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Selected methods of *in vitro* embryo production in felids – a review*

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During the past decade the need for Artificial Reproductive Techniques in felids has greatly increased. Mostly, this is a result of growing expectations that these techniques may be applied in conservation biology and thereby contribute to saving wild felids from extinction. In this article we describe three most common methods of obtaining embryos in vitro in the domestic cat and its wild relatives: classic *in vitro* fertilisation, *in vitro* fertilisation by intracytoplasmic sperm injection and somatic cell nuclear transfer. Each of the methods provides a cleavage rate of around 50% and approx. 20% of embryos develop to the blastocyst stage. After the transfer of embryos produced by these methods, scientists obtained living offspring of the domestic cat, as well as several wild cats: the tiger, serval, fishing cat, caracal, ocelot, wild cat, sand cat, black-footed cat and the oncilla. These successes, in spite of the low efficiency of the discussed methods, are promising and suggest that biotechniques of reproduction will be valuable tools in the protection of wild species. Somatic cell nuclear transfer will allow to sustain the narrow gene pool in the critically endangered felids. For these reasons it is necessary to conduct further research on the optimization of artificial reproduction techniques in cats.

KEY WORDS: feline embryos / *in vitro* fertilization / ICSI / somatic cell nuclear transfer

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Classic *in vitro* fertilization (IVF)

1970 – first embryos obtained *in vitro* [Hamner *et al*. 1970]
1988 – first kitten obtained by IVF with the use of *in vivo* matured oocytes [Goodrowe 1988]
1990 – first wild felid (tiger) born after IVF and intraspecific transfer [Donoghue *et al*. 1990]
1993 – first wild carnivore (Asiatic wildcat) born after IVF and intraspecific transfer (to the domestic cat) [Pope *et al*. 1993]
1997 – first kitten obtained by IVF with the use of in vitro matured oocytes [Pope *et al*. 1997]
2003 – first domestic kittens born after embryo transfer of cryopreserved embryos [Gomez 2003, IVF]

Intracytoplasmic Sperm Injection (ICSI)

1998 – first ICSI derived kittens born from *in vivo* matured oocytes [Pope 1998]
2000 – first ICSI derived kittens born from in vitro matured oocytes [Gomez *et al*. 2000]
2009 – first gender-selected domestic kittens produced from embryos fertilized by sex sorted sperm [Pope 2009]
2011 – first ICSI derived transgenic kittens (green fluorescence) [Wongsrikeao *et al.* 2011]
2012 – first kittens born after transfer of embryos derived from ICSI of vitrified oocytes [Pope *et al.* 2012a]
2012 – first kittens born after transfer of embryos derived from ICSI of vitrified embryos [Pope *et al.* 2012b]
2012 – first kitten born after ICSI with testicular spermatozoa from cryopreserved tissue and after embryo cryopreservation [Tharasanit *et al.* 2012]

Somatic Cell Nuclear Transfer (SCNT, cloning)

2002 – first cloned cat [Shin *et al.* 2002]
2004 – first wild felid (African wildcat) born after SCNT and intraspecific transfer (to the domestic cat) [Gomez *et al.* 2004].
2008 – first transgenic cat (red fluorescence) [Yin *et al.* 2008a]
2008 – first second generation clones [Yin *et al.* 2008b]

During the past decade we have observed a rapid increase in the *in vitro* production of embryos both in human patients and in farm animals. In human medicine, according to the most recent world report [Dyer *et al*. 2016], in 2008-2010 more than 1.1 million babies were born worldwide using assisted reproductive techniques (ART), with an approx. 9% increase from year to year. In the case of animals over 0.5 million bovine embryos were produced in vitro in 2014, of which more than 300,000 were transferred [IETS 2014]. Embryo transfer of in vitro produced equine embryos almost doubled

between 2013 and 2014, and for sheep in 2014 two new countries started to produce and transfer ovine embryos commercially [IETS 2014]. Unfortunately, similar statistics do not apply to the domestic cat, for which in vitro embryo production is still more of a scientific quest than a practical/clinical issue. However, the need to use ART in this species has increased, because the domestic cat is an excellent biomedical model for endangered feline species; thus, that methods of in vitro embryo production developed for the domestic cat might be applied in conservation biology and contribute to saving wild felids from extinction.

The aim of this article is to describe methods of *in vitro* embryo production in domestic and non-domestic cats, to summarise achievements obtained in this field and to pinpoint problems that still need to be solved.

Obtaining feline embryos *in vitro* – why should we do this?

Considering the huge numbers of unwanted kittens born each season and thousands of sterilisation procedures performed each year, it may seem that the goal of veterinarians should be to limit feline reproductive potential instead of supporting it by ART. Although this statement is true for stray cats, the situation is different for purebred cats and wild felids, which suffer from high inbreeding with a negative impact on animal health and fertility.

The lower fertility of purebred animals is a well-known fact that is especially true for companion animals, which are selected for their appearance, not their reproductive value. Although, to the authors' knowledge, there are no large, statistical reports concerning the reproductive status of breeding cats, the increasing number of feline infertility cases admitted into the ambulatory clinic of the Reproductive Department in Wrocław seems to confirm that reproductive problems have become a crucial issue in this species. Some of the infertile males in our clinic showed oligoteratozoospermia (data not shown), while Axnér and Linde Forsberg [2007] reported a higher incidence of sperm abnormalities in cats with poor breeding results. In general, teratospermia (less than 60% normal sperm cells) is a common condition in cats [Axnér and Linde Forsberg 2007, Prochowska *et al.* 2015, Pukazhenthi *et al.* 2006], while additionally purebred cats exhibit poorer sperm quality than household cats [Axnér and Linde Forsberg 2007]. In this situation, in vitro fertilisation may be applied as an advanced method in the treatment of infertility.

Wild cats, both free-ranging and captive, face similar problems. Production of many morphologically abnormal spermatozoa is common and is more severe than in the domestic cat. Extreme examples are Florida panthers (*Puma concolor coryi*), which produce ejaculates with only 6.5% normal sperm cells [Barone *et al.* 1994]. Poor semen quality is thought to be caused by reduced heterozygosity [Barone *et al.* 1994, Wildt *et al.* 1987], which is a consequence of small population sizes, few founding individuals and a limited possibility to exchange genes between populations. Breeding in captivity is difficult, due to the small numbers of animals as well as ethical and legal

restrictions relating to the transportation of individual animals. Additionally, natural breeding in zoos may be challenging due to behavioral incompatibilities, which in some species may be extremely strong. For example, in the clouded leopard (*Neofelis nebulosa*) intersex aggressive behavior may lead to the death of an animal during pairing for mating [Brown *et al.* 1995]. In vitro fertilisation (IVF) and embryo transfer (ET) might help maintain genetic variability by combining gametes from individuals separated by distance or behavioral barriers [Herric *et al.* 2010].

Another approach is the possibility of in vitro embryo production using cells collected from dead animals [Cocchia *et al.* 2010]. This is especially important in the case of genetically valuable individuals and endangered species and will allow us to preserve rare genotypes that normally would be lost when the animal dies.

Obtaining embryos *in vitro* – how can we do it?

There are several methods, by which embryos can be produced *in vitro*. In this article we will focus on three most common methods reported to be used in felids: classic *in vitro* fertilisation, intracytoplasmic sperm injection and somatic cell nuclear transfer. These methods are briefly described below and summarised in Table 1.

Table 1. Comparison of methods of *in vitro* embryo production

Item	IVF	ICSI	SCNT
Cells required	mature oocytes and appropriate number of motile, competent spermatozoa	mature oocytes and few spermatozoa (may be non-motile and non-viable)	somatic cells (mostly fibroblasts) from the donor of any age and gender, oocytes (may be from other, closely related species)
Equipment required	stereomicroscope	inverted microscope with micromanipulators	inverted microscope with micromanipulators, electrofusion chamber (optional)
Time required	few minutes regardless of the number of oocytes	around 0.5-1h for oocyte preparation, ICSI - few minutes per oocyte	around 0.5-1h for oocyte preparation, SCNT - few minutes per oocyte
Difficulty of the procedure	+	++	+++
Efficacy in vitro (domestic cat)			
cleavage rate	from 30 to 80%, typically around 50%	from 30 to 80%, typically around 50%	from 40 to 90%, typically around 65%
blastocyst rate (related to the number of embryos)	from 10 to 60%, typically around 30%	from 10 to 45%, typically around 25%	from 5 to 50%, typically around 15%
Efficacy *in vivo* (pregnancy rate after embryotransfer) (domestic cat)	from 0 to 100%, typically around 50%	from 16 to 66%, data are scarce	from 5 to 25%, typically around 15%

In vitro fertilization (IVF)

This is the oldest and simplest method of *in vitro* embryo production, which involves coincubation of oocytes and spermatozoa outside the female body. In cats, the first embryos were obtained by this method in 1970 [Hamner *et al.* 1970] and the first kitten produced from IVF-derived embryos was born in 1988 [Goodrowe *et al.* 1988]. Since then, several variations of this technique have been introduced by different authors to optimise the procedure. For instance, coincubation may be performed in

400-500 µl of culture medium in a 4-well plastic dish [Pope *et al*. 1998] or in small droplets of medium in a Petri Dish [Gómez *et al.* 2000]. Different culture media are used, including media enriched with stimulants such as caffeine [Eriani *et al.* 2008] or penicillamine-hypotaurine-epinephrine [Zambelli *et al.* 2006]. The germ cells are most often incubated together for 18 h (Fig. 1); however, 3-6 h were enough for feline spermatozoon to penetrate the zona pellucida, fuse with the oolemma and to fertilise the oocyte [Pope *et al*. 1993].

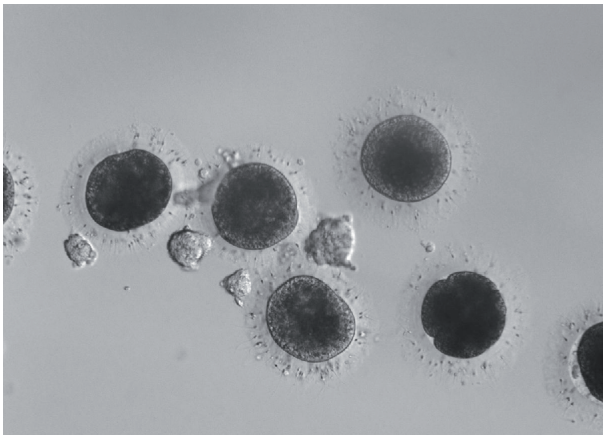

Fig. 1. Presumptive feline embryos after 18 hours of coincubation with spermatozoa.

The IVF method facilitates fertilisation by depositing sperm cells directly next to the oocyte, eliminating any negative impact of an abnormal uterine environment, low sperm number or poor sperm survival. However, to achieve success an appropriate number of competent spermatozoa (motile, morphologically normal, and able to undergo capacitation and the acrosome reaction) is still required [Michelmann 1995]. This makes the classic mode of in vitro fertilisation less efficient or even unsuccessful in the case of very poor semen quality (oligospermy, severe teratospermy, asthenozoospermy, etc.) [Ron-el *et al.* 1991]. For cats, it was proven that spermatozoa from teratospermic donors have a reduced ability to bind to and penetrate the homologous zona pellucida [Howard *et al*. 1991].

Intracytoplasmic sperm injection ICSI

The first kittens produced after embryo transfer of ICSI-derived embryos were born in 1998 [Pope *et al.* 1998]. In this method a spermatozoon is injected with the use of special micropipettes and micromanipulators directly into the cytoplasm of a mature oocyte (Fig. 2). Usually the best, motile and morphologically normal spermatozoon is chosen. However, because the sperm cell is deposited in the oocyte manually, non-motile, non-viable, even immature spermatozoa may be used [Michelmann 1995]. This makes ICSI a good solution in the case of very low sperm quality. Apart from being very popular as a way of overcoming male infertility, it facilitates the invention

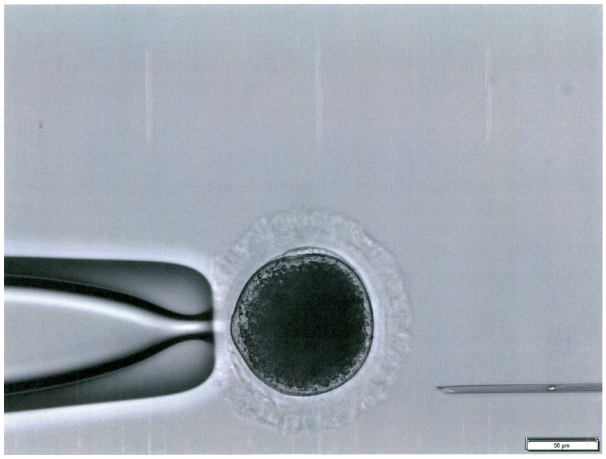

Fig. 2. Intracytoplasmic sperm injection in a domestic cat – an oocyte held by the holding micropipette and a sperm cell inside the injection micropipette.

and application of new sperm collection methods, in which non-motile and immature gametes are recovered. For cats, ICSI has been applied using cells collected from the testicular tissue [Buarpung *et al.* 2013, Comizzoli *et al*. 2006, Tharasanit *et al*. 2012]. Also new methods of sperm conservation may be established – methods that preserve DNA integrity, but not other characteristics of spermatozoa, such as motility and viability. Examples of such alternative sperm storage methods include alcohol storage [Murakami *et al.* 2005] or freeze-drying (lyophylisation) [Choi *et al.* 2011]. One of the main advantages of these alternative techniques is that liquid nitrogen or dry ice are no longer required for the storage and shipment of preserved spermatozoa, because they may be stored at room temperature or 4°C, thereby resulting in enormous reductions in storage and shipping costs and facilitating the creation of gene banks for endangered felids.

Somatic cell nuclear transfer (SCNT)

In this method, commonly known as "cloning", a nucleus obtained from a donor somatic cell is transferred into an enucleated oocyte and then fusion of these two elements followed by further embryo development is activated by electrical or chemical stimuli. The first cloned kitten was born in 2002 [Shin *et al.* 2002] and since then much work has been done to optimise this technique for felids. For example, different types of somatic cells were tested as a source of genetic material for SCNT, e.g. fibroblasts from both adults and fetuses, preadipocytes and cumulus cells [Kitiyanant *et al.* 2003, Shin *et al.* 2002, Tomii *et al.* 2011, Yin *et al.* 2005]. Fetal cells appear to exhibit better potential to support embryonic development than those obtained from adults [Kitiyanant *et al.* 2003, Yin *et al.* 2005].

Although very controversial and less efficient than IVF and ICSI, SCNT has some remarkable advantages that are significant for the protection of endangered species by

sustaining genetic biodiversity and saving rare genotypes. For wild animals, obtaining tissue samples (e.g. a small piece of skin) is usually much simpler than collecting gametes or embryos, so a larger and more diverse collection may be accumulated. Additionally, thanks to this method, genetic material collected from animals that do not produce germ cells (because they are too young or too old, exhibit illness or gonad abnormality, have been sterilised, etc.) may be used to produce embryos. Additionally, performing SCNT from fetal cells allows us to save genetic material in the case of abortion or stillbirth. The common belief that cloned animals are of the same cellular age as the donor, was disproved, e.g. in cats the telomere length was independent of telomere length in donor cells [Imsoonthornruksa *et al.* 2012]. It has been shown that cloned animals are able to reproduce naturally and produce healthy progeny [Kasai *et al.* 2007]. For cats, cloned males possessed semen characteristics within the normal range of values [Choi *et al.* 2010]. Also, the first cloned cat (a female named Copy Cat) was bred naturally and produced 3 healthy kittens. All of the above confirms the opinion that cloned animals may be introduced into the "normal" population and therefore SCNT is a valuable method to safeguard endangered wild cat populations.

There are two versions of this technique involved in rescue programs for felids, i.e. intra-specific or inter-specific (intergeneric). In the former approach the nucleus obtained from one species is transferred into an enucleated oocyte of the same species, e.g. genetic material obtained from a domestic cat fibroblast is fused with an oocyte of another domestic cat. Inter-species somatic cell nuclear transfer (iSCNT) consists in transferring the nucleus of one species into the oocyte of another species within the same genus, e.g. a nucleus from the sand cat (*Felis margarita*) into a domestic cat oocyte [Gómez *et al.* 2008]. A variation of this technique is intergeneric SCNT (igSCNT), in which cells of animals from a different genus are used, e.g. a nucleus from cheetah (*Acinonyx jubatus*) cells is transferred into an enucleated domestic cat oocyte [Moro *et al.* 2015]. Interestingly, it was also possible to produce embryos at the blastocyst stage when domestic cat [Wen *et al.* 2003] or marble cat (*Pardofelis marmorata*) [Thongphakdee *et al.* 2006] nuclei were transferred into rabbit oocytes. However, embryo transfer was not performed and the subsequent viability of such intergeneric embryos is unknown.

Embryo culture (EC)

Regardless of the embryo production method, the zygotes obtained need to be cultured before embryo transfer or cryopreservation. Although the International Embryo Technology Society has prepared a manual for in vitro embryo production in felids [IETS 2011], other laboratories/research groups use different in vitro culture systems. The embryo culture is conducted in an incubator under controlled conditions optimal for cats: temperature of 38.5 °C and a humified atmosphere with 5% CO_2. Some laboratories use lower oxygen tension values (5% O_2) [Filliers *et al.* 2010, Gómez *et al.* 2000], although its beneficial effect depends on the culture medium used [Moro *et al.* 2014]. Embryos are cultured in different media, e.g. feline optimised culture medium

(FOCM) [Herric *et al.* 2007], a medium based on Tyrode's Solution [Pope *et al.* 1998] or Ham's F-10 [Comizzoli *et al*. 2006], Synthetic Oviduct Fluid (SOF) [Freistedt *et al.* 2001, Thongkittidilok *et al.* 2015], or commercial media, which are designed mostly for human clinical applications [Nestle *et al.* 2012, Prochowska and Niżański 2017]. Embryos may be cultured in wells (in a large volume of medium, usually 400-500 µl) or in droplets - individually or in groups. Group culture has provided better results [Spindler and Wildt 2002, Thongkittidilok *et al.* 2014, Thongkittidilok *et al*. 2015], which may indicate the supportive effect of paracrine factors released by embryos. However, the effect of group culture depends on the quality and age of co-cultured embryos [Spindler and Wildt 2002]. The beneficial effect is sustained even when embryos of other species are used for co-culture [Spindler *et al.* 2006]. This fact is important regarding wild cats, where a low number of embryos can be obtained.

Embryo transfer (ET)

Regardless of the embryo production method, embryos need to be transferred into a surrogate mother for fetal development. Embryo transfer can be both autologous, when the donor of the oocyte is simultaneously the recipient of embryos, or heterologous, when embryos are transferred into another female. Heterologous transfer may be intraspecific (embryos transferred into a surrogate mother of the same species) or interspecific (embryos transferred into a female of a different species; most commonly embryos of wild animals are transferred into a closely related domestic animal). The limitation for interspecific embryo transfer is connected with the size of the female, but so far kittens of several small wild felids have been born from domestic cat mothers: the Asiatic wildcat (*Felis silvestris ornata*) [Pope *et al.* 1993], African wildcat (*Felis silvestris lybica*) [Gómez *et al.* 2004], sand cat [Gómez *et al*. 2008] and the black footed cat (*Felis nigripes*) [Pope *et al.* 2012]. Intraspecific embryo transfer has led to the birth of live kittens of the tiger (*Panthera* tigris) [Donoghue *et al.* 1990], serval (*Leptailurus serval*) [Pope *et al.* 2006a], fishing cat (*Prionailurus viverrinus*) [Pope *et al.* 2006b], caracal (*Caracal caracal*) [Pope et al. 2006 b], black footed cat [Pope et al. 2012], ocelot (*Leopardus pardalis*) and the oncilla (*Leopardus tigrinus*) [Swanson and Brown 2004]. In several cases attempts at embryo transfer were made, but they did not lead to pregnancy, as in the case of the marble cat [Imsoonthornruksa *et al.* 2012], flat-headed cat (*Prionailurus planiceps*) [Thongphakdee *et al.* 2010], jungle cat (*Felis chaus*) or the fishing cat [Pope *et al*. 1993]. Also, in some cases pregnancy was diagnosed, but no viable offspring were obtained, as in the leopard cat (*Prionailurus bengalensis*) [Yin *et al.* 2006] or the jaguarundi (*Puma yagouaroundi*) [Pope *et al.* 1998].

Some authors prefer to transfer early stage embryos into the oviduct [Goodrowe *et al.* 1988], while others have transferred morulae/early blastocysts into the uterus [Gómez *et al.* 2000, Pope *et al.* 1993, Pope *et al.* 1998]. Both approaches supported pregnancy and production of live offspring in domestic and wild cats, although for cloned black footed cat embryos more fetuses developed to term after oviductal transfer

of embryos at day 1 [Gómez *et al.* 2004]. Embryos may be deposited via laparotomy [Gómez *et al.* 2000], laparoscopy [Swanson *et al.* 2001] or transcervically [Swanson *et al.* 1994]. Recently, the use of endoscopy for transcervical artificial insemination was described by Zambelli *et al.* [2015], which raises hopes for the application of this method also for embryo transfer.

Typically a large number of embryos is transferred to increase prospects for success, and indeed more pregnancies occurred following transfer of ≥12 embryos per recipient than if <12 embryos per recipient were transferred [Pope *et al.* 1993]. In the African wildcat pregnancies were obtained only if >30 embryos were transferred [Gómez *et al.* 2004]. Interestingly, the first cloned cat was produced when only 3 embryos were transferred [Shin *et al.* 2002].

Obtaining embryos *in vitro* – what have we achieved so far?

Since the first feline embryos were produced in vitro in 1970 [Hamner *et al.* 1970] and the first kitten was born after in vitro fertilisation in 1988 [Goodrowe *et al.* 1988], scientists have obtained healthy domestic cat litters with each of the methods described, using oocytes collected by follicular aspiration (*in situ* collection, in vivo matured) [Goodrowe *et al*. 1988] or ovarian cortex slicing (ex situ collection, in vitro matured) [Gómez *et al.* 2000, Pope *et al.* 2012]; with spermatozoa collected with an artificial vagina [Gómez *et al.* 2000], electroejaculation [Goodrowe *et al.* 1988], epididymal slicing [Galiguis *et al.* 2014] and testicular retrieval [Tharasanit et al. 2012]; after gamete [Galiguis et al. 2014] and/or embryo cryopreservation [Gómez *et al.* 2003, Pope *et al.* 2012] or even with sex-sorted spermatozoa [Pope *et al.* 2009] (see Supplementary material). Using SCNT the first cloned cat was born in 2002 [Shin *et al.* 2002] and nowadays this service is available commercially, at a price of US $ 25,000 (ViaGen).

These successes support claims that ART may be a useful tool for saving endangered wild cat species. Until now, embryos of almost all feline species have been obtained using various techniques. Living offspring after embryo transfer were born in several of them: the tiger (IVF) [Donoghue *et al.* 1990], fishing cat (IVF) [Pope *et al.* 2006b], caracal (IVF) [Pope *et al.* 2006b], African and Asiatic wildcats (IVF) [Pope *et al.* 1993, Pope 2000], serval (IVF) [Pope *et al.* 2006 a], black-footed cat (IVF) [Pope *et al.* 2012], sand cat (IVF [Pope *et al.* 1993] and SCNT [Gómez *et al.* 2008]); also using cryopreserved gametes and embryos [Pope *et al.* 2006b]. These results give hope that reproductive biotechniques may indeed be successfully introduced into conservation programs for wild cats.

Obtaining embryos *in vitro* – why still not commonly used?

Despite the successes described in the previous paragraphs, ART is still rarely used in felids. Several factors may be responsible for this situation. One of them, probably the most important, is the low efficiency of ART procedures.

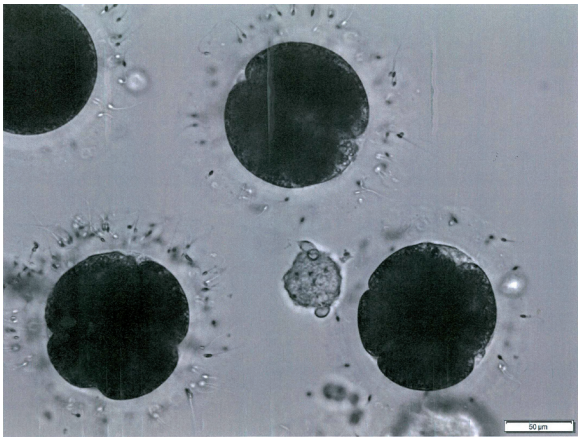

Fig. 3. High cleavage rate obtained after classic *in vitro* fertilisation in our laboratory (domestic cat).

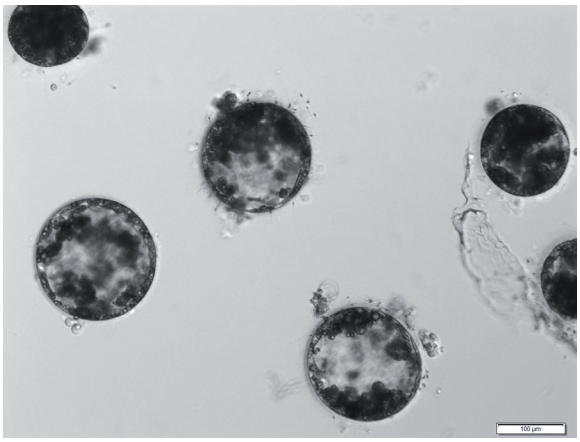

Fig. 4. High blastocyst yield obtained after classic *in vitro* fertilisation in our laboratory (domestic cat).

Some authors achieved good results for in vitro fertilisation in the domestic cat, with a cleavage rate (the percentage of fertiliszed oocytes that developed to embryos 24h after IVF or ICSI) of more than 80% [Pope *et al.* 1998] (Fig. 3) and with a blastocyst rate of over 50% [Freistedt *et al.* 2001, Gómez *et al.* 2003, Thongkittidilok *et al.* 2015] (Fig. 4). However, the average outcome is around 50% for the cleavage rate and less than 20% for the blastocyst rate. SCNT provides higher cleavage rates – commonly >70% [Kitiyanant *et al.* 2003, Wen *et al.* 2003, Yin *et al.* 2005], sometimes more than 90% [Imsoonthornruksa *et al.* 2012, Moro *et al.* 2015]; however, fewer embryos develop to the blastocyst stage – usually less than 10% [Kitiyanant *et al.* 2003, Wen *et al.* 2003, Yin *et al.* 2005].

Results after embryo transfer are highly inconsistent – the pregnancy rate ranges from 0% [Pope *et al.* 1997] up to 83% [Goodrowe *et al.* 1988] or even 100% [Pope

Table 2. Efficiency of embryo transfer in the domestic cat

Procedure	Embryos transferred	Pregnancy rate	Kittens born	Reference
SCNT, recloning	av. 21 per recipient	1/13	1 kitten	Cho *et al.* 2011
IVF, vitrified oocytes	av. 18 per recipient	1/2	1 kitten	Galiguis *et al.* 2014
ICSI	6-14 per recipient	3/18	2 kittens (from two females)	Gomez *et al*. 2000
IVF, cryopreserved embryos	av. 12 per recipient	4/15	3 kittens (from three females), one died	Gomez *et al*. 2003
IVF	6-18 per recipient	5/6	10 kittens (1-4 kittens per female)	Goodrowe *et al.* 1988
SCNT	av. 21 per recipient	2/12	7 kittens (1 and 6 per female), all died	Imsoonthornruksa *et al.* 2012
IVF	> 12 embryos transferred per recipient	11/26	21 kittens (1-5 per female)	Pope *et al.* 1993
	< 12 embryos per recipient	6/23	11 kittens (1-5 per female)	
IVF, cryopreserved embryos	av. 15 per recipient	2/4	3 kittens	Pope *et al.* 1994
IVF Trial 1 Trial 2	 av, 18 per recipient av. 10 per recipient	 0/4 3/3	 - 4 kittens	Pope *et al.* 1997
ICSI	11 per recipient	2/4	3 kittens (from both females)	Pope *et al.* 1998
IVF, sex sorted semen	11per recipient	3/4	12 kittens (1, 4 and 7 per female), 3 of them dead	Pope *et al.* 2009
ICSI, vitrified oocytes	9, 12 and 22 per recipient	2/3	4 kittens (3 and 1 per female)	Pope *et al.* 2012a
IVF, cryopreserved embryos	av. 18 per recipient	4/8	5 kittens, 4 died	Pope *et al.* 2012b
SCNT Trial 1 Trial 2	 av. 11 per recipient, 3 per recipient	 1/7 1/1	 0 1 kitten	Shin *et al.* 2002
ICSI FT embryos, testicular sperm	av. 30 per recipient	3/7	2 kittens (from one female)	Tharasanit *et al.* 2012
IVF	10-35 per recipient	6/6	4 kittens (from three females)	Thongphakdee *et al.* 2011
IVF	15-25 per recipient	5/22	5 kittens (1 per female), 2 died	Wongsrikeao *et al*.
SCNT	30-90 embryos or 120 - 140 reconstructed oocytes per recipient	2/10	3 kittens (2 and 1 per female)	Yin *et al*. 2005
SCNT	av. 16 per recipient	3/11	2 kittens (from two females)	Yin *et al*. 2008a
SCNT, Cloning Recloning	 av. 25 per recipient av. 29 per recipient	 1/5 4/15	 1 kitten 5 kittens (from two females), one died	Yin *et al*. 2008b

Abbreviations: av. – average, IVF – classic *in vitro* fertilization, ICSI – intracytoplasmic sperm injection, SCNT – somatic cell nuclear transfer.

et al. 1997, Thongphakdee *et al.* 2010]. For SCNT pregnancy rates are lower than for IVF/ICSI – around 15-30% [Imsoonthornruksa *et al.* 2012, Yin *et al.* 2005]. Additionally, in many cases (both IVF, ICSI and SCNT) pregnancy was not carried to term, e.g. in the domestic cat [Gómez *et al.* 2003, Thongphakdee *et al.* 2010], leopard cat [Yin *et al.* 2006] and the jaguarundi [Pope *et al.* 1998]. Single fetus pregnancies

are very common [Galiguis *et al.* 2014, Gómez *et al.* 2000, Gómez *et al.* 2003, Gómez *et al.* 2004, Pope *et al.* 2012]. This indicates that the in vivo survival of in vitro derived embryos is impaired. Considering that usually a dozen or even several dozens of embryos need to be transferred into one recipient to support pregnancy and that hundreds of oocytes need to be collected to obtain such numbers of transferable embryos, the overall efficiency of ART in felids is extremely low (Tab. 2).

Low pregnancy and birth rates and small litter sizes following embryo transfer may be a result of a high incidence of embryonic death and resorption during early gestation or fetal abortion in later stages [Gómez *et al.* 2003, Gómez *et al.* 2004, Gómez *et al.* 2008, Thongphakdee *et al.* 2010]. The underlying cause may be associated with the abnormal hormonal environment of the surrogate female, which was reported in felids after gonadotropin treatment [Donoghue *et al.* 1990, Goodrowe *et al.* 1988, Graham *et al.* 2000], in placental dysfunction, especially after SCNT [Gómez *et al.* 2004, Hill *et al*. 2000] or it may reside in the embryos themselves. It was reported that culture conditions [Hribal *et al.* 2013], the adopted fertilisation method (IVF or ICSI) [Waurich *et al.* 2010] or semen cryopreservation [Waurich *et al.* 2010] altered gene expression in embryos, which may influence their further development. For cryopreserved embryos, the reasons for pregnancy failure may lie in embryonic damage inflicted during the freezing and thawing processes [Tharasanit *et al.* 2012].

In addition to problems with maintaining pregnancy, there are relatively high losses of individuals derived from in vitro produced embryos during their perinatal and early postnatal development, especially for kittens developed after iSCNT [Gómez *et al.* 2004, Gómez *et al.* 2008]. The kittens died mainly due to respiratory failure and septicemia [Donoghue *et al.* 1990, Pope *et al.* 2012]. Abnormalities, such as abdominal organ exteriorization, were reported and were the main causes of stillbirths or death in neonatal kittens after cloning [Gómez *et al.* 2004, Gómez *et al.* 2008] and embryo cryopreservation [Pope *et al.* 2012]. The abnormal development may be a result of aberrant epigenetic alterations and gene deregulation [Dean *et al.* 2001, Gómez *et al.* 2008] or chromosomal abnormalities [Gómez *et al*. 2006].

Conclusions

The results for obtaining feline embryos by classic in vitro fertilisation, intracytoplasmic sperm injection and somatic cell nuclear transfer are promising, but still inferior to those achieved in humans and farm animals. From this point of view, the low efficiency of in vitro embryo production together with its high relative costs may place doubts in the usefulness of ART in felids. Some researchers claim that the production of a single viable offspring, unfortunately, cannot justify the expense, labor and animal stress [Swanson 2006]. However, in our opinion, when ART is used in endangered animals, when every single individual is invaluable, those costs are justified. Therefore, the work should be continued to increase the efficacy of the methods described here and to ensure their practical use in safeguarding the survival of wild felids.

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