includes reactive oxygen species, reactive nitrogen species and reactive chlorine species, which can be either radicals or non-radicals. By definition, a free radical is any species capable of independent existence that contains one or more unpaired electrons (Halliwell and Gutteridge 2007). The presence of

these unpaired electrons renders free radicals highly reactive towards other biomolecules. However, their chemical reactivity varies greatly, for example, superoxide ion ($O_2^{\bullet-}$) is less reactive, whereas $^{\bullet}OH$ oxidises everything around it with a half-life of about one trillionth of a second (Halliwell and Gutteridge 2007).

Electron spin resonance (ESR) is the only technique capable of measuring reactive species directly (Buettner 1987; Davies and Hawkins 2004). ESR detects unpaired electrons and thus is used only for detecting free radicals (and not the non-radical reactive species). An unpaired electron, due to its electrical load, behaves like a small magnet. Thus, when a concrete electromagnetic energy is applied, the energy level of the unpaired electron changes and this alteration can be detected by ESR (Halliwell and Gutteridge 2007). The main disadvantage of ESR is that it detects mostly less reactive radicals. The highly reactive radicals cannot be detected because they react rapidly with nearby biomolecules or with antioxidant compounds. A solution was given by substances called spin traps, which react rapidly with free radicals generating other radicals that are more stable and ESR detectable. The most commonly used spin traps are the DMPO, DEPMPO and PBN, which have been used only in animal studies (Berliner et al. 2001). The ability of spin traps to react with radicals led scientists to ponder on them as potential therapeutic antioxidants, attributing to them a more physiological character (Dikalov and Harrison 2014). Unfortunately, whether these spin traps are free of toxic effects is still unknown, since they have not been tested *in vivo* in human studies (Halliwell and Whiteman 2004). For this reason, when free radicals are measured in a particular human tissue, the sample (mostly blood or muscle) is added into a spin trap-solution in order to react with free radicals *ex vivo* (Clermont et al. 2002; Valgimigli et al. 2002). As a result, the highly reactive free radicals cannot be detected by the *ex vivo* technique, because they have already reacted before the sample collection (Halliwell and Whiteman 2004). For human *in vivo* studies, the most frequently used spin traps are of aromatic nature, which are considered not harmful (e.g. salicylate and phenylalanine that can be hydroxylated by $^{\bullet}OH$; Themann et al. 2001). Generally, an ideal spin trap should fulfil the following requirements: (i) react quickly, efficiently and exclusively with the free radical of interest; (ii) generate through its reaction with a free radical a product that is stable and not further metabolised and (iii) the product between the spin trap and the free radical should emit a unique ESR spectrum (Halliwell and Gutteridge 2007). Unfortunately, a spin trap fulfilling all these requirements (if it exists) has yet to be found.

14.2.3 Oxidation Products

This is the most extensively investigated category of redox biomarkers. This category is composed of oxidatively modified biomolecules, namely products generated by the reaction of free radicals with biomolecules. The term 'modified' is preferred instead of the term 'damaged', because recent studies do not support the old point of view, whereby every reaction of free radicals with a molecule was associated with harmful consequences. In this section, we limit our analysis (in most cases) to the two most popular and reliable biomarkers reflecting oxidative modifications to each of the three main molecular targets of free radicals (i.e. lipids, proteins and DNA). Carbohydrates (Benov and Beema 2003) and RNA (Jorgensen et al. 2013) are also
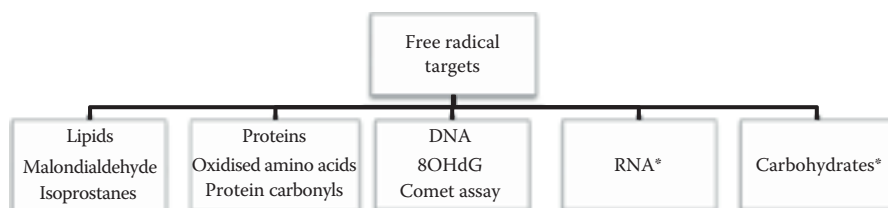
FIGURE 14.2 Free radical targets and two of the most frequently used biomarkers in each category. *No adequate data available.

oxidisable biomolecules; however, only very limited data are available for these targets (Figure 14.2).

14.2.3.1 Lipids

Lipid peroxidation, namely the reaction of reactive species with lipids (e.g. fatty acids, triacylglycerols, phospholipids, sterols) leads to a wide range of products through complex processes. For a long time, lipid peroxidation was considered only a harmful process. Nowadays, it is certain that many beneficial effects accompany lipid peroxidation (Greenberg et al. 2008). Additionally, most studies tended to focus on cell membrane lipid peroxidation, despite the existence of other lipids found in cytosol such as triacylglycerols, which could also be oxidised (Wu et al. 1999). Various methods have been developed to quantify the products of lipid peroxidation reactions. Two of the most popular and reliable biomarkers for the detection of lipid peroxidation are presented below.

The old and simple thiobarbituric acid (TBA) assay seems inadequate in modern research. This is because most of the TBA-reactive substances (TBARS) formed *in vivo* are not related to lipid peroxidation (Halliwell and Whiteman 2004). On the bright side, the measurement of $(TBA)_2$-malondialdehyde (MDA) adduct in human plasma with the use of high-pressure liquid chromatography (HPLC) (in order to separate this adduct from other chromogens) increases markedly the specificity of the assay (Breusing et al. 2010). However, reaction of MDA with TBA still requires treatment at high temperatures for extended incubation times and in strong acidic conditions, which may result in artefactual peroxidation of sample constituents (Mateos et al. 2005). Alternatively, derivatisation of MDA with 2,4-dinitrophenylhydrazine (DNPH) and conversion into pyrazole and hydrazone derivatives has been found to allow specific estimation of this compound if combined with its separation using HPLC (Mateos et al. 2005).

By today's standards, isoprostanes is considered to be the best lipid peroxidation biomarker (Halliwell 2009). Isoprostanes are peroxidation products of polyunsaturated fatty acids. The most frequently measured isoprostane class is F_2-isoprostanes (Montuschi et al. 2007; Nourooz-Zadeh 2008) and particularly the abundant stereo-isomer $15\text{-}F_{2t}\text{-}IsoP$ (frequently also called 8-iso-prostaglandin F_{2a}). In human studies, $15\text{-}F_{2t}\text{-}IsoP$ has been mostly measured in the blood and urine and, to a lesser extent, in muscle biopsies (Karamouzis et al. 2004). The most reliable methods used for F_2-isoprostane measurement are gas chromatography-mass spectrometry
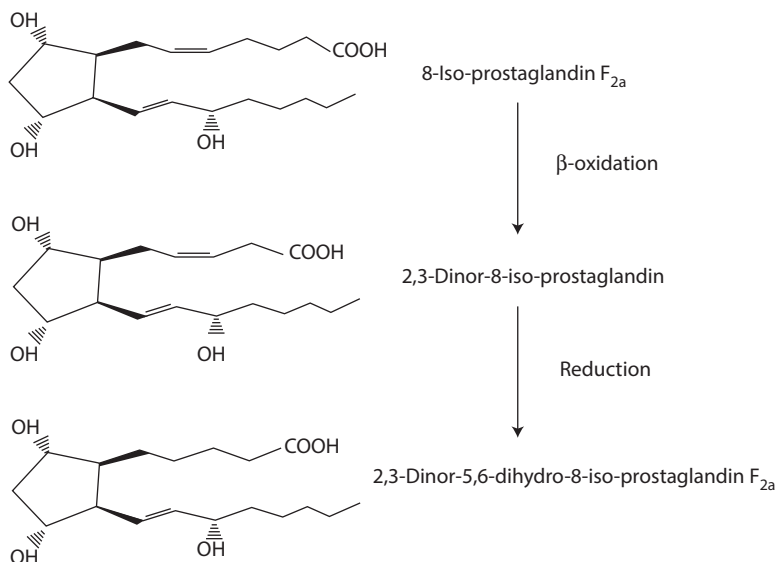
FIGURE 14.3 Metabolism of the most frequently measured isoprostane 8-iso-prostaglandin F_{2a}. Owing to their higher accumulation, it is possible that these two metabolites of 8-iso-prostaglandin F_{2a} will prove to be more valid and reliable biomarkers than the parent molecule. (Structural formulae drawn in Mnova Lite 5.2.5 (Mestrelab Research, Spain).)

(GC-MS) and the most recently developed–HPLC-MS (Nikolaidis et al. 2011). The aforementioned methods offer sufficiently accurate results; nevertheless, they require sophisticated equipment. As a result, most of the exercise physiology and nutritional laboratories have no access to this equipment and for this reason the use of commercially available immunoassay kits for F_2-isoprostanes measurement is very common. It is important to consider that most of the studies suggest that GC- or HPLC-MS and immunoassay kits do not measure the same compounds (Nikolaidis et al. 2011). Therefore, caution needs to be exercised when comparing results from GC- or HPLC-MS and immunoassay kits. A very important advantage of isoprostanes as redox biomarkers is that their levels in plasma or urine seem not to be confounded by isoprostanes present in food (Gopaul et al. 2000). On the contrary, an important drawback of isoprostanes is that they are unstable in plasma with a half-life of only 20 min (Basu 1998). On a positive note, some of their metabolites (β-oxidation products like 2,3-dinor-8-iso-prostaglandin F_{2a} and 2,3-dinor-5,6,-dihydro-8-iso-prostaglandin F_{2a}) are more stable and found in higher concentrations than F_2-isoprostanes [up to 15-fold and 6-fold higher, respectively (Dorjgochoo et al. 2012; Nourooz-Zadeh et al. 2005; Yan et al. 2007)], and thus it is possible that they may prove to be more reliable lipid peroxidation biomarkers in the future (Figure 14.3).

14.2.3.2 Proteins

Protein modifications are caused by the reaction of proteins with free radicals, lipid peroxidation products (e.g. 4-hydroxynonenal) or by glycosylation. Protein modifications have important physiological repercussions because they directly affect the

function of receptors, antibodies or enzymes and may lead to indirect modification of biomolecules (e.g. inactivation of enzymes related to DNA repair) (Halliwell and Gutteridge 2007). Oxidative modifications of proteins can be reversible or irreversible. In the case of irreversible modifications, the oxidised protein should be removed or destroyed because it may result in cell death (Davies 2001). Protein oxidation has been mostly measured through protein carbonyls (Levine et al. 2000) and by quantification of specific amino acid oxidation products (Hawkins et al. 2009).

The protein carbonyl assay is a widely used technique that measures carbonyl groups and provides a general picture of the systemic protein oxidation. This is because protein carbonyls can also be originated by protein glycation or by the binding of aldehydes to proteins, processes that overestimate the actual protein oxidation (Negre-Salvayre et al. 2008). In addition, as it is the case with all biomarkers, what is actually measured is the oxidised protein turnover, namely the balance between production and removal of oxidised proteins (Halliwell and Whiteman 2004). Measurements of protein carbonyls in fluids and tissues can be performed spectrophotometrically (Levine et al. 2000) after the reaction of protein-bound carbonyls with DNPH or by using DNPH antibodies in immunochemical techniques, such as western blotting (Shacter et al. 1994) and enzyme-linked immunosorbent assay (ELISA) (Buss et al. 1997).

Reactive species react individually with all the 22 amino acids in many different ways. Oxidation of amino acids produces molecules such as kynurenines, bityrosines, valine hydroxides and L-DOPA (Halliwell and Whiteman 2004). Bityrosine (or dityrosine) is considered to be one of the most reliable redox biomarkers and it is easily detectable in human plasma and urine using the ELISA technique (Davies et al. 1999; Giulivi and Davies 2001). Since amino acids are also oxidised by reactive nitrogen species and reactive chlorine species (e.g. peroxynitrite and hypochlorous acid), substances like nitro- and chloro-tyrosines have also been considered as redox biomarkers (Buss et al. 2003; Leeuwenburgh et al. 1998). Indeed, some of these oxidation products, for example, 3-nitrotyrosine, are thought to be sensitive and stable biomarkers (Hawkins et al. 2009). For the measurement of all the aforementioned oxidised amino acid products, the tools used are antibody techniques, GC-MS and HPLC. The most sensitive and reliable are the GC-MS and the HPLC techniques (Kaur et al. 1998). However, the required hydrolysis of proteins, in order to separate the amino acids, may lead to artefacts (Halliwell and Whiteman 2004).

14.2.3.3   DNA

Oxidative DNA modifications induced by reactive species and followed by repair occur continuously into cells (Halliwell and Gutteridge 2007). DNA bases can be oxidised by several reactive oxygen (especially by ˙OH) and/or nitrogen (e.g. dinitrogen trioxide) species; thus, many products are generated (Dizdaroglu et al. 2002). However, the repair process of the oxidised DNA bases may be overwhelmed and this imbalance between oxidation and repair may lead to cell death. This is why DNA oxidation more than any other redox biomarker has been associated with diseases (Halliwell 2006, 2007b). DNA oxidation has been mostly investigated in leukocytes (especially in lymphocytes) and in urine by HPLC, GC-MS, HPLC-MS and antibody-based techniques.

The measurement of 8-hydroxy-2′-deoxyguanosine (8OHdG) in urine represents a whole-body DNA oxidation assessment and there is a good agreement among laboratories on the concentration of this biomarker in urine (Halliwell and Gutteridge 2007). It is most commonly measured through ELISA, HPLC and MS-based techniques. The ELISA technique needs caution because it may give falsely elevated levels of DNA oxidation (Halliwell and Whiteman 2004). Specifically regarding the measurement of 8OHdG in urine, it is critical to consider that the procedure requires 'cleaning' of the sample from interfering substrates unrelated to oxidation (Lin et al. 2004). Measurements of 8OHdG have also been performed in DNA isolated from tissues. However, numerous artefacts have been reported and the results of the several studies seem inconsistent (Collins et al. 2004; Gedik and Collins 2005). Most artefacts are formed during the isolation and preparation of the samples, due to sample exposure to oxygen and to traces of transition metals (Halliwell 2000).

The 'Comet assay' (Duthie et al. 1996; Fairbairn et al. 1995), which is applied directly to cells (mostly in leukocytes) and measures DNA strand breaks, is an approach to bypass the artefacts occurring during the isolation and analysis of DNA. This technique is a single-cell gel electrophoresis, easy to perform and does not require DNA isolation. Additionally, since this method is a cell-assay, it has been used for investigating the potential protection effects of an antioxidant specifically to cells (Mastaloudis et al. 2004). Generally, the comet assay indicated decreased levels of DNA oxidation after antioxidant supplementation in humans (Duthie et al. 1996). However, there is no sufficient information whether these effects appear due to minimised artefactual DNA oxidation (i.e. the high reliability of the assay) or due to underestimation of oxidative damage by the assay (Halliwell and Whiteman 2004). This assay is considered to be one of the most reliable in redox biology, although some limitations exist. For instance, strand breaks can be induced not only by DNA damage, but also by enzymatic repair leading to a 'false increased' number of strand breaks (Spencer et al. 1996). Moreover, two strand breaks occurring within a short interval may be perceived incorrectly as one, leading artefactually to minimised DNA oxidation (Collins 2013).

14.2.4 Antioxidants

An antioxidant is defined as any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell and Gutteridge 2007). However, the characterisation of a substance as an 'antioxidant' becomes more constructive when it is followed by the oxidant agent that neutralises (Azzi et al. 2004) and the assay used to measure it is reported (Gutteridge and Halliwell 2010). Additionally, the characterisation of a substance as 'antioxidant' *in vitro* does not automatically lead to its acceptance as an 'antioxidant' *in vivo* (Azzi et al. 2004; Veskoukis et al. 2012). A well-organised antioxidant system exists and works continuously in the human body. Performing a 'functional' categorisation of antioxidants, they are separated in reactive species scavengers (e.g. vitamin C and catalase), molecules that reduce reactive species generation (e.g. a chelator such as EDTA) and molecules that increase the production of antioxidants (e.g. isothiocyanates). On the basis of whether or not they exhibit enzymatic activity, antioxidants are divided into two major categories: the enzymatic and non-enzymatic antioxidants. Obviously, these are artificial divisions

and all antioxidants work as a united complex system throughout the cytoplasm, within organelles (e.g. mitochondria) and in the extracellular and vascular space (Pamplona and Costantini 2011).

The category of non-enzymatic molecules usually includes low molecular weight compounds, such as vitamin C, vitamin E, reduced glutathione (GSH), uric acid, coenzyme Q, lipoic acid, selenium and carotenoids. Although interdependence exists among some of them [e.g. uric acid "stabilises" vitamin C plasma concentrations (Sevanian et al. 1985), which in turn recycles vitamin E (Buettner 1993)], this category is completely heterogeneous, and the only common link among these antioxidants is that they do not display enzymatic activity. The most commonly investigated non-enzymatic antioxidants are vitamin C and uric acid (as the two most important hydrophilic antioxidants), vitamin E (as the most important lipophilic antioxidant) and GSH (as the most important cellular antioxidant). The most commonly used techniques for their determination are various spectrophotometry- and HPLC-based assays.

The major endogenous antioxidant enzymes against oxidation are superoxide dismutase, catalase, glutathione peroxidase and the peroxiredoxins. Most of the enzymatic antioxidants exhibit their normal activity inside the cell, and therefore their measurement in plasma or urine has no apparent physiological value. Hence, given the difficulty in obtaining tissue samples, the most suitable specimens in human investigations are erythrocytes. Virtually, it is unknown which of these enzymes responds better to an oxidative challenge, since (at least theoretically) they are sensitive to different reactive species. Therefore, it is premature to suggest a specific enzymatic antioxidant as the ideal antioxidant biomarker.

14.2.5 REDOX COUPLES

In recent years, there has been an increasing interest in redox couples and particularly the redox potential that is calculated through the Nernst equation (Dimauro et al. 2012; Go and Jones 2011; Kemp et al. 2008). It has been suggested that a cell as a biological entity responds according to redox potential differences (Schafer and Buettner 2001; Jones 2008). The oxidised/reduced pair of some molecules (better known as 'redox couples') are in the core of the 'redox hypothesis' (Jones 2008) and nicely reflect the concept of redox homeostasis (Nikolaidis et al. 2012b). Virtually, electron(s) transfer is taking place in redox couples, where the reduced molecule loses electron(s) becoming the oxidised form and vice versa.

Redox potential is calculated by the Nernst equation:

$$\Delta E = \Delta E^0 - \frac{RT}{nF} \ln Q$$

Here, ΔE^0 is the standard reduction potential, R is the universal gas constant, T is the temperature, n is the number of electrons transferred, F is the Faraday constant and Q is the mass law quotient with the actual concentrations of reaction partners. The most frequently studied redox couple is that composed of glutathione (GSH) and glutathione disulphde (GSSG), mostly because of the GSH high cell content (1–10 mM; Jones 2008). For the GSH/GSSG couple, the reaction quotient is

$$Q = \frac{[GSSG]}{[GSH]^2}$$

The redox potential calculated by GSH and GSSG concentration in cells/fluids has produced values ranging from −300 mV to −140 mV and also been correlated with the biological status of the cell (Kemp et al. 2008). In addition, the less the redox potential (i.e. more negative), the higher the reductive capacity of the cell/fluid.

In most (if not all) of the relevant exercise literature, in which GSH and GSSG were measured, the redox potential was not calculated. Instead, it has been calculated as the ratio of GSH/GSSG. When the ratio decreases, this was an indication of 'oxidative stress', whereas an increased ratio was indicative of 'reductive stress'. However, this kind of conclusion, based on the GSH/GSSG ratio, ignores the large concentration differences between GSH and GSSG in the blood (or in any other tissue). Indeed, the concentration of GSH in the blood is about 100-fold greater than that of GSSG. The following example illustrates how misleading the uncritical use of the GSH/GSSG ratio could be. For the sake of simplicity, if it is assumed that at rest blood GSH concentration is 1 mM and GSSG concentration is 0.01 mM, then the GSH/GSSG ratio is 100. On the basis of this value, it is frequently assumed that a GSH/GSSG ratio equal to 100 is the 'basal redox state', a ratio less than 100 denotes 'oxidative stress' and a ratio higher than 100 denotes 'reductive stress'. Although the ratio and its interpretation work well in cases where acute exercise is employed, it does not seem to work as well with chronic exercise. Acute exercise induces opposite changes in the concentration of glutathione, by decreasing GSH and increasing GSSG (Nikolaidis et al. 2007; Michailidis et al. 2007). As a result, their ratio (GSH/GSSG) magnifies the effect of exercise. However, in a hypothetical scenario after chronic exercise, if the values stated above were doubled at rest, that is, GSH becomes 2 mM and GSSG becomes 0.02 mM, then the GSH/GSSG ratio will remain unaltered, that is 100. In this case, if we adopt the explanation given for the acute exercise, there is no change in the redox state of glutathione. Nevertheless, we believe that this would be a misleading conclusion, because in fact chronic exercise induces redox adaptations by increasing GSH concentration and its antioxidant function. In the case of chronic exercise and based on the changes in the concentrations of GSH and GSSG, we would even conclude that there is a 'reductive stress'. Therefore, in that particular case-scenario, calculating the redox potential through the Nernst equation can possibly provide a more realistic representation of what is happening *in vivo*. This is because in the Nernst equation GSH enters the calculation as GSH squared (GSH^2), and consequently the redox potential depends not only on the GSH/GSSG ratio but also on the absolute concentrations of GSH and GSSG. Without question, the increasing use of the Nernst equation is not derived solely from the sudden acceptance of its ability to reflect the reductive capacity of the cell, but also by the struggle to achieve mathematical realism using equations and paper chemistry. Therefore, transforming the easily understood GSH/GSSG ratio into abstract redox potentials is not without disadvantages. The major limitation of using the Nernst equation stems from the fact that assumes reversible systems and equilibrium conditions, which actually occur rarely *in vivo* (Flohé 2013; Kohen and Nyska 2002).

14.3   QUESTION 2: HOW MANY REDOX BIOMARKERS TO MEASURE?

Aside from the redox biomarkers mentioned above, there are countless others. However, very few of them (including many of the biomarkers presented above) have been subjected to a rigorous test of validity and reliability. Taking into account also the complexity of redox biology, it is not surprising that most researchers measure a battery of biomarkers as a means to provide a more satisfactory view on the general redox status of the human body. However, it should be kept in mind that the results produced by measuring, for example, 10 unreliable biomarkers are equally untrustworthy to those produced by a single unreliable biomarker.

Indeed, even within the same biomarker category (for instance, DNA oxidation or lipid peroxidation), the corresponding biomarkers, which presumably indicate the same type of oxidative modifications, may provide partially or completely different responses to the same oxidant stimulus (Breusing et al. 2010; Watters et al. 2009). This is not surprising, since each biomarker is a product of a specific chemical reaction. Considering also that many different techniques (e.g. HPLC, GC-MS or ELISA) are frequently applied to determine the same biomarker and that many different protocols are available (e.g. the determination of MDA via HPLC can be performed using many different derivatisation protocols; Bird and Draper 1984; Mateos et al. 2005), comparisons among studies become even more difficult (Figure 14.4).

However, assessment of redox homeostasis through only one or few biomarkers is also risky, because almost always the oxidative pathway(s) of the oxidant stimulus are unknown and the chosen biomarker may not respond to this pathway. Unfortunately,
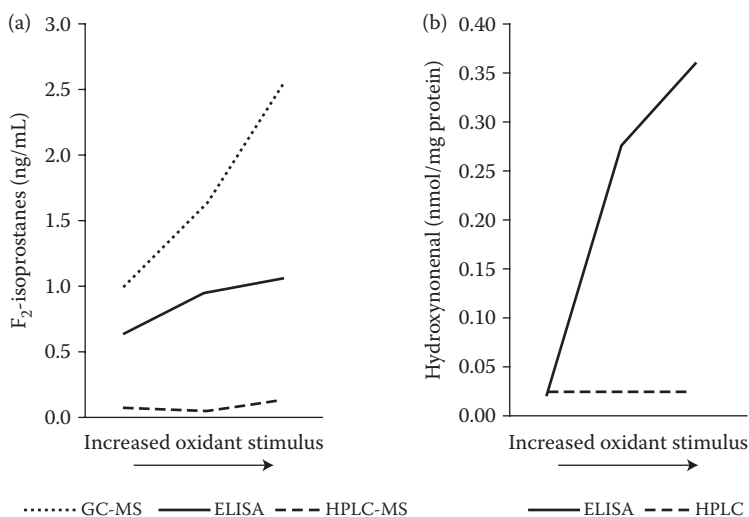
FIGURE 14.4   Different techniques for measuring the same redox biomarkers often produce different results both under non-stressed and stressed conditions. (Adapted from Breusing, N., T. Grune, L. Andrisic et al. 2010. An inter-laboratory validation of methods of lipid peroxidation measurement in UVA-treated human plasma samples. *Free Radic Res.* 44(10):1203–15.)

due to the absence of validation studies, a specific number of biomarkers cannot be suggested. For these reasons, and until an 'ideal' biomarker is found (if it exists), we believe that the safest approach is for each laboratory to validate its own set of redox biomarkers and to choose which biomarkers to measure based on the hypotheses and experimental set-up of their study.

14.4   QUESTION 3: WHICH BODY FLUID TO COLLECT?

One major issue of the studies looking to assess redox homeostasis is the type of sample for analysis. Given the objective difficulties in accessing muscle or other tissue samples in human studies, the present chapter has been focused on body fluids. A key question is in which body fluid (i.e. blood or urine) could alterations in redox biomarkers be more easily and reliably detected (Veskoukis et al. 2009). Blood has a unique place in biomedical science, since its easy collection renders it the most frequently studied tissue (Nikolaidis et al. 2012b). For a long time, researchers have focused on muscle-derived reactive species production (Nikolaidis et al. 2008), particularly in animal studies, disregarding the role of blood. In particular, blood through the circulatory system interacts with almost all organs, tissues and cells of the human body. Clearly, during this interaction, a considerable exchange of redox biomarkers takes place, and this is probably the main reason why biomarkers measured in blood are considered to have been transferred from other tissues.

In addition, blood itself is a noticeable generator of reactive species (Nikolaidis and Jamurtas 2009). In fact, blood plasma contains metal ions that can lead to reactive species generation (e.g. reaction between H_2O_2 with Fe^{2+}) as well as carbohydrates (e.g. glucose), proteins (e.g. albumin) and lipids (e.g. polyunsaturated fatty acids), which are potentially oxidisable components. Moreover, blood cells also contain all the three main types of oxidisable substrates. In particular, erythrocytes have an extensive membrane network, which contains several types of carbohydrates, lipids and proteins (Delaunay et al. 1990; Lemaitre et al. 2008) and a haemoglobin solution inside them, which undergoes autoxidation producing $O_2^{\cdot-}$ (Cimen 2008). Finally, leukocytes (mostly neutrophils) are also a source of reactive species, particularly after muscle-damaging exercise (Cannon et al. 1990; Ookawara et al. 2003). Since reactive species are generated by both blood and tissues, it is reasonable to assume that there is a bidirectional movement of reactive species from the tissues to the blood and vice versa. Supporting the inverse relationship (i.e. from blood to tissues), blood plasma has been reported to considerably affect redox homeostasis of endothelial cells, and particularly this effect was shown to be dependent on the type of exercise activity (Conti et al. 2012).

Compared with blood, the use of urine samples in redox science is more limited, despite its easy collection and the opportunity for repeated measurements offered (spot samples). An advantage of using urine is that it does not contain many reactive substances or catalysts (e.g. metal ions), which implies that the possibility for *ex vivo* oxidation reactions is limited. A further advantage of urine samples is that they allow accumulation of oxidative products for a longer time period than blood (Nikolaidis et al. 2012a). However, renal function may affect the quantity of biomarkers excreted.

TABLE 14.1

Choice of Body Fluid (Plasma vs. Urine) and Type of Biomarker (Esterified vs. Free F$_2$-IsoPs) Can Affect the Time of Body Fluid Collection (More on This Issue under Section 14.6)

Free radical
production

Reaction with PUFA in membranes/lipoproteins → Plasma-esterified F$_2$-IsoPs → (PLA$_2$ / PAF-AH) → Plasma-free F$_2$-IsoPs → Urinary F$_2$-IsoPs

	Plasma-esterified F$_2$-IsoPs	Plasma-free F$_2$-IsoPs	Urinary F$_2$-IsoPs
Normal 'steady state' continuous production of free radicals	$\longleftrightarrow$	$\longleftrightarrow$	$\longleftrightarrow$
Acute oxidative stress (e.g. acute exercise)	↑ Shortly after	↑ Somewhat later	↑ Several hours later
Chronic oxidative stress (e.g. prolonged inflammation)	↑ In steady state	↑ In steady state	↑ In steady state

Source: Adapted from Halliwell, B. and C.Y. Lee. 2010. *Antioxid Redox Signal*. 13(2):145–56.

Note: In the upper panel, the formation of F$_2$-isoprostanes is presented. In the lower panel, possible scenarios of free radical production and how they would potentially affect plasma or urinary F$_2$-IsoPs levels (esterified or free) at different time intervals are presented.

PAF-AH, platelet-activating factor acetylhydrolase; PLA$_2$, phospholipase A$_2$; PUFA, polyunsaturated fatty acids.

We cannot provide definite directions about which body fluid to collect, because insufficient data are available about the analytical and the biological variability of the same biomarker in different fluids. There are also no data available comparing the magnitude of biomarker stress response after an exercise or nutritional intervention. Additionally, it is clear that each body fluid provides different information about a redox alteration depending on the time point and the redox biomarker chosen (Table 14.1). For example, free F$_2$-isoprostane levels are peaked at different time points in blood (4 h) and urine (6 h) after carbon tetrachloride-induced lipid peroxidation (Basu 2003). Therefore, each researcher should validate his/her available biomarkers in the various body fluids in order to draw more reliable and interpretable results.

14.5 QUESTION 4: WHICH TYPE OF EXERCISE TO APPLY?

14.5.1 Non-Muscle-Damaging Exercise versus Muscle-Damaging Exercise

Exercise is probably the most widely studied physiological stimulus to alter redox homeostasis in redox biology research (e.g. Finkler et al. 2013; Neubauer et al. 2008; Radak et al. 2013). However, an important distinction should be made among the different types of exercise and, specifically, between muscle-damaging exercise (e.g. downhill running, containing a strong component of eccentric actions) and
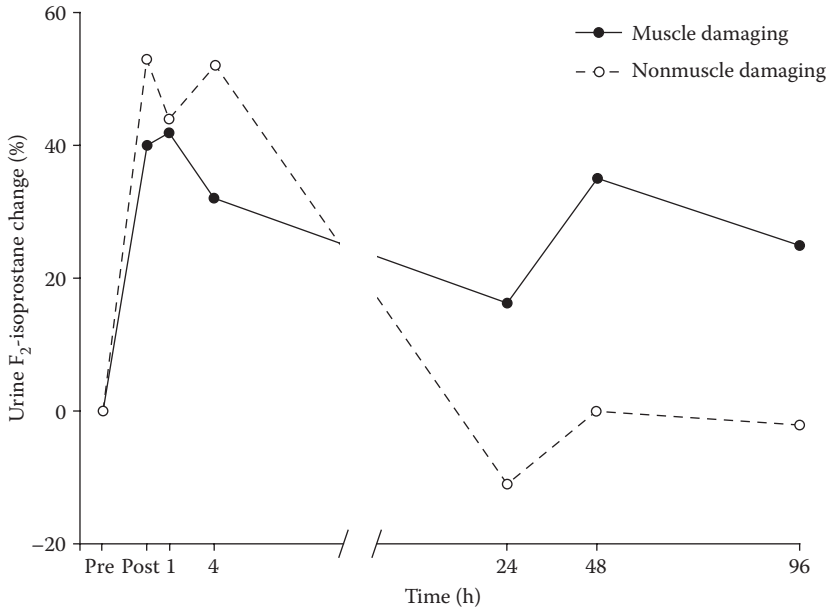
FIGURE 14.5   Urine F_2-isoprostanes after muscle-damaging exercise (solid line) and after non-muscle-damaging exercise (dotted line). Muscle-damaging exercise causes a biphasic wave during the 96 h of recovery, while non-muscle-damaging exercise induces a monophasic wave lasting for a few hours after exercise. (Based on data from Nikolaidis, M.G., A. Kyparos, K. Dipla et al. 2012a. *Biomarkers.* 17(1):28–35.)

non-muscle-damaging exercise (e.g. horizontal running, containing a strong component of concentric contractions). It should be pointed out that even low-intensity exercise may cause some degree of muscle damage and that the term 'non-muscle-damaging exercise' is used only to distinguish between types of exercise that cause limited or extensive muscle damage. It is well established that eccentric exercise causes oxidative stress to a greater extent than common aerobic exercise and this stress is prolonged and lasts up to 4 days (Close et al. 2004; Kerksick et al. 2013; Nikolaidis et al. 2007, 2008; Silva et al. 2011), compared with the few hours-lasting (approximately 4 hours post-exercise) oxidative stress caused by mainly concentric exercise (Bloomer 2008; Fogarty et al. 2013; Michailidis et al. 2007; Nikolaidis et al. 2013). Taking into account that eccentric exercise induces long-lasting and extensive alterations in redox homeostasis, muscle-damaging exercise may be a more suitable model to study the effects of experimental interventions on free radical biology (Figure 14.5).

14.5.2   Acute versus Chronic Exercise

Despite the fact that almost any type of acute exercise causes oxidative stress (Michailidis et al. 2013; Nikolaidis et al. 2012b; Pittaluga et al. 2013), chronic exercise seems to induce reductive stress (i.e. the opposite of what acute exercise does;
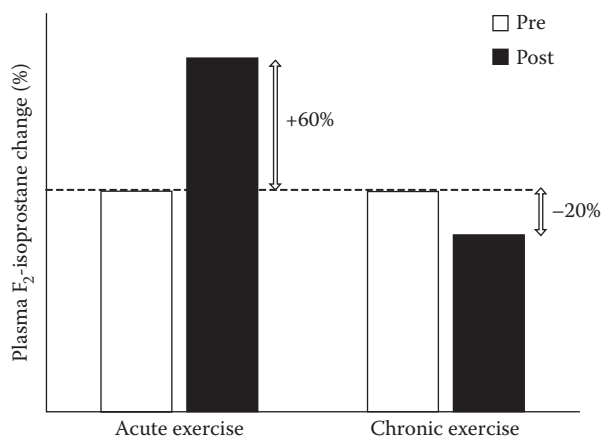
FIGURE 14.6 Acute exercise induces greater changes in plasma F$_2$-isoprostane concentration compared to chronic exercise. (Based on data from Nikolaidis, M.G., A. Kyparos, I.S. Vrabas. 2011. *Prog Lipid Res*. 50(1):89–103.)

Gomez-Cabrera et al. 2008a). For example, plasma levels of F$_2$-isoprostanes are markedly increased after acute exercise (Nieman et al. 2003), whereas F$_2$-isoprostanes are slightly decreased (Galassetti et al. 2006) or even unchanged (Watson et al. 2005) after chronic exercise. Specifically, changes after acute exercise can be almost up to three times greater compared with those after chronic exercise (56% increase compared with 17% decrease, respectively; Figure 14.6). Therefore, if the aim of a study necessitates the use of a redox stimulus, the use of acute exercise instead of chronic exercise seems more suitable since it causes greater redox alterations.

14.6 QUESTION 5: WHEN TO COLLECT BODY FLUIDS AFTER EXERCISE?

Most of the redox modification biomarkers do not remain in their initial form for a long time, but they are either metabolised to other products or participate in further reactions often unrelated to redox status. Therefore, ideally, the half-life of a biomarker should be known and remain large enough to allow sufficient accumulation of the biomarker after exposure to an oxidant stimulus. In addition, it would be useful to know the time point at which the biomarker reaches its peak value. The most common oxidant biomarkers exhibit their maximum (or minimum) values at different time points. For example, after an aerobic non-muscle-damaging exercise session, GSH exhibits its minimum value after 2 hours of recovery, TBARS exhibit highest changes after 1 hour, protein carbonyls after 4 hours and catalase immediately after exercise (Michailidis et al. 2007) (Figure 14.7). It is therefore presumed that only one 'spot' sampling, if not misleading, surely cannot provide comprehensive information about the time course of a biomarker and, consequently, the effect of an oxidant stimulus. For example, in order to describe the effects of aerobic exercise in more complete dimensions, multiple measurements should be planned over
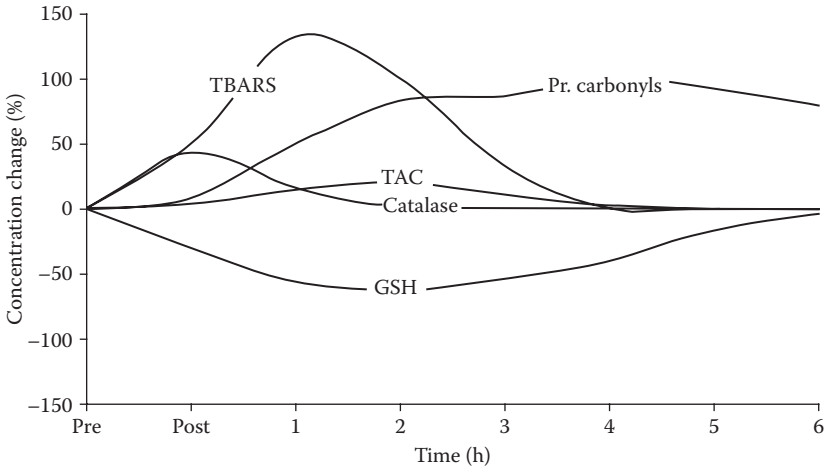
FIGURE 14.7 Redox biomarkers exhibit different time-courses after the same exercise stimulus indicating the need for multiple sampling points after exercise. (Based on data from Michailidis, Y., A.Z. Jamurtas, M.G. Nikolaidis et al. 2007. Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. *Med Sci Sports Exerc*. 39(7):1107–13.)

time, the first one taking place immediately after exercise and the following ones during the next few hours.

14.7 QUESTION 6: HOW TO CHOOSE AN ANTIOXIDANT MOLECULE?

A frequently used approach to find out the most effective antioxidant *in vivo* is by measuring its total antioxidant capacity (TAC) *in vitro*. Several assays have been developed over the years (Lopez-Alarcon and Denicola 2013; Magalhães et al. 2008) to estimate the TAC of several nutrients (e.g. polyphenol-rich foods) as well as of body fluids and tissues (Buico et al. 2009; Prior and Cao 1999). The major drawback of these 'TAC' assays is that they measure antioxidant capacity against only one reactive species or oxidant stimulus (often not normally found *in vivo*) in a non-physiological medium. Moreover, when investigating the antioxidant capacity of polyphenol-rich foods or plants extracts, there are some further limitations. In particular, most of these substances show high instability and low bioavailability in the human body (Manach et al. 2004), while it is still uncertain if any of their effects arise from their antioxidant activity or from the many other bioactive components they possess (Fraga 2007; Halliwell 2007a). Hence, the approach of screening the antioxidant capacity of a substance *in vitro* in order to draw conclusions for *in vivo* conditions is better avoided (Figure 14.8).

A multitude of substances have been used as antioxidant agents in redox biology research. However, the objective of each particular study is the crucial determinant of which antioxidant to choose. In nutritional and clinically oriented studies, nutrients derived from the diet should be preferred. However, the low bioavailability

The question	The approach	The answer
Which is the most effective antioxidant *in vivo*?	*In vitro* measurement of TAC	On the basis of the findings of the 'TAC trial', the antioxidant with the highest TAC score is chosen for the *in vivo* experiments
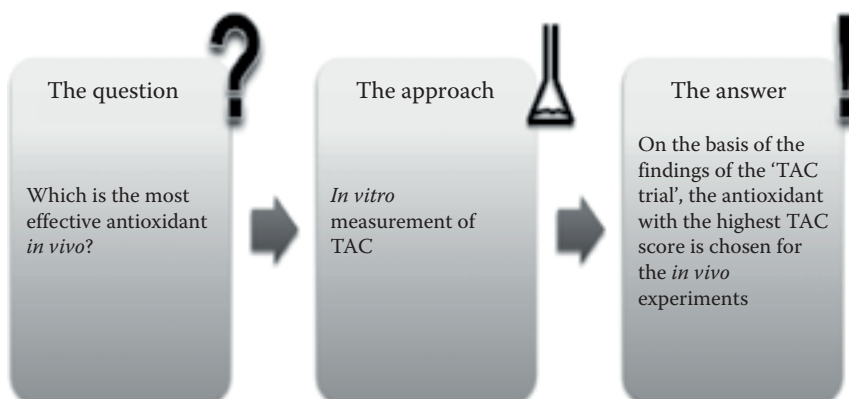
FIGURE 14.8 A controversial reductional approach often performed by researchers in order to decide on the most effective antioxidant *in vivo*.

and/or bioefficacy of most of the nutrient antioxidants (Halliwell and Gutteridge 2007) render them not appropriate in mechanistic studies. In fundamental research, the researchers may use better characterised pharmaceutical substances like allopurinol or mitoQ.

14.8   QUESTION 7: WHICH DOSE OF THE ANTIOXIDANT MOLECULE TO ADMINISTER?

In the following analysis, we limit our discussion to vitamins C and E since the number of molecules with acclaimed 'antioxidant' properties is vast and consists of substances with completely different characteristics. Vitamins C and E are probably the most extensively studied antioxidants. In addition, vitamins C and E are two of the most commonly used supplements by athletes, and thus their antioxidant effects on exercise-induced oxidative stress have been investigated both after acute (Lamprecht et al. 2009; Nieman et al. 2004; Sureda et al. 2013) and chronic exercise (Gomez-Cabrera et al. 2008b; Ristow et al. 2009; Strobel et al. 2011). They act via a common mechanism (Njus and Kelley 1991) and dependency exists between them, since vitamin C recycles vitamin E through the tocopheroxyl radical (Buettner 1993).

A standard dose-protocol about the most efficient amounts of the various antioxidants (even for the well-studied vitamins C and E) does not exist. In most of the recent studies, a combination of these two vitamins is preferred, administering daily about 500–1000 mg of vitamin C and 200–400 mg of vitamin E. However, since antioxidant doses do not follow a specific protocol, the findings of these studies remain partly incomparable. The most important factor for determining the dose of the antioxidant is the research objective of the study set by the investigator. When investigating the physiological role of an antioxidant, the dose should be similar and close to the amounts normally consumed. However, when investigating the potential pharmacological functions of an antioxidant (e.g. supraphysiological amounts of
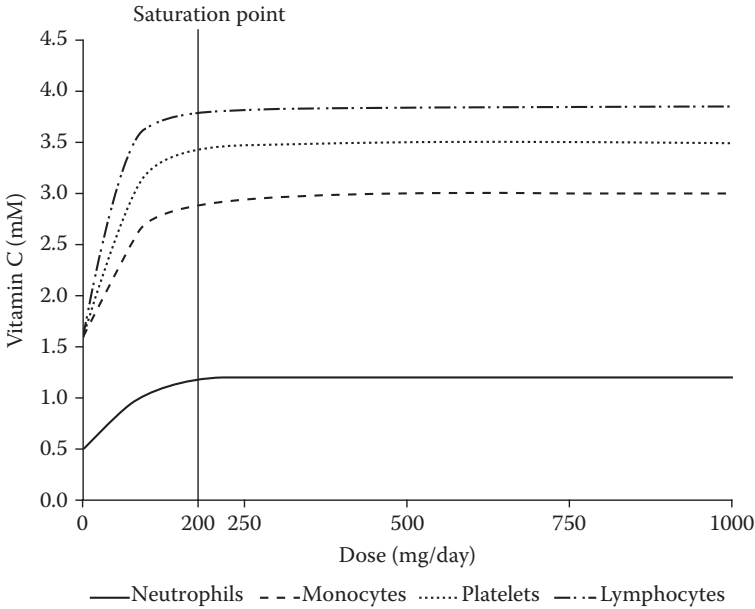
FIGURE 14.9   Vitamin C concentration in circulating cells as a function of dose. Daily administration of approximately 200 mg vitamin C seems to be sufficient in order to achieve a concentration plateau in all blood cells. (Adapted from Levine, M., Y. Wang, S.J. Padayatty, J. Morrow. 2001. *Proc Natl Acad Sci USA*. 98(17):9842–6.)

vitamin C against cancer cells), attention should not be focused on the dose administrated, but on the possible desirable result and the mediated pathway. Hence, in fundamental/pharmacological studies, the dose administered can be much higher than the normal daily administration. Another important factor influencing the dose is when the administered antioxidant reaches its peak value in the tissue of interest. For example, daily administration of 200 mg vitamin C seems to be sufficient for all circulating cells (Levine et al. 2001; Figure 14.9).

14.9   QUESTION 8: WHEN TO ADMINISTER THE ANTIOXIDANT MOLECULE?

The administration time point of an antioxidant during the day could greatly affect the results of a study. For example, vitamin C is absorbed in a few hours after administration. Considering that oxidative modifications occur continuously, it is better that vitamin C administration should take place not only once (e.g. in the morning) but every few hours (e.g. every 6 hours) in order to achieve high blood concentration throughout the daytime (Padayatty et al. 2004). Moreover, when investigating the acute ingestion of more than one antioxidant (let us consider again the convenient example of vitamins C and E), their administration should take place at different time points in order to exhibit their peak values at the same time point in the fluid and/or tissue of interest (Figure 14.10). This signifies that the sampling point
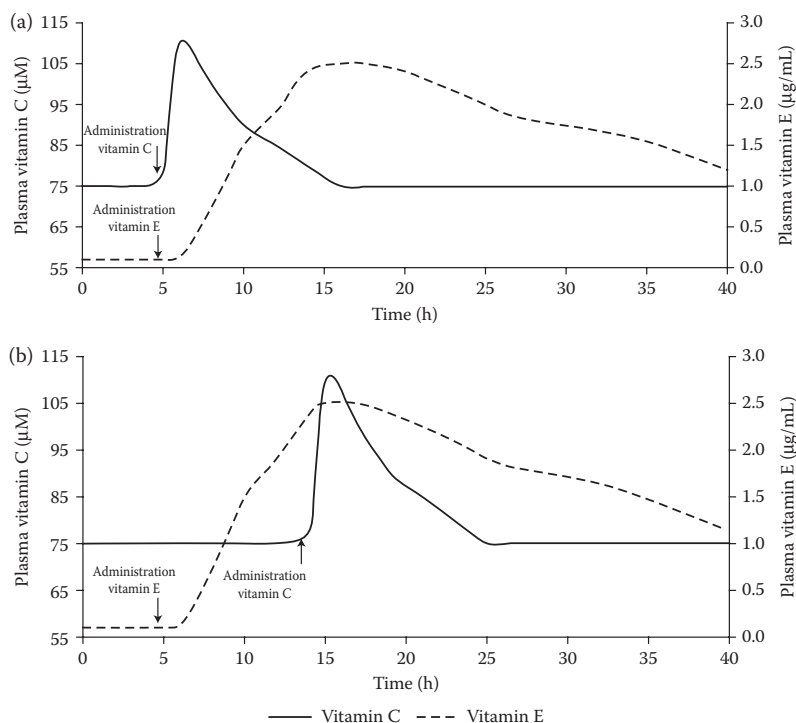
FIGURE 14.10 (a) Concentration of plasma vitamin C and vitamin E peak at different time points after a simultaneous administration. (b) Concentration of plasma vitamin C and vitamin E peak at the same time point after asynchronous administration. In a hypothetical asynchronous administration experiment, the best sampling point is about 10 h after vitamin E administration. (Based on data from Levine, M., C. Conry-Cantilena, Y. Wang et al. 1996. *Proc Natl Acad Sci USA*. 93(8):3704–9; Julianto, T., K.H. Yuen, A.M. Noor. 2000. *Int J Pharm*. 200(1):53–7.)

in studies administering a single dose of antioxidants depends on the antioxidant supplement administered. Additionally, the route of administration (intravenously or orally) seems to play a crucial role for the time point at which plasma peak levels appear (Padayatty et al. 2004). Finally, several antioxidant products used in studies do not exhibit the same level of absorption (Julianto et al. 2000). In any case, researchers are encouraged to perform pilot pharmakokinetic studies to confirm the appropriateness of their administration scheme.

14.10 CONCLUSIONS

Certainly, many papers published in exercise redox biology have employed inappropriate methodologies leading to doubtful data. The aim of this chapter was to highlight some of the most important questions a researcher faces when setting up an experiment in this field. The careful reader will notice that we avoided giving a definitive answer to any question that we raised. This was mainly for

three reasons: first, because redox biology is a relatively new field and not having established maturity. As a result, it still lacks a solid theoretical framework and valid experimental tools. Second, because we are sceptical about the feasibility of defining firm criteria for physiological studies with a huge variety in animals, tissues/fluids, redox biomarkers, antioxidant molecules and exercise protocols. Third, because we believe that establishing 'gold standards' in any field of science is implausible. Experimental standardisation implies definite answers and particularly current redox biology is much far from that. We think that, instead of a dogmatic approach, researchers should establish their own reference methods based on scientific evidence and decide which experimental variables are most critical when dealing with a specific question in the vast field of 'antioxidant' nutrition and exercise biology. This *ad hoc* approach does not facilitate the comparison of results obtained in different studies, though it will potentially provide more satisfactory answers to the questions of each specific study. We hope that the limitations of the many usual adopted procedures presented in this chapter, along with the potential solutions provided, will lead to better designed and more carefully executed studies.

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15 Biomarkers Part I
Biomarkers to Estimate Bioefficacy of Dietary/ Supplemental Antioxidants in Sport

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CONTENTS

15.1 INTRODUCTION

Physical exercise and sport increase the formation of free radicals and reactive oxygen and nitrogen species (RONS). This leads to an imbalance in favour of pro-oxidative processes plus damage to biomolecules, namely oxidative and nitrosative stress, especially in individuals undergoing strenuous exercise. Oxidative and nitrosative stress is expressed in significantly increased generation of RONS against a sophisticated regulated enzymatic and non-enzymatic antioxidant system, depending

on the stress intensity, exposure time and frequency (Bloomer and Goldfarb 2004). It is important for athletes as well as scientists to know how oxidative stress affects health and sport performance via the intake of antioxidant-acting substances, such as vitamins, trace elements and micronutrients (Bloomer et al. 2008; Halliwell 1996; Lamprecht et al. 2009; Radak et al. 2013).

It is also of great importance to develop analytical methods that would show the biological absorption and bioavailability of supplemented micronutrients, as well as their bioactivity and effectiveness (Bloomer et al. 2008). However, it is unclear whether supplementation shows an antioxidant-acting regulatory. Therefore, oxidative and nitrosative stress markers are well-known opportunities to estimate effects in the redox biology of exercise.

In a recent report, we listed some analytical methods which are used for oxidative stress evaluation of different specimens such as cells (erythrocytes, leukocytes), serum or plasma, urine and tissue. Besides the listed analytic methods of blood, this review describes the influence of antioxidant supplementation, such as of vitamins or micronutrients, on athletes. Positive, negative or no effect of supplemented substances can be displayed on the basis of the measurement of oxidative stress and nitrosative stress markers (Lamprecht et al. 2004).

15.2 BLOOD PROTEIN MODIFICATION AS A BIOMARKER FOR BIOEFFICACY

In a plasma or a serum, the major oxidative damage occurs with protein modifications, such as reactive carbonyl proteins (CP), free nitrated tyrosine residues (NT) or nitrotyrosine proteins (NTP) and oxidised albumin fractions (Halliwell and Guterridge 1999; Berlett and Stadtman 1997).

In some cases, proteins lose their natural function, for example, enzymatic function, transport function and structural function. During conversion of the modified protein structure, it could also be that enzymes produce reactive molecules like free radicals. It is known that some modified enzymes in the Krebs cycle are able to produce free radicals and RONS (Halliwell and Guterridge 1999). Oxidatively modified NO synthases are able to produce $NO^{\bullet}$ and oxygen anion radicals ($O_2^{\bullet-}$) and peroxynitrite, which reacts with further proteins, lipids, carbohydrates and nucleic acids. This results in further inactivation of enzymatic antioxidative systems, such as superoxide dismutase, glutathione peroxidase, catalase and a loss of vitamin-scavenging substances and micronutrients.

15.2.1 Carbonyl Proteins

The determination of CP is a widely used oxidative stress marker generated from different sources such as free radicals, trace amounts of metals, lipid peroxidation from polyunsaturated fatty acids, carbohydrate degradation and direct oxidation of free radicals or RONS. In recent years, ELISA techniques are commonly used for the determination of reactive CP because they seem to be more sensitive and specific than the old spectrophotometric assay (Winterbourn and Buss 1999, Dalle-Donne et al. 2003). Both techniques used the derivatisation of carbonyl groups on proteins

with 2,4-dinitrophenylhydrazine (DNPH) forming, under acid conditions, dinitro-phenyl-hydrazine (Levine et al. 1990).

The spectrophotometric assay is known to be a time-consuming method and not applicable to a large number of samples. We have reported about a new immunoas-say for a reliable determination of CP (Immundiagnostik AG, Bensheim, Germany) with a high throughput of samples used in sport and exercise studies (Lamprecht et al. 2009, 2012). Samples containing protein standard, controls and plasma samples react with DNPH; then the non-protein constituents and unconjugated DNPH are separated and washed by ultracentrifugation (11,000 g, three times by addition of 10 mM phosphate buffered saline pH 7.4). The proteins are adsorbed to an ELISA plate and incubated with an anti-DNPH antibody followed by antibody-linked horse-radish peroxidase. Absorbances are related to a standard curve prepared with oxi-dised serum albumin. The CP content is calculated from the estimated carbonyl concentration and the total protein content of the same sample using the BCA protein technique (Pierce, USA). This method contains the following improvements:

- No negative absorbance by free DNPH
- No cross reaction by antibodies to free DNPH
- Exact estimation of the protein content of DNPH proteins in parallel with the estimation of carbonylated proteins
- No estimation of free carbonyl groups of organic compounds in plasma

Using this technique, it was shown that CP increased after 80% of individual VO_{2max} intensity (Lamprecht et al. 2008), whereas long-term supplementation with a juice powder concentrate (JPC) was able to reduce the generation of CP before exercise, immediately after exercise and 30 h post-exercise (Lamprecht et al. 2009). In the latter study, CP did not change after cycling exercise at 80% of individual VO_{2max} when subjects were supplemented with the JPC. Additionally, the JPC group had lower baseline CP levels after 16 and 28 weeks compared to the placebo group.

Bloomer and colleagues reported that 6–24 h blood collection after an acute 30 min aerobic and anaerobic exercise CP content increased significantly (Bloomer et al. 2005). Also, an increase of CP by nearly 12% after strenuous exercise in trained men was shown (Morillas-Ruiz et al. 2006). Long-time supplementation of poly-phenolic antioxidants decreased CP effectively by 23%.

Short-time supplementation with vitamins had no effect on CP as reported (Gatterer et al. 2013): While CP increased significantly in well-trained male volunteers under hypoxia, supplementation of antioxidants [vitamin cocktail concentrate versus alpha-ketoglutarate (AKG)/5-hydroxy-methylfurfural (5-HMF) concentrate] showed no effect in reducing CP compared to the control group. Only the special cocktail of AKG and 5-HMF was able to reduce free carbonyl groups during hypoxia compared to the vitamin-supplemented group or the control group. This special micronutrient cock-tail was also used in pre-operative supplementation to decrease post-operative stress syndromes compared to a control group (Matzi et al. 2007). CP values of the supple-mented AKG/5-HMF group showed no increase during surgery, while pre-operative measurements of VO_{2max} increased by 20–25% compared to the control group.

Another study deals with the influence of exercise on a fruit powder concentrate (FBV: fruit, berry, vegetable powder) supplemented obese women group compared

to obese women with placebo over 8 weeks (Lamprecht et al. 2013). There were no differences between the groups at baseline, pre- and post-70% VO_{2max} walking exercise. After 8 weeks of supplementation, there was a significant difference in CP levels between the FBV and the placebo group pre- and post-exercise in favour of the supplemented group: The FBV group had significantly lower CP concentrations compared to the placebo group. The model of exercise had no influence on CP concentrations.

Using probiotic supplementation in trained men, there were no differences between the supplemented group and the placebo group at baseline (Lamprecht et al. 2012). The post-exercise increase of CP was significant in both groups. *Post hoc* analysis revealed that this exercise-induced increase did not reach significance after 14 weeks of probiotic treatment. After 14 weeks, the supplemented group showed decreased CP concentrations pre- and post-exercise compared to placebo.

Bloomer et al. (2008) reported CP levels between male and female volunteers and furthermore between untrained volunteers. They found that CP levels did not differ significantly between trained men and women or between untrained men and women. Interestingly, there was only a positive correlation between CP concentrations of untrained men before and after dietary intake and untrained men.

15.2.2 Nitrotyrosine and Nitrotyrosine Protein

Nitric oxide is released by the vascular endothelium and plays an important role in the maintenance of normal vascular endothelial function. Peroxynitrite is a highly reactive oxidant metabolite of nitric oxide ($NO^{\bullet}$), generated in the vasculature from the reaction of $NO^{\bullet}$ with superoxide anion radicals ($O_2^{\bullet-}$), when both are present in low concentrations under inflammatory and oxidative stress conditions (Johansen 2000). Peroxynitrite nitrates free and protein bound tyrosine residues to yield NT or NTP, which are closely involved in intra- and inter-cellular signal transduction, in the activation of apoptosis and cell death by suppressing oxidative phosphorylation. Therefore, estimation of NT is not only used for the non-direct identification of nitrating reagents like $ONOO^-$ but also for the bioactivity of these nitrating and oxidising compounds (Vandervliet et al. 1995). Less is known about the generation of NT or NTP during sport and exercise as markers of peroxynitrite and NO action. Measurement of NT was mostly done by two different ELISA techniques, namely competitive ELISA or sandwich-ELISA (Khan et al. 1998). Both techniques seem to be not applicable because NT should be measured from urine instead of blood, and sandwich ELISA using two antibodies against NT is also not suitable for the detection of free NT.

A new ELISA technique catching plasmatic NTP (Immundiagnostik AG, Bensheim, Germany) is more reliable because larger protein molecules are able to circulate for a longer time in blood. The assay utilises the 'sandwich' technique. Standards, controls and diluted samples, which are assayed for NT, are added into the wells of a micro-plate coated with a polyclonal goat anti-nitrotyrosine antibody. During the first incubation step, the immobilised primary antibody binds nitrated proteins. Then a peroxidase-conjugated polyclonal goat anti-human serum protein antibody is added to each microtitre well and a 'sandwich' of a primary antibody – nitrated a protein – peroxidase conjugate is formed. Tetramethylbenzidine (TMB) is used as a peroxidase substrate. Finally, an acidic stop solution is added to terminate

the reaction. The colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the concentration of NT. A dose–response curve of the absorbance unit (optical density, OD, at 450 nm) versus standard concentration is generated using the values obtained from the standard.

Recently, it was reported for the first time that faster O_2 extraction at the ventilatory threshold (VT) was associated with an effective decrease of NTP under hypoxia conditions (Gatterer et al. 2013). Short-time supplementation of vitamins or other antioxidative-acting substances showed no change of NTP. It was suggested that long-term supplementation might have some beneficial effect in preventing peroxynitrite generation. Sureda et al. (2013) supplemented amateur runners with a combination of vitamins C and E, compared them against a placebo group and estimated NT and CP. NT and CP were increased only in the placebo group after exercise and remained high in the recovery period.

Bjork et al. (2012) showed no relation between NT of active and sedentary young healthy men, but when age groups were compared the younger volunteers had lower NT levels than the older ones, independent of exercise.

15.2.3 Albumin SH (Alb-SH) Groups

The redox state of human serum albumin (HSA) is a potential approach to investigate the extracellular redox state in exercise. This is the main protein in extracellular fluids, and its redox state appears to be influenced by physical exercise (Imai et al. 2002). In HSA, cysteine-34 can exist in several forms: the reduced form with a free thiol group (human mercaptalbumin, HMA); a reversibly oxidised form, wherein cysteine-34 forms a disulphide with low-molecular-weight thiol compounds such as cysteine (human non-mercaptalbumin 1, HNA1); or a further oxidised form, such as a sulphenic or sulphonic acid state (human non-mercaptalbumin 2, HNA2). The main fraction of serum albumin, HMA, is thought to participate in maintaining an appropriate redox potential in blood or interstitial fluid. Albumin was fractionated using high-performance liquid chromatography to give three peaks corresponding to the cysteine-34 redox state: the free thiol form (HMA), as a mixed disulphide (HNA1) or more oxidised (HNA2).

It was shown that the reduced fraction of HSA, HMA decreased significantly after cycle tests at 70%, 75% or 80% of VO_{2max} exercise intensity (Lamprecht et al. 2008). HMA values returned to pre-exercise values after 30 h post-exercise. This study demonstrated that HSA is reversibly shifted to a more oxidised state by a recent intense exercise. In another study, Lamprecht et al. (2009) showed that over a period of 4, 16 and 28 weeks of supplementation with JPC or placebo no significant effect of supplementation was observed in HMA. Obviously, exercise exerts more effects on the redox state of HAS than antioxidant treatment.

15.3 BLOOD LIPID PEROXIDATION MODIFICATION AS A BIOMARKER FOR BIOEFFICACY

Reactive intermediates produced under conditions of oxidative stress cause the oxidation of polyunsaturated fatty acids (PUFAs) in membrane lipid bilayers, leading

to the formation of aldehydes. The most intensively studied aldehydes so far, formed after lipid hydroperoxides breakdown in biological systems, are 4-hydroxynonenal (4-HNE), isoprostanes and malondialdehyde (MDA). Reaction with biomolecules like proteins leads primarily to CP.

Susceptibility of lipoproteins to oxidation *in vitro* and quantification of lipid peroxidation products generated *in vivo* have long been used as markers for oxidative stress. Alternatively, lipid oxidation may be measured directly in a simply diluted serum without isolation of lipoproteins. Therefore, diene formation may be followed spectrophotometrically, or in reaction products of aldehydes. These methods have been applied in exercise studies as well (Esterbauer et al. 1992). Quantification of oxidised LDL resulting from lipid peroxidation *in vivo* has also been used for the detection of exercise-related oxidative stress (Kaikkonen et al. 2002).

15.3.1 MALONDIALDEHYDE, 4-HYDROXYNONENAL AND OXLDL

Free radicals produced during lipid peroxidation have some very local effects because of their short life. In contrast to the short-living free radicals, the breakdown products of lipid peroxides may serve as 'oxidative stress second messengers', due to their prolonged half-life and their ability to diffuse from their site of formation. Owing to their chemical reactivity, those breakdown products can make covalent modifications on macromolecules such as nucleic acids, protein and lipids and exert some biological effects. Aldehydes have received much attention because they are reactive and toxic (Esterbauer et al. 1991).

A relatively stable intermediate of lipid peroxidation is MDA, but it could also be generated during oxidation of glucose. The quantification of this compound in serum or plasma as an indicator of lipid peroxidation has been used frequently. The technique can be performed by a simple measurement of thiobarbituric acid-reactive substances or in combination with high-performance liquid chromatographic (HPLC) separation and fluorescence detection. Because the formation of thiobarbituric acid-reactive substances is quite unspecific and the HPLC technique is simple, the latter is clearly preferred and more valid (Esterbauer and Zollner 1989). 4-Hydroxynonenal (4-HNE) is a lipid peroxidation endproduct of polyunsaturated fatty acids. Its HPLC analysis is not as simple as that for MDA (Esterbauer et al. 1986).

Lamprecht et al. (2009) showed significantly increased MDA concentrations in trained men after supplementation with a high-dosed encapsulated antioxidant concentrate at rest, before and after strenuous exercise, but there were no differences between treatments after the strenuous exercise bouts. Another study observed obese women with placebo or JPC supplementation before and after walking exercise at 70% of VO_{2max} (Lamprecht et al. 2013). There were no differences in MDA between the groups at baseline and after 8 weeks of supplementation, pre- and post-exercise, with all concentrations within the reference interval. The model of exercise had no influence on MDA concentrations.

Bloomer et al. (2008) found that dietary intake correlated to MDA as a marker of oxidative stress in trained men. Furthermore, MDA levels of young healthy adults seemed to be lower in trained women than men compared with untrained individuals.

Franzoni et al. (2005) evaluated MDA levels between older and young healthy men with long-term physical activity. Sedentary older men showed higher MDA levels compared to the young subgroup. Examination of the relationship between aerobic power and MDA as a marker of oxidative stress showed no differences between high aerobic power (HAP) and low aerobic power. Plasma HNE levels of sedentary obese men decreased significantly after endurance exercise, as did CP.

15.3.2 ISOPROSTANE (F2-ISOPROSTANE)

Estimation of F2-isoprostane is formed from oxidative modification of arachidonic acid (C20:4) by free radicals which could be estimated by the ELISA technique or GC-MS (Sacheck et al. 2003).

The latter is more sensitive but time consuming, is not applicable to a large number of samples and is mostly detectable in urine. The ELISA technique is much more comfortable for both urine or plasma isoprostane, but sensitivity is not as much as for GC-MS.

F2-Isoprostane was estimated before and after exercise between a placebo athlete group and a quercetin and resveratrol supplemented athlete group. Supplementation did not influence the F2-isoprostane level in both groups before exercise, but post-exercise the quercetin and resveratrol supplemented group showed significant lower levels compared to the placebo group (McAnulty et al. 2013).

Sacheck et al. (2003) showed that in young men 72 h after eccentric exercise, F2-isoprostane was significantly lower compared to older men. Supplementation of vitamin E decreased F2-isoprostane levels and suppressed the 24 h post-exercise increase in older men, but not in young men. These data indicate that estimation of F2 isoprostanes seems to be a sensitive marker of exercise-induced lipid peroxidation.

15.3.3 OXIDISED LDL

Measurement of oxidised LDL (oxLDL) is based on a direct sandwich ELISA technique which is commercially available using either monoclonal antibodies (Mercodia AB, Sweden) or polyclonal antibodies (Immundiagnostik AG, Bensheim, Germany) with similar results (data not shown). Whereas monoclonal antibodies seem to be specific for one epitope expressed on oxidatively modified LDL, polyclonal antibodies have the opportunity to react with several epitopes on oxLDL.

Using the ELISA technique, Lamprecht et al. (2013) effectively showed a decrease in oxLDL in FBV supplemented obese women compared to the placebo group after 8 weeks. However, no influence of exercise was estimated in the amount of oxLDL in both groups.

Comparing active with inactive men, oxLDL levels were higher in the inactive group as reported by Bjork et al. (2012).

15.4 BLOOD ANTIOXIDATIVE CAPACITY AS A BIOMARKER FOR BIOEFFICACY

Antioxidants, for example, vitamins and micronutrients, play an important role in preventing oxidative and nitrosative stress-mediated injury, and also in exercise.

The knowledge of antioxidants in nutraceutical components allows taking decisions for dietary interventions in sport and exercise, and increasing the commercial value of antioxidant-rich natural products. Detection of the antioxidative capacity in preventing oxidative and nitrosative stress are used by different techniques which are recently available. The most frequently used methods are the determination of the total antioxidative capacity or the total oxidative status (TAC or TOS), the oxygen radical absorptive capacity (ORAC), the ferric reducing ability of plasma (FRAP) and the trolox equivalent antioxidative capacity (TEAC).

These methods differ mostly in the generation of free radicals by enzymatic reactions, metal-induced radical formation or peroxides.

15.4.1 Total Oxidative Status, Total Antioxidative Capacity and Total Antioxidative Status

The TOS assay determines total lipid peroxides by the detection of a coloured product spectrophotometrically from the reaction of a peroxidase with the peroxides in the sample, followed by the conversion of TMB.

Using this technique the plasma total oxidation status of lipids in overweight and obese women ($n = 42$) before and after 8 weeks of supplementation with FBV, and pre- and post-30 min of walking exercise was measured (Lamprecht et al. 2013). There was a significant effect of treatment after 8 weeks, but with no influence of exercise.

In the probiotic study (Lamprecht et al. 2012), the measured TOS values were above normal at all time points in the probiotic supplemented athlete group as well as in the placebo athlete group. There were no differences between groups at any time point assessed, either with treatment or with exercise.

Sharmen et al. (2004) examined the relationship between aerobic power and TAC of healthy young men at high- or low-intense aerobic exercise. High-intensity aerobic exercise significantly decreased TAC levels compared to low-intensity aerobic exercise. They suggested that a decreased antioxidative capacity induces a higher susceptibility to oxidative damage, but measurements of MDA failed to underline this hypothesis.

Supplementation of polyphenolic antioxidants showed no effect in exercise-induced oxidative stress in 30 sportsmen before and after sub-maximal aerobic exercise as measured by TAS, whereas TBARS increased at a slower rate in the supplemented group compared to the placebo group (Morillas-Ruiz et al. 2006).

Finally, Franzoni et al. (2005) measured the plasma antioxidant capacity (TOS) between active young and older healthy men. Surprisingly, the antioxidative capacity was even higher in the sedentary older men than in the group of young men. Similar results were obtained for MDA.

15.4.2 FRAP, TEAC and ORAC

The estimation of total antioxidant activity is described by the ferric-reducing antioxidant power (FRAP) assay of Benzie and Strain (1999). At low pH, reduction of ferric tripyridyltriazine measures the change in absorption at 593 nm and can

monitor complex formation to an intense blue coloured ferrous form. The change in absorbance is therefore directly related to the combined or total reducing power of electron donating antioxidants present in the reaction mixture, for example, of human serum compared to the standard with ascorbic acid.

The TEAC assay is based on the suppression of the absorbance of radical cations of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulphonate) (ABTS) by antioxidants in the test sample when ABTS incubates with a peroxidase (metmyoglobin) and H_2O_2. If the inhibition time is fixed with 3 min, the added antioxidants quench ABTS radicals in a non-linear dose–response fashion. To optimise the incubation period for the complete inhibition of ABTS radical formation in the system, Wang et al. (2004) extended the reaction up to 40 min and monitored the absorbance changes at 3 min intervals at 600 nm.

The ORAC assay is based largely on the work reported by Glazer (1990) in which the decrease in fluorescence of B- or R-phycoerythrin (PE) is measured in the presence of 2,2′-azobis(2-amidinopropane) dihydrochloride, and the lag phase or rate constant for PE fluorescence decay is used to determine the antioxidant capacity of the added sample. The ORAC method is the only method that takes free radical action to completion and uses the area under the curve for quantification; it thus combines both the percentage of inhibition and the length of inhibition of free radical formation by antioxidants into a single quantity.

In a resveratrol/quercetin supplemented study with a double-blind crossover design, three different methods to estimate antioxidative capacities, namely FRAP, TEAC and ORAC, were used (McAnulty et al. 2013). FRAP, ORAC and TEAC increased significantly after exercise in 14 athletes, but treatment did not show any effect. The same result was found in inflammation markers like cytokine interleukin 8 and C-reactive protein. Treatment with resveratrol–quercetin reduced exercise-induced lipid peroxidation without any change in inflammation and antioxidative status.

Sacheck et al. (2003) used the ORAC test to estimate the role of vitamin E supplementation in eccentric exercise of young and elderly men. The ORAC levels in young men decreased significantly 72 h post-exercise (45 min downhill running) and correlated with the rise of MDA and isoprostane, but supplementation of vitamin E showed no effect in the ORAC assay. The FRAP assay was also used by Kappus et al. (2011) for comparing supplementation of multi-flavanoids with placebo in athletes before, immediately and 30 min after an acute bout of aerobic exercise. The FRAP assay showed a significant increase after supplementation compared to the placebo group.

An increased need of nutritive antioxidants was estimated by Neubauer et al. (2010) after an ultra-endurance exercise by estimation of ORAC, whereas FRAP and TEAC failed. ORAC levels of these triathlon athletes correlated inversely with lymphocyte DNA damage.

15.5   BLOOD DNA DAMAGE AS A BIOMARKER FOR BIOEFFICACY

Early measurements of urine 8-hydrox-deoxoguansoine (8-OHdG) should show that an increase in exercise-induced RONS also injures DNA. However, the oxidative damage to DNA is not accumulated by consecutive exercise, although it is sustained

as long as the exercise is repeated (Okamura et al. 1997). Different HPLC techniques have been used for the measurement of 8-OHdG (Shigenaga et al. 1994; Bogdanov et al. 1999; Loft and Poulsen 1999).

The major analytical disadvantage is the requirement of 24-hour urine collection to obtain reliable 8-OHdG results. As urine collection seemed to be time consuming, not stable and valid, estimation of plasma or serum 8-OHdG was used to estimate oxidative stress-induced modification of DNA damage by competitive ELISA techniques with specific monoclonal antibodies raised against 8-OHdG protein.

Several studies using serum 8-OHdG ELISA methods failed to show any effect of exercise and supplementation with different antioxidants. Also the influence of sex, exercise training status and dietary intake showed no change of 8-OHdG levels (Bloomer et al. 2008).

Another specific method to determine DNA damage from peripheral blood cells is by using the comet assay for 8-OHdG determination. Different types of comet assay versions can be used:

- An alkaline and/or neutral typical version to identify different types of DNA damage
- An Fgp-modified version to measure the 8-oxo-dG level
- An Atypical comet assay by adding peroxide with or without antioxidants directly on agarose-embedded whole blood cells

The last one was considered for estimating the antioxidative vitality of lymphocytes (Tomasello et al. 2012).

Alkaline and neutral comet assay: Lymphocytes are harvested from whole blood using gradient density centrifugation and are cryopreserved till measurement. Lymphocytes are embedded in a thin agarose gel on a microscope slide, and lysed in high salt and detergent to remove membranes and soluble constituents of the cytoplasm and the nucleoplasm, leaving DNA still attached to the nuclear matrix, as 'nucleoids'. After incubation in alkali, the embedded nucleoids were subjected to electrophoresis. Migration of loops of relaxed DNA towards the anode creates a comet tail, which is visualised by staining with ethidium bromide. The DNA damage is determined as the percentage of DNA content in the comet tail, which is considered as a validated measure of DNA damage (Klaude et al. 1996).

The Fpg FLARETM assay kit (Trevigen, Inc., Gaithersburg, MD) can be used to detect 8-oxo-dG levels. DNA (or nucleoid) is digested with the Fpg DNA repair enzyme that recognises and cuts the site corresponding to oxidised guanine bases. For each sample, 35 μL of agarose embedded whole blood cell suspensions is dropped on slides. After 20 min at 4°C in the dark, the slides are immersed in a pre-chilled lysis solution at 4°C for 60 min. After lysis, 100 μL of the enzyme dilution buffer and/or Fpg enzyme solution is added to the samples and then placed in the humidity chamber at 37°C for 45 min. The slides are immersed twice in pre-chilled alkaline electrophoresis solution and then electrophoresed. The DNA damage is determined as the percentage DNA content in the comet tail, which is considered as a validated measure of DNA damage.

By using this assay, DNA damage was estimated in runners (Tomasello et al. 2012). DNA damage by measuring 8-oxo-dG was significantly higher in runners than in control subjects. Another report showed that exhaustive aerobic exercise induced DNA damage (Davison et al. 2005), whereas supplementation of antioxidant cocktail had no effect before and after exercise to prevent DNA damage in cells. Furthermore, no difference in DNA damage could be estimated between the supplemented group and the placebo group.

Mastaloudis et al. (2004) studied the exercise-induced DNA damage in ultra-marathon runners: one group was supplemented for 6 weeks with antioxidants (vitamins C and E) before the marathon and the second group received a placebo. Leukocyte collection was conducted pre-, mid- and 2 h post-race and daily for 6 days post-race. DNA damage increased significantly at mid-race in men and women. One-day post-race, the women taking antioxidants had significantly lower DNA damage than the women with placebo, but this was not observed in men.

15.6   CONCLUSION

There is evidence to suggest that severe and prolonged exercise induces oxidative and nitrosative stress. The extent of occurrence of oxidative and nitrosative stress depends strongly on the model of exercise. Free radicals and RONS are scavenged by enzymatic or non-enzymatic antioxidative defences. Overwhelming this antioxidant network by extensive exercise induces a direct free radical and RONS attack on proteins, lipids, carbohydrates and DNA structures in blood cells and tissues. Supplementation of antioxidative acting substances has the primary role to prevent oxidative stress-induced modification of biomolecules and to increase the antioxidative capacity of non-enzymatic and enzymatic acting antioxidative systems during sport and exercise to maintain the healthy conditions in humans (Radak et al. 2013).

When planning studies concerning oxidative stress, exercise and supplementation with antioxidative micronutrients, one has to consider

1. The number of participants, exercise intensity and duration
2. Time point of blood collections
3. Sample preparations (temperature, source, addition of anticoagulation substances such as citrate, heparin, EDTA)
4. Determination of enzymatic antioxidative systems (superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase, etc.) and non-enzymatic antioxidants (vitamins, micronutrients)
5. Measurement of damaged biomolecules such as modified proteins, lipids, carbohydrates and nucleic acids
6. Valid analytical methods for the determination of oxidative stress markers (Lamprecht et al. 2004)

These factors influence the generated results found and also the interpretation of the studies performed on the bioefficacy of antioxidants and micronutrients in sport and exercise.

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16 Biomarkers Part II
Biomarkers to Estimate Bioefficacy of Dietary/ Supplemental Antioxidants in Sport

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CONTENTS

16.1 INTRODUCTION

Free radicals are normally produced during numerous physiological processes and play important roles as regulatory mediators in signalling processes (Strobel et al. 2011). Under physiological conditions, the body has adequate antioxidant defences to cope with the production of free radicals. However, oxidant species can become toxic when generated in excess or in the presence of a deficiency in the naturally occurring antioxidant defences. Specifically, the imbalance between free radical generation and antioxidant defence leads to an oxidative stress state, which may be involved in ageing processes and in many pathological conditions (e.g. cardiovascular and neurodegenerative disease, and cancer) (Valko et al. 2007).

Exercise can have positive or negative effects on redox biology depending on the type (acute or chronic), and on training specificity, load and the basal level of training. Beneficial changes on multiple physiological and laboratory parameters have

been generally observed as a result of regular moderate training (Haskell et al. 2007). Conversely, acute and strenuous exercise may paradoxically induce oxidative stress and adverse effects on health (Neubauer et al. 2008, Suzuki et al. 2006). Antioxidant nutrients by diet and exogenous supplementation may be helpful to cope with adverse implications on health and performance (Whayne and Maulik 2012, Braakuis 2012). However, caution should be taken against excess antioxidant supplements.

The complexity of the human antioxidant defence/oxidant system and their delicate balance renders it extremely difficult to estimate the oxidative stress status (Lee et al. 2012). A number of oxidative stress biomarkers (belonging to both antioxidant and oxidant counterparts and also to inflammatory processes) have been identified and measured in biological fluids (Lee et al. 2012). The choice of a specific biomarker and the analytical method used may have a great impact on the results obtained (Lee et al. 2012). Moreover, it is clear that the choice of specific markers depends on the function affected by the exercise and/or by the deficit of the nutrient intake. At the moment, no shared consensus exists on which biomarkers or group of biomarkers must be used to estimate exercise effects as well as bioefficacy of dietary/supplemental antioxidants in sport.

In this chapter, aspects related to the effect of exercise on oxidative stress, diet/antioxidant supplementation and biomarkers in trained subjects will be discussed. Moreover, possible future strategies to answer still open questions and to solve problems in the assessment of these complex issues will also be proposed.

16.2 EFFECT OF EXERCISE ON HEALTH: GOOD AND BAD OF TRAINING

There is a large amount of evidence on the fact that regular and moderate physical exercise improves prognosis and quality of life in patients with cardiovascular disease, hypertension and some types of cancers as well as in those affected by cognitive diseases (Mellett and Bousquet 2013, Briet and Schiffrin 2013, Jones and Alfano 2013, Raichlen and Polk 2013). Performance is related to training and physical adaptation, and correct nutrition in individuals with specific genetic characteristics can facilitate such adaptations. Beneficial effects on multiple physiological laboratory parameters have generally been observed as a result of regular moderate training (Banfi et al. 2012).

However, the effect of sustained and strenuous exercise, as in competitive athletes, is still controversial. In fact, while some studies report that strenuous and sustained exercise induces oxidative stress, inflammatory response and even structural damage of skeletal and cardiac muscle cells, other data show that training is able to confer resistance against oxidative stress and reduce inflammation (Neubauer et al. 2008, Pinto et al. 2012, Nunn et al. 2010).

Increased reactive oxygen species (ROS) generation is considered to be the hallmark of ageing and the major determinant of lifespan (Vina et al. 2013). On an epidemiological basis, elite athletes, in particular those performing endurance aerobic exercise, live longer than the general population and an inverse linear dose–response relationship has been reported between the volume of physical training and all-cause mortality (Corbi et al. 2012, Lee et al. 2001). Thus, an apparent contradiction exists

in professional athletes between the evidence of oxidative and inflammatory effects of exercise and the beneficial effect of physical activity on health and survival. This contradiction might be explained by a hypothetical adaptive response of the organism to repeated oxidative stress induced by physical training, leading to the acquired ability to cope with oxidative stress of different origins (Teramoto and Bungum 2010). The adaptive response to repetitive and potentially harmful stressors is commonly called 'hormesis' (Radak et al. 2008). According to this hypothesis, regular physical exercise could act as a stimulating stressor, which induces physiological and biochemical systemic adaptation (Radak et al. 2008). Such a repeated exposure to increased ROS production from repeated exercise bursts leads to the up-regulation of antioxidants and the shift towards a more reducing environment (Radak et al. 2008). This adaptation might provide protection from increased oxidative stress during successive exercise sections, ultimately leading to improvement in health and/or physical performance. Actually, several experimental data show that regular exercise is associated with reduced oxidative damage and enhanced resistance to oxidative stress, supporting the requirements of hormesis theory. Accordingly, regular exercise leads to up-regulation of gluthatione levels and reduces the generation of ROS and inflammation in rats (Radak et al. 2008, 1999). We have previously reported lower levels of malondialdehyde in endurance athletes with respect to sedentary controls (Vassalle et al. 2002). Interestingly, in accordance with the ROS-hormesis hypothesis, exercise-induced ROS production may play an important role in cell signalling involved in the antioxidant defence network. This response to ROS stress, called mitochondrial hormesis, is supposed to be responsible for the respective lifespan-extending and health-promoting effects of physical exercise (Radak et al. 2008).

Another interesting issue emerging among aspects related to exercise is the overtraining syndrome (OTS) (Meeusen et al. 2013). This condition frequently occurs in athletes who train longer and harder beyond the body's ability to recover (Meeusen et al. 2013). Its relevance is evidenced by marked decrements in performance and profound fatigue of athletes (Meeusen et al. 2013). The diagnosis of OTS is differential because the underlying causes are essentially unknown, and remains difficult (Meeusen et al. 2013). Different factors that affect performance and mood status must be excluded, including anaemia, magnesium deficiency, viral infections, muscle damage evidenced by creatine kinase levels, eating and endocrinological disorders (e.g. diabetes, thyroid disorders and adrenal dysfunction), depression, allergies, asthma and cardiovascular disease (Purvis et al. 2010). Interestingly, overtraining drives a marked response of oxidative stress biomarkers, which in some cases appeared proportional to the training load (Margonis et al. 2007). Recent data on proteomic profiling of skeletal muscle in animal models of overtraining confirm the role of proteins of oxidative phosphorylation complexes and antioxidants, together with proteins related to lipid metabolism, and chaperones in response to OTS (Gandra et al. 2012). Thus, oxidative stress biomarkers may be helpful as a tool for OTS diagnosis. Moreover, oxidative stress may represent a potential target of future antioxidant interventions, although at the moment there is a lack of data on the effects of diet/supplemental antioxidants in OTS.

An important question to correctly interpret the response of an oxidative stress system to exercise is the complex interrelationship between this system and inflammation, neuroendocrine and immunitary systems. Recent findings from *in vitro*

studies have shown that the inflammatory cytokine interleukin (IL)-8 is significantly and inversely related to antioxidant levels (Vidyashankar et al. 2013). Moreover, ROS generation and cellular redox status are known to lead to the activation of nuclear factor-κB (NF-kB), which in turn leads to the expression of proinflammatory cytokines. Importantly, NF-kB represents the main transcription factor for the expression of IL-8 in response to oxidative stress (Vidyashankar et al. 2013). Among the various proinflammatory cytokines, IL-8 is identified as the most sensitive indicator responding to intracellular ROS intensity (Vidyashankar et al. 2013, Tsuji et al. 2011, Pei et al. 2002, Park et al. 2001). Recently, we showed a close relationship among variations in oxidative stress, IL-8 and N-terminal pro-brain natriuretic peptide (NT-proBNP) in Ironman athletes early after strenuous exercise consisting of a 3.8 km swim, 180 km bike and 42.2 km run (Pingitore et al. 2011a,b). This result most likely reflects a common pattern regulating the physiological acute response to exercise and the hormetic process.

In this context, also intriguing is the recent hypothesis that elevated mitochondrial ROS can exert beneficial effects by triggering hypoxia-inducible factor 1α (HIF-1α) signalling, which in turn stimulated the immune system to improve protection against infective and neoplastic stimulus, thus reducing molecular damages (Hekimi 2013). However, there are still no data corroborating this hypothesis in the adaptive response to exercise.

16.3 AVAILABLE BIOMARKERS OF OXIDATIVE STRESS

In vivo, oxidative stress is a dynamic condition amplified by a continuing vicious cycle leading to increased free radical generation and reduced defence systems that further exacerbate oxidative damage.

The direct measurement of oxidative stress in complex biological systems is extremely difficult, because free radicals are highly reactive and have a very short half-life. Thus, major advances have been made in the development of indirect assays, and it is generally based on the measurement of different oxidised compounds or antibodies directed against oxidised epitopes. Most commonly used oxidative biomarkers include conjugated dienes, hydroperoxides, malondialdehyde, 4-hydroxynonenal, hydrocarbons such as pentane and ethane (in breath), F2-isoprostanes and oxLDL (Valko et al. 2007, Lee et al. 2012) (Table 16.1).

The human antioxidant defence consists of antioxidant enzymes (including catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic antioxidants (such as vitamins E, A, C, and glutathione and uric acid) (Table 16.2) (Pinchuk et al. 2012).

Indeed, there are situations in which knowledge of the individual levels of a single, specific antioxidant may be useful, as when determining the antioxidant contribution of specific dietary components and their relationship with antioxidant composition and activities, or during studies of decreased antioxidant capacity in subjects under oxidative stress in specific patho-physiological states. However, global approaches to measure the total antioxidant activity are generally used to overcome the complexity of this system (Somogyi et al. 2007). These tests are easy to perform and measure in a single step the so-called total antioxidant capacity (TAC), a parameter that represents the cumulative effects of all antioxidants present in a sample (food,

TABLE 16.1
Main Oxidative Stress Biomarkers

Lipids
Isoprostanes
Malondialdehyde
Thiobarbituric acid-reactive substances
4-Hydroxy-2-nonenal
Conjugates dienes

Proteins
Protein carbonyl content
3-Nitrotyrosine
Advanced oxidation protein products

Advanced glycation end products

Nucleic acids
8-Hydroxyguanosine (8-OHG)
8-Hydroxydeoxyguanosine (8-OHdG)
Comet assay

Reactive oxygen species
Hydrogen peroxide/peroxidase
Nitric oxide
Nitric oxide synthetase activity

Breath hydrocarbons
Exhaled pentane and ethane

blood or tissue). However, in this case, the contribution of the antioxidant enzymes may be very small. Moreover, whether this integrated approach allows to assess the capacity of both known and unknown antioxidants and their synergistic interaction, the assumption that an *ex vivo* exposure of the sample mimics the complexity of a physiological context, clearly represents the main limitation of such assays.

The principle on which these assays are based is the capacity of antioxidants in the sample to inactivate the oxidants added in excess (Pinchuk et al. 2012, Somogyi et al. 2007). The degree of colour change is proportional to the capacity of all anti-oxidants present, and the reaction endpoint is reached when colour change stops. In these tests, it is assumed that antioxidant capacity is equal to reducing capacity. However, these assays may differ from each other in terms of substrates, reaction conditions and quantification methods (Pinchuk et al. 2012, Somogyi et al. 2007). This fact may render it difficult to compare the results obtained by different assays.

New biological markers of oxidative stress are emerging. The effect of physical activity on molecular biomarkers associated with chronic degenerative diseases has been recently reviewed (Izzotti 2011). Among molecular markers, the gene poly-morphisms of glutathione S-transferase, GSTM1/T1, which is involved in antioxi-dant defence, appear to be closely related to physical activity and consequently to clinical outcomes (Izzotti 2011). Specifically, this gene may present a homozygous deletion polymorphism that results in a lack of gene activity ('null' polymorphism).

TABLE 16.2
Main Antioxidant Biomarkers

Enzymes
Catalase
Glutathione peroxidase
Superoxide dismutase
Paraoxanase
Glutathione S-transferase
Glutathione reductase
Heme-oxygenase

Non-enzymes
Uric acid
Glutathione
Melatonin
Ubiquinol

Vitamins
Vitamin C
Vitamin E
Vitamin A

Metal-binding proteins
Ceruloplasmin
Ferritin
Lactoferrin
Transferrin
Total antioxidant capacity

This condition is correlated to higher oxidative DNA alterations (8-oxo-dG) and the mtDNA 4977 deletion in arterial smooth muscle cells of null polymorphism carriers compared to wild-type carriers for sedentary subjects (Izzotti 2011). However, the effect of this polymorphism appeared to be strongly influenced by physical activity. In fact, no significant difference was observed between the wild-type and double null genotype carriers in terms of the level of these oxidative biomarkers (8-oxo-dG and mtDNA 4977 deletion) in physically active subjects (Izzotti 2011).

Interestingly, available evidences give support to the existence of cardioprotective genotypes for the haeme oxygenase gene, which catalyses the degradation of heme antioxidant, in its adaptive response to exercise. In particular, recent results suggest that after endurance training, subjects carrying a CC genotype presented higher values in different cardiac function biomarkers and better cardiac adaptation than those having CT and TT genotypes (He et al. 2008).

Beneficial effects induced by physical activity at the molecular level are also evidenced by the reduction of oxidative DNA damage to nuclear DNA in terms of 8-oxo-dG and DNA adducts and to mitochondrial DNA in terms of the mtDNA 4977 deletion (Izzotti 2011). Thus, subjects lacking antioxidant defences due to their adverse genetic polymorphism (i.e. the double null carriers) might receive the

benefit of physical activity and/or antioxidant supplementation, thereby replacing the lack of endogenous antioxidant defences due to their genetic profile.

Moreover, in view of the close correlation of oxidative stress with inflammatory and other physiological processes, an integrative approach including biomarkers such as cytokines (such as ILs such as IL-8), neuroendocrine biomarkers (such as NT-proBNP) or physiological tests (such as those used to assess endothelial function; e.g. flow-mediated dilation) could be additively conducted to indirectly assess the effects of dietary/antioxidant supplementation in athletes.

16.4 HYPER-HOMOCYSTEINEMIA

Mild hyper-homocysteinemia (HHcy) is an independent marker of cardiovascular diseases. A dose response between level and risk, even within the reference interval, is reported. Homocysteine (Hcy)-induced oxidative damage may contribute to increase the risk of vascular events. Only a few studies have been performed on exercise and homocysteinemia.

Bambaeichi et al.'s study on the influence of aerobic exercise on plasma Hcy levels in young men showed that 8 weeks incremental exercise had no significant effect on reducing Hcy as a risk factor for cardiovascular diseases (Bambaeichi et al. 2010). The relationship between physical exercise and plasma Hcy levels, metabolically related to folate and vitamin B12, was investigated in well-trained male triathletes (Konig et al. 2003). After a 30-day endurance training period, athletes had a significant decrease in Hcy levels and a significant increase in folate but not in vitamin B12 levels. Conversely, intense exercise (sprint triathlon) acutely increased Hcy levels (Konig et al. 2003).

Marked circulating HHcy occur in homocystinuria, a recessively inherited disorder of methionine metabolism, due to cystathionine-β-synthase deficiency, associated with a greatly enhanced cardiovascular risk. The mechanisms mediating Hcy-induced vascular changes are not completely defined; however, subjects, also young, with homocystinuria or with HHcy from other causes (e.g. folate or vitamin B12 deficiency) have impaired endothelial function and, consequently, an oxidative stress condition. In Wilcken et al.'s study, the positive relationship between Hcy and superoxide dismutase, an important component of the endogenous antioxidant defence in vascular tissue, could represent a protective antioxidant response to Hcy-induced oxidative damage and contribute to reducing cardiovascular risk in homocystinuric patients (Wilcken et al. 2000).

16.5 SUPPLEMENTAL ANTIOXIDANTS, HEALTH AND RISK

An active debate still exists on the effect of antioxidant supplementation on exercise-induced oxidative stress. Typical treatment generally includes vitamins A, C and E, at various dosages, administered alone or in combination, chronically or acutely (Braakuis 2012, Askari et al. 2012, D'Adamo et al. 2013, Simar et al. 2012, Bobeuf et al. 2011, Fisher-Wellman and Bloomer 2009, Nikolaidis et al. 2012a,b, Bohlooli et al. 2012). Of these, vitamins C and E have been used more frequently in clinical and experimental studies, mostly because of their safety profile and easy availability

(Braakuis 2012, Nikolaidis et al. 2012b). Bohlooli et al.'s study showed a protective role of a moderate dose of vitamin C (500 mg), acutely administered in a non-trained male group compared with the placebo group, on exercise-induced lipid peroxidation (MDA and TAC) and muscle damage (creatine kinase); conversely, vitamin C did not show any effect on inflammatory markers (total leukocytes, CRP, IL-6) (Bohlooli et al. 2012). Other less used antioxidants include quercetin, coenzyme Q10 and N-acetylcysteine (Bloomer et al. 2012, Díaz-Castro et al. 2012, Michailidis et al. 2013). Concerning the endpoints, the antioxidant might be effective in particular conditions in terms of exercise and training, such as one type of sport with respect to another one (e.g. aerobic vs. anaerobic) or specific moment of the training (e.g. before, or after the race, during OTS). Thus, the selection and detailed description of the appropriate training stimulus is needed, and/or the monitoring of the athlete during training phases.

Concerning the biomarker used, some authors did not report any significant changes in levels of markers of lipid peroxidation, DNA damage and glutathione redox status following long duration protocols, whereas others reported reductions in F2-isoprostanes, thiobarbituric acid reactive substances, DNA damage as well as inflammatory biomarkers (Fisher-Wellman et al. 2009, Nikolaidis et al. 2012a,b, Bloomer et al. 2012). Indeed, some studies reported that antioxidant supplementation may induce no effect or even an adverse pro-oxidant response (Rytter et al. 2010, Theodorou et al. 2011, Tomasello et al. 2012). In the light of contrasting results in the literature, there are several arguments in favour of or against antioxidant supplementation. One argument against the use of antioxidants is based on the evidence that ROS production during exercise is fundamental to promote the expression of several skeletal muscle proteins, including antioxidants enzymes, mitochondrial and heat shock proteins that represent the molecular basis of the exercise-induced hormetic response. Interestingly, recent experimental data evidenced that ROS mitochondrial production upregulates HIF-1α that is an important regulator of the immune response and that is elevated in heterozygote Mclk1$^{+/-}$ mutant mice. Mclk1$^{+/-}$ encodes a mitochondrial protein necessary for ubiquinone biosynthesis (Hekimi 2013). These mice have increased mitochondrial ROS production but have a reduced development of oxidative biomarkers or ageing, a decrease in the age-associated loss of mitochondrial function and increased lifespan. The use of antioxidants may therefore blunt the ROS-induced adaptive response to exercise. Further data from a recent meta-analysis showed that beta-carotene, vitamins A and E given singly or combined with other antioxidant supplements significantly increase overall mortality, whereas there was no evidence that vitamin C may increase longevity (Bjelakovic et al. 2007). Conversely, selenium given singly or in combination with other supplements significantly decrease mortality (Bjelakovic et al. 2007).

One of the main arguments in favour of antioxidant supplementation strengthens the benefit in terms of reduction of muscular fatigue and improvement of performance (Powers et al. 2011, Bjelakovis et al. 2011). In particular, N-acetylcysteine may delay muscular fatigue (Kelly et al. 2009, Cobley et al. 2011). More recently a new antioxidant agent, pycnogenol, administered 4 h before exercise has been found to improve performance in trained cyclists increasing maximal oxygen consumption (Bentley et al. 2012).

Currently, there is no a general agreement to the use of antioxidants for athletes. This is clearly stated in a recent position statement on the maintenance of immune

health in athletes, where the more common antioxidants are not recommended due to the absence of documented benefit when compared with placebo (Walsh et al. 2011). Nonetheless, the benefit of antioxidant intake can be more reasonable in athletes not consuming a balanced diet (Machefer et al. 2007). Polyphenols, such as quercetin, curculin, resveratrol and luteolin, are recommended especially when mixed with other flavonoids and nutrients, for their antioxidative, anti-inflammatory, cardioprotective and anticarcinogenic and mitochondrial stimulatory activities (Walsh et al. 2011). In a recent study, 500 mg quercetin + 250 mg vitamin C supplementation for 8 weeks was effective in reducing oxidative stress and inflammation among subjects with regular exercise, and 3 weeks quercetin (1000 mg/day) lowered the incidence of upper respiratory tract pathologies in athletes (Askari et al. 2012, Nieman et al. 2007).

16.6 ANTIOXIDANT SUPPLEMENTATION IN EXERCISE: LIMITS AND CONSIDERATIONS

Marked divergence and often contradiction among studies addressing the effect of antioxidant supplementation on exercise adaptation may be explained by a variety of factors (Table 16.3).

At the moment, the use of different exercise protocols, different outcomes, in different physically trained subjects, and the use of a variety of laboratory parameters to evidence such effects still make it difficult to evaluate effects of physical activity on health. Thus, in any case, a detailed description of the type of exercise (e.g. aerobic or anaerobic), subject characteristics, oxidative stress biomarkers used and training endpoints examined is always necessary to allow data interpretation.

Subjects presenting higher levels of oxidative stress may clearly benefit more from the antioxidant treatment. An initial screening of the oxidative stress status is therefore essential. Clearly, individual susceptibility related to the presence of specific genetic variants in key enzymes for ROS detoxification can be another important factor (Izzotti 2011, He et al. 2008). Possible drug interaction may be considered, for example, it being known that antioxidant treatment may blunt the effectiveness of hypolipidemic therapy with statins and niacin. Moreover, evaluation of the hydration status could be helpful (Kenefick and Cheuvront 2012). It would be useful to consider the integrated effect of diet and exogenous antioxidant supplementation.

TABLE 16.3

Antioxidant Supplementation in Exercise: Main Endpoints to Be Considered

Selection and detailed description of the appropriate training stimulus

Type of antioxidant used: the 'cocktail approach'

Dose and duration of treatment

Interaction with dietary supplementation and drugs

Choice of oxidative stress parameters and other biochemical/physiological biomarkers

Initial screening of the oxidative stress status

Evaluation and reporting of hydration status

Complexity of redox reactions *in vivo* and the potential for a paradoxical increase in oxidant generation
  by antioxidants themselves

The use of proper oxidative stress biomarkers, as well as valid and reliable procedure, and techniques and assays remains critical to evaluate results. Evaluation of oxidative stress implied careful attention to pre-analytical aspects, which include procedures for collecting and storing samples. In fact, appropriate procedures adopted during blood collection and sample storage are essential for consistent and accurate results. The best conditions include that samples, unless immediately dosed, must be kept on ice soon after collection and rapidly separated by centrifugation at 4 °C. Then haemolysis-free serum samples should be frozen and preferably maintained at −80°C until assayed (Vassalle 2008).

Oxidative stress represents a dynamic situation of balance between oxidants and antioxidants (Vassalle 2008). Thus, estimate of redox status should include an appropriate measure of both components, in most cases (Vassalle 2008). However, this principle is not true in all situations. There may be a difference in the contribution of different oxidant/antioxidant classes in different states, potentially limiting the effectiveness of a given antioxidant under certain conditions. As an example, TAC appears to be increased in patients with chronic renal failure (Jackson et al. 1995). This finding may not be representative of the real status of oxidative stress, because the elevation of this biomarker is principally due to high urate concentrations. Thus, in this specific case, other parameters may be more useful, such as malondialdehyde, which resulted high, and ascorbate, whose levels fell in these patients (Jackson et al. 1995). Moreover, much evidence suggests an increased oxidative stress, but the lack of TAC decrease in obese subjects (Vassalle et al. 2013a, Puchau et al. 2010, Mancini et al. 2008, Vigna et al. 2010). Thus, the measure of TAC in such subjects might be less important or negligible.

The number of different antioxidants present in serum or other body fluids makes it difficult to measure how much each contributes separately. Conversely, the possible interactions between different antioxidants *in vivo* make the measure of each individual component not representative of the entire antioxidant status.

Clearly, lack of consensus may also be partially explained by variability of redox processes. In this context, and last but not least, it is important to consider the complexity of redox reactions *in vivo* and that the antioxidant itself may act as a pro-oxidant under certain circumstances (Tomasello et al. 2012).

16.7   VITAMIN D: TO BE SUPPLEMENTED OR NOT TO BE SUPPLEMENTED?

The case of vitamin D [25(OH)D] in exercise is of particular interest. In fact, of the well-known effects in maintaining normal calcium–phosphorus homeostasis, this molecule has been involved in many extra bone conditions—including cancer, diabetes, cardiovascular and autoimmune diseases, and osteoporosis (Pludowski et al. 2013).

Vitamin D retains antioxidant properties and affects inflammatory and immunity processes, synthesis of proteins, cell growth and proliferation, and regulates the expression of over 1000 genes in a variety of tissues (Pludowski et al. 2013). In regard to oxidative status, experimental studies (cellular and animal models) indicated a significant reduction in oxidative stress damage and chromosomal aberrations, as well

as prevention of telomere shortening and inhibition of telomerase activity following treatment with vitamin D (Nair-Shalliker et al. 2012). Moreover, vitamin D influences the poly-ADP-ribose polymerase activity, affecting the cell cycle to prevent propagation of damaged DNA, and apoptosis to promote cell death (Nair-Shalliker et al. 2012). Treatment with paricalcitol alone or in combination with enalapril protected against inflammatory and oxidative endothelial damage in mice atherosclerotic aorta (Husain et al. 2010). In an *in vitro* study, a vitamin D analogue prevented neuronal damage caused by H_2O_2-induced toxicity (Tetich et al. 2004). Other data showed that vitamin D reduced lipid peroxidation and induced SOD activity in a hepatic anti-oxidant system in a rat model (Garcion et al. 1999). Calcitriol increases intracellular glutathione pools and reduces nitrite production that is induced by lipopolysaccharides in astrocytes (Bao et al. 2008). Moreover, the activation of 1α-hydroxylase in macrophages increases the level of calcitriol, which inhibits iNOS expression and reduces nitric oxide (NO) production within lipopolysaccharide-stimulated macrophages, providing protection against the oxidative injuries caused by the NO burst (Chang et al. 2004).

Total 25(OH)D is the recognised laboratory parameter accepted to estimate the status of the overall vitamin D status in the clinical field (Vassalle and Pérez-López 2013b).

It is known that the majority of the population the world over presents 25(OH)D deficiency, whose levels are also highly dependent on seasonality (Shoben et al. 2011, Holick et al. 2011). Thus, emerging evidence indicates that 25(OH)D deficiency exists in some athlete categories, especially in players practising indoor sport, such as basketball. In fact, a high prevalence of 25(OH)D insufficiency has been recently reported in Israelian basketball athletes and dancers, with a very higher rate of 25(OH)D insufficiency among participants who practise indoors, and during the winter (Constantini et al. 2010). A high prevalence of 25(OH)D insufficiency/deficiency has been also observed among elite Irish athletes, suggesting that vitamin D supplementation is an appropriate regime to ensure 25(OH)D sufficiency in athletes during winter and early spring (Magee et al. 2013).

However, other evidence also suggests that outdoor athletes may be at risk of 25(OH)D deficiency during winter, and that supplementation may be advisable to maintain adequate 25(OH)D concentrations (Galan et al. 2012).

Vitamin D effects on athletic performance have been recently reviewed (Cannell et al. 2009). Available data suggest that 25(OH)D are related to the size and number of muscle fibres (Type II fast twitch-muscle fibres) (Cannell et al. 2009). Since the 1950s, it has been reported that vitamin D-producing ultraviolet light may improve athletic performance. Moreover, more recent results suggest the seasonality of physical and athletic performance, enhanced or reduced in parallel with 25(OH)D levels variation (Cannell et al. 2009).

Recent data found a correlation between vitamin D levels and bone mass and muscle strength in a cohort of adolescent girls (Foo et al. 2009). Other results, obtained in adolescent girls, evidenced a significant association between 25(OH)D and muscle power, force, velocity and jump height (Ward et al. 2009).

Additional data evidenced that supplementation with vitamin D increased variables related to power, jumping velocity and height increase and consequently the

efficiency of the jump, although it did not increase the maximum muscle force and power (Ward et al. 2010). Thus, authors suggest an effect of vitamin D on lower limb function through improvements in the mechanical efficiency of the muscle (Ward et al. 2010).

Vitamin D is essentially produced by skin exposure to ultraviolet irradiation (Holick et al. 2011). However, vitamin D can also be supplied in part from the diet by the intake of a limited number of aliments (e.g. some fish, egg, mushrooms) (Holick et al. 2011). Supplementation is safe, because intoxication for elevated levels of vitamin D is rare, and it may be considered in categories at risk of deficiency (Holick et al. 2011). Actually, there is no shared consensus on the use of vitamin D in athletes; thus the role of vitamin D in athletic performance still has to be determined. Data are also expected on the relationship between vitamin D and oxidative stress in sport studies, an interesting area of research so far still not investigated. Nonetheless, based on the evidence that many athletic cohorts are vitamin D deficient or insufficient, vitamin D could be monitored in athletes to decide whether they would benefit from vitamin D supplementation.

16.8   DIET: THE IMPORTANCE OF BEING HEALTHY

A well-balanced and appropriate diet is essential for sport performance. Actually, there are more existing position papers on athletic basic nutrition adopted in many countries (Rodriguez et al. 2009). However, this matter is not simple, as there is no such thing as 'one-size-fits-all plan'. Indeed, nutrition requirement in athletes is reasonably influenced by the number of variables that could influence an individual's diet, including the type of exercise and individual characteristics (sex, age, hormonal changes in women, etc.) and training status. Generally, the quality of the food composition plan for trace elements, carotenoids and flavonoids is still considered to be poor (Soriguer et al. 2007, Bernardi et al. 2007). Moreover, food habits as well as quantities of nutrients within food may vary among countries and studies are not always comparable.

One of the most intensively emerging aspects in nutrition is the development of the so-called 'functional food' (Sirò et al. 2008). Functional foods are fortified, enriched or enhanced foods that give additional health benefits (as health-promotion or disease prevention) exceeding the capacity based on their content of nutrients, when consumed as part of a varied diet on a regular basis. The category of functional foods includes processed food or foods fortified with additives like 'vitamin-enriched' products. Several such foods have been demonstrated to improve sport performance at a higher level than the one expected with a well-balanced diet (Deldique et al. 2008). However, there is still the need for additive rigorous scientific evidence to classify these food types on the basis of safety and efficacy (Deldique et al. 2008). The Mediterranean diet is characterised by a high consumption of monounsaturated fatty acids, primarily from olives and oil, and daily consumption of fruits, vegetables, whole-grains and other low-fat dairy products, with a relatively low consumption of red meat.

A recent meta-analysis evidenced that adhering to the Mediterranean diet protected against overall mortality and incidences of various chronic degenerative

diseases, including cardiovascular incidence or mortality, cancer incidence or mortality and neurodegenerative diseases (Sofi et al. 2010). The Mediterranean diet is recognised to exert a significant anti-inflammatory, cardioprotective and anticancer action (Benetou et al. 2008).

In particular, a diet low in trans fatty acids and glycaemic load, high in cereal fibre, marine omega-3 fatty acids and folate with a high ratio of polyunsaturated fatty acids, non-smoking and moderate to vigorous exercise (≥30 min/day) significantly lowered the relative risk for coronary events in women (Stampfer et al. 2000).

Adhesion to the Mediterranean diet has been found to enhance the regenerative capacity of the endothelium and fitness in subjects with the metabolic syndrome trained with moderate-to-high-intensity endurance (Fernández et al. 2012). Other data indicate that adherence to a Mediterranean dietary pattern in healthy subjects increases the circulating plasma levels of carotenoids, vitamins A and E, and reduced oxidative stress and inflammation (evaluated as levels of uric acid, SH groups, SOD and GPx activities, FRAP and TRAP, tumour necrosis factor-α and IL-10 cytokines, and malondialdehyde in the erythrocytes as a marker of lipid peroxidation) (Azzini et al. 2011).

However, there is a lack of clear results in the field of Mediterranean diet and athlete well-being and exercise performance. Nonetheless, available results will most likely help to stimulate the interest of researchers to better tailor forthcoming studies in the field of nutrition in exercise, and to make uniform and standardise the score and its components.

16.9 CONCLUSIONS

Available data still do not allow us to define the optimal type and intake of antioxidants, as well as better laboratory biomarkers to evaluate these effects. The alternative possibility that antioxidant supplements modulate selective inhibitors of various enzymatic sources of ROS could also be considered (Olukman et al. 2010).

The specificity of the adaptive response is a function of the specific individual characteristics, such as the training period, the type of physical activity performed, the training level, age and sex of the subjects, environmental conditions and interindividual differences. It is important to determine the individual antioxidant need of each athlete performing a specific sport or in a specific phase of training. Antioxidant supplementation could help athletes with initially low antioxidant levels to improve their antioxidant status. High-intensity training periods are other critical periods for nutritional requirement. In such cases, the risk of deficit can be reduced by a rational application of recommended training loads and the requirement can be satisfied by well-balanced dietary/supplemental help.

For the biomarkers to be used, one possibility could be the shared agreement on some critical oxidative stress biomarkers, which may allow better standardisation and comparison of data. However, given the complexity of the relationship between oxidative stress, antioxidant diet/supplementation and exercise, the careful choice of a specific panel of biomarkers may be more appropriate in different cases in the research area as well in clinical practice. Moreover, available evidence supports that the development of additive integrative and comparative biomarkers/physiological

tests could shed light on the interactions of key redox responses at multiple levels of biology/environment interactions, and assist clinicians in selecting the optimal treatment/monitoring on an individual basis.

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Index