Current and future perspectives of reproductive technologies in domestic and wild canids

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Abstract

Due to their unique reproduction, reproductive technology advances in canids have lagged behind compared to other mammalian species. Currently, semen cryopreservation and artificial insemination have been widely used in domestic dog. However, artificial insemination in wild canids is still limited due largely to poor semen quality, high susceptibility of sperm to cryopreservation procedures, and inability to noninvasively predict ovulation and timed insemination. For female canids, inability to consistently mature oocytes in vitro has limited the use of embryo technology in these species. However, birth of the first domestic dog puppies produced via in vitro fertilization has reinvigorated research interests in this area. This presentation will summarize the status of assisted reproductive technologies in domestic and wild canids and will discuss new, exciting research in fertility preservation and application of reproductive technologies in wild canid conservation.

Keywords: Canids, artificial insemination, in vitro maturation and fertilization, in vitro folliculogenesis, somatic cell nuclear transfer

Introduction

Reproductive science has critical roles in species conservation and management.[1,2] Specifically, advances in understanding species' reproductive biology facilitate development of reproductive technologies that are useful for ensuring genetic and demographic viability of ex situ wildlife populations and assist in development of strategies to control overpopulated species.[1] Of the 37 *Canidae* family (includes domestic dog [*Canis familiaris*]) species, 5 are listed as 'endangered' or 'critically endangered' by the International Union of Conservation of Nature. Therefore, development of assisted reproductive technologies (ARTs) would certainly be useful for conservation and management of these threatened canids.

Currently, existing knowledge on canid reproductive biology is mostly gleaned from domestic dog studies.[3-7] It is well recognized that reproductive biology of female canids is unique compared to other mammalian species. Specifically, female reproductive cycle is characterized by an extended proestrus followed by protracted estrus with each period lasting on average of 1 week.[8] Estrous period is characterized by an estrogen peak that coincides with rising progesterone concentrations before ovulation.[8] Estrus is followed by diestrus, a luteal phase averaging 2 months in duration irrespective of pregnancy. Diestrus is succeeded by anestrus, an extended interval of ovarian quiescence.[8] Whereas domestic dogs exhibit nonseasonal monoestrus once or twice a year,8 most wild canids breed seasonally. The onset of breeding season in wild canids varies among species and is dependent on environmental factors, such as latitudes and rainfalls.[9,10] Furthermore, although most canids are spontaneous ovulators, there has been evidence of induced estrus or ovulation in the Island fox (*Urocyon littoralis*), maned wolf (*Chrysocyon brachyurus*),[11,12] and seasonal polyestrus in dholes (*Cuon alpinus*) and bush dog (*Speothos venaticus*), indicating diversity in reproductive mechanisms within *Canidae* family.

Canid oocyte is also unique compared to that of other mammalian species. Specifically, domestic dog ovaries contain a higher proportion (7 - 11%) of polyovular follicles than those (4%) of the domestic cat.[13,14] Although polyovular follicles release multiple oocytes, there is evidence that only 1 gamete is capable of undergoing maturation and fertilization.[14] Another unique feature of canid oocytes is that they contain large amount of cytoplasmic lipids compared to other mammalian species, including cat and pig.[15] To date, the extent to which cytoplasmic lipids have roles in oocyte development is unknown. Nevertheless, it has been suggested that the challenges in applying conventional in vitro oocyte maturation systems to the dog is partly associated with the unusually large amount of cytoplasmic lipid in this species.[16] Finally, the most striking feature of dog gamete biology is that the oocyte ovulates in an immature stage requiring up to 48 - 72 hours to complete nuclear maturation within the oviduct,[6,15,16] and this characteristic undoubtedly contributes to challenges in developing in vitro maturation (IVM) system for dog oocytes.

Due to inability to reliably mature dog oocytes in vitro, ARTs that require the use of in vitro matured gametes have lagged behind compared to other species. Nevertheless, a handful of live offspring have been produced from in vitro derived embryos produced from in vivo matured oocytes via in vitro fertilization (IVF) or somatic nuclear transfer (SCNT),[17-19] and thousands of pups were born from AI with fresh and frozen-thawed sperm.[20,21] This review will summarize the status of reproductive technologies in domestic and wild canids and will discuss exciting research in fertility preservation and the application of reproductive technologies in wild canid conservation.

Semen collection and artificial insemination

Apart from domestic dog and farmed fox species, semen collection is normally performed by electroejaculation (EEJ) in wild canids.[22-27] Maned wolf,[28] gray wolf (*Canis lupus*),[29,30] and crab-eating fox (*Cerdocyon thous*)[31] ejaculates also have been obtained using digital stimulation, although this is not a routine method. The limitation of digital stimulation technique is that it requires preconditioning of animals to physical restraint, and thereby, relies on the availability of animal trainers. However, this method does not require anesthesia and can be performed more frequently than EEJ (multiple collections per week versus once or twice during breeding season).[28] Recently, urethral catherization (after medetomidine treatment) developed for felids[32,33] has been successfully applied to the domestic dog[34] and red wolf (*Canis rufus*).[27] Like EEJ, this method requires anesthesia; however, it does not require specialized equipment, and therefore, can be applied to individuals living in situ or under conditions where EEJ is not feasible. Semen characteristics of domestic and wild canid ejaculates collected using various techniques are summarized (Table).

A major challenge in semen collection in canids, especially via EEJ and urethral catheterization is urine contamination.[24,27,34] The prevalence of urine contamination varies among species, collection method and time of the year. Specifically, this author has observed urine contamination as a common feature in the maned wolf, regardless of collection methods. For red wolf, urethral catheterization often results in urine contamination compared to EEJ.[27] As a result, it is recommended that urethral catheterization semen collection method should not be used in red wolf for cryopreservation of sperm.[27] Urine alters osmolarity and pH of semen samples that, in turn, increases the proportions of sperm with bent and coiled tail, decreases motility,[27,35] and increases the susceptibility of sperm to osmotic stress. For the African wild dog (*Lycaon pictus*), urine contamination in semen samples collected via EEJ occurred more often when samples were collected from subordinate males during prebreeding season

Table. Seminal traits of domestic dog and wild canids

Species	Volume (ml)	Concentration (x 106 sperm/ml)	Motility (%)	Morphologically normal sperm (%)	Citations
Digital manipulation					
Domestic dog	1-30	300 - 1000	70	70	36
Maned wolf[a]					28
Breeding season	1.3 ± 1.2	73.9 ± 87.2*	76.1 ± 23.9	36.5 ± 24.0	
Non-breeding season	0.4 ± 0.6	6.1 ± 4.9*	80 ±14.5	20.8 ±19.8	
Gray wolf[b]	1.7 ± 0.2	290.8 ± 53.5	91.7 ± 1.5	N/A	30
Blue fox[b]	0.39 ± 0.26	491.8 ± 594.4	N/A	89.9 ± 4.4	37
Crab-eating fox[a]	0.39 ±0.18	463.7 ±84.3	86.0 ±16.9	2.0 ±1.0	31
Electroejaculation					
Domestic dog	1.8	129.6	30.1	N/A	38
Coyote[b]	1.67 ± 0.4	549.2 ± 297.7	90.4 ± 4.5	78.0 ± 13.5	26
Red wolf[a]	6.15 ± 5.6	96.7 ± 178.7	80.8 ±16.9	46.5 ±14.1	27
Red wolf[b]	4.7 ± 0.7	146.5 ± 25.7	71.2	73.6 ± 3.2	39
African wild dog[b]	0.6 ± 0.1	212.3 ± 87.3	69.5 ± 3.3	76.2 ±6.2	25
African wild dog[b]					24
Breeding season	NA	32.3 ± 9.2	47.4 ± 6.7	50.9 ± 5.2	
Non-breeding season	NA	27.4 ± 11.5	17.3 ± 10.2	40.6 ± 9.8	
Maned wolf[b]	2.0 ± 0.6	43.4 ± 18.2	59.8 ± 4.9	28.1 ± 4.4	22
Urethral catheterization					
Domestic dog[b]	0.09 ± 0.03	1,186.67 ± 304.66	58.3 ± 8.7	53.2 ± 5.6	34
Red wolf[a]	0.36 ± 0.08	50.4 ± 23.5	~40%	NA	27

*Total sperm per ejaculate
[a]Mean ± standard deviation
[b]Mean ± standard error of mean

than breeding season.[24] To circumvent this issue, a common practice for semen collection in wild canids is to remove urine and flush the urinary bladder with saline prior to EEJ.

To date, AI with fresh, chilled and frozen-thawed sperm has been widely used in the domestic dog[21] and farmed foxes.[40] Offspring were produced from AI with fresh or frozen-thawed sperm in gray wolves,[41,42] Mexican gray wolves,[40] and red wolves.[39] However, AI has not been routinely applied to genetic management of threatened and endangered canids, likely due to the lack of knowledge on species' reproductive biology, the challenges in predicting ovulation onset and effectively manipulating female reproduction as well as poor seminal quality (Table 1).[40] Therefore, future research should focus on developing a noninvasive method to predict ovulation in endangered canids. For example, the ability to assess luteinizing hormone in urine samples would be extremely useful for ovulation prediction and timed insemination when frequent blood sampling is not feasible.

Sperm cryopreservation

First records of live birth after AI with cryopreserved sperm were reported in 1969[43] for domestic dog and in 1975 for gray wolf.[42] Since these first successes, canid sperm cryopreservation has been widely studied in both domestic dog[21,44-46] and wild canids, including gray wolf,[47,48] red wolf,[27,39,49] maned wolf,[22] African wild dog,[25] red fox,[50] and blue fox.[50] Glycerol has been generally used as a cryoprotectant for canid sperm.[21,25,27] However, in 1 study, dimethyl sulfoxide was superior to glycerol for cryopreservation of maned wolf sperm.[22] Interestingly, dimethyl sulfoxide was toxic for dog sperm,[51] indicating species differences in the response to cryoprotective additives. Like pig[52,53] and horse,[54] there appears to be male-to-male variations in the susceptibility of sperm to cryopreservation that is independent of the quality of fresh semen and normal fertility at natural mating.[21,46,55] Whelping rate and litter size obtained from frozen-thawed dog sperm are about 23 - 30% less than fresh sample.[21] Nevertheless, AI with frozen-thawed sperm has been applied in domestic dog breeding and has resulted in thousands of puppies.[21] Unlike domestic dog, studies in wild canids, including African wild dog[25] and red wolf have demonstrated precipitous decreases in motility and viability of frozen-thawed sperm following incubation despite acceptable viability immediately after postthaw.[49] Our laboratory recently evaluated the effects of extracellular vesicles (EVs) from domestic dog oviducts on postthaw survival of red wolf sperm. EVs contain proteins, RNA, and DNA messages that deliver to neighboring cells, that in turn regulate recipient cells' function.[56] We reported that thawing red wolf sperm in medium containing dog oviductal EVs supports sperm motility and acrosomal membrane integrity after 2 hours of incubation compared to nonEVs control.[57] Proteomic analysis also revealed that dog oviductal EVs contain several proteins that influence mitochondria function, plasma and acrosomal membrane integrity, and stress responses,[57] indicating the useful potential of oviductal EVs in improving posthaw survival and longevity of wild canid sperm.

In vitro oocyte maturation and fertilization

In vitro oocyte maturation (IVM)

Due to their unique reproductive and gamete biology, development of IVM systems for canids has been far from successful.[16] Investigations have included impacts of stage of reproductive cycle, culture medium and protein, and hormone (gonadotropins and gonadal steroids) and growth factor supplementation (see review[16]). Nevertheless, on average, only 15 - 20% of cultured oocytes achieve the metaphase II (MII) stage after 48 - 72 hours of in vitro culture.[16] Supplementing the culture medium with 10 mM caffeine during the first 24 of 72 hours of incubation increased maturation rates compared to unsupplemented controls (42.2 versus 25.5%) with small proportion (4%) of gametes from the former treatment developing to morula stage after IVF.[58] Interestingly, incubating dog oocytes with caffeine during 24 - 48 or 72 hours of culture did not improve maturation rate. The beneficial effect of caffeine on dog IVM is likely due to activation of maturation-promoting factor and mitogen-activated protein kinase (MAPK),[59] 2 kinases with critical roles in chromatin reconfiguration during oocyte maturation.[60] Supplementation of insulin like growth factor-1,[61] growth differentiation factor-9 and bone morphogenetic protein-15[62] also enhanced nuclear maturation of dog oocytes compared to unsupplemented control. Yet, overall MII rates in those studies were still < 20%.

During the past several years, interest in examining the roles of reproductive EVs in regulating gamete function has substantially increased.[56] Oviductal exosomes (the smallest EVs) stimulated cumulus cell proliferation by activating epidermal growth factor receptor (EGFR)/MAPK signaling pathway.[63] This indicates the potential role of oviductal EVs in regulating dog oocyte maturation, as cumulus cells are known to induce meiotic resumption and support cytoplasmic maturation in several mammalian species.[64] Dog's oviductal and cumulus cells recovered during estrus had higher levels of MAPK1 than cells recovered during anestrus and diestrus.[65] Yet, coincubation of dog oocytes with oviductal cells from estrus resulted only in 10% of the cultured gametes developing to MII.[65] Coincubation of estrous oocytes with oviductal cells significantly improved MII rate after 72 hours IVM compared to controls (47 versus 11%).[66] Furthermore, in vitro matured estrous oocytes developed to 8-cell stages (66%) following parthenogenetic activation and in vitro culture at a rate comparable (85%) to in vivo matured gametes.[66] Despite the discrepancy in the above studies, findings to date are encouraging and emphasize the need to further explore the roles of reproductive EVs (from follicle fluid and/or oviduct) in development and maturation of dog oocytes.

Most IVM studies recover dog oocytes from tissues obtained during routine ovariohysterectomy. Follicle size significantly influenced developmental competence of the dog oocyte.[67] Specifically, ~ 80% of oocytes from follicles > 2 mm in diameter complete nuclear maturation in vitro compared to only 16 to 38% of those from smaller (0.5 - < 2 mm) source follicles.[67] Because > 2 mm diameter follicles only appear during proestrus and estrus, it is hypothesized

that the overall low IVM success in canids is likely due to that the oocytes from smaller follicles have not fully acquired developmental competence and therefore, are not able to mature under culture conditions developed for fully grown gametes.[67] Exposure of dog oocytes for 48 hours to meiotic inhibitor compounds (e.g., roscovitine and butyrolactone) inhibited meiotic resumption of dog oocytes in vitro.[68] Such oocytes were able to resume meiosis at a higher rate than those not been exposed to meiotic inhibitor compounds.[68] Therefore, it may be useful to explore the influence of short-term inhibition of meiotic resumption followed by coincubation with oviductal cells or EVs on dog oocyte development. Such studies may provide insights in mechanisms regulating gamete maturation, information that is critical for the development of an effective IVM system for this species.

In vitro fertilization

The initial report of IVF using in vitro matured dog oocytes was published more than 40 years[69]ago, although embryonic development was not reported. Since then, several investigators have attempted to perform IVF of incubated oocytes, albeit with limited success.[16,58,70-77] Thus far, there is only a single report demonstrating the production of 1 blastocyst from IVF from in vitro matured oocytes,[72] and 1 non-term pregnancy after transferring in vitro derived presumptive zygotes into recipient females.[73] In vitro maturation and fertilization have also been conducted in silver fox; however, embryonic development was not reported.[78] To date, there have been a few studies on dog and fox IVF using in vivo matured gametes. Only 1 oocyte reached morula (144 hours after IVF) when blue fox oocytes (n = 36) were inseminated with frozen-thawed sperm.[79] Approximately, 12% of oocytes collected 4 days after ovulation developed to 2-cell stage post IVF; 5 embryos developed further but arrested at the 4-cell stage. Only recently was the first litter of pups produced from cryopreserved, in vitro derived embryos.[17] Two factors contributing to that success included supplementation of magnesium to the sperm capacitation medium and use of day 6 (post LH surge) oocyte for IVF.[17,80] Interestingly, the presence of progesterone during IVF did not impact fertilization and embryonic development.[17]

Oocyte and embryo cryopreservation

Large amounts of intracellular lipid within the canid oocyte presents an additional challenge in developing ARTs. To date, a handful of studies have been conducted on canid oocyte cryopreservation.[81-84] Due to the lack of effective IVM systems to assess developmental competence of cryopreserved gametes, morphological assessment or vital staining have been used to determine cryopreservation success in most studies. By using the open-pull straw technique, the percentage of vitrified-warmed dog oocytes completing nuclear maturation was similar to that of fresh control, although more cryopreserved gametes were arrested at the GV stage than those of fresh counterparts.[83] Approximately, 90% of blue fox oocytes vitrified using the two-step open-pulled straw method exhibited normal morphology post-warming and 11% of these gametes developed to the MII stage, comparable to the fresh control.[84] Finally, 60% of dog[81] and Mexican gray wolf oocytes[82] maintained viability (based on vital staining) after vitrification using the cryotop technique.

Successful embryo cryopreservation in canids either by vitrification[85] or slow freezing method[86] has been reported in the domestic dog, demonstrating a stage-dependency in the susceptibility to cryopreservation. Specifically, blastocysts are more sensitive to vitrification than those at the earlier stages of development (1-cell to morula stages).[85] Although dog blastocysts cryopreserved using the slow freezing method were able to re-expand during in vitro culture, transferring these embryos did not result in offspring production.[86] To date, live births have been produced from embryos that had been frozen during cleavage stages (2 - 16 cells). For example, transfer of 77 vitrified-warmed 4 - 16-cell dog embryos resulted in the birth of 7 live offspring (9.1%).[85] Furthermore, 16% birth rate were achieved after transferring 6 embryos vitrified using a closed vitrification system (Vit kit).[87] This same vitrification method has been used to cryopreserve in vitro derived embryos resulting in live birth.[17]

In vitro folliculogenesis

Each ovary contains thousands of immature follicles enclosing oocytes that are never ovulated and thus never contribute to reproduction. The ability to activate and grow immature follicles to a mature stage producing a competent oocyte would help preserve genetically valuable dog models of human diseases and endangered canids.[88] During the past decade, advances have been made in development of in vitro culture system for dog ovarian tissue and isolated follicles.[89-96] Compared to domestic cat, dog ovarian tissues are highly susceptible to in vitro culture, likely due to the highly rigid cortex that limits nutrient supply to the enclosed follicles.[97] Studies have examined effects of growth factors, including epidermal growth factors and vascular endothelial growth factors,[92,98]as well as an anti-apoptotic agent, Z-VAD-FMK[99] on the activation and survival of enclosed follicles, with varying results. The current in vitro culture protocol can maintain the viability of enclosed follicles for only 7 days.[92] Nevertheless, the culture system developed for dog has been applied to the maned wolf with comparable results. These findings underscore the importance of the dog model for developing ARTs for threatened and endangered canids.

Follicle stimulating hormone (FSH) is essential for in vitro growth of isolated dog follicles;[90,93]although they increase in size and produce steroids in the presence of FSH, gonadotropin supplementation does not support the growth and survival of the resident gamete.[90] This was attributed to disruption of the communication between the oocyte and the surrounding granulosa cells.[90] Our laboratory has demonstrated that supplementing culture medium with 100 ng/ml activin promotes dog follicle growth and antral cavity expansion and supports oocyte's chromatin integrity by maintaining the transzonal projection for 12 days.[100] Most recently, it was reported that supplementing culture media with 2 cAMP modulators, cilostamide and forskolin can sustain viability of cultured oocytes by promoting cAMP production and gap junction activity.[101] Because

the communication between the oocyte and surrounding granulosa cells is critical for folliculogenesis and oogenesis, future work on in vitro culture of isolated follicles should focus on developing approaches that support the maintenance of the cell-cell communication during long-term incubation.

Somatic cell nuclear transfer and transgenesis

Since the first report on live birth in 2005, there have been several studies on SCNT in domestic dogs.[19,102,103] The protocol developed for the dog has also been applied to gray wolves[104,105] and coyotes,[106] resulting in production of live offspring. Due to the inability to in vitro mature dog oocytes, studies to date have utilized in vivo matured gametes as the recipient cells for a variety of donor cell types, including fetal and adult fibroblasts as well as adipose derived mesenchymal stem cells.[103,107] Despite the low success rate (<5% live birth from numbers of transferred embryos), cloned individuals appear to have normal health and reproductive competence.[103] Specifically, health and reproductive assessment of cloned individuals (n = 3 dogs) revealed that age-related hematological and serum biochemical parameters as well as circulating hormone concentrations of cloned dogs are similar to non-cloned counterparts.103 Furthermore, cloned dog ovaries exhibit morphological changes in the same manner as non-cloned individuals. Finally, live puppies have been produced after AI of cloned females with fresh semen from a cloned male dog.[108]

The successful production of SCNT dog embryos has facilitated the application of transgenesis technology in this species.[109-111] The first transgenic dog that expressed a red fluorescent protein gene was produced in 2009.[18] Since then, there have been several reports on the production of transgenic offspring as models for studying human disorders, including type 2 diabetes[112] and Alzheimer's disease.[113]

Summary and future perspectives

Due to their unique female reproductive characteristics, the development of ARTs in the domestic dog and their wild cousins has proven to be extremely challenging. Nevertheless, substantial progress has been made during the past decade, including the first production of IVF puppies and numerous offspring from SCNT and transgenesis. Recent studies on the influence of reproductive EVs on the function and cryosurvival of dog and wild canid gametes, and utilization of organ-on-a-chip technology in growing dog ovarian cortices and isolated follicles in vitro[114] have provided encouraging results. Such studies will likely provide insights into mechanisms regulating gamete formation and function and generate information useful for development of effective tools to preserve/extend fertility of domestic and wild canids. So far, the domestic dog has served as a valuable model for establishing reproductive technologies in wild canids and dog protocols have been successfully applied to their endangered cousins. Yet, there are still needs for species-specific research due to the enormous diversity in reproductive biology within the family *Canidae*.[10]

Conflict of interest

There are no conflicts of interest to declare.

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