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Evaluation of an ovary-on-a-chip in large mammalian models: Species specificity and influence of follicle isolation status

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Abstract

The ability to grow oocytes from immature ovarian follicles *in vitro* has significant potential for fertility preservation; yet, it has proved challenging in large mammalian species due to the complex metabolic needs and long-term culture requirements. Currently, follicular incubations are based on a ‘static’ system with manual exchange of medium. Despite the numerous advantages of conventional culturing approaches, recapitulating the native microenvironment and supporting the survival of ovarian follicles still represent challenges. In this study, we utilized an innovative microfluidic dynamic system to support the *in vitro* survival of domestic cat and dog follicles enclosed within the ovarian cortex or isolated from ovarian cortex. Results indicate both species and tissue type-specific differences in response to microfluidic culture. Domestic cat but not dog ovarian cortical tissue maintained viability under flow similar to conventional agarose gel controls. Preantral stage isolated follicles from both species grew most favorably in conventional alginate bead culture, but overall there was no influence of culture system on expression of follicle development or oocyte health markers. This system represents an important exploration toward the development of an improved ovarian *in vitro* culture system of large mammalian species (*e.g.*, cats and dogs), which has potential applications for fertility preservation, reproductive toxicology, and endangered mammal conservation efforts.

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Author Contributions

J.N., R.E., N.S., and U.D., developed the idea; J.N., R.E., N.S., and U.D., designed the experimental approach; J.N. and R.E., performed the experiments; J.N., analyzed the data; J.N., R.E., N.S., and U.D., wrote the manuscript.

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Competing Financial Interests

Dr. U. Demirci is a founder of, and has an equity interest in: (i) DxNow Inc., a company that is developing microfluidic and imaging technologies, (ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions, and (iii) LEVITAS Inc., a company that develops biotechnology tools for genomic analysis in cancer. U.D.'s interests were viewed and managed in accordance with the conflict of interest policies.

Introduction

Each ovary contains hundreds of thousands of immature or ‘primordial’ follicles consisting of a single layer of flattened granulosa cells enclosing oocytes. The vast majority of oocytes from these primordial follicles are never ovulated, and therefore never contribute to reproduction (Gougeon, 2010). The ability to rescue and grow these follicles *in vitro* to a mature stage with a competent oocyte would be enormously beneficial. For example, with advances in cancer research, numbers of patients surviving increase yearly; yet, the treatments themselves often put the patients at risk for permanent infertility (Woodruff, 2007, Woodruff and Snyder, 2008, Asghar et al., 2014). Thus far, the laboratory mouse is the only species in which ovarian follicles have been grown from the primordial stage through to ovulation (folliculogenesis descriptions: Supplemental Table S1), fertilization, and the birth of live young (Eppig and Schroeder, 1989, Eppig and O’Brien, 1996). Although this is a good step in understanding some of the universal mammalian mechanisms of folliculogenesis, this research has not been translated from mice to larger mammalian species (Smitz and Cortvrindt, 1999, Smitz et al., 2010) or humans (Picton et al., 2008). There are two key reasons for this: (i) the longer duration of follicle development in larger animals (10 to 12 days for the mouse compared to at least 2 to 3 months for larger mammals (Picton et al., 2008)), and (ii) the very large sizes reached by follicles from larger animals (~3.6 mm, 6.5, and 6 mm for the cat (Reynaud et al., 2009b), dog (Reynaud et al., 2009a), and human (Picton et al., 2008), respectively, versus 0.5-0.6 mm for the mouse (Picton et al., 2008)). As result, successful *in vitro* culture of follicles from larger species may require a more dynamic system to address these complex needs.

Currently, most incubations are based on a static system with manual exchange of medium every 24 to 72 hours. Examples of such approaches include the culture of ovarian cortical tissue on agarose gel blocks (Fujihara et al., 2014, Fujihara et al., 2012), or alginate-encapsulated isolated follicles (Kreeger et al., 2005, Xu et al., 2009, Nagashima et al., 2015). A comparison of the approaches relevant to this study is supplied in Supplementary Table S2. Ovarian tissue culture allows for the maintenance of the physical and hormonal microenvironment for follicles, but is unable to monitor their development over time. Further, nutrient availability is a problem. Domestic cat and dog ovarian tissues can survive up to 2 weeks and 3 days *in vitro*, respectively; however, a significant reduction in follicle viability occurs (Fujihara et al., 2012), particularly in the central portion of the tissue section. Tissue culture on an agarose gel block remains a sharp contrast to what occurs *in vivo*, where there is a constant flow of blood through the body’s organs. Mouse ovaries (Winkler-Crepaz et al., 2014) and human ovarian cortices (Liebenthron et al., 2013) cultured under flow show improved follicle survival and growth. Follicles isolated from cortical tissue can maintain their three-dimensional (3-D) structure and grow *in vitro* when encapsulated in alginate hydrogel (Nagashima et al., 2015), but development of advanced stages from small follicles have not been consistently achieved in large mammals (Hovatta et al., 1999, Jewgenow, 1998, Jewgenow and Göritz, 1995). Recently, a significant recapitulation of reproductive organs-on-chip was reported using human fallopian tube, endometrial, cervical, and liver cells, along with murine follicles (Xiao et al., 2017). These data are promising; however, as we are aware of the limitations of the murine model for

development of *in vitro* folliculogenesis technologies for other species, there is still a need to prove empirically that a dynamic system improves survivability and growth of follicles from large mammalian species *in vitro*. The ability to mature ovarian follicles *in vitro* is urgently needed to improve the clinical practice of reproductive medicine.

In this study, we utilized a microfluidic device to investigate the influence of flow on domestic cat and dog ovarian follicles. Cortical tissue and isolated follicles from both domestic cats and dogs were cultured in a microfluidic chip under various flow rates compared with conventional, static culture systems. Follicle density and diameter in cultured tissue, as well as growth and expression of growth differentiation factor 9 (*GDF9*) and follicle stimulating hormone receptor (*FSHR*) in isolated follicle culture were evaluated to assess the effect of culture system and isolation status on *in vitro* folliculogenesis in these species.

Results and discussion

To achieve a dynamic *in vitro* culture system, a microfluidic chip was fabricated via assembling acrylic sheets of polymethyl methacrylate (PMMA) and double sided adhesive (Rizvi et al., 2013) to incorporate three channels (Figure 1A,B) for the culture of either pieces of ovarian cortical tissue or isolated ovarian follicles (Figure 1D). This straightforward design and fabrication process is particularly beneficial as it can be adapted to evaluate the influence of fluid motion for multiple applications (Rizvi et al., 2013, Guven et al., 2015, Asghar et al., 2015), and is particularly accessible for more widespread utilization. As recent research has indicated that rigidity of the microenvironment surrounding follicles may contribute to their survival (Woodruff and Shea, 2011), we first sought to recreate this rigidity *in vitro* by filling the channel with a 1% alginate hydrogel. Permeability of media (mixed with food coloring dye) through the alginate layer was assessed, to confirming follicles embedded in alginate in the chip would maintain access to nutrients (Figure 1C).

Next, we applied on chip culture toward our overall objective of investigating the influence of flow on domestic cat and dog ovarian follicles. In Study #1, ovarian tissue from both cats and dogs, were cultured in a microfluidic chip under various flow rates compared with conventional, agarose gel culture system. Histological analyses of cultured cat ovarian cortical tissue revealed increased presence of degenerate or pyknotic nuclei and morphologically abnormal follicles in 4 day cultured tissue (Figure 2A). Agarose gel cultured sections commonly displayed more structurally normal tissue and follicle density on the outer edges, with degeneration and abnormal somatic cell patterns in the center of the tissue. Number of normal morphology primordial stage follicles per mm² tissue was somewhat reduced after culture, regardless of system, compared to fresh controls (Figure 2B, Primordial follicles: $n_{\text{fresh}} = 897$, $n_{\text{agarose}} = 1186$, $n_{2\mu\text{L}/\text{min}} = 1003$, $n_{10\mu\text{L}/\text{min}} = 571$). There were no significant differences in density of normal primary stage follicles among treatment groups (Primary follicles: $n_{\text{fresh}} = 45$, $n_{\text{agarose}} = 240$, $n_{2\mu\text{L}/\text{min}} = 120$, $n_{10\mu\text{L}/\text{min}} = 170$, Figure 2B). Transitional follicles are follicles in the process of developing from the primordial (*i.e.*, with flattened granulosa cells) stage to the primary (*i.e.*, with cuboidal granulosa cells) stage. These follicles were only observed in tissue from a single cat, and

only when cultured on the agarose gel control and in the 10 $\mu\text{l}/\text{min}$ flow systems ($n_{\text{agarose}} = 2$, $n_{10 \mu\text{l}/\text{min}} = 3$). A significant difference ($P < 0.05$) in average primordial follicle diameter was detected, wherein morphologically normal follicles in cultured tissue were larger than fresh, uncultured controls (fresh/uncultured = $36.15 \pm 0.36 \mu\text{m}$ diameter (mean $\pm$ standard error of the mean), $n = 183$ follicles). Primordial follicles cultured on the agarose gel were significantly larger ($40.42 \pm 0.42 \mu\text{m}$, $n = 173$) than those cultured under 2 $\mu\text{l}/\text{min}$ flow ($38.02 \pm 0.43 \mu\text{m}$, $n = 135$), with 10 $\mu\text{l}/\text{min}$ flow ($39.51 \pm 0.85 \mu\text{m}$, $n = 73$) not significantly different from either (Figure 2C).

For the domestic dogs, ovarian cortical tissues were cultured for 2 days. Histological analyses revealed dramatic disorganization or loss of somatic/stromal cells in all treatment groups (Figure 3A). No significant differences in density of normal morphology primordial or primary stage follicles were observed among culture groups ($P > 0.05$; Figure 3B; Primordial follicles: $n_{\text{fresh}} = 650$, $n_{\text{agarose}} = 599$, $n_{2 \mu\text{l}/\text{min}} = 710$, $n_{10 \mu\text{l}/\text{min}} = 391$; Primary follicles: $n_{\text{fresh}} = 87$, $n_{\text{agarose}} = 85$, $n_{2 \mu\text{l}/\text{min}} = 192$, $n_{10 \mu\text{l}/\text{min}} = 36$); however, there was an overall trend toward reduction in normal follicles under increasing flow. A few transitional follicles were observed in all treatment groups, with the most in tissues cultured on the agarose gel block (Figure S1; $n_{\text{fresh}} = 5$, $n_{\text{agarose}} = 18$; $n_{10 \mu\text{l}/\text{min}} = 6$; and $n_{2 \mu\text{l}/\text{min}} = 6$). Unlike in the cats, primordial follicle sizes did not increase with culture, but were larger in tissues cultured on the agarose gel block compared with under 10 $\mu\text{l}/\text{min}$ (Figure 3C; fresh = 31.65 ± 0.88 , $n = 159$; agarose gel = 32.64 ± 0.62 , $n = 155$; 2 $\mu\text{l}/\text{min}$ flow = 32.18 ± 0.88 , $n = 48$; and 10 $\mu\text{l}/\text{min}$ flow = 28.84 ± 1.28 , $n = 38$; $P < 0.05$).

In accordance with previous work, there is a significant species-specific response to tissue culture. Specifically, domestic dog ovarian follicles embedded in cortical tissue responded poorly to culture compared with similarly cultured cat tissues (Fujihara et al., 2012). The cause of this differential response is not known, but based on knowledge of the unique physiology of the domestic dog we propose two possible drivers. First, we have previously demonstrated that dog ovarian tissues have different biochemical requirements, as they respond more favorably in culture medium with high vitamin and non-essential amino acid content (alpha-MEM rather than MEM) compared with the cat (Fujihara et al., 2012). In rodent *in vitro* embryo work, these amino acids have been shown to promote the maintenance of normal rates of glycolysis and pyruvate oxidation, reducing the cellular stress in culture that typically results in loss of cellular viability (Lane and Gardner, 1998). Our data suggest the need for yet additional biochemical support to maintain tissue viability in the dog *in vitro*. Second, we have observed that domestic dog cortical tissue was more rigid than that of the cat (unpublished observations). This likely influences nutrient availability *in vitro*, and may also explain our observation of improved (albeit not statistically significant) maintenance of normal primordial follicle density in agarose gel controls in this species. Further, this same aspect of tissue enclosure – though providing structural and paracrine support – may also have restricted gas exchange to the follicles, particularly when the tissues were submerged in culture medium within the chip system. Thus, we were eager to evaluate the dynamic culture system on follicles freed from the ovarian cortex as well.

In Study #2, we evaluated the influence of flow on isolated ovarian follicles. The benefit of culturing isolated follicles in microfluidic chip is the ability to monitor the development of individual follicles over time. As a preliminary investigation, isolated primordial through small pre-antral stage cat follicles were embedded in alginate hydrogel ($n_{\text{total}} = 81$ follicles) in the microchip. These follicles were cultured under either static (no flow) or 10 $\mu\text{l}/\text{min}$ flow for 2 or 6 days (Figure S2). Individual follicle outcome was highly variable in the cat, with some follicles displaying modest growth over the culture periods (Figure S2A, B), and others losing basement membrane integrity and displaying a dark, granular appearance consistent with degeneration (Figure S2D, E). The 100-160 μm follicles in the flow system displayed statistically significant growth on Day 2 of culture compared with the smaller size group ($P < 0.05$); however, this was not maintained over 6 days culture.

As damage to cat follicle basement membranes occurred in both treatment groups, it was unlikely to have been an effect of shear force on the follicles by flow. Instead, the loss may have been related to isolation. We had previously assessed two ways of isolating immature follicles. Embedded in ovarian cortical tissue, follicle isolation can be achieved enzymatically, as with collagenase (Hovatta et al., 1999), or by physically disrupting the tissue via mechanical or mesh filtration (Jewgenow and Göritz, 1995). Both methods were successful in isolating follicles, but mechanical isolation was selected for the current study to reduce potential impact of collagenase digestion on follicle basement membranes. Our results are in line with other studies in domestic cats, wherein viability of preantral stage follicles has been shown to drop dramatically after isolation (Jewgenow and Göritz, 1995). One additional possible method would be to use a gentle enzymatic method. We have previously utilized liberase blendzyme (Dolmans et al., 2006) (rather than the harsher collagenase) for the isolation of preantral stage domestic dog follicles (Nagashima et al., 2015). Some damage to membrane integrity was observed with this method as well, but direct comparisons have not been made between these enzymatic methods for primordial stage follicles in the dog and cat. Additional studies are needed to optimize the isolation of these smallest stage follicles. Isolated follicles ($n = 28$, average size $167.32 \pm 6.57 \mu\text{m}$ diameter) from a dog were similarly embedded in alginate and cultured in the microchip for 6 days. Follicles cultured in the presence of flow displayed slightly increased growth rates on average; however, there was no statistically significant difference in growth by 6 days culture, as follicles under flow grew on average 10% in diameter, whereas statically cultured follicles grew on average 8% from day 0 sizes (Figure S3).

We next considered that it is likely that the benefits of dynamic culture increase as follicles develop into advanced stages. *In vivo* follicles would experience increased vascularization surrounding them over the progression of folliculogenesis (Jiang et al., 2002, Jiang et al., 2003), resulting in increased nutrient availability by perfusion from vasculature. Thus, our preliminary experiments were expanded in Study #2 to incorporate a larger range of ovarian follicle stages. At this time, we also compared “forward” versus “reverse” flow. The flow methods we had utilized to this point were designated as “forward”, wherein culture medium was pumped from a syringe to the chip. This is in contrast with “reverse” flow, wherein culture medium was drawn through the chip from a gas-permeable reservoir housed inside the tissue culture incubator, to promote oxygen and carbon dioxide exchange in the medium prior to arrival at the chip. Ovarian follicles isolated from domestic cats and domestic dogs

(Supplementary Table S3) were cultured under four different conditions: statically while (i) embedded in a bead of 1% alginate hydrogel in a 4-well dish, or (ii) embedded in a 60 μ l layer of alginate (approximately 0.5 mm thick) within a channel of the chip. In the same alginate layer, follicles were also cultured either under (iii) “forward” flow at 2 μ l/min, or (iv) “reverse” flow at 2 μ l/min. In cats, primordial through antral stage follicles were cultured, whereas in the dog preantral (small secondary) through antral stage follicles were cultured. At the end of 6 days culture follicle were grouped according to the culture system, and gene expression was evaluated for follicle stimulating hormone receptor (*FSHR*), as a marker for normal follicle functionality, and growth differentiation factor 9 (*GDF9*), as an indicator of oocyte quality.

Isolated domestic cat follicles of all stages experienced growth (preantral through antral: Figure 4A, primordial: S4A), which was most notable in preantral and early antral stage follicles. Preantral follicles cultured in the ‘static’ alginate bead yielded the most rapid growth rate, which was significantly different from those cultured under similarly static conditions on the chip, but not different than either flow condition. A single antral stage follicle cultured under forward flow experienced dynamic fluctuation in diameter over the course of the six day culture, resulting in the growth pattern observed in Figure 4C. Two potential explanations exist. First, that although the follicle still appeared morphologically normal it was actually undergoing atresia in the second half of culture. Second, that this follicle was experiencing normal fluctuations in diameter as part of antral cavity expansion. Pulsatile growth (*i.e.*, contraction and re-expansion) has been well described in another cavity-forming reproductive blastocyst-stage embryo (Massip et al., 1982, Cole, 1967, Niimura, 2003). Our laboratory has observed fluctuations in diameter in these stage ovarian follicles previously (unpublished observations), but not typically to the amplitude observed here. Therefore, the first explanation appears more likely, although longer-duration culture would be necessary to confirm. Further, while the 1% alginate hydrogel utilized in this study is within range for what we have previously utilized for domestic dog follicles (Songsasen et al., 2011), it may have been prohibitively rigid for domestic cat follicles, particularly at advanced stages where we would expect to see significant changes in diameter owing to expansion of antral cavities.

Expression of follicle function marker *FSHR* yielded no significant differences among treatment groups (Figure 4D). Expression of *GDF9* was reduced in the static chip compared with all other culture systems (Figure 4E); however, low levels of total RNA were obtained from this group in two of the three replicates. Therefore, there are no sufficient samples for full statistical evaluation of this treatment group. Both the low RNA and reduced *GDF9* expressions were, perhaps, unsurprising given the abnormal morphology of follicles cultured statically in the chip after only 3 days (Example with primordial and primary stage follicles: Figure S4B). While no statistically significant difference was observed among either flow or alginate bead group with regard to gene expression, more robust expression of *GDF9* in the ‘forward’ flow system was observed compared with ‘reverse’ flow system.

In direct contrast with results from the ovarian cortical culture experiments, there was a significant dog follicle growth in the various systems after even 3 days in culture (Figure 5A). Preantral stage experienced the most significant growth over the culture period overall,

and, similar to the domestic cat preantral follicles, grew the best in conventional alginate bead system, although this was not statistically different (Figure 5A). Antral stage follicles experienced the most modest growth overall, with no influence of culture system on growth over the 6 day period (Figure 5A). This follicle stage-specific response to culture is not surprising, as we have previously observed improved growth in terms of percent increase in diameter, in preantral stage follicles over those in later stages of development (Nagashima et al., 2015). Again, the 1% alginate was likely more restrictive to the growth of antral stage follicles than the smaller stages. No statistically significant differences in expression of *FSHR* or *GDF9* were observed among treatment conditions ($P > 0.05$; Figure 5B).

The use of microfluidic approach for the purpose of maturing ovarian follicles of large animals has not been well studied or applied, although it could potentially provide a powerful tool to preserve fertility. One challenge is the ovarian tissue species-specific sensitivity to culture observed in both the current study and previous research (Fujihara et al., 2012). Ovarian tissue from domestic dogs is notably more rigid than that of the domestic cat or laboratory mouse, which leads us to hypothesize this tissue stiffness may have contributed to the very different culture outcomes observed between the two species. Future studies should investigate the feasibility of this microfluidic culture system in long-term culture and identify optimal flow rates specific for cat and dog ovarian tissues and isolated follicles.

Conclusion

In this study, we report the ability to culture cat and dog ovarian follicles using a microfluidic dynamic *in vitro* culture system. Results were comparable to the conventional/static alginate bead system in supporting isolated preantral through antral stage ovarian follicle growth and markers of development in a short-term culture. Conversely, for ovarian tissue culture in the domestic cat and dog, conventional (static) agarose gel best maintained normal primordial follicle morphology and diameter. Further, this study suggests that the status of the follicles – either embedded in ovarian cortical tissue or encapsulated in alginate hydrogel, influences follicular responsiveness to flow. The ovary-on-a-chip holds broad promise. Such a system for *in vitro* folliculogenesis will allow us to improve our understanding of the mechanisms of folliculogenesis, particularly primordial follicle activation and the interaction between microenvironment rigidity and follicle development. Use of large mammalian animal models are relevant to the development of new fertility preservation techniques for women, as well as in the development of assisted reproductive technologies for endangered felid and canids.

Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO), and base medium from Irvine Scientific (Irvine, CA).

Microchip Production

A laser cutter (VLS2.3 X-660, Universal Laser Systems, Inc., Scottsdale, AZ) was used to cut chip components (acrylic sheets polymethyl methacrylate (PMMA), and double sided

adhesive (DSA) as previously described (Rizvi et al., 2013). Briefly, the base layer was cut from PMMA then stick to 1.5 mm PMMA with DSA to accommodate channels. Finally, the upper layer of the chip, containing in/outlet holes was aligned with the ends of the three channels cut into the lower layers were cut from 3 mm PMMA. The base and channel PMMA layers were adhered via DSA and the assembled portions as well as the in/outlet layer were sterilized under UV light prior to final assembly and culture.

Ovarian Cortex and Follicle Isolation

Domestic cat and dog ovaries were collected at local spay clinics from routine ovariohysterectomies and transported to the laboratory in L-15 medium containing 10 mM HEPES, 100 µg/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate on ice. Tissue was processed within 6 hours of surgery. Cortical slices (0.5-1 cm thick) were dissected from each ovary's surface, then trimmed into 2 mm² sections for cortical tissue culture. Fresh tissue (2 pieces) from each individual were collected and fixed to evaluate follicle populations and morphology. For isolated follicle culture, small follicles were isolated from ovarian slices via mechanical dissection and mesh filtration (Jewgenow and Göritz, 1995).

Study #1: Ovarian Cortical Tissue Culture

For each individual animal, 2 pieces of ovarian cortical tissue were placed in the bottom of the microchannels and on the control agarose gel block. For the chip, the channel was then closed by covering with a final layer of DSA and the upper chip layer. The entire chip was squeeze-clamped to ensure close adhesion. Sterilized tubing was inserted and epoxy-secured into each end of the channels, with one end connecting to a syringe containing the medium and the other terminating in a tube to collect waste. Flow was tested then syringes loaded into dispensing machines and set with the appropriate flow rate. The chips were maintained in an incubator at 38°C and 5% CO₂ for the duration of culture (2 or 4 days for dog or cat tissue, respectively). Dog tissues were subjected to an abbreviated culture compared with cats (2 versus 4 days), as we have previously found a significant loss in viability after 3 days culture in conventional culture in the dog (Fujihara et al., 2012). As a control, tissue sections for each animal were also cultured in a conventional culture system on an agarose gel block (Fujihara et al., 2014).

Domestic cat tissue was cultured in modified eagle's medium (MEM) supplemented with 4.2 µg/ml insulin, 3.8 µg/ml transferrin and 5 ng/ml selenium, 2 mM L-glutamine, 100 µg/ml penicillin G sodium and streptomycin sulfate, 0.05 mM ascorbic acid, and 0.1% w/v polyvinyl alcohol. Further, 10 ng/ml FSH and 100 ng/ml EGF, which has previously been utilized for cat ovarian tissue culture (Fujihara et al., 2014), was supplemented. Dog ovarian cortical tissue was cultured in alpha-MEM supplemented with 3 mg/mL BSA, 4.2 µg/ml insulin, 3.8 µg/ml transferrin and 5 ng/ml selenium, 2 mM glutamine, 10 IU/ml of penicillin G and 10 µg/ml of streptomycin, and 10 µg/ml follicle-stimulating hormone (FSH) (Nagashima et al., 2015). Previous work in our laboratory also identified MEM as the preferred base medium for cat ovarian tissue over alpha-MEM, and the opposite preference for dog tissues (Fujihara et al., 2012). For both media, hormone/growth factor (FSH and EGF) supplements were added to the medium immediately prior to warming before use.

Cultured ovarian cortical tissue as well as fresh controls were embedded in paraffin, cut in 6 μm thick sections, and processed for hematoxylin and eosin staining for morphological assessment. Morphologically normal follicle counts were evaluated in a total of 6 slices spaced every 10 sections (*i.e.*, 60 μm tissue depth apart, so as to prevent the double-counting of primordial follicles as well as to capture the sections with highest density of primordial follicles). Primordial stage follicles were defined as a centralized oocyte surrounded by a single layer of flattened granulosa cells. Primary follicles contained a single layer of cuboidal granulosa, and secondary stage follicles had at least two layers of granulosa cells and, together with primary stage follicles, were considered “preantral”. Follicles observed to have a combination of flattened and cuboidal granulosa cells in a single layer were classified as activated, ‘transitional’ follicles. Only follicles with visible nuclei were counted. Follicles with pyknotic nuclei were considered non-normal morphology or “atretic”.

Preliminary Experiments—Approximately 2 mm² pieces of cat ovarian tissue were first cultured in the microfluidic channel for 6 days, under two flow rates (*i.e.*, 2 $\mu\text{l}/\text{min}$ vs. 5 $\mu\text{l}/\text{min}$). Representative histological sections from an individual animal are shown in Figure 1E. No significant differences in follicle densities or tissue morphologies were observed between the two flow rates, thus 2 $\mu\text{l}/\text{min}$ was chosen as the flow rate to culture the ovarian tissues. This flow rate is supported by our previous work, which utilized a flow rate of 2 $\mu\text{l}/\text{min}$ (Guyen et al., 2015, Rizvi et al., 2013) on monolayer cultured cells. Furthermore, we chose a higher flow rate (10 $\mu\text{l}/\text{min}$) to evaluate influence of flow rate on follicles in tissue culture for our primary experiment in Study #1.

Primary Experiments—Cortical tissue was collected from three cats and three domestic dogs, and two pieces per animal were subjected to 4 (cat) or 2 (dog) day culture under either flow or in an agarose gel block control system, as described earlier. Every animal was represented in every treatment except one dog with 10 $\mu\text{l}/\text{min}$ flow. Based on our preliminary experiments, flow rates of 2 $\mu\text{l}/\text{min}$ (Guyen et al., 2015, Rizvi et al., 2013) and 10 $\mu\text{l}/\text{min}$ flow were selected for comparison.

Comparisons of normal follicle density among treatment groups was performed by comparing number of morphologically normal follicles of each stage (primordial and primary) out of the total area of tissue. Transitional stage follicles were observed infrequently; therefore, were reported but not subjected to statistical analyses. For each individual and treatment group, follicle diameters were measured in the section with the highest follicle count. As there were fewer primary stage follicles in the tissues, the subset diameter evaluation resulted in too small a sample size for assessment. Statistical evaluation of differences in follicle counts and primordial follicle diameters were performed using nonparametric Wilcoxon’s test with JMP software (Cary, NC). Significance was set at $P < 0.05$.

Study #2: Isolated Follicle Culture

Chips were loaded with 60 μl 1% alginate. Isolated follicles ($n = 10\text{--}15$ follicles/channel) were added to the alginate solution at regular intervals along the channel using a wiretrol®, then alginate was allowed to crosslink in 5 mM calcium chloride solution for 5 min prior to

closing the channel. The channel was filled with the appropriate culture medium while tubing was connected as previously described. Once complete, fresh warmed medium was flowed through to test the connection, which acted simultaneously to wash out any remaining calcium solution, then flow rate was set to 10 $\mu\text{l}/\text{min}$ (Preliminary Experiments) or 2 $\mu\text{l}/\text{min}$ (Primary Experiments) for culture.

Preliminary Experiments—Isolated ovarian follicles from domestic cats (5 individuals, aged 6-7 months, $n_{\text{total}}=81$ follicles) were cultured, with 58 cultured for 6 days ($n_{<100\text{ }\mu\text{m}}=38$ follicles, $n_{100-160\text{ }\mu\text{m}}=20$, with approximately half of the follicles from each group cultured under flow versus under ‘static’ conditions – similarly embedded in the microchannel, but with medium exchanged every other day rather than dynamically) and the remainder cultured only 2 days in the same MEM-based cat follicle culture medium ($n_{\text{flow}}=5$, $n_{\text{static}}=18$). Images of individual follicles were taken to determine follicle diameters on Day 0 (time of isolation), 2, and 6. Isolated follicles were small preantral, but could not be classified as either primordial or primary based on stereoscope assessments. For initial isolated domestic dog follicle assessments, 28 preantral stage follicles from one dog were cultured under 10 $\mu\text{l}/\text{min}$ flow ($n=15$) and under static conditions ($n=13$) in the microchip for 6 days. Growth data (percent diameter increase from Day 0 or time of isolation) was evaluated using nonparametric Wilcoxon (Kruskall-Wallis) test with JMP software. Significance was set at $P<0.05$.

Primary Experiments—For the primary evaluation of culture system on isolated ovarian follicles, follicles were isolated from domestic cats (5 individual cats, $n_{\text{primordial and primary}}=53$, $n_{\text{preantral}}=46$, $n_{\text{early antral}}=23$, and $n_{\text{antral}}=5$) and domestic dogs (4 individual dogs, $n_{\text{preantral}}=49$, $n_{\text{early antral}}=77$, and $n_{\text{antral}}=24$). These were cultured under four different conditions: (1) embedded in a bead of 1% alginate hydrogel in a 4-well dish (Alginate Bead), (2) embedded in a 60 μl layer of alginate (approximately 0.5 mm thick) within a channel of the microchip (Static Chip), or in the same alginate layer, cultured under (3) “forward” flow, being pumped via syringe through the chip at 2 $\mu\text{l}/\text{min}$ or, (4) “reverse” flow, with medium being drawn through the chip from an internal cell culture flask reservoir at 2 $\mu\text{l}/\text{min}$. Initial sizes of follicles in each treatment group are listed in Supplementary Table S3. Follicle growth was assessed on days 3 and 6 of culture, as previously described, and analyzed based on stage at isolation as well as culture condition in JMP software.

At the end of culture, follicles were collected and stored frozen for gene expression analysis. Total RNA was isolated using Agilent Absolutely RNA Nanoprep kit with on-column DNase digestion, according to the manufacturer’s instructions. Synthesis of cDNA was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) with 20 ng (cat) and 30 ng (dog) total RNA, also per manufacturer’s instructions. Reverse-transcription polymerase chain reaction (RT-PCR) was performed with FastStart Essential DNA Green Master (Roche), using β -actin as a reference gene. In the both species, mRNA expression evaluated included follicle stimulating hormone receptor (*FSHR*), as a marker for normal follicle functionality, and growth differentiation factor 9 (*GDF9*), as an indicator of oocyte health. Primers used are outlined in Supplementary Table S4, using primers for GDF9 for the cat (Veraguas et al., 2017) and dog (Palomino and De los Reyes, 2016), and feline FSHR

(Hobbs et al., 2012) and canine β -actin (Jais et al., 2011) as previously published. RT-PCR data were analyzed via Pfaffl method, accounting for primer efficiency (Pfaffl, 2001) and relative to alginate-encapsulated follicles, followed by post hoc Wilcoxon test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Dynamic microfluidic *in vitro* system for culturing cat and dog ovarian tissue and follicles

A,B) Schematic of microfluidic chip components (A) and assembled microchip (B) using double sided adhesive. **C)** Permeability test of dye (food coloring) through 1% alginate hydrogel crosslinked within chip channel. **D)** Ovarian tissue and isolated follicle isolation via enzymatic digestion (3 mg/ml collagenase for 40 min at 38.5°C, ending reaction with 20% fetal bovine serum) and mechanical dissection. **E)** Preliminary assessments of ovarian tissue culture in the microchip under 2 µL/min flow or 5 µL/min flow for 6 days, with solid black arrows (►) indicating morphologically normal, live follicles and white arrows (◻) indicating dead follicles.

Figure 2. Cat ovarian tissue culture in the microfluidic chip

(A) Histology images of sections from a single cat at 40 $\times$ in the different treatment groups, with solid black arrows (►) indicating morphologically normal, live follicles and white arrows (◄) indicating dead follicles. (B) Mean $\pm$ S.E.M. of the proportion morphologically normal primordial and primary stage follicles out of total follicles of each stage (normal + atretic) per mm² ovarian tissue (N = 3 cats, $n_{\text{primordial}} = 3657$ and $n_{\text{primary}} = 575$ follicles). (C) Box-and-whisker plot of primordial follicle diameters ($n_{\text{total}} = 565$ follicles) for each treatment group with black dots (●) signifying outliers. Asterisks (*) indicate significant differences between treatment groups ($P < 0.05$).

Figure 3. Dog ovarian tissue culture in the microfluidic chip

(A) Histology images of sections from a single dog at 40 $\times$ in the different treatment groups, with solid black arrows (►) indicating morphologically normal, live follicles and white arrows (◄) indicating dead follicles. (B) Mean $\pm$ S.E.M. of the proportion morphologically normal primordial and primary stage follicles out of total follicles of each stage (normal + atretic) per mm² ovarian tissue (N = 3 dogs, $n_{\text{primordial}} = 2350$ and $n_{\text{primary}} = 400$ follicles). (C) Box-and-whisker plot of primordial follicle diameters for each treatment group ($n_{\text{total}} = 400$ follicles) with black dots (●) signifying outliers. Asterisks (*) indicate statistically significant differences among treatment groups ($P < 0.05$).

Figure 4. Growth and gene expression of domestic cat ovarian follicles cultured in forward versus reverse flow microchips

Percent growth $\pm$ SEM of (A) preantral ($n = 46$), (B) early antral ($n = 23$), and (C) antral stage ($n = 5$) follicles over 6 days in culture in various systems. Asterisk (*) and pound sign (#) indicating significant differences between culture systems for that day and follicle stage ($N = 5$ cats, $n_{\text{total}} = 74$ follicles, and $P < 0.05$). Messenger RNA expression, normalized to β -actin and relative to alginate bead culture control for (D) *FSHR* and (E) *GDF9*.

Figure 5. Growth and gene expression of domestic dog ovarian follicles cultured in forward versus reverse flow microchips

Follicle percent growth $\pm$ SEM of (A) preantral ($n = 49$), (B) early antral ($n = 77$), and (C) antral stage ($n = 24$) follicles over 6 days in culture in various systems ($N = 4$ dogs, $n_{\text{total}} = 150$ follicles, and $P < 0.05$). Messenger RNA expression, normalized to β -actin and relative to alginate bead culture control for (D) *FSHR* and (E) *GDF9*.