Thirty years of assisted reproductive technology in the domestic cat: A selected summary

Trinta anos de tecnologia de reprodução assistida no gato doméstico: Um resumo selecionado

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Abstract

The first kittens to be born after embryo transfer (ET) in the cat (Schriver and Kraemer, 1978) were reported four decades ago. The births of kittens after in vitro fertilization (IVF)/ET (Goodrowe et al., 1988) and after embryo cryopreservation/ET (Dresser et al., 1988) were described 10 years later. In an early report it was established that embryo donors had the ability ot exhibit repeated ovarian stimulatory response to multiple gonadotropin treatments (porcine FSH; Dresser et al., 1987). After studies on gonadotropin-induced hyperstimulation of follicular development for recovery and ET of fresh and cryopreserved in vivo derived uterine stage embryos from mated donors (Dresser et al., 1988; Pope et al., 1989), we transitioned into developing methods for *in vitro* production of domestic cat embryos for the purpose of applying the technology to support conservation of threatened and endangered felid species (Pope et al., 1993). Since then, techniques for the *in vitro* production of cat embryos have been developed sufficiently to allow births of kittens after transfer of embryos derived by an assortment of *in vitro* techniques, including cryopreservation (Pope et al., 1994; Gómez et al., 2003a; Pope et al., 2012a, b; Galiguis et al., 2014), intracytoplasmic sperm injection (ICSI; Pope et al., 1998; Gómez et al., 2000; Pope et al., 2012a), gender selection (Pope et al., 2009) and somatic cell nuclear transfer (SCNT) (Gómez et al., 2004; 2008; 2009).

Keywords: domestic cats, embryo transfer, cryopreservation, IVF, assisted reproduction.

Introduction

The present paper is a brief overview of research on developing assisted reproduction technology (ART) in domestic cats over a 30 year period at the Center for Research of Endangered Wildlife (CREW) at the Cincinnati Zoo from the mid-1980s through 1995 and subsequently, at the Audubon Center for Research of Endangered Species, a facility of the Audubon Nature Institute, in New Orleans, LA from 1996 until 2015. In the paper I describe the innovation and progression of specific ARTs in the domestic cat that are required to achieve the goal of producing live offspring. The focus of the research described in the present paper is primarily embryo-centric. Even so, from the early studies to the more recent, our lab maintained consistent activity in improving methods for evaluating, handling and storing both epididymal and ejaculated sperm (Pope et al., 1991; Harris et al., 2002; Pope et al., 2009; Saenz et al., 2012). Importantly, most of the same ARTs were shown to be applicable to several species of non-domestic cats. In addition to successful intra-species ET, non-domestic kittens derived from IVF and SCNT have been produced by inter-species ET using the domestic cat as a recipient (Pope, 2000; Pope et al., 2000; 2012b; Gómez et al., 2004; 2008). The results we obtained by applying the ARTs that were developed in domestic cats to rare and endangered cats are included in a companion paper in these proceedings.

Gamete sources

The two primary sources of female domestic felid gametes are 1) ovaries obtained from local veterinary hospitals following ovariohysterectomy (*in vitro* maturation, IVM), or 2) aspiration from preovulatory follicles of gonadotropin-treated oocyte donors (in vivo maturation). There are obvious advantages and disadvantages to each source, and the ideal situation is convenient access to both. Often, that is not the case; however, our laboratory, both in Cincinnati and New Orleans, was fortunate to have the human, physical and economic resources to acquire oocytes from each source. The availability of domestic cat gametes from veterinary hospitals, at least in some countries, provides a valuable and practical source of material for further research on fundamental characteristics of *in vitro* oocyte maturation, fertilization and early embryo development (Pope et al., 1997; Gómez et al., 2000; Gómez et al., 2003a).

Another advantage to having access to gonadal tissue following extirpation is that knowledge gained from domestic cat gametes can be used to potentially expand the otherwise unavailable pool of genetic material from wild felids either postmortem or due to a medical or management requirement for gonadectomy. Lastly, to advance the goal of enhancing propagation of genetically valuable females requires the ability to obtain maximal numbers of viable oocytes from a nominal number of animals. Certainly, animal welfare is a primary consideration; accordingly, the methods must present minimal short- and long-term health risks and allow the ability to do multiple procedures on individual animals while maintaining ovarian response to gonadotropin treatment (Pope et al., 2006a; Pope, 2014).

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In vitro oocyte maturation (IVM)

The effects of several specific factors on *in vitro* maturation and subsequent *in vitro* development of domestic cat oocytes are described briefly in the first portion of this section. Some results on production of in vivo matured oocytes from gonadotropin-treated donor females over a 15 y period in New Orleans are presented in the next section.

Oocyte quality and gonadotropin source

In an early study to determine optimal conditions for IVM of cat oocytes, cumulus oocyte complexes recovered from ovaries after ovariohysterectomy were classified by morphology of ooplasm and cumulus (A = good, B = fair, C = poor) and cultured for 24 h in TCM 199 + BSA with different gonadotrophins (eCG, FSH, hCG or FSH/hCG) in a 5% O_2 atmosphere at 38°C (Pope et al., 1997). From the total oocyte population (~ 1000) selected for culture the percentages that were designated into each morphology type were similar — 37%, 30% and 33% for types A, B and C, respectively A higher percentage of type A oocytes (52%) underwent IVM (metaphase II) than type B (41%) or type C (17%). Gonadotrophin source did not affect IVM frequency of type A (50—53%) or type B (38—44%) oocytes, but IVM of type C oocytes in hCG or FSH/hCG (27%/19%) was double that in eCG or FSH alone (13%/10%). After IVF, frequency of cleavage for type A (54%), B (41%) and C (26%) oocytes was comparable to IVM rate of the equivalent type. After 7 days IVC, development to the morula (M) stage was similar among types (47 to 58%); however, higher percentages of type A and B oocytes developed to blastocysts (Bl), 31% and 29%, respectively, than did type C (15%) oocytes. In two trials, a total of 102 Day 5 and Day 6 embryos was transferred to seven recipients, four in trial 1 and three in trial 2. The three recipients in trial 2 established pregnancies after ET, producing a total of four live kittens.

Cysteine/gas atmosphere

We examined the effects of adding an anti-oxidant to the IVM medium and reducing atmospheric oxygen concentration (20% *vs*5%) during IVM/IVC on *in vitro* development (Pope et al., 1999). Cumulus oocyte complexes were split into four treatment groups: 0.1 mg/mL cysteine/5% CO_2 in air; no cysteine/5% CO_2 in air; 0.1 mg/mL cysteine/5%O_2, 5% CO_2, 90% N_2; no cysteine/5% O_2, 5% CO_2, 90% N_2. In good/excellent quality oocytes, neither addition of cysteine or O_2 concentration affected frequency of cleavage (46% to 49%). Day 7 blastocyst development was higher ($P < 0.05$) in the cysteine/reduced 0_2 group (59%) than in the control/5% CO_2 in air group (35%) but was not different from the control/reduced O_2 group (50%) or the cysteine/5% CO_2 in air group (47%).

Epidermal growth factor (EGF)

The beneficial effects of supplementing oocyte maturation and embryo culture medium with growth factors, particularly epidermal growth factor (EGF) and insulin-like growth factor (IGF-1), have been widely demonstrated in several species; however, there are fewer reports on their use in cats. So, in a follow-up to the previous experiment, in which cysteine and a reduced O_2 atmosphere were integrated into the IVM protocol, we compared *in vitro* development after oocytes underwent IVM in medium with or without EGF (Gómez et al., 2001). Although the addition of 10 ng/ml of EGF to IVM medium did not improve cleavage frequency after IVF of high-quality oocytes (53% vs. 48%), the percentage of embryos that developed into blastocysts was increased (55% *vs*. 43%; $P < 0.05$).

Freshly collected vs. 'stored' ovaries

Here, we evaluated the developmental competence of oocytes collected from ovaries after storage for 24 to 28 h at 4ºC (Pope et al., 2003). For comparison, oocytes from freshly collected ovaries were recovered and placed in culture within 2 to 5 h after ovariohysterectomy. Cleavage frequency after IVF was comparable in the fresh *vs*. stored oocytes. *In vitro* development to the blastocyst stage on Day 7 was also similar in the two groups, 46 % *vs.* 46%, after culture in a three-step system. The in vivo developmental ability of embryos generated from IVM/IVF/IVC of oocytes recovered from ovaries after storage at 4ºC for ≥ 24 h is described in the section on Embryo transfer.

In vivo oocyte maturation-laparoscopic follicular aspiration

The following section is a brief overview of our results on oocyte production in domestic cats after multiple treatments with pituitary-derived gonadotropins during the interval from late 1998 through June 2013. The gonadotropin treatment (porcine FSH and LH), and laparoscopic oocyte retrieval protocols were described by Gómez et al. (2000) and Pope (2004). The data presented below were published previously by Pope (2014).

From 1998 until 2013 we did >1600 laparoscopic oocyte retrievals (LOR) on a total of 337 cats and recovered an average of 24.1 mature oocytes/LOR at an average interval between gonadotropin treatment/LOR of ~ 7 mo. At the time the data were summarized in 2013, of all cats used, 59 (17.5%) were still active oocyte donors, while the remaining 278 (82.5%) were retired after an average of 4.8 LORs/donor. Of all 337 cats, 27 had≥ 10 LORs (8%) and eight had 12 LORs (2.4%). In the latter sub-group of eight cats that had 12 LORs, an average of 28.5 mature oocytes were recovered at the first LOR, but decreased to slightly < 20 at the 6^{th}, 7^{th}, and 11^{th} LORs for a total of 3041 oocytes (mean = 31.7/LOR).

From 2000 through 2012 we did an average of 114 LORs/y (85 to 144) and recovered an average of 2798 mature oocytes/y (2024 to 3686). Fewer oocytes were recovered/LOR in June (20.8) and September (20.7) ($P < 0.05$) than during January, April, May, October, November and December (from 24.7 to 26.4). The reason that fewer oocytes were recovered during those two months is not obvious since the cats lived in an environmentally controlled facility without seasonal changes in light or temperature. During the 15-y period the total number of LORs/mo. ranged from 74 (January) to 164 (March) with an overall average/mo. of 133.6.

Of 142 cats that were between 6 and 12 mo. of age at their first treatment/LOR, a higher ($P < 0.05$) average number of oocytes was recovered from 6 and 7 mo. old females (54.0 and 46.7, respectively) than from 9, 10 and 12 mo. old females (26.2, 29.9 and 28.8, respectively). The presence of corpora lutea (in addition to preovulatory follicles) from 'spontaneous' ovulations increases during the interval from 6 to 12 mo. of age, as the cats progress through the peri-pubertal to the post-pubertal stage and this transition may have contributed to the difference in oocyte production (unpublished observation).

Embryo transfer-uterine *vs.* oviductal

For the first 15 y we did uterine ET, usually on Day 5, because it was considered to be less technically difficult and more likely to have practical applicability. During those years, we successfully transferred both 'fresh' (Pope et al., 1993) and cryopreserved embryos—in vivo derived (Dresser et al., 1988) and in vivo matured/IVF (Pope et al., 1994). Then, kittens were born following uterine transfer of embryos produced *in vitro* (IVF or ICSI) from IVM oocytes (Pope et al., 1997; Gómez et al., 2000; Gómez et al., 2003a). While these studies represented important steps in the development of cat ART, their future application would require significant improvements both in pregnancy rates and embryo survival.

One approach to improving the efficiency of *in vitro* production and transfer of embryos is to minimize the potentially compromising effects of *in vitro* culture is by transferring embryos into the oviduct at early cleavage stages using a laparoscopic technique. In our initial report on SCNT (Gómez et al., 2003b), embryos derived from nuclear transfer of African wildcat (*Felis silvestris lybica*) somatic cells into enucleated domestic cat oocytes were transferred into the uterus of domestic cat recipients on Day 5 or 6, but no pregnancies resulted. Subsequently, recognizing that most SCNT embryos were developmentally deficient and that an extended period of *in vitro* culture may further increase the pregnancy failure rate, Gómez et al. (2004) evaluated the effects of transferring low (≤ 25) *vs*. high (≥ 30) numbers of embryos either into the oviduct on Day 1 by laparoscopy or uterus by laparotomy on Day 5 or 6. No pregnancies were seen in the 'low' group, whereas, 43% of the 'high' recipients and 50% of the 'high' Day 1 recipients were pregnant at Day 21. While the pregnancy rate did not differ between the 'high' Day 5 or 6 and the 'high' Day 1 group, implantation rate (total implantations/total pregnancies) was higher ($P < 0.05$) in the latter (7/6 *vs.* 17/6).

Later, we demonstrated the improved efficiency of laparoscopic oviductal ET by producing a total of 12 domestic kittens (all female) in three recipients after transfer of Day 2 embryos derived by IVF with presumptive X-chromosome bearing sperm into four recipient females (75%) (Pope et al., 2009). After that, to determine in vivo viability of cryopreserved cat embryos produced by IVF with flow-sorted sperm, on IVC Day 2, 35 embryos were transferred by laparoscopy to the oviducts of four Day 1 gonadotropin-treated recipients. Three recipients (75%) established pregnancies and delivered, without assistance, a total of six live kittens, of which, four were female (Pope et al., 2011). Overall, of 18 kittens--12 from fresh (Pope et al., 2009) + six from frozen embryos--born to six recipients (of eight, 75%) after transfer of embryos produced by IVF with sex-sorted sperm, 16 (89%) were female. The variation in sex ratios between kittens born from fresh *vs*. cryopreserved embryos reflected the sort purity of the different sperm samples used for IVF.

Other studies in which cat zygotes/embryos were transferred into the oviduct by laparoscopy are described in later sections of the present paper (Oocyte vitrification I and II and Embryo vitrification) and in a companion paper in the present proceeding on ART in nondomestic mammalian species. Of the recipients that received zygotes/embryos by laparoscopic oviductal transfer, 19 of 21 had also been oocyte donors, usually one day earlier. Nearly 70% of the donor/recipients (13/19) produced ET kittens. Others also have shown that laparoscopic oviductal embryo transfer is an efficient technique for producing live offspring in felids (Swanson *et al.,* 2012). A summary of our domestic cat ET results is shown in Table 1.

Table 1. Embryo transfer (ET) in domestic cats at the Cincinnati Zoo (1985-1995) and the Audubon Nature Institute, New Orleans, LA (1996-2015).

Oocyte/Emb. Source	Fertil. Type	ET Site	Fresh/ Cryo	Embryos/ Recipient, mean	Recipients, n		Kits born, n	Reference
					Total	Preg.		
In vivo develop	Natural mating	Ut[d]	Fresh	7.8	6	3	8	Pope et al., 1989
			Cryo	12.4	11	5	17 2 dead	Dresser et al., 1988
In vivo[a]	IVF[b]	Ut	Fresh	< 12	23	6	12	Pope et al., 1993
				> 12	26	11	22	
In vivo	IVF	Ut	Cryo	14.5	4	2	2	Pope et al., 1994
IVM[c]	IVF	Ut	Fresh	Tr 1=15.2	4	0	--	Pope et al., 1997
				Tr 2=13.6	3	3	4	
In vivo	ICSI[f]	Ut	Fresh	10.7	4	2	3	Pope et al., 1998
IVM	ICSI	Ut	Fresh	9.0	D4=9	0	--	Gómez et al., 2000
					D5=9	3	3	
IVM	IVF	Ut	Cryo	FzD4=12.2	4	2 1resorb	1	Gómez et al., 2003a
				FzD5=12.0	6	2	2	
In vivo	IVF Sperm Sex-sorted	Ovi[e]	Fresh	11.3	4	3	12	Pope et al., 2009
			Cryo	8.8	4	3	3	Pope et al., 2011
IVM	Ovaries 24 h at 4 °C IVM/IVF	Ut	Fresh	10.0	3	3 1resorb	3	Pope et al., 2003
			Cryo	10.0	2	1	1	Pope et al., 2006b
IVM	ICSI	Ovi	Vitrif oocyte	18.5	2	2	5	Pope et al., 2012a
IVM	IVF Delipid.	Ovi	Vitrif oocyte	Part 12.5	2	2	1	Galiguis et al., 2014
				Full 28.7	4	1	0	
In vivo/ IVM	IVF	Ovi	Vitrif emb	16.6/22.5	8/2	4/1	5/1	Pope et al., 2012b
In vivo	SCNT[g]	Ovi	Fresh	40.0	4	1	4 1 dead	Gómez, Pope, 2015
In vivo	Transgen, SCNT	Ovi	Fresh	36.5	8	3 1resorb	3 2 dead	Gómez et al., 2009

[a]preovulatory/gonadotropin treatment, [b]*in vitro* fertilization, [c]*in vitro* maturation, [d]laparotomy, [e]laparoscopy. [f]intracytoplasmic sperm injection, [g]somatic cell nuclear transfer

Embryo culture-by steps

After a series of experiments comparing *in vitro* development of cat embryos in different culture media (Pope et al.,1993), we settled on a single-step system consisting of modified Tyrode's medium containing 10% FBS from immediately post-fertilization until Day 7 (Pope et al., 1994; 1997). Then, we began using a two-step system, in which, after IVF, both essential and nonessential amino acids were included throughout the seven-day culture period (Pope et al., 1998). Until Day 2 or 3, medium contained BSA (3 mg/mL), when it was replaced with 10% FBS for the remaining culture period (Two-step I). With both the one-step and the two-step I methods, the gas atmosphere was 5% CO_2 in air. After a while, the first step was modified by 1) including only NEAA (1%) and 2) extending the duration until Day 3 or 4. The only change in step-two was that it was delayed by one or two days (two step II; Gómez et al., 2000; 2003b). In 2003, we began using a three-step system in which 1) a lower concentration of EAA (1% MEM) was added from Day 2 until Day 5 and the duration of culture in BSA was extended until Day 5, after which FBS was used until the end of culture on Day 7 or 8 (Pope, 2004; Gómez *et al.,* 2004). In the two most recently used methods (two-step II and three-step) the gas atmosphere was 5% CO_2, 5% O_2, and 90% N_2. In a comparison of *in vitro* development of cat embryos derived from *in vitro* matured oocytes in the two latter methods, frequency of blastocyst formation was higher ($P < 0.05$) in the three-step (101/219, 46%) than in the two-step II (85/241, 35%) and the mean number of cells per blastocyst was 157 (± 12) *vs*. 145 (± 10), respectively.

Controlled-rate cryopreservation of embryos

In vivo embryos

The first kittens produced from transfer of cryopreserved embryos were reported ~ 30 years ago (Dresser et al., 1988). In vivo derived embryos were recovered from gonadotropin-treated females after natural mating. Morulae

and blastocysts were frozen using a standard controlled rate method with glycerol as the cryoprotectant. Of a total of 137 embryos (mean =12.5) transferred to 11 gonadotropin treated recipients, five pregnancies were established that produced a total of 17 term kittens.

In vitro derived embryos (IVF/IVC)

Embryos at the two to four cell stage generated by IVF of mature oocytes recovered from gonadotropin-treated donors were used in the initial study on cryopreservation of *in vitro* derived embryos. Embryos were frozen using a modified version of a method used successfully on early cleavage staged human, mouse and rabbit embryos with propylene glycol as the cryoprotectant and a slow cooling rate of 0.3ºC/min to ~30ºC (Lassalle et al.,1985). After thawing, most embryos retained their potential for *in vitro* development to the morula/blastocyst stages (Pope et al., 1994). In vivo viability was demonstrated by births of three live kittens in two of four recipients following uterine transfer of 58 embryos on Day 5.

In vitro produced embryos (IVM/IVF/IVC)

After establishing that IVF/IVC derived cat embryos could be successfully cryopreserved, the next step was to evaluate the developmental ability of cryopreserved embryos produced by IVM/IVF/IVC (Pope *et al.,* 1997; Gómez *et al.,* 2003a). In the first report, the effects of stage at cryopreservation (Day 2, 3 or 4) and plunge temperature (-30ºC *vs.* -150ºC) on *in vitro* survival and development were examined. *In vitro* development to the morula stage of IVM/IVF embryos cryopreserved at early cleavage stages was similar (64 to 69%) to that of cohort controls (64%), but blastocyst formation was reduced (13 to 17% *vs*. 32%; $P < 0.05$). Damage to the zona pellucida after plunging into liquid nitrogen at -30°C was higher (11%) than that of embryos cooled at 10°C/min from -30°C to -150ºC before plunging into liquid nitrogen (2%; $P = 0.06$). Then, Gómez et al. (2003a) further evaluated the effect of stage at cryopreservation in embryos derived from in vivo matured *vs*. *in vitro* matured oocytes on *in vitro* development and on viability after transfer to recipients (IVM oocytes only). The results showed that survival rate of IVM/IVF derived cat embryos was not affected by the stage at cryopreservation (four to eight cells, early-morula or morula) and was not different from that of non-frozen embryos, 89% *vs*. 93%, respectively. In contrast to our earlier study on cryopreservation of IVM/IVF embryos (Pope et al., 1997), frequency of blastocyst development of embryos that survived after cryopreservation (53%) was not different from non-frozen controls (52%), and there was no effect of day at freezing on blastocyst development. The improved development seen by Gómez *et al.* (2003a) was attributed to modifications made in the cryopreservation protocol and culture medium. After uterine transfer of cryopreserved IVM/IVF embryos, four recipients were diagnosed as pregnant, two each from embryos frozen on Day 4 and Day 5, respectively. Pregnant recipients each had from one to three fetuses, for a total of seven. Three term kittens were produced from three recipients. Four fetuses were resorbed at 40 to 45 days of gestation, including all three fetuses in one recipient.

The ability to produce progeny by transfer of cryopreserved embryos derived *in vitro* (IVM/IVF/IVC) from stored ovaries (≥ 24 h at 4°C) would provide even greater opportunities for preserving valuable genetic material. Subsequently, a total of 20 Day 6 and 7 embryos were transferred after controlled-rate cryopreservation (on Day 5) to two synchronous recipients. The recipient of 10 Day 6 embryos (morulae) established pregnancy and produced a single kitten, thereby demonstrating the feasibility of producing kittens by ET of cryopreserved embryos produced *in vitro* from ovaries after 24 h storage at 4°C (Pope et al., 2006b).

Oocyte vitrification I

In 2012, we reported the birth of domestic kittens after transfer of embryos derived by ICSI of in vivo and *in vitro* matured oocytes after minimal volume vitrification by the Cryotop® method (Pope et al., 2012a). As assessed by normal morphological appearance after liquefaction, survival rate of both in vivo- and *in vitro*-matured oocytes was >90%. For *in vitro* matured oocytes, cleavage frequencies after IVF of control and vitrified oocytes were 73% (16/22) and 53% (30/57), respectively, as compared to 68% (19/28) after ICSI of vitrified oocytes ($P > 0.05$). For in vivo matured oocytes, cleavage frequencies after IVF of control and vitrified oocytes were 55% (18/33) and 35% (6/17), respectively, *vs*. 50% (10/20) after ICSI of vitrified oocytes ($P > 0.05$). At 18 to 20 h after ICSI, 18 presumptive zygotes and four–two cell embryos derived from vitrified *in vitro* matured oocytes and 19 presumptive zygotes produced from seven in vivo matured and 12 *in vitro* matured oocytes were transferred by laparoscopy into the oviducts of two recipients at 24 h after oocyte retrieval. The two recipients delivered four live kittens, without assistance, on day 63 and day 66 of gestation, all of which developed into healthy adult cats.

Oocyte vitrification II

The capability of cat oocytes to survive cryopreservation is affected by several factors, but their comparative sensitivity to cooling may be due to the relative concentration of intracellular soluble macromolecules. Thus, in a later effort to improve the developmental capacity of cat oocytes after vitrification, we evaluated whether

1) delipidation of *in vitro* matured domestic cat oocytes by high-speed centrifugation before vitrification enhanced cryosurvival and, 2) embryos derived from oocytes delipidated before vitrification can produce healthy kittens after transfer to recipient females (Galiguis et al., 2014). Cryosurvival and development to the blastocyst stage was enhanced after removal of intracellular lipids before vitrification. Nonetheless, complete intracellular lipid removal (delipidation) was detrimental to survival of ET embryos. When embryos derived from fully delipidated/vitrified oocytes were transferred, two recipients became pregnant, but all implanted embryos were subsequently reabsorbed. In contrast, when embryos derived from partially delipidated/vitrified oocytes were transferred, one recipient became pregnant and produced one live healthy kitten. Although embryos derived from fully delipidated/vitrified oocytes developed to the blastocyst stage at higher rates than those of partially delipidated/vitrified or non-centrifuged/vitrified oocytes, *in vivo* developmental competence was highly compromised. Our results indicated that additional approaches to altering intra-cellular lipid levels in cat oocytes should be evaluated.

Embryo vitrification

Subsequently, in a complementary study, we compared development of early cleavage stage cat embryos produced from *in vitro* and in vivo matured oocytes after cryopreservation by minimal volume vitrification *vs*. our standard slow-controlled rate method *vs.* non-frozen (fresh) controls (Pope et al., 2012b). Blastocyst development was similar in both groups of cryopreserved embryos (22 to 26%), but it was lower ($P < 0.05$) than that of fresh embryos (50%). After laparoscopic oviductal ET, four (of eight) recipients of vitrified embryos established pregnancies--three of six (50%) and one of two (50%) that received embryos from in vivo and *in vitro* matured oocytes, respectively. Five kittens weighing from 51 to 124 g (mean = 88 g) were delivered on Days 61 to 65 of gestation.

Intracytoplasmic sperm injection (ICSI)

Occasionally, poor sperm quality is a limiting factor in generating *in vitro* embryos by standard methods of IVF. In such cases, ISCI is an important alternative approach for embryo production and is a widely used technique in human fertility clinics. In our initial study, we evaluated the *in vitro* development of cat embryos produced by ICSI *vs*. IVF of in vivo matured oocytes. Cleavage frequency was similar in the IVF and ICSI groups, 88% *vs*. 84%, respectively, and, both were higher ($P < 0.05$) than the sham ICSI group (37%). Likewise, incidence of blastocyst development was not different in the IVF *vs*. ICSI groups, 53% *vs*. 43%, respectively. Sham-ICSI oocytes did not develop past the morula stage. Kittens were born after transfer of ICSI derived embryos to recipients (Table 1; Pope et al., 1998). In a subsequent study, we compared development after IVF or ICSI of 1) *in vitro* and in vivo matured oocytes, and 2) the in vivo viability of ICSI derived embryos produced from *in vitro* matured oocytes (Gómez et al., 2000). Frequency of cleavage was affected by the type of maturation (*in vivo vs*. *in vitro*), but not by the method of fertilization (IVF *vs*. ICSI). The percentage of in vivo matured oocytes that cleaved after ICSI and IVF, 72% and 61%, respectively, was higher than that after ICSI and IVF of *in vitro* matured oocytes, 57% and 30%, respectively ($P < 0.05$). A lower frequency of blastocyst development was found after ICSI and IVF (19% and 42%) of *in vitro* matured oocytes than after ICSI and IVF (30% and 48%) of in vivo matured oocytes ($P < 0.05$). From six to 14 morulae produced by ICSI of *in vitro* matured oocytes were transferred to each of 18 Day 4 or Day 5 recipients. On Day 21, three recipients (of nine) were pregnant after ET on Day 5. Two pregnant cats delivered two normal, healthy live male kittens on Day 68, and the remaining cat delivered an unresponsive male kitten on Day 62. An additional study involving ICSI of *in vitro* and in vivo matured oocytes after vitrification was described in an earlier section (Oocyte vitrification I).

Transgenic Cloned Kittens

In 2009, Gómez et al. reported the birth of transgenic cloned kittens produced by LV vector-mediated transduction of donor cells and showed that cloned kittens expressed the EGFP reporter transgene in all body tissues. In that study, an LV vector-based transgene delivery approach for producing live transgenic domestic cats by SCNT was established. It was shown that cat fetal fibroblasts could be transduced with EGFP-encoding LV vectors bearing various promoters including human cytomegalovirus immediate early (hCMV-IE) promoter, the human translation elongation factor 1 (hEF-1) promoter and the human ubiquitin C (hUbC) promoter. Blastocysts reconstructed with donor cells transduced with a LV-vector carrying the hUbC promoter displayed sustained transgene expression while embryos reconstructed with LV vector-transduced cells containing hCMV-IE-EGFP or hEF-1-EGFP cassettes did not. Three of eight domestic cat recipients were pregnant at Day 21 after oviductal transfer of 291 transgenic cloned embryos (mean 36.5 ± 10.1). Of six embryos (2.1%) that implanted, one live male kitten was delivered by Cesarean section on Day 64, and two kittens were born dead after premature delivery on Day 55.

Summary

The present paper is a selected synopsis of research on developing ARTs in domestic cats that was done in Cincinnati from the early 1980s through 1995 and New Orleans from 1996 to 2015. The driving force behind the studies was the expectation that the technology developed and knowledge gained in domestic cats would be implemented for the conservation and genetic management of threatened and endangered felids. Much of our research could be properly described as 'embryo-focused' and, accordingly, developing effective methods for *in vitro* production and intra-and interspecies transfer of embryos were precedent to our ultimate goal. To exemplify the evolution of our research on embryo transfer in cats, in the first experiments, in vivo derived uterine stage embryos recovered from gonadotropin treated females following natural mating were surgically transferred to synchronous recipients, while in the most recent study, black-footed cat kittens were born after successful laparoscopic transfer of cryopreserved IVF derived embryos to the oviducts of a black-footed cat and a domestic cat. The two studies--the earliest and the latest-were separated by a period of thirty years of research on cat ART by a dedicated team of scientists, technicians, graduate students and support staff. I am truly fortunate to have been a part of the early development of cat ART and am encouraged by the continuing progress of techniques for assisting conception in felids.

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