Review of assisted reproduction technologies in small animal theriogenology

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Summary (article adapted from chapter in press [The practical veterinarian: small animal theriogenology])

The emerging technologies of assisted reproduction encompasses a wide range of possible and potential manipulations to produce kittens or puppies from a reproductively compromised female or male. While there are multiple examples of successes in other species it is evident from ongoing research that canine gamete and embryo manipulations are not very successful. The use of emergent technologies in cat, however, has been far more successful than the dog. There are significant and inherent differences between the physiology of the two species, which probably explains the differences. However, it is my personal feeling that mastery of the unrelenting portions of the canine gamete physiology is only a matter of time.

The goal of any of these technologies is to provide an avenue for fertility salvage in the face of catastrophic reproductive insufficiencies or damage. The argument can be submitted that this is a valid and laudable endeavor. However, I feel it is important that we never loose sight of the fact that a significant amount of small animal breeding involves strategies that lead to genetic convergence rather than increased genetic vigor. As advocates of the new technologies, we must always ensure that we strive for the health of the animals through genetic vigor rather than a genetic convergence.

Introduction

Oocyte collection and maturation

Oocyte morphology

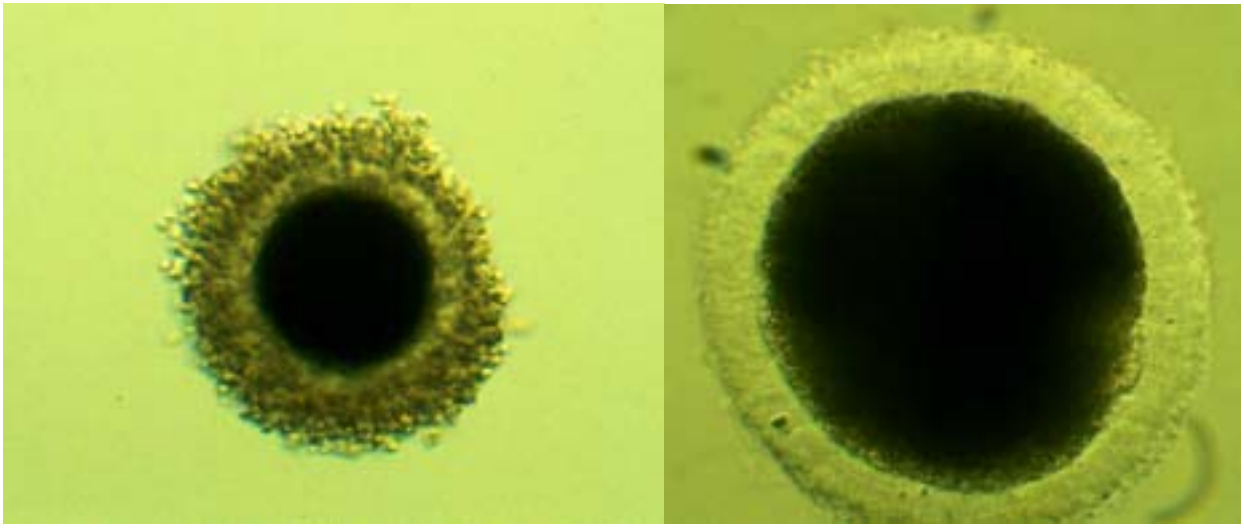
The canine and feline oocytes consists dense cytoplasm bound by a plasma membrane. The cytoplasm contains the nucleus, or condensed chromosomes, and various organelles. While the oocyte is within the ovary, the Golgi apparatus plays an important role in yolk synthesis and formation of cortical granules. The cortical granules are a key factor in preventing polyspermy after fertilization[1]. The presence of marginated cortical granules along the plasma membrane are also an indication of cytoplasmic maturity[2,3]. Spindles of microtubules form during meiosis and mitosis to aid in the alignment of chromosomes[2]. Cumulus cells are in close cellular contact, gap junctions, during maturation and provide nourishment to the oocyte. These cells also provide a surface for oviductal cilia while the egg is being transported.

Morphologic changes of the canine oocyte during different stages of the estrus cycle may be a direct result of the ability of the canine oocyte to communicate with cumulus cells[4]. The gap junctions between the oocyte and cumulus cells are involved in the regulation of oocyte meiotic differentiation and maturation[5]. Microinjected 3% Lucifer Yellow into canine oocytes collected during different stages of the estrus cycle allowed the evaluation of cumulus-oocyte gap junction communications[4]. Of the oocytes collected during late proestrus, 89% (16/18) exhibited functional cumulus-

oocyte communications through gap junctions. This was in contrast to 0% (0/20) for oocytes collected during anestrus[4].

Oocytes collected from ovaries are graded on a scale of 1 to 3 with grade 1 being darkly pigmented and completely surrounded by one or more layers of cumulus cells, grade 2 lightly pigmented with incomplete layers of cumulus cells, and grade 3 being degenerate and exhibiting pale color and frequently misshapened and without any cumulus cell attached[6]. Stained oocytes were classified as either germinal vesicle (GV, tight compact nucleus), germinal vesicle break down (GVBD, nucleus less compact), metaphase I/anaphase I (MI/AI, presence of a metaphase plate/ chromatids separating), metaphase II (MII, extrusion of a polar body) or unidentifiable (U, unclassifiable nuclear material).

Figure 1. Various stage canine oocytes demonstrating dark cytoplasm and difficulty to visualize nuclear structures with light microscopy.



General oocyte maturation

For the two cycles of the meiotic cell division of an oocyte there are four phases. The phases are prophase, metaphase, anaphase and telophase. Interphase is the time between cell cycles and is when DNA replication occurs. Prophase of the first meiotic division is divided into five stages: leptotene, zygotene, pachytene, diplotene and diakinesis. Diakinesis is also referred to as germinal vesicle stage. The prophase of the first meiotic division contains two periods when development is arrested. In most species the oocyte present at the beginning of in vivo maturation reaches metaphase II (MII) prior to ovulation. However, this is not the case for the dog and fox. Canidae ovulate a oocytes containing an intact germinal vesicle (primary oocyte). These oocytes maturation while in the oviduct.

Figure 2. In vitro matured canine oocyte matured in TCM 199 with recombinant FSH at the germinal vesicle stage stained with Hoechst 33342.

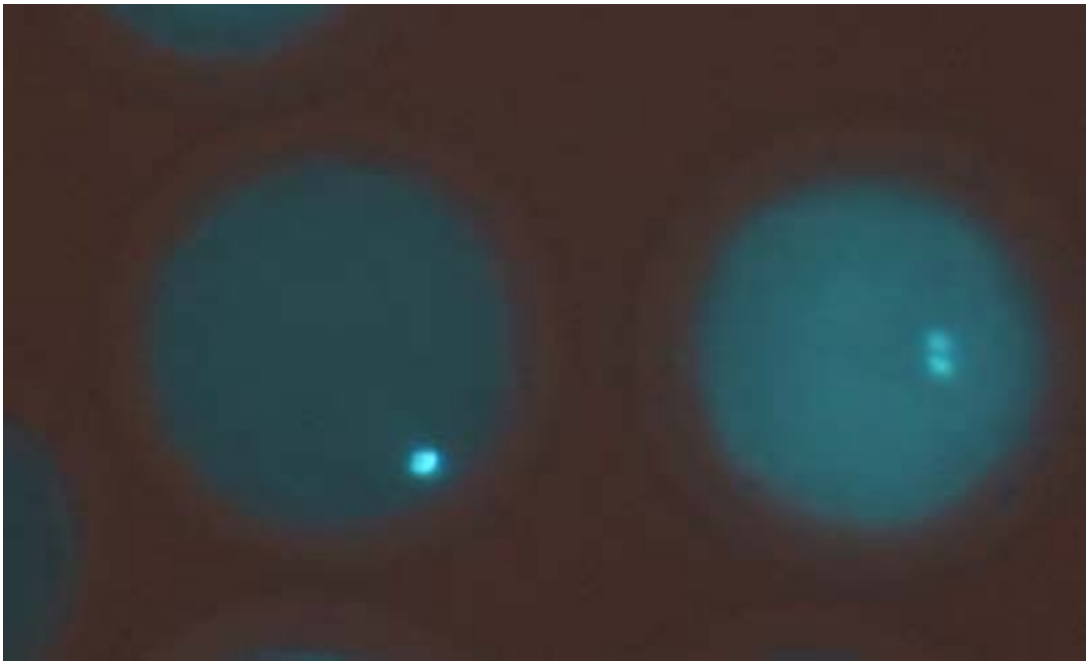


Figure 3. In vitro matured canine oocyte at the germinal vesicle break down stage matured in TCM 199 with recombinant FSH stained with Hoechst 33342.

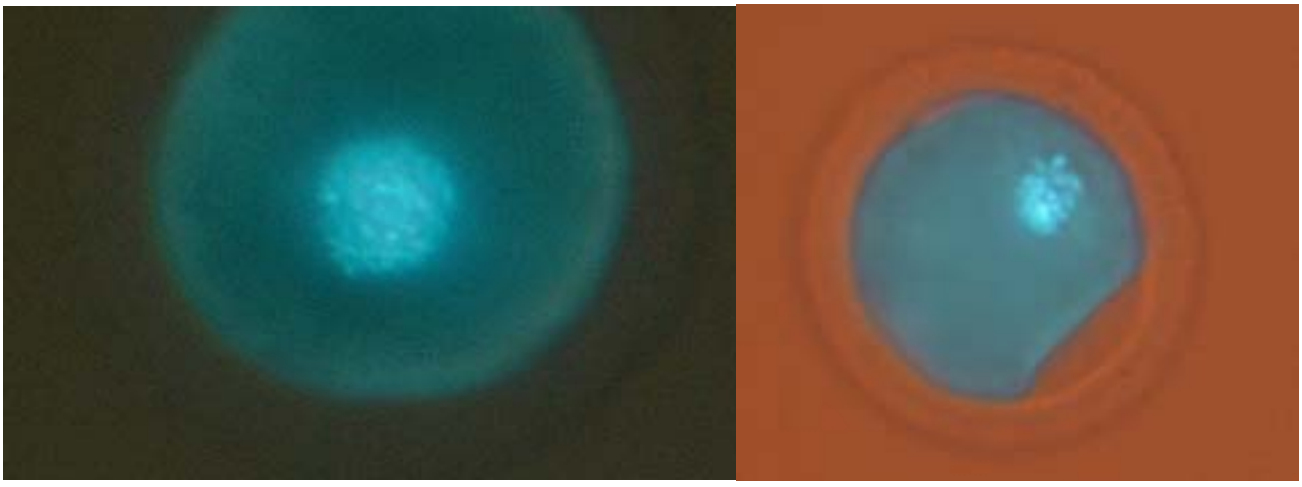
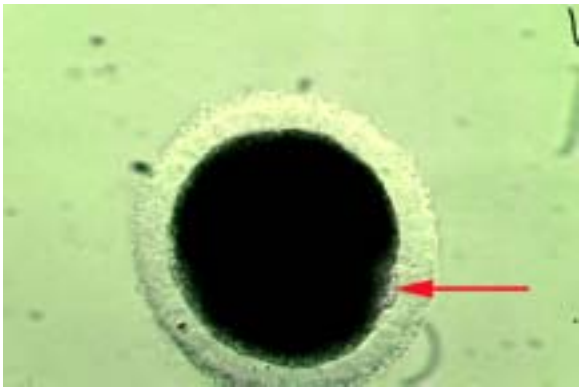


Figure 4. In vitro matured canine oocyte at the metaphase II stage matured in TCM 199 with recombinant FSH.



In vivo maturation

There are no reports of the in vivo maturation of dog oocytes with successful embryo production with IVF.

Innovative technologies

Canine oocyte in vitro maturation (IVM)

In the dog there have been a series of studies[7,8,9,10,11,12]. During a recent study[13] a total of 8,536 canine oocytes were matured in vitro and examined. The data reported here from this study demonstrates the current difficulties associated with IVM. The maturation rates of oocytes cultured in any of the rFSH treatments was statistically significant ($p<0.05$) compared to the control treatment although the effect of concentration of rFSH was not shown to be significant. The oocytes cultured for 96 hours in a rFSH supplemented media were significantly more likely to mature to MII than the control group. Medias supplemented with estradiol, progesterone, FSH, eCG, LH, L-cysteine and arachidonic acid did not significantly effect mature rates for canine oocytes when compared to the control treatment oocytes.

The effects of progesterone or estrogen on oocyte culture in vitro was evaluated with groups of 30 to 50 grade I oocytes cultured in 1.5 ml of basic culture media. Basic culture media is composed of TCM 199 without phenol red, but with 100 IU/ml penicillin, 50 μg/ml streptomycin, 10 ml/L sodium pyruvate, 3 g/L BSA, 5.8 g/L Hepes, 100 ml/L L-glutamine. Treatments of progesterone or estradiol were added to obtain a final concentration of 0.1 μg/ml, 1.0 μg/ml or 10.0 μg/ml. Cultures were incubated at 38.8˚C with 5% CO_2 for 24, 48, 72 or 96 hours. After each 24 hour period, one ml of the culture media was removed and immediately replaced with fresh, warmed media with or without hormone supplement. Control oocytes with no hormone supplement were cultured for the same length of time. For this study a total of 3,421 oocytes were cultured, stained and classified. There was no effect ($p>0.05$) of treatment, culture time, or treatment and culture time interaction. The highest rate of maturation to MII was 5/115 (4.35%) from the 96 hour culture control treatment.

The 96 hour culture control treatment also resulted in the highest percentage of MI/AI, 19/115 (16.52%).

The effects of recombinant FSH (rFSH) on oocyte culture were examined, where the treatment groups were supplemented with rFSH at a concentration of 0.1 IU/ml, 1.0 IU/ml or 10.0 IU/ml. A control group of oocytes receiving no hormone supplement was cultured for the same length of time. Oocytes cultured in the presence of rFSH were significantly ($p<0.05$) more likely to mature to metaphase II than oocytes in the control treatment. The highest rate of maturation to MII was 5/113 (4.42%) from the 96 hour culture with 1.0 IU/ml rFSH treatment. Although the 0.1 IU/ml rFSH and control treatments also resulted in MII oocytes. While statistical significance was found in the length of culture time ($p<0.05$), with 96 hours being the most likely group to extrude a polar body, no statistical significance was found between the varying concentrations of rFSH examined for this study. With a limited supply of ovine luteinizing hormone (LH), reduced numbers of groups were could be studies. Groups of 25-30 oocytes were cultured in 50 µl droplets of IVM supplemented with ovine LH to reach final concentrations of 0.1 IU/ml or 1.0 IU/ml. The media droplets were submerged in embryo tested mineral oil. A control group of oocytes received no hormone supplementation and was cultured for the same length of time. Cultures were incubated at 38.8˚C with 5% CO_2 for 96 hours. A total of 426 oocytes were cultured and no significant difference between LH treated oocytes and controls was found.

Subsequently the effect of equine chorionic gonadotropin (eCG) on oocyte maturation in vitro was examined. The treatment groups were supplemented with eCG to obtain a final concentration of 0.1 IU/ml, 1.0 IU/ml or 10.0 IU/ml, for 24, 48, 72 or 96 hours. A total of 1,384 oocytes were cultured and the control treatment was shown to have a significantly ($p<0.05$) greater effect of maturing oocytes to MII compared to eCG treated oocytes.

The potential deleterious effect of oxygen radicals was examined using L-cysteine at a final concentration of at 0.1 mM/ml or 1.0 mM/ml. For this study a total of 1,096 oocytes were tested and L-cysteine supplemented groups oocyte maturation was not statistically improved when compared to oocytes. The 0.1 mM/ml concentration of L-cysteine resulted in the largest percentage of MI/AI oocytes (2.78%) and MII oocytes (1.85%) of any group including the control group. In some species arachidonic acid has been shown to have a beneficial effect on oocyte maturation. In addition, the effect of media supplemented with arachidonic acid was examined. A final concentration of 10 µM/ml or 100 µM/ml was used and oocytes were incubated at 38.8˚C with 5% CO_2 for 72 hours. Meiotic maturation was assessed by staining cultured oocytes with the fluorescent dye Hoechst 33342, and a total of 422 oocytes were cultured. Only the control treated oocytes reached the MII stage, thus control oocytes were significantly ($p<0.05$) more likely to result in MI/AI oocytes when compared to the 10 µM/ml arachidonic acid treatment oocytes.

Cat oocyte in vitro maturation

Oocyte maturation has been shown to be very successful, even with superovulatory protocols.

An is universally accepted as being a practical and viable technology for mature oocyte production for all associated technologies.

Collection of oocytes from xenotransplanted ovaries

Xenotransplantation might provide some glimer of hope in cases where the female dog or cat is terminally ill and it is imperative that oocytes are produced. The protocol involves the collection of ovarian cortex and transplantation to unique locations in another species. Research to date has involved the implantation to areas such as under the capsule of the kidney. Here they are protected and should become viable after vascularization[15,16,17.] No xenotransplanation of canine ovarian tissue has been performed, but in the cat[18] results demonstrate that xenografts in SCID mice can lead to follicle development.

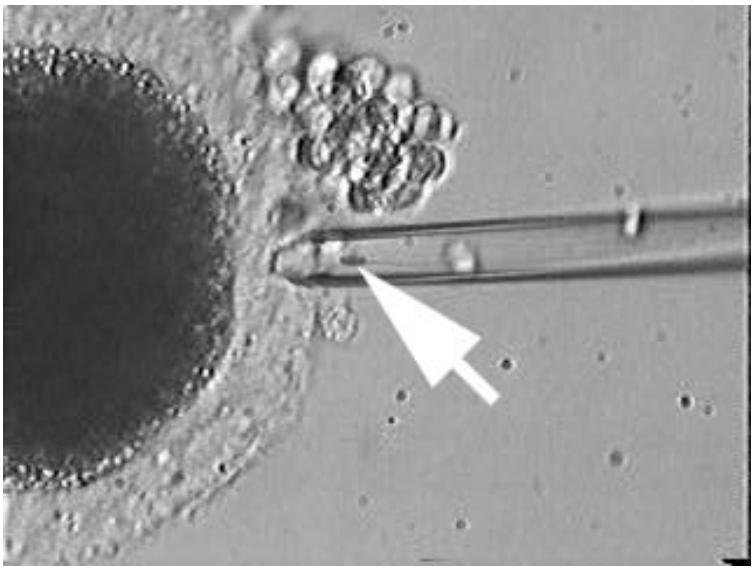
Intracytoplasmic Sperm Injection (ICSI)

ICSI has been generally successful in other species. In fact it is a primary basis for human in vitro fertilization clinics that allow circumvention of the process of fertilization.

Although we[12] have had success with ICSI in the dog, no puppies have been produced by this technology. Oocytes were collected from the ovaries obtained by ovariohysterectomy. The cortex of the ovaries were sliced to release the oocytes. The minced ovarian tissue was incubated and gently agitated in a solution of 500 units collagenase/ml and 75 units DNase/ml (2 ml per gram of tissue) at 37˚C for 1 hour. The tissue was then filtered and washed with phosphate buffered saline containing 0.1% bovine serum albumin over 40 μm mesh screen to collect the oocytes and inactivate the enzymes. The oocytes were graded and only grade 1 oocytes were placed in culture. The oocytes were cultured in TCM 199 for 72 hours. The oocytes were matured in TCM-199 culture media on agar with penicillin, streptomycin, FSH, LH and fetal calf serum and incubated at 38˚C under 5% CO_2 and 100% humidity for 72 hours.

Of the oocytes 38.5% (82/213) reached the metaphase II (MII) stage. The oocytes were injected with sperm cells obtained from percoll gradient separated fresh semen. Of the ICSI oocytes 7% (6/82) showed evidence of pronucleus formation.

Figure 5. In vitro matured canine oocyte at metaphase II being injected with sperm cell obtained from frozen-thawed semen.



Proven or practical successes in the cat

ICSI cats has proven to be far more successful with the production of several litters of kittens[19,20,21]. Dr. Earle Pope has kindly provided a picture of the first kittens produced by ICSI.

Figure 6. The first litter of kittens from transfer of embryos produced by intracytoplasmic sperm injection (ICSI), the oocytes were in vivo matured at the time of retrieval from gonadotropin treated donors at 24-26 hours after hCG. The embryos were cultured in vitro to the morula stage before transferred to the uterus (kind permission Dr. Earle Pope).

Dr. Pope's team has also produced a kitten using subzonal microinjection of spermatozoa into the perivitelline space and kittens after ICSI of IVM oocytes[20].

Canine superovulation

Yamada et al reported obtaining the following embryos after IVF in his 1992 experiment[7] (1 eight-cell, 2 seven-cell, 2 six-cell, 1 five-cell, 4 four-cell, 1 three-cell, and 4 two-cell embryos). Ovaries were collected from fifty-four bitches that had treated with estrone (100-400 μg/day) daily until vaginal discharge was detected. For three days after onset of vaginal discharge, 400 IU eCG and 1000 IU were injected subcutaneously. Then injections of estradiol (10 μg/day) were given on days 3 and 4 the eCG and hCG injections. Estrus was determined by the percent of vaginal cornification, and on the first day of estrus, hCG (1000 IU) was injected intravenously. Ovaries were collected 72 to 78 hours after hCG and ovulation had occurred in the bitches 72 hours after hCG. The oocytes were placed in a culture media containing 10% FBS and by 72 hours IVM, 31.9% of oocytes had matured to MII. Sperm cells were concentrated by centrifuging and capacitated with BSA. These sperm cells were then added to the culture media containing the IVM oocytes for 18 to 20 hours. The fertilized oocytes were cultured for an additional 48 hours, by which time 14% of the fertilized oocytes had developed into embryos at the two to three cell stage. Seventy-two hours post insemination 4.8% (3 out of 62) of fertilized oocytes had resulted in five to eight cell embryos, 29% were two to four cell. Ninety-six hours post insemination 13.3% of fertilized zygotes had developed beyond the 4 cell embryo stage. No development of blastocysts or morulas occurred.

Canine in vitro fertilization (IVF)

Hewitt and England have reported development of one two cell embryo obtained during a study of canine oocyte penetration[6]. The first day plasma progesterone levels exceeded 3 ng/ml, ovario-hysterectomy was performed[6] and after co-incubation for 12 hours with sperm that had been treated with canine capacitation media a single two cell embryo was produced IVF. The media was modified M199 with 0.3% BSA and the culture conditions were 39°C for 72 hours[6]. Hewitt found that 75% of oocytes remaining at GV stage were penetrated by the sperm[6]. Of oocytes that had resumed or completed nuclear maturation (GVBD, MI, AI, MII), 88% were penetrated by sperm[6]. The ability of spermatozoa to penetrate canine oocytes appears to have no dependence on nuclear maturation of the oocyte, but it is important to note that oocytes were primarily in vivo matured.

Using oocytes collected from bitches in various stages of the estrous cycle a single blastocyst has been produced by IVF[23]. These oocytes had been conditioned on a feeder layer of bovine cumulus cells in a culture medium that had supported in vitro fertilized bovine embryos for 13 to 14 days. Oocytes were cultured for 72 hours at 37.5˚C, 5% CO_2 and inseminated by spermatozoa from a Brackett-Oliphant medium containing bovine serum albumin (3 mg/ml), heparin (10 μg/ml) and caffeine (2.5 mM)[23]. After co-incubation with sperm, COCs were moved to a feeder layer of bovine

cumulus cells for 10 days of culture and one blastocyst was produced[23].

The most advanced report of an IVF and gestation was reported by England[24] with a 22 day pregnancy[24]. The oocytes resulting in the pregnancy were obtained from research bitches originally in anestrous and treated daily with cabergoline for 12 to 15 days, until the onset of vulva bleeding. From the bleeding onset daily vaginal cytology and plasma progesterone levels were recorded. When plasma progesterone concentrations exceeded 5.0 ng/ml, ovariohysterectomy was performed to obtain oocytes. The ovaries produced 169 grade I cumulus oocytes complexes which were cultured in TCM 199 containing 0.3% BSA for 24 to 78 hours[24]. Sperm cells were treated with canine capacitation media and added to the oocytes 12 hours[24]. Subsequently the oocytes were cultured for 48 hours and two out of 90 oocytes were at the two-cell embryo stage. All 90 oocytes/embryos were surgically transferred to the uterine lumen of a surrogate bitch whose plasma progesterone was above 5.0 ng/ml. Ultrasonographic pregnancy evaluation was performed 20 days post embryo transfer and one conceptus was in the proximal region of the uterine body and two conceptuses in the left uterine horn. All conceptuses were considered to be undersized for their anticipated age and ultrasonographic examination at 22 days post embryo transfer showed that all conceptus were absent. The surrogate bitch's serum levels for progesterone, relaxin and fibrinogen were noted as normal for early pregnancy.

Embryo transfer

Standard embryo transfer from one dog that has produced embryos through in vivo fertilization to a recipient dog has been successful. Kraemer et al[38,39], produced puppies from the transfer of embryos. The transfer of canine embryos which were in vivo fertilized in one bitch, were transferred to the uterus of an naturally synchronized surrogate bitch produced 3 live puppies[39]. The technique would thus be viable in cases of female gestational infertility and once IVM/IVF has been developed the transfer to an estrus synchronized bitch is a clinical reality.

Cloning

Current technological expectations in other species

To date cloning of individuals has been successful in sheep [26,27], cattle [26,28], goats [29,30], mice [31,32,33], and pigs [34]. Cloning in all cases has been an extremely inefficient process with a 1-4% success rate [35,36]. To the current time there has been no live cloned puppies have been produced. This is despite a significant and focused volume of research. For the cat cloning has been successful[37], but as with other species this has also been an inefficient process.

Summary

What techniques can be practically achieved and what techniques are not realistic for fertility salvage and offspring production? In the dog, semen freezing and standard embryo transfer have realistic expectations of success. However, IVF, xenotransplantation, ovarian cortical (oocyte) slice freezing and cloning are not realistic technologies. In the cat semen freezing, ET, IVF, ICSI and cloning are functional and realistic technologies.

References

1. Sato K. Polyspermy-preventing mechanisms in mouse eggs fertilized in vitro. *J Exp Zool* 1979;210(2)353-9.

2. Hyttel P, Callesen H and Greve T. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle.
J Reprod Fertil 1986;76(2)645-56.

3. Barber MR, Lee SM, Steffens WL, Ard M and Fayrer-Hosken RA. Immunolocalization of zona pellucida antigens in the ovarian follicle of dogs, cats, horses and elephants. *Theriogenology* 2001;55(8)1705-17.

4. Luvoni GC, Luciano AM, Modina S and Gandolfi F. Influence of different stages of the oestrous cycle on cumulus-oocyte communications in canine oocytes: effects on the efficiency of in vitro maturation. *J Reprod Fertil Suppl* 2001;57141-6.

5. Phillips DM and Dekel N. Maturation of the rat cumulus-oocyte complex: structure and function. *Mol Reprod Dev* 1991;28(3)297-306.

6. Hewitt DA and England GC. The canine oocyte penetration assay; its use as an indicator of dog spermatozoal performance in vitro. *Anim Reprod Sci* 1998;50(1-2)123-39.

7. Yamada S, Shimazu Y, Kawaji H, Nakazawa M, Naito K and Toyoda Y. Maturation, fertilization, and development of dog oocytes in vitro. *Biol Reprod* 1992;46(5)853-8.

8. Yamada S, Shimazu Y, Kawano Y, Nakazawa M, Naito K and Toyoda Y. In vitro maturation and fertilization of preovulatory dog oocytes. *J. Reprod. Fertil. Suppl* 1993;47227-9.

9. Bolamba D, Borden-Russ KD and Durrant BS. In vitro maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles. *Theriogenology* 1998;49(5)933-42.

10. Durrant BS, Pratt NC, Russ KD and Bolamba D. Isolation and characterization of canine ad-

vanced preantral and early antral follicles. *Theriogenology* 1998;49(5)917-32.
11. Farstad W. Reproduction in foxes: current research and future challenges. *Anim Reprod Sci* 1998;53(1-4)35-42.
12. Fulton R, Durrant B, Keskintepe L, Brandon C and Fayrer-Hosken R. Assisted reproductive techniques for the treatment of canine infertility. *Society for Theriogenology Newsletter* 1998;21(1).
13. Dew E. In vitro maturation (IVM) of the canine oocyte. *Department of Physiology and Pharmacology* 2002;56.
14. Spanel-Borowski K. Vascularization of ovaries from golden hamsters following implantation into the chick chorioallantoic membrane. *Exp Cell Biol* 1989;57(4)219-27.
15. Gook DA, McCully BA, Edgar DH and McBain JC. Development of antral follicles in human cryopreserved ovarian tissue following xenografting. *Hum Reprod* 2001;16(3)417-22.
16. Wolvekamp MC, Cleary ML, Cox SL, Shaw JM, Jenkin G and Trounson AO. Follicular development in cryopreserved Common Wombat ovarian tissue xenografted to Nude rats. *Anim Reprod Sci* 2001;65(1-2)135-47.
17. Weissman A, Gotlieb L, Colgan T, Jurisicova A, Greenblatt EM and Casper RF. Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. *Biol Reprod* 1999;60(6)1462-7.
18. Gosden RG, Boulton MI, Grant K and Webb R. Follicular development from ovarian xenografts in SCID mice. *J Reprod Fertil* 1994;101(3)619-23.
19. Pope CE, Johnson CA, McRae MA, Keller GL and Dresser BL. Development of embryos produced by intracytoplasmic sperm injection of cat oocytes. *Anim Reprod Sci* 1998;53(1-4)221-36.
20. Gomez MC, Pope CE, Harris R, Davis A, Mikota S and Dresser BL. Births of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured in vitro. *Reprod Fertil Dev* 2000;12(7-8)423-33.
21. Bogliolo L, Leoni G, Ledda S, Naitana S, Zedda M, Carluccio A and Pau S. Intracytoplasmic sperm injection of in vitro matured oocytes of domestic cats with frozen-thawed epididymal spermatozoa. *Theriogenology* 2001;56(5)955-67.
22. Goodrowe KL, Wall RJ, O'Brien SJ, Schmidt PM and Wildt DE. Developmental competence of domestic cat follicular oocytes after fertilization in vitro. *Biol-Reprod* 1988;39(2)355-72 issn: 0006-3363.
23. Otoi T, Murakami M, Fujii M, Tanaka M, Ooka A, Une S and Suzuki T. Development of canine oocytes matured and fertilised in vitro. *Vet Rec* 2000;146(2)52-3.
24. England GC, Verstegen JP and Hewitt DA. Pregnancy following in vitro fertilisation of canine oocytes. *Vet Rec* 2001;148(1)20-2.
25. Goodrowe KL, Howard JG and Wildt DE. Comparison of embryo recovery, embryo quality, oestradiol-17 beta and progesterone profiles in domestic cats (Felis catus) at natural or induced oestrus. *J-Reprod-Fertil* 1988;82(2)553-61 issn: 0022-4251.

26. Willadsen SM. Cloning of sheep and cow embryos. *Genome* 1989;31(2)956-62.
27. Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;385(6619)810-3.
28. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA and Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts.
Science 1998;280(5367)1256-8.
29. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW and Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol* 1999;17(5)456-61.
30. Keefer CL, Baldassarre H, Keyston R, Wang B, Bhatia B, Bilodeau AS, Zhou JF, Leduc M, Downey BR, Lazaris A and Karatzas CN. Generation of dwarf goat (Capra hircus) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. *Biol Reprod* 2001;64(3)849-56.
31. Wakayama T, Rodriguez I, Perry AC, Yanagimachi R and Mombaerts P. Mice cloned from embryonic stem cells. *Proc Natl Acad Sci U S A* 1999;96(26)14984-9.
32. Wakayama T and Yanagimachi R. Cloning the laboratory mouse. *Semin Cell Dev Biol* 1999;10(3)253-8.
33. Wakayama T and Yanagimachi R. Cloning of male mice from adult tail-tip cells. *Nat Genet* 1999;22(2)127-8.
34. Prather RS. Cloning. Pigs is pigs. *Science* 2000;289(5486)1886-7.
35. Niemann H and Rath D. Progress in reproductive biotechnology in swine. *Theriogenology* 2001;56(8)1291-304.
36. Wolf DP, Mitalipov S and Norgren RB. Nuclear transfer technology in Mammalian cloning. *Arch Med Res* 2001;32(6)609-13.
37. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L and Westhusin ME. A cat cloned by nuclear transplantation.
Nature 2002;723(1038)10.
38. Kinney GM, Pennycook JW, Schriver MD, Templeton JW and Kraemer DC Surgical collection and transfer of canine embryos. *Biology of Reproduction* 1979;20(supplement 1):96A (abstract).
39. Kraemer D, Kinney G, Schriver M. Embryo transfer in dogs and cats. *Embryo transfer and in vitro fertilization,* Second world conference. 1989;223-233.