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The domestic dog and cat as models for understanding the regulation of ovarian follicle development *in vitro*

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Abstract

The culture of ovarian follicles is an important tool for understanding of the mechanisms controlling follicle development and differentiation of its oocyte. The benefit of recovering meiotically and developmentally competent oocytes from early stage follicles (primordial, primary, preantral and early antral) also would be significant, ranging from rescue of genomes from endangered species to preserving fertility in women facing cancer treatments. This field of research is at an early stage of scientific discovery. To-date, live offspring from cultured primordial follicles that produced fertilizable oocytes has occurred only in the mouse. Progress in other more complex species has been limited because larger animals have longer durations of natural folliculogenesis, thereby requiring more culture time to generate fully grown follicles and oocytes. We believe the dog and cat are excellent models for understanding more about folliculogenesis *in vitro*. This review highlights what is known about this topic for these two species as well as future priorities. In brief, it is more challenging to maintain viability of primordial follicles within ovarian tissues *in vitro* in the dog than the cat. Nonetheless, it is possible to grow both isolated cat and dog preantral follicles in culture. Although the follicles of both species have the capacity to increase in size and produce steroids, only cat oocytes are morphologically normal. This striking difference between the dog and cat is an area of high research priority. While much more fundamental data are required, we envision advanced technology that will allow harvesting oocytes from the vast, unused follicle stores sequestered within carnivore ovaries. These gametes have utility for reproducing genetically valuable dogs and cats that are ‘companions’ or biomedical models for investigating human disorders or for salvaging the genomes of rare canid and felid species that die before contributing to genetic management programs.

Keywords

ovarian tissues; primordial follicles; secondary follicles; *in vitro* follicle culture

INTRODUCTION

The ability to maintain follicular structure *in vitro* while manipulating the surrounding biochemical and mechanical environment has improved our understanding of the mechanisms regulating the intimate relationship between the ovarian follicle and its maturing and differentiating oocyte (Eppig 2001, Kreeger et al 2005). Most of these pioneering studies have been conducted in the laboratory mouse, establishing, for example,

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that bidirectional communication between the oocyte and surrounding granulosa cells is crucial for follicle and gamete development (Eppig 2001).

Beyond improving fundamental knowledge, this area of research has practical potential. Because the ovary contains thousands of primordial and primary follicles, *in vitro* culture could provide access to enormous numbers of oocytes that, if viable, could be matured and fertilized *in vitro* to produce embryos for offspring production. In general, there are at least two target groups, the most prominent being young women (or girls) whose germplasm is at risk from toxic cancer treatments (Smitz et al 2010). The second includes genetically valuable animals that have failed to reproduce naturally and then suddenly die or must undergo an ovariectomy for medical reasons. For carnivores, there are three possible subgroups that could benefit: 1) companion dogs and cats; 2) special genotypes of these two species used as biomedical models for investigating human diseases; and 3) diverse wild carnivore species that are being managed to create sustainable populations to reduce extinction potential.

Our laboratory conducts folliculogenesis research in both the dog and cat for all of these reasons. We are especially excited about the role of such studies for contributing to human reproductive health and wildlife propagation. For example, for the latter, six of the 36 extant canid and 16 of 37 extant felids are listed formally as threatened by extinction, mainly due to habitat loss, persecution and disease (IUCN 2011). This is one of the primary rationales for attempting to maintain viable populations of rare species *ex situ*, a complex process that can retain the necessary genetic diversity to ensure species integrity (Ballou 1997). However, these intensive management programs require moving animals or, alternatively, germplasm between institutions. The latter approach has been an incentive for adapting assisted reproductive technologies (so successful in humans and livestock) (Comizzoli et al 2010) to selected wildlife species.

But embryo-related strategies have not been used extensively in carnivores. Methods for estrous cycle stimulation and synchrony as well as gamete collection and culture that are so effective in cattle do not readily translate to canids and felids with their own unique (and more complex) reproductive anatomy and physiology. One of the most challenge issues is accessibility to mature oocytes capable of fertilization. For example, the canid ovary is encapsulated in an ovarian bursa that makes it difficult to recover oocytes from preovulatory follicles and the oviduct (Wildt et al 1977; England and Allen 1989). Combined with the challenge that both canids and felids resist (or are exquisitely sensitive) to exogenous gonadotropins given to provoke ovulation (Kutzler 2005, Pelican et al 2010), it is not surprising that embryo technologies currently play a negligible role in carnivore reproductive management.

While conventional embryo strategies remain problematic, we believe it is timely to explore the feasibility of generating viable ova from the vast store of follicles within carnivore ovaries, most of which never ovulate. A prerequisite step to applying *in vitro* follicle culture to practical dog and cat reproduction is learning more about the basics of follicle (and corresponding oocyte) development. Such research is facilitated by easy access to routinely discarded ovariectomy material from veterinary hospitals and spay clinics. Using these freshly excised ovaries, our laboratory has been exploring the impact of differing microenvironments on the ability to grow primordial, pre- and early antral follicles in both species. Although the eventual goal is to secure significant numbers of fertilizable oocytes, current objectives focus mostly on understanding the regulatory elements and timelines for differing stage follicles. Here, we highlight recent advances in both the dog and cat for the purpose of demonstrating not only potential, but the substantial challenges remaining.

***In vitro* culture of primordial follicles within ovarian tissue**

The first *in vitro* culture studies of intraovarian primordial follicles were published in the mouse (Eppig and O'Brien 1996) and cow (Wandji et al 1996) in the mid-1990s. The biological feasibility of the concept (i.e., the ability to nurture such premature follicles to the point of producing viable, fertilizable oocytes) was proven by O'Brien and colleagues (2003) with the production of 72 live mice pups. Since then, there has been progress through studies of other species, including observations of some sustained follicle viability and related oocyte growth in the baboon (Wandji et al 1997), human (Telfer et al 2008) and goat (Matos et al 2010). In these species, primordial follicles have been able to advance to primary and secondary, and in the case of humans, to the antral stage. However, to-date, no offspring have been produced in species other than the mouse.

We are interested in understanding the mechanisms regulating primordial follicle activation in the dog and cat, information crucial for formulating a consistently effective and long-term *in vitro* culture system. The first step has been to begin determining the basic requirements for maintaining living ovarian tissue and corresponding primordial follicles. Briefly, our protocol has involved the recovery of fresh ovaries within 6 hours of excision followed by preparing cortical pieces (1 to 2 mm²) that are cultured on an agarose gel in a 24-well dish at 38.5°C for 14 days (Fujihara et al., in press). Studies to-date have revealed remarkable species-specificity requirements for the *in vitro* microenvironment. For medium preference, α -minimum essential medium (MEM) has been found to sustain the viability of dog ovarian follicles in culture for up to 14 days compared to only 3 days for the cat (Fujihara et al., in press). By contrast, cat ovarian tissue prefers MEM over α -MEM with the former resulting in follicle viability being retained for up to 2 weeks versus only 3 days for the latter. The difference between these two media largely is the much higher amino acid concentration in the α -MEM. Therefore, perhaps the species difference is related to markedly varying amino acid metabolism for dog compared to cat follicles. This conjecture probably is supported by our earlier studies demonstrating species variation in glutamine metabolism patterns for the dog versus cat oocyte (Spindler et al 2000; Songsasen et al 2007).

Our comparative evaluations also have shown that cat ovarian tissue is more amenable to *in vitro* culture than dog ovarian tissue (or conversely dog tissue is more susceptible to degeneration under the described microenvironment conditions). Specifically, with the existing incubation protocols, nearly 40% of cat primordial follicles (n evaluated = 110) exhibit normal morphology 15 days later compared to <5% from the dog (n = 265) (Fujihara et al., in press). Histology also revealed extensive injury (i.e., degeneration of stromal cells) to dog ovarian tissues along with shrunken oocytes. These indicators of degeneration were far less apparent in the cat. It is known that communication between the gamete and surrounding somatic cells is crucial for follicle growth and viability (Picton et al 2008). Therefore, we suspect that the high incidence of apoptosis in the dog is related to an inability of the current system to sustain somatic cell survival that, in turn, diminishes nutrient support to the primordial follicle and oocyte. For this reason, future carnivore studies should examine the value of a bioreactor culture system (Heise et al., 2009) for *in vitro* follicle development. This approach, which involves a suspension system consisting of orbiting test tubes and rotating-wall vessels, enhances nutrient and oxygen transport from the microenvironment into the ovarian tissues and has improved growth and viability of rat preantral follicles (Heise et al 2009). Compared to a conventional static system (similar to what is now used in the dog and cat), the bioreactor approach has increased oxygen and nutrient supplies to rat follicles and allowed resident oocytes to more readily achieve meiosis (Heise et al 2009).

There has been minimal effort at culturing ovarian cortical tissues from other canid or felid species. We have had one opportunity to culture ovarian cortical pieces from a maned wolf (*Chrysocyon brachyurus*) using the static incubation system routinely used in the domestic dog. We determined that the dog microenvironment retained viability for ~30% of the maned wolf primordial follicles ($n = 100$; based on calcein-AM and ethidium homodimer staining) at 9 days of culture (M. Fujihara, unpublished observations). Such opportunistic studies should continue in collaboration with zoos to determine how conserved folliculogenic mechanisms are across species, especially those within the same family.

***In vitro* culture of isolated pre- and early antral follicles**

Compared to primordial counterparts, much more information is available on *in vitro* incubation of isolated, later stage dog and cat follicles. The first such studies were conducted in late 1990s using a two-dimensional culture system (Bolamba et al. 1998, Jewgenow 1998). Bolamba et al. (1998) demonstrated that 35% of dog oocytes recovered from pre- and early antral follicles (cultured for 72 h) resumed meiosis, with the majority arresting at the germinal vesicle breakdown stage. Meanwhile, Jewgenow (1998) studied the domestic cat and, in a comparison of four media, determined that Dulbecco's MEM (DMEM) was superior in sustaining structural integrity of preantral follicles (primordial, primary and secondary stages) through 10 days of incubation. This same investigation also demonstrated that supplementing DMEM with pyruvate and/or lactate had a suppressive influence on DNA synthesis of cat preantral follicles (Jewgenow 1998).

Recent studies in our laboratory have focused on testing the potential of a three-dimensional culture system using alginate hydrogel for supporting growth of isolated pre- and early antral dog follicles. Our findings have revealed that the physical microenvironment (including gel concentration and composition) significantly influences follicle growth and steroidogenesis *in vitro* (Songsasen et al 2011). Specifically, follicles encapsulated in 0.5% alginate grow two times faster than those in a 1.5% concentration, probably because the latter results in conditions that are too physically restrictive for follicle development. Recently, we have determined that a more dynamic alginate-fibrin matrix further enhances dog follicle growth in culture compared to alginate alone (Songsasen et al 2012). In this combination, the degradation of fibrin (by cell-secreted proteases) facilitates more room for follicle expansion, whereas the non-degradable alginate offers adequate physical support to the encapsulated cells (Shikanov et al 2009).

Similar to what has been discovered for the mouse (Kreeger et al 2005) and rhesus macaque (Xu et al 2010), follicle stimulating hormone (FSH) clearly is essential for *in vitro* folliculogenesis in the dog (Songsasen et al, 2011). Follicles cultured in a microenvironment void of FSH grow slower and secrete less estrogen and progesterone than counterparts incubated in the presence of this gonadotropin (Songsasen et al 2011). Luteinizing hormone also accelerates ($P < 0.05$) growth and steroid production in cultured dog follicles in the absence of FSH (Nagashima et al 2010). There appears to be no benefit from combining the two gonadotropins in the incubation system.

Protein supplementation within the culture media also influences dog follicle growth. In one study, dog preantral follicles encapsulated in 0.5% alginate were cultured for 30 days in α -MEM supplemented with 2 mM glutamine + 5.5 μ g/ml insulin + 5.5 μ g/ml transferrin + 6.7 ng/ml selenium + 1 μ g/ml FSH (basic medium). Test media then included the basic with additions of 0.1% (w/v) polyvinyl alcohol (protein-free; $n = 13$ follicles), 10 mg/ml fetuin ($n = 10$), 1% ($n = 10$), 3% ($n = 12$) or 10% ($n = 9$) canine serum (CS). Culturing preantral dog follicles in the presence of 10% CS was detrimental ($P < 0.05$), with growth stopping within 2 days of culture onset. By contrast, all other conditions promoted increased follicle size

over a 7 to 14 day period (Fig. 1). The adverse effects of a high (10%) serum homologous concentration on pre- and early antral follicle survival may be linked to presence of proliferation inhibitors inherent to serum (Harrington and Godman 1980). Meanwhile, the incidence of follicle growth (mean range, 40 - 80%) and overall mortality (mean range, 10 - 40%) over the 30 day experiment were no different ($P > 0.05$) among the protein-free, 10 mg/ml fetuin or 1% versus 3% CS-supplemented media. However, morphology of oocytes recovered from cultured follicles was different. Oocytes incubated in CS-containing medium retained the characteristic, normative dark cytoplasm of the species (Songsasen and Wildt 2007) after 8 days *in vitro* culture; eggs exposed to the other microenvironments were pallid by comparison (Fig. 2). By the end of the 30 day follicle culture, all recovered oocytes were pale in coloration and denuded of cumulus cells. We have interpreted this to mean that homologous CS likely contains key factors essential for lipid metabolism in dog oocytes. Additionally, clearly the current static *in vitro* culture system fails to support intercellular communication between somatic cells and the corresponding oocyte that, in turn, appears to disrupt transportation of nutrients from the granulosa cells to the gamete.

For the cat, we recently have tested the alginate hydrogel culture system using pre- and early antral cat follicles (Fig. 3A), largely to explore the influence of FSH on *in vitro* development. In this study, individual follicles were encapsulated in 0.5% alginate and cultured in MEM supplemented with 2 mM glutamine + 5.5 $\mu\text{g/ml}$ insulin + 5.5 $\mu\text{g/ml}$ transferrin + 6.7 ng/ml selenium + 3 mg/ml bovine serum albumin in the presence ($n = 23$ follicles) or absence ($n = 20$) of 1 $\mu\text{g/ml}$ FSH for 14 days. Like what was observed in the dog, the hormonal microenvironment influenced *in vitro* growth of cat follicles. Specifically, follicles cultured in the presence of FSH grew more quickly ($P < 0.05$) than those incubated without this gonadotropin (Fig. 4). Furthermore, 50% (4/8) of preantral follicles exposed to FSH formed an antral cavity (Fig. 3B) compared to only 25% (3/12) without this gonadotropin. Regardless, with or without FSH, those oocytes that appeared morphologically normal ($n = 13$ per group) increased ($P < 0.05$) in size (Fig. 3C; FSH absent, from $80.6 \pm 4.1 - 91.7 \pm 3.5 \mu\text{m}$; FSH present, from $84.3 \pm 3.7 - 93.8 \pm 3.7 \mu\text{m}$) and displayed normal chromatin structure after 14 days *in vitro* culture (Fig. 3D). These average sizes of oocytes recovered from cultured follicles was comparable to the mean diameter of eggs developing *in vivo* in early antral stage cat follicles ($93.9 \pm 3.5 \mu\text{m}$) (Comizzoli et al 2011).

Although current methods have allowed successful growth (i.e., increased size) of isolated dog and cat follicles *in vitro*, oocyte viability (based on morphology and chromatin structure) is sustained only in the latter. The egg mortality challenge in the dog may be related to how the follicles are isolated. Cat follicles are easily segregated by mechanical microdissection (Jewgenow and Goritz 1995; Comizzoli et al 2011), whereas, due to a more dense cortex, the dog counterpart must first be introduced to enzyme digestion (Songsasen et al 2011). Exposing mouse follicles to a proteolytic enzyme is known to deplete theca cells and degrade the basement membrane, both of which contribute to reduced follicle and oocyte development (Demeestere et al 2002). Alternatively, dog follicles simply may be more sensitive to a non-physiological concentration of FSH in the *in vitro* microenvironment. For example, high FSH dosage in the cow *in vitro* system is known to trigger premature retraction of transzonal projections that subsequent disrupts essential communication between the granulosa cells and oocyte (McLaughlin and Telfer 2010). As the FSH dosage used for *in vitro* incubation of dog follicles has been 1,000 fold higher than 'physiological' (Beijerink et al 2004), it seems prudent that future studies explore a reduced FSH dosage (i.e., $< 10 \text{ ng/ml}$) on *in vitro* dog follicle and oocyte viability.

Conclusion and priorities

Given the challenge of recovering large numbers of mature, fertilizable oocytes from carnivores, especially canids, it makes sense to explore recovering the maternal genome by growing intraovarian follicles in the laboratory. The production of mouse pups from oocytes recovered from cultured primordial follicles provides incentive. However, clearly carnivore reproductive physiology, morphology and function as well as the specific mechanisms driving follicle and oocyte development are more complex than in rodents. Each species also seems to vary significantly in their adaptability to culture methods as well as what regulates folliculogenesis. Nonetheless, early data demonstrates the potential of sustaining both live dog and cat ovarian tissue at least for days if not weeks *in vitro*. However, before becoming a practical propagative and management tool, far more basic research is required. For the dog, near term priorities include identifying an *in vitro* microenvironment that sustains oocyte viability. For the cat, there is a need to focus on determining the explicit paracrine elements that promote expansion of the antral cavity and oocyte growth, especially to the size of eggs normally recovered from preovulatory stage follicles. Finally, both species could benefit from exploring the potential of a dynamic, bioreactor system that permits enhanced exchange of nutrients and gas as well as removal of metabolic wastes.

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Fig. 1.

Percent growth in size (mean $\pm$ SEM) of dog preantral follicles encapsulated in 0.5% (w/v) alginate and cultured in a protein-free medium or the presence of fetuin, 1%, 3% or 10% canine serum. *, ** Different symbols indicate differences in overall follicle growth ($P < 0.05$).

Fig. 2.

Dog preantral follicles (A, C and E) immediately after recovering from the ovary (within 6 hours of excision) and then after 8 day *in vitro* culture in a protein-free (B), fetuin (D) or 1% canine serum supplemented medium. Bar indicates 100 μm .

Fig. 3. Cat follicles (A) immediately after recovery from the ovary; (B) a follicle after 14 days of *in vitro* culture; (C) a cat oocyte recovered from follicle cultured for 14 days; (D) a cat oocyte exhibiting normal nuclear chromatin structure after recovery from a follicle incubated for 14 days. Bar indicates 100 μm .

Fig. 4.

Percent growth in size (mean $\pm$ SEM) of cat pre- and early antral follicles encapsulated in 0.5% (w/v) alginate and cultured in the absence (control) or presence of 1 μ g/ml FSH. *,** Different symbols indicate differences in overall follicle growth ($P < 0.05$).