In vitro growth and steroidogenesis of dog follicles are influenced by the physical and hormonal microenvironment

N Songsasen, T K Woodruff¹ and D E Wildt

Department of Reproductive Sciences, Center for Species Survival, Smithsonian Conservation Biology Institute, 1500 Remount Road, Front Royal, Virginia 22630, USA and ¹Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago 60611, Illinois, USA

Correspondence should be addressed to N Songsasen; Email: songsasenn@si.edu

Abstract

The present study examined the influences of the physical and hormonal microenvironment on in vitro growth and steroidogenesis of dog follicles. Follicles were enzymatically isolated and individually encapsulated in 0.5% (w/v; n = 17) or 1.5% (n = 10) alginate and cultured with 0.5 IU/ml equine chorionic gonadotropin for 192 h. Follicle diameter and steroid production were assessed every 48 h in both studies. Follicles encapsulated in the 0.5% alginate grew faster (P < 0.05) than those cultured in the 1.5% alginate. Oestradiol (E₂) and progesterone (P₄) increased consistently over time in both studies. Follicles cultured with 0 (n = 22), 1 (n = 23), 10 (n = 20) or 100 (n = 21) µg/ml FSH for 240 h. Follicle diameter and steroid production were assessed every 48 h in both studies. Follicles encapsulated in the 0.5% alginate grew faster (P < 0.05) than those cultured in the 1.5% alginate. Oestradiol (E₂) and progesterone (P₄) increased consistently over time in both studies. Follicles cultured with 0 (P < 0.05) P₄ than those in the 0.5% solution. Follicles cultured in the highest FSH concentration (100 µg/ml) increased 100% in size after 240 h compared with 50 to 70% in lower dosages. E₂ concentration remained unchanged over time (P > 0.05) across FSH dosages. However, P₄ increased (P < 0.05) as culture progressed and with increasing FSH concentration. Results demonstrate that dog follicles cultured in alginate retain structural integrity, grow in size and are hormonally active. Lower alginate and increasing FSH concentrations promote in vitro follicle growth. However, the absence of an E₂ rise in follicles cultured in FSH alone suggests the need for LH supplementation to support theca cell differentiation and granulosa cell function.

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Introduction

The ability to successfully culture whole ovarian follicles in vitro and to recover viable oocytes has potential for preserving the reproductive capability of women (Smitz et al. 2010) and for rescuing the genome of rare genotypes or even endangered species that die unexpectedly (Comizzoli et al. 2010). Basic studies in this area also can enhance our understanding of the dynamic, spatio-temporal regulation of folliculogenesis, including the pathways for cellular signals regulating this process (Woodruff & Shea 2009). There have been significant advances over the last 2 decades in establishing an in vitro culture system for ovarian follicles, especially in the mouse (Eppig & Schroeder 1989, Eppig & O’Brien 1996, Eppig et al. 1998, Smitz & Cortvrindt 2002, Kreeger et al. 2005, Xu et al. 2006a, 2006b, West-Farrell et al. 2009). Live births have resulted from embryos produced by in vitro maturation and fertilisation of oocytes derived from mouse follicles grown in vitro (Eppig & Schroeder 1989, Eppig et al. 1996, Cortvrindt et al. 1998, Dela Pena et al. 2002, Xu et al. 2006a). Although attempted in the cow (Gutierrez et al. 2000, Itoh et al. 2002, Thomas et al. 2007, McLaughlin & Telfer 2010), goat (Figueiredo et al. 2011, Magalhães et al. 2011), sheep (Arunakumari et al. 2010), pig (Mao et al. 2002, Wu & Tian 2007, Wu et al. 2007), buffalo (Gupta et al. 2008), cat (Jewgenow & Paris 2006), non-human primate (Xu et al. 2009b, 2011) and human (Xu et al. 2009a, Telfer et al. 2008), live offspring have not been produced from in vitro grown follicles. To date, there are few studies in carnivore species on the responsiveness of ovarian follicles to in vitro culture (Bolamba et al. 1998, Jewgenow & Paris 2006, Serafin et al. 2010).

Original studies conducted in the mouse involved a two-dimensional (2D) culture system, where pre-antral follicles are placed on plastic surfaces coated with collagen or polylysine (Eppig & Schroeder 1989, Eppig & O’Brien 1996). More recently, Telfer et al. (2008) reported that human pre-antral follicles cultured within a V-shape microwell plates in the presence of activin were able to develop to the antral stage. Nevertheless, in the 2D approach, the granulosa cells usually migrate, causing disrupted communication with the adjacent oocyte that, in turn, compromises gamete development (Oktem & Oktay 2007, West et al. 2007a). More recently, a three-dimensional (3D) system has provided encouraging results (Xu et al. 2006a, 2009a, 2009b, 2011),...
especially in large mammals, including the buffalo (Gupta et al. 2008), non-human primate (Xu et al. 2009b, 2011) and human (Xu et al. 2009a). This approach consists of culturing follicles in a collagen gel matrix (Oktem & Oktay 2007, Sharma et al. 2009) or alginate hydrogels (Kreeger et al. 2006, Xu et al. 2006a, 2009a, 2009b, 2011) that physically retain the follicular unit and the cell-to-cell interaction while supporting pre-antral follicle growth, antral formation and steroidogenesis.

It is known that the physical properties of alginate itself can influence the follicle’s in vitro capacity to develop (Xu et al. 2006b, West et al. 2007b). Specifically, low gel concentrations have less rigidity and appear more permissive for allowing normative follicle growth and steroidogenesis (Xu et al. 2006b, West et al. 2007b). Nonetheless, optimal alginate concentration appears to be species specific (e.g. 0.25% in the mouse (Xu et al. 2006b) versus 0.5% in the non-human primate (Xu et al. 2009b)). In the mouse system, FSH is also known to regulate pre-antral follicle growth and steroidogenesis in vitro in a dose-dependent fashion (Kreeger et al. 2005, Xu et al. 2009b, Figueiredo et al. 2011). Yet, there are stage-specific responses of pre-antral follicles cultured in alginate to FSH stimulation (Kreeger et al. 2005). For example, >70% of two-layered, secondary mouse follicles survive in the absence of FSH compared with only 40% of the multi-layered counterparts. Moreover, LH is not required for in vitro follicle development in the mouse or non-human primate (as would be predicted by the two-cell, two-gonadotropin hypothesis (Liu & Hsu 1986, Hillier et al. 1994)).

The dog is a particularly challenging model for follicle and oocyte studies. To date, in vitro embryo production using cultured oocytes has been unsuccessful, mostly due to the unique reproductive biology of the species (Songsasen & Wildt 2007). The dog, and canids in general, are distinctive in that the female unpredictably enters oestrus generally only once and rarely more than twice annually and at a 5–12 months interval (Concannon et al. 1989, Concannon 2009). The cycle is notable for a protracted prooestrous of 3 days to 3 weeks (average, 1 week) with elevated circulating oestrogen (Wildt et al. 1978, 1979, Concannon et al. 1989, Concannon 2009) followed by oestrus that lasts from 2 to 3 days to 3 weeks or more (Concannon et al. 1989). With or without pregnancy, oestrus is followed by dioestrus, a luteal phase of elevated circulating progesterone (P_4) averaging ~2 months. Toward the end of dioestrus, corpus luteum function declines as the bitch enters a prolonged anoestrus (~2 to 10 months) characterized by lack of gonadal activity (Concannon et al. 1989). It is also known that the dog oocyte acquires meiotic competence intrafollicularly when the host follicle is >2 mm in diameter (Songsasen & Wildt 2005). Ovaries from anoestrous or dioestrous females contain mostly pre- and early-antral follicles of <0.5 mm in diameter, whereas larger antral follicles are apparent only during prooestrus or oestrus (Songsasen & Wildt 2005). While less studied, there is general consensus that there is prolonged ovarian quiescence in wild canids, most of which are seasonal, monoestral species (Wildt et al. 2009). As a result, small follicles containing meiotically incompetent oocytes are the norm for canids, meaning that each ovary contains tens of thousands of oocytes that never become available for reproduction.

Our ultimate interest is in the ability to rescue oocytes (and thus the maternal genome) from canid pre- and early-antral follicles that never naturally develop and ovulate in situ. The capacity to recover the maternal genome would offer enormous opportunities for the enhanced propagation of domestic dogs used as models for examining human diseases (Schneider et al. 2008), as well as for improved management and conservation of wild canids, of which 7 of 36 species are officially listed as threatened with extinction (IUCN Red List of Threatened Species, version 2010.3, http://www.iucnredlist.org. Downloaded on 23 October 2010). However, a prerequisite to these applied benefits is a better understanding of folliculogenesis in the domestic dog model – in this case, via 3D in vitro follicle culture using alginate hydrogels. Our specific objectives were to examine the influence of 1) alginate rigidity and 2) FSH concentration in the microenvironment on dog follicle survival, growth and steroidogenesis.

**Results**

**Study 1: influence of alginate rigidity**

All follicles retained a 3D structure (Fig. 1B). Overall, follicle survival was unaffected (P>0.05) by the alginate concentration with 12 of 17 (70.6%) follicles retaining viability after 192 h in 0.5% concentration versus 8 of 10 (80%) in the 1.5% solution. Despite an increasing trend over time, overall follicle sizes at culture onset (226.7±21.4 μm for those in 0.5%; 290.0±28.7 μm for 1.5%) and after 192 h (377.3±30.5 μm; 393.5±52.0 μm, respectively) were not different (P>0.05) between treatment groups. However, follicles in the lower alginate concentration grew faster (P>0.05) than those cultured in the higher concentration; the former increased 70% in overall size within 96 h compared with 50% by 96 h in the higher alginate solution (Fig. 2). Oestrogen concentrations increased over time in both culture solutions of alginate (Fig. 3). Although the oestradiol (E_2) production pattern in vitro was comparable (P>0.05; Fig. 3A), follicles encapsulated in 1.5% alginate produced ~5–10 fold more P_4 (P<0.05) than those cultured in the 0.5% solution (Fig. 3B).
Study 2: influence of FSH concentration

The overall mean follicle size at culture onset was 218.2 ± 21.6, 231.8 ± 21.6, 205.0 ± 22.9 and 204.4 ± 10.1 mm for 0, 1, 10 and 100 μg/ml FSH, respectively (P > 0.05). There were no subsequent differences among FSH treatment groups (P > 0.05) in follicle survival in vitro (20/22 for 0 μg; 19/23 for 1 μg; 15/20 for 10 μg; 14/21 for 100 μg). It was confirmed that all follicles judged as ‘survivors’ (via the light microscopy) fluoresced green of calcein AM (Fig. 1C) at the end of culture period; thus, these follicles were considered viable. FSH concentration influenced (P < 0.05) the kinetics of follicular growth. Follicles cultured in the highest FSH concentration (100 μg/ml) increased 100% in size after 240 h compared with a 50–70% increase in size in lower dosages over the same culture period (P < 0.05; Fig. 4). Patterns of E₂ remained static and at baseline over time (P > 0.05) regardless of FSH concentration (Table 1). In contrast, FSH supplementation enhanced (P < 0.05) P₄ production, and in a dose-dependent fashion, with the highest concentrations occurring in the 10 and 100 μg groups (Table 1).

Discussion

The paucity of information on folliculogenesis in canids, including the domestic dog, is related to unique, taxon-specific characteristics associated with prolonged periods of reproductive shutdown followed by unpredictable onset of variable, yet protracted periods of ovarian activity. The trigger for provoking a quiescent dog ovary to grow its follicles to the antral stage remains unknown, although it must be complex given the many historical failures at reliably stimulating oestrus/ovulation using exogenous hormonal therapies (Kutzler 2007). Studying the canid ovary in situ is also complicated by anatomical challenges, especially an encapsulating ovarian bursa that prevents access to the gonad, for example, to monitor follicle growth or recover oocytes (Wildt et al. 1977, England & Allen 1989, England et al. 2009). These complexities mean that it is essential to identify alternative approaches for studying the dog follicle, most likely via an in vitro system. We were encouraged that the follicle culture system developed for the mouse (Xu et al. 2006a), macaque monkey (Xu et al. 2009b) and baboon (Xu et al. 2011) relying on the alginate hydrogel also showed good results for the dog. Specifically, encapsulated pre- and early-antral dog follicles survived in vitro for 10 days while maintaining an inherent spherical architecture, increasing in size 75 to 100% and being hormonally active. Our investigation

Figure 1 Dog pre-antral follicles and enclosed oocytes immediately after isolation from the ovary (A) and 192 h after in vitro culture in alginate (B). A viable follicle stained with calcein AM/ethidium homodimer-1 after 240 h of in vitro culture (C). Note that follicles with an oocyte centrally located and surrounded by complete layers of granulosa cells (arrow) were cultured.

Figure 2 Percent growth in size (mean ± S.E.M.) of dog pre- and early-antral follicles encapsulated in 0.5% or 1.5% (w/v) alginate and cultured in vitro for 192 h. a,b,c,d,e Different superscripts indicate significant differences among time points within each treatment (P < 0.05).
also revealed the significance of the physical and hormonal microenvironment. Specifically, alginate appeared to exert its influence via follicular growth, and dog follicles responded to FSH in a dose-dependent fashion.

Pre-antral follicles can be isolated from the ovary by either mechanical isolation or enzymatical digestion (Picton et al. 2006). Mechanical isolation is a time-consuming process; however, the follicles recovered by this method maintain their structurally integrity (i.e. contain the basement membrane and theca cells; Picton et al. 2006). By contrast, enzymatical digestion is less laborious, but exposure of follicles to a proteolytic enzyme also results in the removal of theca cells and degradation of the basement membrane (Demeestere et al. 2002, Picton et al. 2006). However, it has been shown in the mouse that theca cells or their precursors are still present after enzymatic isolation, and these cells are able to develop, differentiate and secret androgen (Dolmans et al. 1998, Durrant et al. 2007). Nevertheless, one potential pitfall of the 3D system is that as follicles continue to grow in vitro, oxygen and nutrients may not be readily accessible to the oocyte, which in turn disrupts gamete development (Desai et al. 2010). After being developed initially in the mouse (West et al. 2007a), the 3D system relying on alginate has been shown to support follicle growth and differentiation in macaque monkey (Xu et al. 2009b) and human (Xu et al. 2009a) follicles for as long as 4 weeks. Recently, this system has been employed to grow pre-antral follicles of the baboon, which resulted in the production of meiotically competent oocytes (Xu et al. 2011). These observations have been important as primates have protracted (90 to 150 days) folliculogenesis, with follicles failing consistently to develop in a 2D culture environment (West et al. 2007a). However, the 3D alginate approach has produced primate follicles that form an antrum and are capable of producing steroids (Xu et al. 2009b) and meiotically competent oocytes (Xu et al. 2011). We encountered similar findings in the dog. In a preliminary study, we cultured 20 dog pre-antral follicles in a 2D system, and all degenerated within 24–48 h (N Songsasen, unpublished data). In contrast, a high proportion of the same stage follicles exposed to the described 3D culture environment thrived for at least

Figure 3 Oestradiol-17β (A) and progesterone (B) profiles (mean ± S.E.M.) for pre- and early-antral dog follicles encapsulated in 0.5 or 1.5% (w/v) alginate and cultured for 192 h. Different superscripts indicate significant differences among time points within each treatment (P<0.05). *Symbol indicates statistical differences between alginate concentrations within each culture period.

Figure 4 Percent growth in size (mean ± S.E.M.) of dog pre- and early-antral follicles encapsulated in 0.5% (w/v) alginate and cultured in medium supplemented with 0, 1, 10 or 100 µg/ml FSH for 240 h. Follicles cultured with 100 µg/ml FSH grew faster (P<0.05) than those cultured in the absence of this gonadotropin. With the exception of the 0 µg/ml FSH treatment, all follicles increased (P<0.05) in size as culture period progressed.
10 days, were sustained in a spherical conformation and had the capacity to produce ovarian steroids.

We determined that the lower percentage solution (0.5%) promoted a faster rate of dog follicle growth than the higher (1.5%) concentration. This finding was consistent with earlier studies of the mouse where lower gel concentrations (0.25 and 0.5%) enhanced pre-antral growth and differentiation and improved developmental competence of the resident oocyte over higher alginate (1.0 and 1.5%) solutions (Xu et al. 2006b). The advantage of a reduced gel concentration has been explained on the basis of the mechanical properties of this biomaterial. That is, a lower alginate concentration confers less rigidity and is more elastic, thereby allowing more ready follicle expansion and differentiation (West et al. 2007b). Furthermore, decreasing solid concentrations of alginate increases diffusion rate and allows macromolecules in the culture medium to easily pass through, becoming more accessible to the follicle (West et al. 2007a). Nevertheless, the optimal alginate dosage appears dependent on developmental stage of the targeted follicle as well as species (Xu et al. 2006b, 2009b). For example, live mice have been produced from IVF oocytes derived from multi-layered secondary follicles (150–180 μm) that were cultured in 1.5% alginate, a concentration that fails to support smaller (100–130 μm) follicles (Xu et al. 2006a). In the nonhuman primate model, a 0.5% concentration appears more appropriate than 0.25% for sustaining the survival, growth and maturation of secondary follicles (100–300 μm; Xu et al. 2009b). Therefore, it may well be that larger size follicles require higher alginate concentrations to achieve a greater rigidity needed to support follicular architecture, while still permitting size expansion. Nonetheless, there are interesting species differences in inherent tissue firmness as demonstrated by less flexibility in the ovarian stroma of the primate ovary compared with that of the mouse; therefore, the former is more likely to prefer a higher alginate concentration to mimic in vivo conditions (Xu et al. 2009b). Dog follicles, as evaluated in the present study, are larger (averaging 200–300 μm), and the ovarian stroma of this species is much more rigid than the mouse (Tesoriero 1984). Therefore, it is legitimate to predict that an alginate concentration <0.5% would be ineffective in promoting the levels of follicle survival and growth (>70%) we observed at 0.5%. Certainly, there are extensive opportunities to examine the influence of altering the physical properties of the microenvironment on dog in vitro folliculogenesis. For example, others have tested an alternative matrix by combining alginate and collagen (Kreeger et al. 2006) or, more recently, an alginate–fibrin mixture (Shikanov et al. 2009), the latter giving a 15% advantage over alginate alone for in vitro grown oocytes to achieve nuclear maturation.

**Table 1** Oestradiol-17β and progesterone concentrations (mean±S.E.M.) for pre- and early-antral dog follicles encapsulated in 0.5% (w/v) alginate and cultured in medium supplemented with 0, 1, 10 or 100 μg/ml FSH for 240 h.

<table>
<thead>
<tr>
<th>Culture periods (h)</th>
<th>Oestradiol-17β (ng/ml)</th>
<th>Progesterone (ng/ml)</th>
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<td></td>
<td>FSH concentrations (μg/ml)</td>
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<td></td>
<td>0</td>
<td>1</td>
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<tr>
<td>48</td>
<td>0.07±0.00</td>
<td>0.61±0.42</td>
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<tr>
<td>96</td>
<td>0.24±0.11</td>
<td>0.14±0.06</td>
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<tr>
<td>144</td>
<td>0.19±0.07</td>
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<tr>
<td>192</td>
<td>0.34±0.18</td>
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<tr>
<td>240</td>
<td>0.08±0.00</td>
<td>0.10±0.03</td>
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a,b,cDifferent superscript letters indicate statistical differences among culture periods within each treatment (P<0.05). *Different symbols indicate significant differences among FSH concentrations within each culture period (P<0.05).

FSH is critical for the follicle growth starting from mid/late pre-antral stage as this gonadotropin promotes granulosa cell proliferation and enhances gap junction formation in the somatic cells (McGee & Hsueh 2000, van den Hurk & Zhao 2005, Kreeger et al. 2005, Knight & Glister 2006). Our observations that supplemental FSH exerted a positive influence on dog follicle growth
and steroidogenesis *in vitro* and in a dose-dependent manner were important, again because little attention has been directed at the role or patterns of this gonadotropin in this species. Our finding is consistent with that of Serafim et al. (2010) who demonstrated that sequential addition of FSH (from low to high concentrations) to culture medium promotes survival, growth, and antrum formation of dog pre-antral follicles. It is known that circulating FSH rapidly increases during the last 60 days of the anoestrus (Concannon 2009), which coincides with increasing numbers of small follicles (1–3 mm in diameter) in the ovaries (England et al. 2009). Furthermore, FSH receptor is detectable in granulosa cells of pre-antral dog follicles (Saint-Dizier et al. 2008). Therefore, although other empirical evidence is lacking, one could reliably predict that this gonadotropin plays an essential role in recruiting a cohort of small follicles to develop to the antral, preovulatory stage (Concannon 2009). Our study supported this assertion in that pre- and early-antral dog follicles cultured in medium containing a comparatively high FSH concentration (100 µg/ml) grew at least 30–50% faster than in the absence of FSH or at a very low (1 µg/ml) dosage. Furthermore, our repeated microscopic assessments clearly revealed a mass increase in these follicles (Fig. 1B). In this context, the dog was similar to a host of other mammals, including the mouse (O’Brien et al. 2003, Kreeger et al. 2005), goat (Matos et al. 2007, Lima-Verde et al. 2010), cow (Gutierrez et al. 2000, Itoh et al. 2002) and macaque monkey (Xu et al. 2009b).

When follicles in FSH alone were evaluated for steroidogenesis, it appeared that P₄ production was FSH dependent, whereas overall E₂ concentrations were low compared with the level assessed in Study 1 (in the presence of chorionic gonadotropin (eCG)). This difference was no doubt due to the use of eCG as a gonadotropin surrogate in Study 1 compared with the use of a purified FSH in Study 2 (the experiment designed specifically to evaluate the influence of this explicit gonadotropin). eCG is well known to exert both FSH- and LH-like activities (Stewart & Allen 1981). Therefore, the significant E₂ production in Study 1 was likely due to the significant presence of LH activity (from the eCG) that allowed converting P₄ into androgen and, thus, into E₂. In *vivo* studies in the dog have demonstrated that termination of anoestrus coincides with increased frequency of high-amplitude LH pulses that probably stimulate the synthesis of precursor androgen for E₂ production (Concannon 2009). Because the amplitude of LH pulse increases >300 fold above baseline 7 to 14 days before proestrus onset, it has been suggested that LH also may play a role in selection of dominant (and eventually ovulatory) follicles (Concannon 2009). Therefore, in planning our next research steps, we predict value in supplementing our dog follicular culture with exogenous LH, especially given its proven ability to enhance developmental competence of oocytes recovered from incubated pig follicles (Wu et al. 2007). In that study, oocytes recovered from pre-antral follicles cultured in the presence of FSH and LH developed to blastocyst at higher rates (23%) than those cultured in FSH alone (14%).

Although we have demonstrated that dog follicles increase in size and produce steroids, the microenvironment described in the present study does not support development of the enclosed oocytes. Most oocytes obtained from the cultured follicles were denuded or pale in colour instead of containing dark cytoplasmic lipid, a characteristic of a fully grown gamete (data not shown; Durrant et al. 1998, Songsasen & Wildt 2007). This indicates that the oocytes either had not completed their growth and/or lost the association with the surrounding granulosa cells. Therefore, future studies should include identifying factors that promote *in vitro* oocyte growth. It has been shown that activin promotes *in vitro* growth of cattle and human oocytes (Telfer et al. 2008, McLaughlin & Telfer 2010). Specifically, oocytes obtained from pre-antral follicles cultured in the presence of this growth factor were morphologically normal (having spherical shape) and larger than those grown in the absence of activin.

To date, several strategies of female fertility preservation have been explored. These include ovarian tissue cryopreservation and/or transplantation, as well as *in vitro* follicle culture (within a cortical piece or isolated follicles; Comizzoli et al. 2010). Transplantation of cryopreserved ovarian tissues had been considered as a viable approach to restore fertility in women suffering from premature ovarian failure associated with cancer treatment (Donnez et al. 2006). However, this approach carries the risk of reintroducing cancer to the patient when the tissues are transplanted back to the donor (Shaw & Trounson 1997). Xenotransplantation of cryopreserved ovaries or cortical tissues to immuno-deficient animal hosts has been suggested as an alternative approach for preservation of female gametes. In addition to the human, this strategy has also been used in a wide variety of mammalian species, including the pig, dog, cat and cow (see review Paris et al. (2004) and Bols et al. (2010)). While recruitment of primordial follicles can be achieved, there has been limited success in terms of development of antral follicles after transplantation, largely due to inappropriate communication between the host’s hypothalamic–pituitary glands and the grafted tissue (Bols et al. 2010). Thus, *in vitro* follicle culture has emerged as an exciting strategy because this technique circumvents the need for an appropriate animal host, and allows basic research to be conducted in a way that will greatly enhance our understanding of folliculogenesis, especially in large mammalian species.

In summary, the 3D hydrogel culture system previously applied to ovarian follicles of the mouse, macaque monkey and human sustained the pre- and
early-antral dog follicle for 10 days while promoting growth and steroidogenesis. Concentration of the alginate component affected success, probably via rigidity tolerance and/or expansion needs of the follicle. Supplemental FSH in the microenvironment appeared essential to optimizing growth and (at least) P₄ secretion. The lack of E₂ responsiveness in the presence of FSH alone suggested that the dog follicle likely required LH for complete theca cell differentiation and normal steroidogenesis. This system has abundant potential for understanding the fundamental, complex regulation and cellular signalling pathways of follicular development. While applicable across an array of mammalian species, this approach appears especially informative for species like the dog, where ovarian follicle activity is triggered erratically and then proceeds over protracted intervals like the dog, where ovarian follicle activity is triggered erratically and then proceeds over protracted intervals before ending in ovulation. Besides generating scholarly information, there are enormous practical benefits for having the ability to provoke hundreds of normally ‘lost’ follicles to grow and develop in vitro. For example, we could envision laboratories dedicated to rescuing follicles from ovaries removed expeditiously from valuable animals that die unexpectedly or undergo ovarioectomies for medical reasons. As sperm cryopreservation has become routine for many mammals, including a diversity of carnivores (Goodrowe et al. 2000, Crosier et al. 2006, Thiangtum et al. 2006, Asa et al. 2007), viable oocytes recovered from cultured follicles could be used to generate embryos in vitro. This capacity would enhance the management of precious genotypes representing dogs used as models for studying human disorders (Schneider et al. 2008) or the conservation of a growing list of wild canid species threatened in nature (IUCN Red List of Threatened Species, version 2010.3, http://www.iucnredlist.org. Downloaded on 23 October 2010). The next step priority for advancing this concept for the Canidae is optimizing culture condition to allow the ovarian follicle to survive long term (likely 30–60 days) to allow producing a developmentally competent oocyte.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise indicated.

Sources of follicles

Follicles were recovered from the ovaries of dogs (age 6 months to 5 years) undergoing routine ovariohysterectomy at local veterinary clinics. Freshly excised ovaries were transported at room temperature (≈22 °C) to the laboratory within 4–6 h of surgery in PBS medium supplemented with 10 µg/ml penicillin G sodium and streptomycin sulphate.

Follicle isolation, encapsulation and culture

Ovarian cortical slices (1 mm thick) were excised from the ovarian surface, cut into 1–2 mm width pieces and digested in 0.7 IU/ml liberase (Roche Applied Science, Indianapolis, IN, USA) at 38 °C for 75 min and individual pre- and early-antral follicles isolated. Only follicles containing a spherical oocyte(s) with homogeneous cytoplasm centrally located and surrounded by completed granulosa cell layers enclosed in the basement membrane were included in the study (Fig. 1A). Each follicle was encapsulated in a bead of alginate solution as previously described (Xu et al. 2006b) with the first step being transfer into a 2–3 µl droplet of 0.5 or 1.5% (w/v) alginate on a propylene mesh (0.1 mm opening; McMaster-Carr, Atlanta, GA, USA). Droplets were immersed in a solution containing 50 mM CaCl₂ and 140 mM NaCl, and the alginate beads were allowed to cross-link in the calcium solution for 2 min and then rinsed for 10 min in α-MEM (Irvine Scientific, Santa Ana, CA, USA) supplemented with 3 mg/ml BSA, 1 mg/ml bovine fetuin and 2.5 µg/ml insulin–transferrin–selenium (hereafter designated as ‘growth medium’). Each encapsulated follicle was cultured in a 100 µl droplet of growth medium supplemented with appropriate gonadotropin concentrations (see below) at 38.5 °C in 5% CO₂ in humidified air for up to 192–240 h. Half the medium was exchanged every 48 h, and the conditioned medium was stored at −80 °C until shipped to the laboratory for hormonal analysis (see below).

Assessment of follicle survival and growth

Follicle survival and diameter were measured every 48 h using an inverted microscope (Leitz DM-IL, Research Instrument Limited, Falmouth, Cornwall, UK) equipped with a heated stage and a calibrated optical micrometer. A follicle was categorized as degenerate (i.e. did not survive in vitro culture) when 1) it continually decreased in diameter (from that measured on previous cultured days), 2) it contained no (or a degenerate) oocyte and/or 3) the granulosal cells were dark and fragmented (Xu et al. 2009b). Each follicle was sized from the outer layer of the somatic cells, and the measurements included the widest diameter and the perpendicular width to the initial assessment. The mean of these two metrics was calculated and reported as ‘follicle diameter’. In Study 2, at the end of the culture period, a proportion of follicles (both somatic cells and oocyte) was evaluated for viability using calcein AM/ethidium homodimer-1 staining (Invitrogen, Carlsbad, CA, USA; Santos et al. 2008). Follicles were considered viable when the oocyte and surrounding granulosal cells fluoresced green of calcein AM.

Evaluation of steroid production

Evaluation of ovarian steroid production was performed at the Endocrine Technology and Support Laboratory, Oregon National Primate Research Center/Oregon Health & Science University. Validated RIAs were used to detect E₂ and P₄ content after the two steroids were separated and purified by extraction-chromatography using previously described methods (Rasmussen et al. 1984). Briefly, a 50 µl aliquot of

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medium used for culturing each dog follicle was aspirated at defined time intervals (see individual studies below), stored frozen, later thawed and then extracted with 5 ml of redistilled diethyl ether. Extractant was dried under an air stream, reconstituted in 200 μl hexane:benzene:methanol mixture (62:20:13) and subjected to chromatography on 1.0 g Sephadex LH-20 (GE Healthcare, Pittsburgh, PA, USA) in glass columns to isolate E₂ and P₄. Collected fractions were dried, dissolved in assay buffer (0.1% gel-PBS) and assayed for each steroid. Assay sensitivity was ~ 3 and 5 pg/tube for E₂ and P₄, respectively. Hormonal values were corrected for extraction and chromatography losses (~70% recovery). The intra- and inter-assay variation were <10 and 15%, respectively.

Experimental design

Study 1: influence of alginate rigidity

Pre- and early-antral follicles (100–500 μm diameter) were obtained from three dogs (age: 2–5 years; reproductive cycle: anoestrus, dioestrus and pro-oestrus) and randomly distributed and encapsulated in 0.5% (n = 17) or 1.5% (n = 10) alginate. Each was then separately cultured in 100 μl of growth medium supplemented with 0.5 IU/ml equine eCG (eCG as a source for both FSH and LH) in 5% CO₂ at 38.5°C for 48 h. Follicle survival, growth and steroidogenesis were assessed every 48 h (as described above). The experiment consisted of three replicates.

Study 2: influence of FSH concentration

Pre- and early-antral follicles (150–400 μm) were obtained from ten dogs (6 months to 2 years; prepubertal, diestrus and anestrus) and capsulated in 0.5% alginate and cultured in 100 μl of growth medium that was supplemented with 0 (n = 22), 1 (n = 23), 10 (n = 20) or 100 (n = 21) μg/ml FSH (Folltropin-V, Bioniche Animal Health, Belleville, ON, Canada) for 240 h. Follicle growth was assessed every 48 h. Culture medium was collected every 48 h from 11 follicles of each treatment group and assessed for E₂ and P₄ production (as described above). A proportion of follicles was evaluated from viability at the end of culture period. Six replicates were included in this experiment.

Statistical analysis

All data were expressed as mean ± S.E.M. Follicle survival among groups was compared using the χ² test (SigmaStat, SPSS, Inc., Chicago, IL, USA). Proportional data indicative of developmental rate were subjected to arcsine transformation. Two-way ANOVA followed by Holm–Sidak multiple comparisons were performed to determine the effect of treatments (i.e. alginate or FSH) and culture periods on follicle growth and steroid production. Growth rate and steroid production of follicles within each alginate or FSH group were also compared among in vitro culture periods using a repeated, measured ANOVA followed by Holm–Sidak multiple comparison. The level of significance was set at 95%.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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