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

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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. In a separate experiment, follicles were encapsulated in 0.5% alginate and cultured with 0 ($n=22$), 1 ($n=23$), 10 ($n=20$) or 100 ($n=21$) $\mu\text{g/ml}$ 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% concentration. Oestradiol (E_2) and progesterone (P_4) increased consistently ($P<0.05$) over time, and follicles in the 1.5% alginate produced more ($P<0.05$) P_4 than those in the 0.5% solution. Follicles cultured in the highest FSH concentration (100 $\mu\text{g/ml}$) increased 100% in size after 240 h compared with 50 to 70% in lower dosages. E_2 concentration remained unchanged over time ($P>0.05$) across FSH dosages. However, P_4 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 E_2 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, Serafim *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 & Hsueh 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 proestrus 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 (P₄) 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 proestrus 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, monoestrous 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 ($226.7\pm21.4\text{ }\mu\text{m}$ for those in 0.5%; $290.0\pm28.7\text{ }\mu\text{m}$ for 1.5%) and after 192 h ($377.3\pm30.5\text{ }\mu\text{m}$; $393.5\pm52.0\text{ }\mu\text{m}$, 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 (E₂) production pattern *in vitro* was comparable ($P>0.05$; Fig. 3A), follicles encapsulated in 1.5% alginate produced 5–10 fold more P₄ ($P<0.05$) than those cultured in the 0.5% solution (Fig. 3B).

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.

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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$) 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 E_2 remained static and at baseline over time ($P > 0.05$) regardless of FSH concentration (Table 1). In contrast, FSH supplementation enhanced ($P < 0.05$) P_4 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 2 Percent growth in size (mean $\pm$ 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 ($P < 0.05$).

Figure 3 Oestradiol-17β (A) and progesterone (B) profiles (mean $\pm$ 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. ^{a,b,c,d} 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.

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 enzymatic digestion (Picton *et al.* 2006). Mechanical isolation is a time-consuming process; however, the follicles recovered by this method maintain their structural integrity (i.e. contain the basement membrane and theca cells; Picton *et al.* 2006). By contrast, enzymatic 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 secrete androgen (that aromatized into estrogen) within 4 days of *in vitro* culture (Demeestere *et al.* 2002). Enzymatic isolation employing collagenase and DNAse has been successfully used to isolate late pre-antral and early-antral follicles of the dog (Bolamba *et al.* 1998, Durrant *et al.* 1998). In the present study, we utilized liberase to isolate dog follicles. It has been shown that liberase is better than collagenase in terms of maintaining normal morphology and oocyte–granulosa cell interaction in isolated primordial and primary human follicles (Dolmans *et al.* 2006). In the present study, we did not observe a rapid disruption of the basement membrane during *in vitro* culture as that previously described in

enzymatically isolated mouse follicles (Demeestere *et al.* 2002), suggesting that degradation of the follicle basement membrane is minimal in our system. Secretion of E_2 by cultured dog follicles suggests that enzymatic isolation employed in the present study did not impact theca cell development.

The 3D approach allows follicles to maintain structure, cell-to-cell contact and cell-to-extracellular matrix interactions (West *et al.* 2007a). Such characteristics are essential because maintaining bidirectional communication between the oocyte and the surrounding granulosa cells to permit exchanges of endocrine and paracrine factors is the prerequisite for normal folliculogenesis (Eppig *et al.* 1996). 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 4 Percent growth in size (mean $\pm$ 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.

Table 1 Oestradiol-17 β and progesterone concentrations (mean $\pm$ 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 μ g/ml FSH for 240 h.

Culture periods (h)	Oestradiol-17 β (ng/ml)				Progesterone (ng/ml)			
	FSH concentrations (μ g/ml)				FSH concentrations (μ g/ml)			
	0	1	10	100	0	1	10	100
48	0.07 $\pm$ 0.00	0.61 $\pm$ 0.42	0.13 $\pm$ 0.03	0.18 $\pm$ 0.08	0.12 $\pm$ 0.02	0.13 $\pm$ 0.03 ^a	0.30 $\pm$ 0.11	0.21 $\pm$ 0.06 ^a
96	0.24 $\pm$ 0.11	0.14 $\pm$ 0.06	0.13 $\pm$ 0.03	0.14 $\pm$ 0.07	0.10 $\pm$ 0.00*	0.64 $\pm$ 0.34 ^{*,a,b,†}	2.16 $\pm$ 1.16 ^{*,†}	1.58 $\pm$ 0.68 ^{a,b,†}
144	0.19 $\pm$ 0.07	0.10 $\pm$ 0.02	0.29 $\pm$ 0.14	0.09 $\pm$ 0.01	0.23 $\pm$ 0.06*	0.83 $\pm$ 0.46 ^{*,a,b,†}	1.97 $\pm$ 1.18 ^{*,†}	2.95 $\pm$ 1.07 ^{a,b,†}
192	0.34 $\pm$ 0.18	0.17 $\pm$ 0.08	0.39 $\pm$ 0.21	0.14 $\pm$ 0.03	0.17 $\pm$ 0.03*	0.88 $\pm$ 0.38 ^{*,a,b,†}	6.94 $\pm$ 4.60 ^{†,‡}	3.60 $\pm$ 1.4 ^{b,c,‡}
240	0.08 $\pm$ 0.00	0.10 $\pm$ 0.03	0.09 $\pm$ 0.01	0.48 $\pm$ 0.19	0.26 $\pm$ 0.09*	1.72 $\pm$ 0.67 ^{*,b,†}	2.56 $\pm$ 0.9 ^{†,‡}	6.88 $\pm$ 3.05 ^{c,‡}

^{a,b,c}Different 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 ($P < 0.05$).

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 μ m) that were cultured in 1.5% alginate, a concentration that fails to support smaller (100–130 μ m) 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 μ m; 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 μ m), 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.

Xu *et al.* (2006b) demonstrated that alginate concentration influences *in vitro* steroidogenesis in the mouse follicle (Xu *et al.* 2006b). The lower hydrogel concentration (and the concomitantly less rigid microenvironment) resulted in consistently higher E₂, but less P₄ production compared with counterpart follicles in the 'less flexible' culture system (Xu *et al.* 2006b). In contrast, cultured dog follicles continued to yield rising concentrations of E₂ regardless of alginate solution. Although P₄ continued to increase as culture period progressed in both alginate concentrations, follicles cultured in 1.5% secreted consistently more hormone than those encapsulated in 0.5%. The increasing E₂ concentration suggested that both alginate concentrations support production and conversion of P₄ to androgen and then to E₂ (Rosenfeld *et al.* 2001). Additionally, the similarity in E₂ concentration and kinetic patterns combined with the marked levels of P₄ in follicles encapsulated in 1.5% alginate were probably indicative of premature, spontaneous luteinization of granulosa or theca cells. The elevated P₄ will be a target for additional study as this condition can be reflective of poor follicle health (Fauser & van Heusden 1997).

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 & Glistler 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 prooestrous 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 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 ovariectomies 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.iucn-redlist.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 granulosa 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 granulosa 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

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 192 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 (Follitropin-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 χ^2 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 a 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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