Follicle growth, oocyte maturation, embryo development, and reproductive biotechnologies in dog and cat

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Abstract

Although the dog and cat both belong to the order Carnivorous, their reproductive physiology is quite different. The dog is a nonseasonal, monoestrus species with spontaneous ovulation only twice a year and with an atypical, postovulatory oocyte maturation. Furthermore, the application of reproductive in vitro biotechnologies such as in vitro maturation (IVM), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) or cloning remains quite a challenge in dogs. By contrast, the cat is a polyestrus seasonal species, with ovulation induced several times a year and typical preovulatory oocyte maturation. As a result, manipulation of ovarian physiology is feasible and in vitro reproductive biotechnologies are as efficacious as in cattle. The first part of this review describes the main facts associated with folliculogenesis in the dog and cat (initiation of growth of primordial follicles, appearance of the zona pellucida, formation of antrum, nuclear and cytoplasmic maturation of oocyte, expression of receptors to gonadotrophins, and expression of steroidogenic enzymes). Second part focuses on oocyte maturation, fertilization, and in vivo early embryo development in both species. Last part discusses in vitro reproductive biotechnologies (IVM, IVF, and IVD), embryo transfer, and more recent biotechnologies (cloning, in vitro folliculogenesis, and vitrification of oocytes and follicles). Innovations in the dog are still limited by various characteristics (long ovarian cycle, difficult in vitro oocyte maturation) whereas in the cat, many in vitro techniques are applicable that may also be extended to wild feline species.

Keywords: Folliculogenesis, biotechnologies, embryo transfer, dog, cat

Introduction

Although the dog and cat belong to same order (Carnivorous), their reproductive physiology is quite different. Dog is nonseasonal monoestrus, with spontaneous ovulation only twice a year. Thus, manipulation of ovarian physiology (e.g. to reduce interval between ovulations, to induce ovulation or superovulation, or to produce embryos) is generally difficult. Furthermore, application of reproductive in vitro biotechnologies (in vitro maturation [IVM], in vitro fertilization [IVF], intracytoplasmic sperm injection [ICSI], and cloning) remains quite a challenge in the dog and only a few research teams have mastered them. By contrast, the cat is seasonally polyestrus with ovulation induced several times a year. As a result, manipulation of ovarian physiology (e.g. stimulation or induction of ovulation) is feasible and in vitro reproductive biotechnologies are as efficient as in cattle. Our purpose is to review available information on: 1) main events occurring during in vitro follicle and oocyte growth, in vivo fertilization, and early embryo development; 2) in vitro biotechnologies and embryo transfer; and 3) other biotechnologies.

In vivo follicle and oocyte growth, fertilization, and early embryo development in dog

In vivo follicle and oocyte growth

Contrary to what occurs in many mammals (e.g. mouse, cow, woman, and others), in dog, ovarian follicles appear after birth. Only oogonia are present in the fetal ovary. First primordial follicles (oocyte surrounded by granulosa cells) appear ~ 1 month after birth.[1] These follicles make up the definitive stock of oocytes. Some of them will start growing only years later. When growth starts, oocyte diameter

increases and granulosa cells appear modified (rounded/cuboidal). As they increase in number, first follicles with an antral cavity appear at 4 months of age. As an antrum is formed, granulosa cells differentiate, distinguishing external cells, close to basal membrane from cells in the cumulus, close to the oocyte. As in other mammalian species, a number of changes take place as the oocyte and follicle increase in size. Changes (Figure 1) include formation of zona pellucida around oocyte, multiplication of granulosa cells (200 - 400 cells when antrum appears), [8] and first cells in theca, expression of FSH and LH receptors and ability to produce steroids (steroidogenic enzymes).

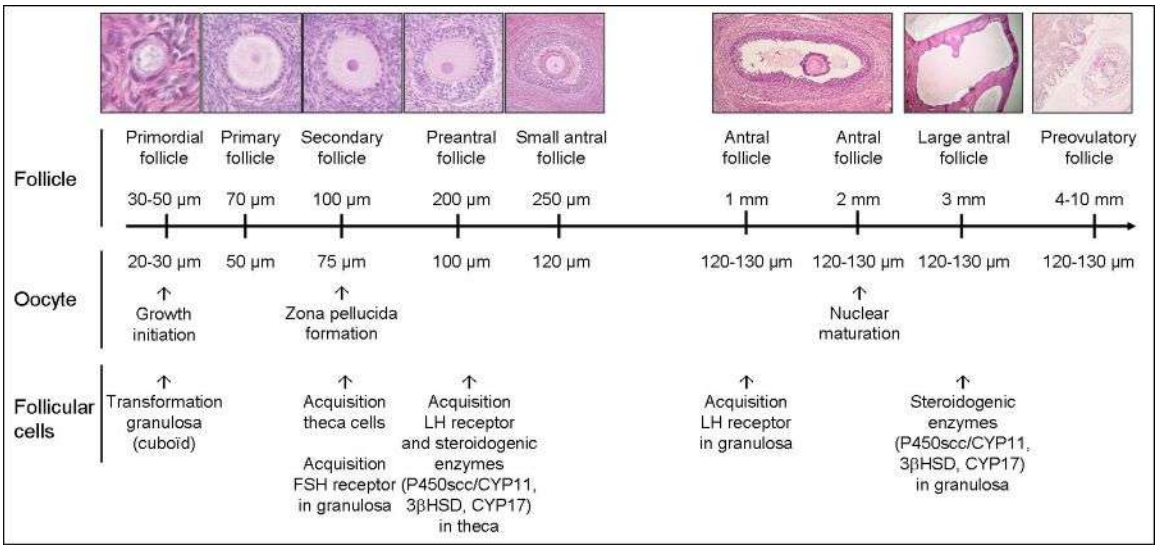

Figure 1. Main events in dog folliculogenesis.[1-7]

During anestrus, some follicles reach 1 mm in diameter.[1] Oocyte is surrounded by granulosa cells of cumulus, with many contacts between them and oocyte, leading to continuous transfer of many compounds (pyruvate, growth factors, RNAs, and organelles) from somatic cells to oocytes (Figure 2).

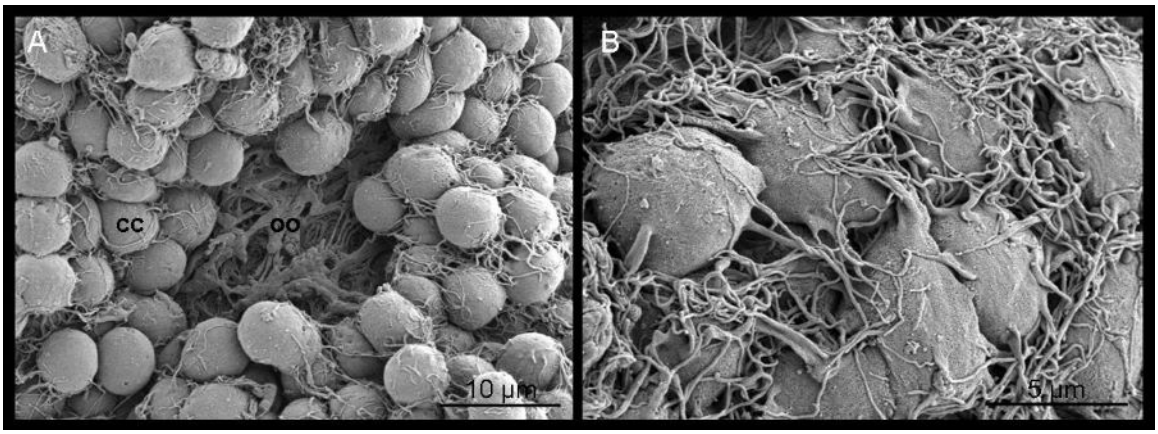

Figure 2. Scanning electronic microscopy image of a dog cumulus-oocyte complex showing numerous "pseudopod-like" connections between the cumulus cells (cc) and the oocyte (oo) (A) and between the cumulus cells (B).

Inside a follicle, the oocyte gradually acquires its ability to resume meiosis (nuclear maturation) and later acquires ability for fertilization and early embryo development (cytoplasmic maturation). A unique feature is the number of follicles with multiple oocytes (polyoocytic follicles) in the ovaries of dogs and

cats.[1,9-11] In dog, as many as 14% of follicles have several oocytes (with a maximum of 17 oocytes in 1 follicle) while it represents < 1 - 2% in other mammals.[12] They may reach ovulation but represent only 4 - 7% of preovulatory oocytes. Among them, a single oocyte in the follicle appears to be of good quality.[13] After puberty, at each cycle, larger follicles with antrum are being recruited to initiate terminal growth. Growth of this follicle may be visible by ultrasonography towards the middle and end of anestrus.[14] Once estrus begins, follicle growth accelerates and under stimulation by LH at the preovulatory stage, follicle wall becomes thicker. Immediately prior to ovulation, a protuberance may be visible at the very site where the oocyte is released (Figure 3). Right after ovulation, follicles are transformed into corpora lutea.

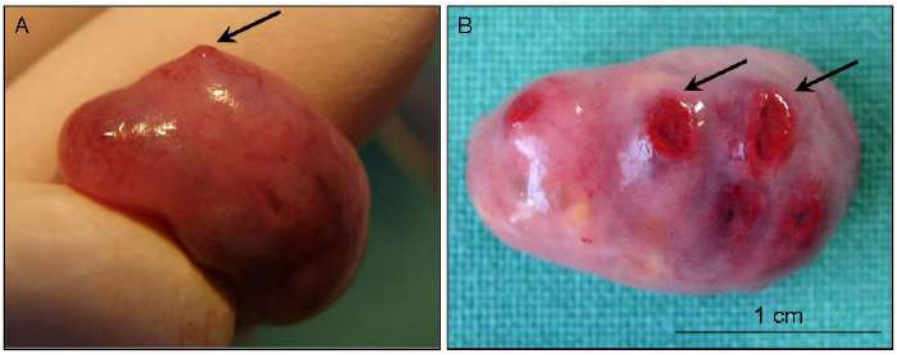

Figure 3. Dog ovaries during periovulatory stage, site (arrow) of future oocyte expulsion is visible immediately before ovulation (A), whereas small haemorrhagic corpora lutea (arrows) are visible right after ovulation.

Monitoring ovulation in dogs is possible by various means[15] such as LH or endocrine assays, possibly coupled with ultrasonography, depending on the veterinary clinic. Indeed, as a result of preovulatory luteinization of ovarian follicles, blood progesterone concentrations gradually rise from basal concentrations to 2 ng/ml at LH peak and 3 - 10 ng/ml at ovulation.[16-17] Thus, it is possible to predict ovulation and have the followup in real time by ultrasonography.[18,19] With 2 ultrasonographies a day, disappearance of follicles can be confirmed. However, not all follicles will transform into corpora lutea; some become filled with blood right after ovulation and may thus appear anechogenic. Follicle size at ovulation is related to dog breed and may vary from 4.5 mm in small breeds to 8 - 10 mm in larger breeds.[20,21]

At ovulation, oocytes are surrounded by mucified granulosa cells and are drawn into the infundibulum to enter the oviduct, but are still unfertilizable. Then, the oocyte loses the external layers of cumulus and migrates rapidly to middle section of oviduct (total length, 5- 10 cm). Several important events take place in oviduct: resumption of oocyte meiosis, selection of sperm,[22,23] and initiation of embryo development.[24] Resumption of meiosis leads to first polar body expulsion (2 - 3 days after ovulation) from oocyte leading to metaphase II stage that is ready for fertilization.[25,26] At that stage, it becomes possible to collect in vivo-matured oocytes. Two research teams have been able to routinely collect oocytes in vivo and have achieved in vitro fertilization and generated genetically modified animals or clones.[27-30] Ability to reach the fertilizable metaphase II stage after extraction from the follicular environment increases from 17 to 80% for oocytes from small follicles (< 500 µm) versus those from larger follicles (~ 2 mm).[7] Maturation of oocyte cytoplasm is more difficult to investigate, as it would require examination of oocytes from various follicle sizes (e.g. 2- 4, 4 - 6, and 6 - 8 mm). After fertilization, these oocytes should be examined through fertilization and embryo development until blastocyst stage. Several papers reported results obtained with oocytes from large follicles.[27,31] Unfortunately, in vitro culture conditions are still suboptimal, so that the exact time of cytoplasmic maturation acquisition remains uncertain.

As mentioned earlier, blood concentrations in progesterone in dog at ovulation and shortly thereafter are extremely high (> 10 times those in cattle or women). Presumably, these high concentrations of progesterone have significant biological effects. Thus, progesterone receptors (e.g.

aglepristone) treatment several days before ovulation induced a delay in resumption of meiosis and increased oocyte survival. It also inhibited the upward movement of sperm in oviduct.[32] Since oocytes are not readily fertilizable at ovulation, artificial insemination (AI) of thawed sperm, in order to obtain embryos or a pregnancy, should be done up to 2 days after ovulation. By contrast, in natural breeding, sperm survive much longer (up to 11 days),[25,33] making much earlier breeding before ovulation possible. Following fertilization, embryo development not only relies on oocyte reserves, but also requires exchanges with the environment in oviduct to obtain metabolites needed for further development. Hormone assays, coupled with ultrasonography, made it possible for us to describe precise timing of embryo development after AI in the dog[26,34] (Figure 4 and Table). Following morula stage, 8.5 - 9 days after ovulation, embryos crosses utero-oviductal junction and start uterine entry.[35] Prior to implantation, embryos migrate frequently to uterine horn on opposite side[36]. Implantation takes place ~ 17 days after ovulation.[37]

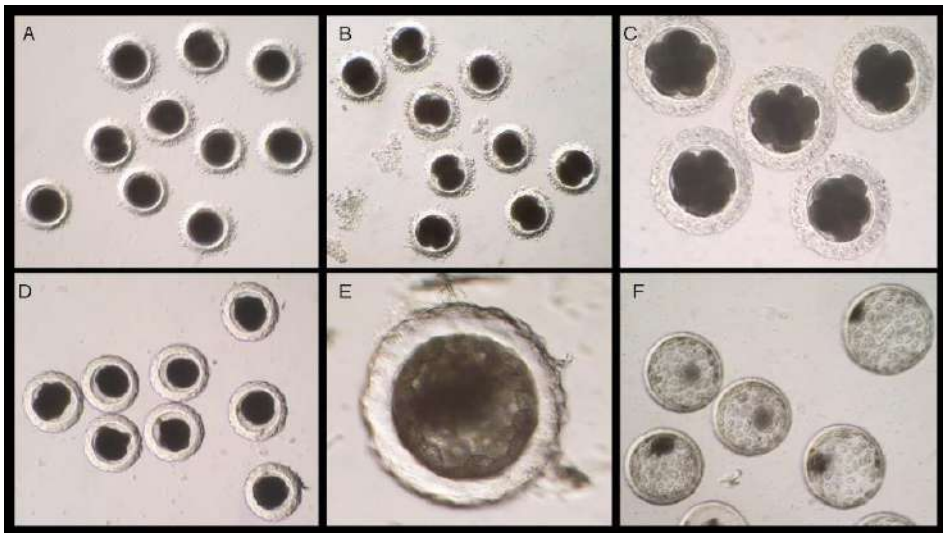

Figure 4. Dog embryos collected in vivo at 2 pronuclei (A), 2 or 4 cell (B), 8 cell (C), morula (D), early blastocyst (E), and expanded blastocysts (F) stages.

		Stage of embryonic development					
		2-PN	2-cell	4-cell	8-cell	Morula	Blastocyst
	n embryos (n bitches)	37 (7)	33 (8)	12 (5)	34 (7)	22 (4)	22 (5)
Time after ovulation	hours	102-132	120-161	133-154	153-225	230-266	230-274
	days	4.5-5.5	5-7	5.5-6.5	6.5-9.5	9.5-11	9.5-11.5

Table. Timing of embryo development *in vivo* in the dog (note: fertilization takes place 2-3 days after ovulation)

Challenges to in vitro maturation, fertilization, and embryo development

In vitro oocyte maturation and fertilization in dog have been investigated for several decades[38] and many obstacles were encountered. However, major progress has been achieved in the last 20 years. Reproductive in vitro biotechnologies represent an important research tool in that they may elucidate the biological mechanisms of oocyte maturation, fertilization, and embryo development, in particular the respective role of environmental factors (e. g. endocrine control, metabolism, growth factors, proteins, and intercellular communications). These biotechnologies also make it possible to preserve genetic resources (e.g. freezing of oocytes, sperm, embryos, ovarian cortex, and testicular pulp) for valuable dog

models in biomedical area (refer Online Mendelian Inheritance in Animals website [OMIA.org] that lists animal models for human diseases, 450 canine models) or to preserve threatened wild canid species.

In vitro maturation of dog oocytes

Purpose of in vitro oocyte maturation (IVM) is to obtain a large number of fertilizable oocytes. After ovariectomy and careful dissection of ovary, oocytes are selected and cultured in vitro in a maturation medium, using bovine models. In other mammals, when oocytes are collected from ovaries/follicles, there is an immediate resumption of meiosis in all competent oocytes (oocytes that reached a sufficient degree of nuclear maturation). For example, in cattle and mice, > 80% of oocytes have resumed their meiosis and become fertilizable after 24 hours. In dog, by contrast, oocyte reaches metaphase II stage and thus becomes fertilizable after 3 - 4 days in culture and with < 20% yield.[39] As mentioned above, folliculogenesis and thus oocyte maturation within the follicle is a process lasting for months. Final folliculogenesis, at ovulation, makes it possible to collect good quality oocytes but takes place only twice a year in dog. Collecting oocytes from an ovary during anestrus results in oocytes that are still quite immature. Furthermore, ovaries collected by ovariectomy may still be at prepubertal stage, that is 2 - 3 months only after appearance of first follicles with antrum. These oocytes are even more immature than anestrous oocytes, with poor synthesis of proteins and limited connections with granulosa cells.[40]

Many research teams have attempted to produce in vitro-matured fertilizable canine oocytes and to identify oocytes that should be used (according to age, breed, stage of cycle), to determine which media, oxygen supply, growth factors, proteins, hormones should be introduced and which cells to use in co-culture (reviewed [39,41,42]). Overall, the results have been disappointing. Major problem appears to be oocyte size compared to follicle size.[43] Thus, using oocytes from large follicles makes it possible to increase the proportion of fertilizable oocytes after in vitro maturation.[44] Yet, rates of success remain low, which may be related to cytoplasm quality (e.g. number and organization of organelles such as mitochondria and cortical granule) in those in vitro-matured oocytes reaching the metaphase II stage, which remain largely below those obtained with oocytes ovulated in vivo.[45]

Current investigations attempt to mimic in vivo intraovarian environment with maturation in pure follicular fluid[46]or in diluted follicular fluid,[47] or within an oviduct. Research on oviductal environment, which started 20 years ago,[48] is now designed to determine the composition of oviductal fluid and physiology of cells in oviductal epithelium[31,49,50] since exposure of oocytes to oviductal environment is essential for normal fertilization.[51] Variety of culture conditions have been tested such as synthetic oviductal fluid medium[52], use of microvesicles produced by oviductal cells,[50,53] cell cultures on plastic dishes or porous inserts,[49] oviductal explants[54] (fragments of oviductal epithelium), or ligated oviduct.[55] However, maturation rates hardly reach 20% and canine oocytes collected during anestrus, even transferred in vivo into an oviduct of dogs in estrus, are not able to adapt, despite having a potentially optimal environment.[34]

Some investigations were conducted in cattle, to increase the rate of metaphase II oocytes and primarily the rate of fertilization and embryos at the blastocyst stage (currently reaching 30 - 60%). These investigations were designed to temporarily block meiosis resumption, right after rupture within the follicle, in order to give the oocyte a chance to complete its cytoplasmic maturation. Several compounds such as roscovitine and dbcAMP were tested in anestrus canine oocytes without significant success.[57,57] Thus, the problem of immaturity of anestrus oocytes is far from being solved. As a result, up to this day, a single early pregnancy was reported after IMV-IVF-IVD[58] but no puppy was born. Birth of pups following embryo transfer (from oocytes, matured in vivo, and fertilized in vitro) was a major progress.[27]

In vitro fertilization and embryo development

In vitro fertilization is now routinely carried out in a number of species. Progress in reproductive biotechnologies in dog is in part from applying results obtained in wild canids (e.g. silver fox). Large numbers of ovaries collected on farms rearing foxes for fur production made it possible to describe maturation of oocytes (ovulated at an immature stage like in dog), in vitro fertilization and transfer.[59-62]

Altogether, rates of in vitro maturation and fertilization in the silver fox are close to those obtained in dog. Surgical embryo transfer has led to the birth of progeny.[63] However, in vitro fertilization and embryo development remain problematic in the dog, due to a number of causes. Cytoplasm quality of canine oocytes is generally poor, zona pellucida undergoes changes during culture and sperm capacitation has not been perfected.[64-65] Microinjection of sperm into oocyte (or ICSI), allowing a normal monospermic fertilization, has been reported in dog, however, is not routinely used in laboratories.[66,67]

Several teams have described in vitro fertilization and occasionally obtained embryos and even puppies.[27] However, bringing together dog sperm and oocytes in vitro does not routinely lead to successful fertilization, because sperm can enter immature oocytes,[64,68] typically ending in a high rate of polyspermia[44,69] and sperm pronucleus abnormalities.[37,65] However, oocytes collected at follicular phase stage have been used successfully to obtain embryos with as many as 30% undergoing cleavage after fertilization using oocytes from follicles > 2 mm.[70] Following fertilization, embryo development is initiated; however, it is blocked at the 8 cell stage when the embryo becomes autonomous and develops its own gene expression (stage of maternal to zygotic transition).[71] Blocking at 8 cell stage suggests that conditions of embryo culture are still not optimal. However, it appears that co-culture with embryonic fibroblasts may improve development of canine embryos to 16 cell/morula stages[72] and that some recently described culture media might enable development of embryos up to the blastocyst stage.[73,74]

In vivo collection, freezing, and transfer of canine embryos

As stated above, in vitro production of canine embryos is quite problematic. Another possibility is to produce in vivo embryos after ovulation, followed by natural breeding or artificial insemination (reviewed[69,75]). Collecting embryos in vivo may be achieved by flushing the oviduct to collect embryos at an early stage (2 cell - morula) or rinsing uterus to obtain embryos at morula and blastocyst stages. A major difficulty remains in getting access to oviduct without ovariectomy, since oviduct is hidden in a lipid-rich ovarian bursa and infundibulum is difficult to handle. By contrast, collecting embryos in uterus is simple and requires only a laparotomy, followed by uterine rinsing.[76,77] It may also be achieved by a nonsurgical endoscopic technique, with the introduction of a suitable catheter into cervix, which may allow to keep the female donor as a reproducer.[78,79] Embryos obtained this way may then be transferred to a recipient female or can be frozen. Canine embryos from 1 donor female are difficult to obtain in large numbers, as well in vitro (as described above) as in vivo because female dogs do not respond to superovulation treatments and because the number of embryos obtained is limited by ovulation rate.

Pioneering research in canine embryo transfer was started in the '80s.[25,76,80,81] Transfer of fresh embryos required optimal synchronization between donor and recipient cycles. At that time, method to synchronize cycles was not available, hence many experimental kennels were needed to routinely obtain females in estrus that could be used as embryo recipients. Ten years ago, only 45 puppies were born in the world after transfer to 57 recipient females (reviewed[69]). Since then, a number of puppies were born after oocyte micromanipulation (cloning), and after IVF and transfer.[51,82] Currently, estrus control, suppression or stimulation, can be obtained using GnRH agonists. These treatments are efficient and free from negative impacts on subsequent fertility.[83-85] Thus it becomes possible, towards end of anestrus period, to induce estrus in a dog within a few days.[86] Generally, embryo is transferred into uterus because oviductal access is difficult. Even at oviductal stages (2 - 16 cells), embryos can be successfully implanted in uterus.

In the absence of efficient synchronization treatment, cryopreservation of embryos may allow postponement of embryo transfer until a recipient female is available. Conditions for optimal cryoconservation of dog embryos remain an area of investigation, including: duration of exposure to cryoprotectants and concentration of cryoprotecting agents, evaluation of cryotolerance beyond morphological evaluation and conditions of embryo transfer after freezing and thawing to obtain pregnancies remain to be well defined.

Two freezing processes are used, namely, slow freezing and vitrification. Regarding slow freezing, glycerol and ethylene glycol were evaluated and gave contradictory results. In a study involving 20 embryos, it appeared that glycerol alone was able to preserve zona pellucida structure.[87] However,

similar post-thaw viability was established in glycerol and ethylene glycol when 50 blastocysts were used.[88]

In vitrification, high concentrations of cryoprotectant agents are being used, embryos are placed in a 1 - 5 µl microdrop and lowering of temperature is very rapid, with direct immersion in liquid nitrogen. This ultrarapid freezing is designed to avoid crystal development within cells and appeared proper for dog embryos. Thus, after vitrification in 1 µl microdrops in a Cryotop system, early canine embryos (zygote to 16 cells) survived freezing better (90 - 100% of survival after thawing) than embryos at morula (50%) or blastocyst (40%) stages.[78] These investigators also transferred 77 embryos to 9 recipient females and obtained 5 pregnancies, 4 to term, and 7 puppies were born. A lag time of 1 or 2 days between donor and recipient cycle stage of the donor was not a deterrent to pregnancy.[78] Furthermore, a recent study[89] compared slow freezing and Cryotop method vitrification in 89 in vivo collected embryos at various stages (8 cell to blastocysts). After cryopreservation (30 embryos by slow freezing and 35 by vitrification) and surgical transfer in recipient females (1 - 6 embryos per recipient), they succeeded in obtaining 2 pregnancies (1 puppy per recipient), but only with vitrified embryos.

Reproductive biotechnologies under development for preservation of dog genetics

Somatic cloning

Somatic cloning, also termed somatic cell nuclear transfer, which consists of injecting somatic cell into a mature oocyte, was first described in the dog in 2005.[28] Indeed, the first dog obtained by cloning, Snuppy, a male Afghan hound, was recloned recently.[90] Since then, the Korean research team has specialized in the collection of mature oocytes followed by micromanipulation. Right after transfer of nucleus, potentially cloned embryos are transferred into an oviduct using an appropriate catheter (Tom cat 3.5Fr) in order to avoid blocking of canine embryos in culture at 8 - 16 cell stage.[91] The technique has been considerably improved and its yield in number of puppies born related to the number of injected oocytes has made progress. South Korean investigators have applied cloning to a number of situations: pet dogs, working dogs (e.g. scent detection dogs), preservation of breeds (e.g. Sapsaree and the Gyeongju Donggyeong dogs), preservation of wild species (e.g. grey wolf and coyote), not to mention the creation of transgenic and canine biomedical models of human disease (reviewed[82]). Currently, it is possible to obtain cloning of a dog or a cat in South Korea, in China or in US for approximately 30,000€ per cat and 45,000 - 50,000€ per dog. Thus, the Sinogene company in Beijing reported that it had cloned as many as 40 dogs and cloned its first cat in 2019. Similarly, the South Korean Sooam company reported it had cloned 800 pet animals. In parallel, the US company Viagen Pets also offers cloning.

Cryopreservation of ovarian cortex/oocytes and in vitro folliculogenesis

Some other reproductive biotechnologies could be used for purposes of fundamental research or for preservation of biodiversity. In vitro folliculogenesis (dissection of ovarian follicles followed by culture for several days or weeks) may answer a number of questions regarding the growth of follicles and oocytes.[92-95] For instance, in mice, one may, over an interval of 3 weeks, culture primary follicles until the preovulatory stage and obtain newborns.[96] However, in dog, as in large mammals, folliculogenesis is a very lengthy process and culturing follicles for months remains quite a challenge and no progeny has been obtained so far anywhere in the world. However, in women, cryoconservation of the whole ovary or of fragments of ovarian cortex followed by autografting has been successfully used to preserve fertility prior to some gonadotoxic anticancer therapy. Following the termination of cancer therapy, the ovary or cortex fragments can be reimplanted and can restore ovarian function, leading to growth of the uterus and eventually pregnancy and the birth of a baby. This procedure for fertility preservation (freezing + grafting) has successfully generated dozens of babies.[97] In dogs, these techniques are still in an experimental stage. However, canine follicles survived in frozen ovaries and autografting was possible with resumption of follicle growth.[99] Another way to preserve female genetic material is cryoconservation of oocytes. In humans, this technique is widely used since the development of

vitrification. Two attempts of vitrification have been reported in dogs. Freezing immature oocytes has already been tested and 65% of the cumulus-oocyte-complexes were reported to have adequate morphology after vitrification by the Cryotop method.[100] Similarly, wolf oocytes survived vitrification.[101]

Further studies are needed to examine oocyte survival and developmental potential after vitrification, according to the presence of cumulus cells and maturation stage at cryopreservation. Research in this area may benefit from the results of vitrification in pigs,[102] whose oocyte is also rich in lipids, like that in the dog. Another technique commonly used in cattle, in vivo follicle puncture, also called Ovum Pick Up (OPU), may be quite useful in dogs. It is feasible in dog; however, it requires having a dog at similar preovulatory stage, good ultrasound equipment to clearly visualize follicles, and a laboratory close by to manipulate the oocytes (observation, culture or micromanipulation). Following OPU, oocytes or cumulus-oocyte-complexes might be cryopreserved or kept for maturation.

In vivo follicle and oocyte growth, fertilization, and embryo development in cat

Similar to dogs, a cat ovary starts developing during fetal life (oogonia migration/oogenesis); however, first primordial follicles appear approximately 1 month after birth.[103] Histology of follicle and oocyte growth have been reported.[103-106] Primordial follicles, primary, secondary, and preantral follicles measure 50, 80, 130, and 150 µm respectively and contain an oocyte whose size is 40, 60, 90, and 100 µm respectively.[106] Zona pellucida appears in secondary follicle and antral cavity appears when follicle reaches 220 µm.[106-107] At that stage, oocyte reaches 110 µm (together with zona pellucida) and nuclear maturation is completed. At the time of ovulation, oocyte size is ~ 125 - 130 µm.[108] Receptors for FSH in granulosa cells and for LH in theca cells can be detected in 200-µm small antral follicles.[109] In granulosa cells, LH receptors appear when antral follicles reach at least 800 µm. Similar to dog, cat ovary contains a large number of polyoocytic follicles, ~ 4%, with a recorded maximum of 10 oocytes in a single follicle.[9,103,110] Preovulatory diameter is 3 - 4 mm and may be measured via laparotomy or by laparoscopy.[111,112] Ultrasonography may be used[113,114] without anaesthesia, particularly, in docile animals. Regular assessment of behavior, vaginal smears and/or ultrasonography is needed, however, as these parameters are poorly correlated.[114] Ovulation can be induced by vaginal stimulation, inducing a peak in LH concentration, or may be obtained by hCG injection. Ovulation may also be spontaneous, without coitus.[115] Meiosis resumption resumes within ovary and fertilization may take place as soon as oocyte reaches oviduct. After in vivo fertilization, embryos at 1 - 4, 5 - 8, 9 - 16 cell, and morula stages were present in oviduct ~ 28 - 34, 40- 46, 64 -70, and 88 - 94 hours respectively after ovulation.[116] Beyond 112 - 118 hours postovulation, compact morulas and blastocysts were present in uterus.[116] Prior to implantation, embryos migrate frequently to uterine horn on the opposite side.[116]

In vitro oocyte maturation, fertilization, and embryo development in cat

Contrary to dog, cat is a species in which oocyte maturation, fertilization and in vitro embryo development can be achieved with a rate of success similar to those obtained in cattle. Thus, already more than 30 years ago, the first cat embryos were obtained after in vitro fertilization (but with in vivo matured oocytes).[108,117] Using oocytes collected from 55 female cats after ovarian stimulation (PMSG + hCG) made it possible to obtain as much as 80% of matured oocytes and 35 - 45% fertilization rate, with < 5% polyspermia.[108] After transfer of embryos into oviduct following laparotomy (54 embryos in 5 recipient cats), 4 litters and a total of 10 kittens were born. Pregnancy rates were satisfactory after transfer of embryos into uterus. However, embryo survival and thus the number of kittens obtained were higher after transfer into an oviduct.[118] Furthermore, using in vivo matured oocytes, the rate of embryo development was high: out of 100 oocytes, 75% divided after in vitro fertilization, 66% developed to the morula stage, and 18% to blastocyst stage.[119]

Even with oocytes collected with no hormonal stimulation after ovariectomy, results can be favourable. Thus, > 45% of oocytes reach metaphase II stage after 32 - 38 hours of maturation,[120] and after in vitro fertilization (46 - 56% of fertilization rate), 30 - 40% of embryos develop.[121] Furthermore, in vitro development is similar to in vivo.[122] However, since cat is a seasonal species, further testing was carried out to evaluate impact of period of the year on developmental competence of oocytes used for in

vitro fertilization. In a study[123] on nearly 7000 cumulus cell oocyte complexes, a seasonal effect was observed, with 45 - 55% embryos cleaved after fertilization and 50 - 70% of these embryos developing up to the blastocyst stage, but with lower rates of success in fall and winter seasons (October - March). In ICSI, even with epididymal or testicular sperm, 37% of embryo developed to morula stage and some births of kittens were reported.[125] After cryoconservation of testicular tissue followed by microinjection of sperm into oocytes matured in vitro, embryos could be obtained, which were then frozen and transferred to several females, leading to birth of several kittens.[126] Ease of assisted reproductive technologies achievement in cat has led to research on wild felids (reviewed[127-130]). For example, 30 years ago, embryos of a leopard cat (*Felis bengalensis*) were obtained after ovarian stimulation by PMSG + hCG treatment followed by follicle puncture via laparoscopy, in vivo fertilization and early embryo development.[131] Very recently, the first Cheetah pups were born as the result of in vitro fertilization and embryo transfer achieved by scientists of the Smithsonian Conservation Biology Institute (https://www.si.edu). Some other teams, such as that of Bill Swanson (Cincinnati Zoo and Botanical Garden), have become real experts in followup/induction of ovulation in domestic cats and also wild felids. This research was applied to the production of animals in zoos and in the wild, to building genetic stocks of sperm and embryos and to maintenance of animals of biomedical interest. Indeed, as in dog, a number of feline models can be used in biomedical research (OMIA.org, 218 feline models of human disease), and the international conference on canine and feline genetics and genomics is devoted to these specific models.

Other reproductive biotechnologies for the preservation of cat genetics

Somatic cloning

The first cat obtained by somatic cloning, "CopyCat", was born in 2001.[132] Genetics Saving and Clone company started its business and produced a first cat, "Little Nicky" sold for $50,000 in 2004. This company kept producing cloned cats until 2006. In the world, cat cloning can be done in South Korea, in China or in US (refer above, section "dog cloning "). A cloned cat can, as the dog, be re-cloned.[133] Furthermore, numerous attempts at interspecific cloning were carried out. Somatic cells of wild felids were injected into oocytes of domestic cats and some small wild felids (e.g. African wild cat[134]) were obtained this way (reviewed[135,136]).

Collection and preservation of the female genetic potential

In vitro folliculogenesis in the cat was also explored. It is a powerful tool to study biological mechanisms and one way to stock genetic potential in a biobank.[137,138] Indeed, preantral follicles are quite numerous in ovaries and can survive cryopreservation.[139] Cryoconservation of immature cat oocytes by vitrification with the Cryotop system has been described.[140] High rates of oocyte survival were reported with resumption of maturation after thawing in ~ 25 - 40% of oocytes. After ICSI (freezing may alter zona pellucida, and thus prevent fertilization), as many as 20% of embryos may reach the morula stage. Some kittens could be obtained using vitrification technique.[141]

Conclusion

Altogether, despite the complexity of canine model (2 ovulations per year, limited number of mature oocytes available and requirement of an experimental kennel), research efforts in reproductive biotechnologies are in full development, with the efforts of a few research teams in the world. On the contrary, in cat, numerous in vitro techniques are applicable that may also be extended to wild feline species. Development of worldwide communications gave the scientific community a chance to collaborate. Thus international embryo technology society created a committee on companion animals, nondomestic, and endangered species with the objective of sharing research progress in this area (https://www.iets.org/comm_candes.asp).

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