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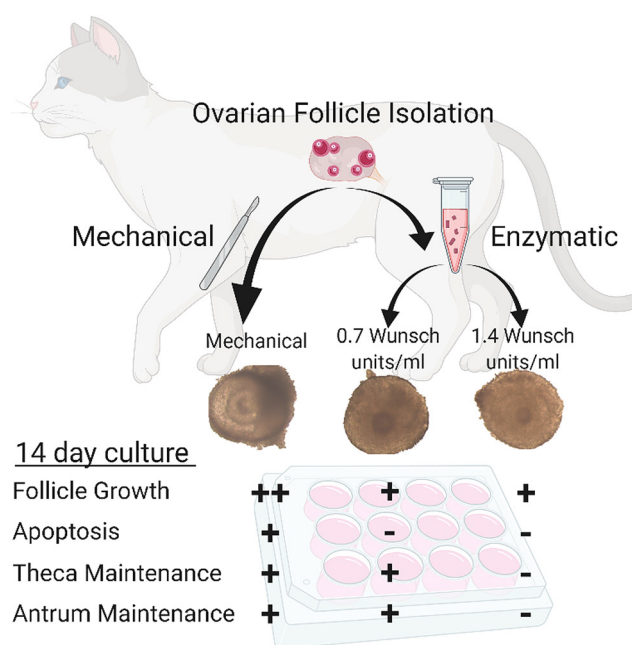
In vitro development of mechanically and enzymatically isolated cat ovarian follicles

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Graphical Abstract



Abstract

Isolation of ovarian follicles is a key step in culture systems for large mammalian species to promote the continued growth of follicles beyond the preantral stage in fertility preservation efforts. Still, mechanical isolation methods are user-skill dependent and time-consuming, whereas enzymatic strategies carry increased risk of damaging theca cell layers and the basement membranes. Here, we sought to determine an optimal method to rescue domestic cat (*Felis catus*) early antral and antral stage follicles from ovarian tissue and to evaluate the influence of isolation strategy on follicle development, survival, and gene expression during 14 days of *in vitro* culture in alginate hydrogel. Mechanical isolation was compared with 90 min digestion in 0.7 and 1.4 Wünsch units/mL Liberase blendzyme (0.7L and 1.4L, respectively). Mechanical isolation resulted in improved follicle growth and survival, and better antral cavity and theca cell maintenance *in vitro*,

compared with 1.4L ($P < 0.05$) but displayed higher levels of apoptosis after incubation compared with enzymatically isolated follicles. However, differences in follicle growth and survival were not apparent until 7+ days *in vitro*. Expressions of *CYP19A1*, *GDF9*, *LHR*, or *VEGFA* were similar among isolation-strategies. Cultured follicles from all isolation methods displayed reduced *STAR* expression compared with freshly isolated follicles obtained mechanically or via 0.7L, suggesting that prolonged culture resulted in loss of theca cell presence and/or function. In sum, early antral and antral stage follicle development *in vitro* is significantly influenced by isolation strategy but not necessarily observable in the absence of extended culture. These results indicate that additional care must be taken in follicle isolation optimizations for genome rescue and fertility preservation efforts.

Lay summary

The ovary contains hundreds of eggs with only a select few developing from an immature stage through to ovulation over the course of an animal's lifetime. Rescue of eggs from this pool, and the ability to grow them in culture to a mature stage, would be incredibly valuable for fertility preservation efforts in both humans and endangered species. Currently, the isolation of ovarian follicles (eggs with their surrounding helper cells) is a key step in culture systems for large mammalian species, to promote continued growth. Yet, isolation methods may affect the follicle's future developmental capacity. We evaluated two isolation strategies, mechanical micro-dissection (needle/scalpel blade) and enzymatic digestion (using Liberase blendzyme) on ovaries of domestic cats obtained via routine spay procedures. Mechanically isolated follicles displayed improved growth, survival, and indications of developmental competence in 14-day culture, compared with high concentration (1.4 Wünsch units/mL) enzyme-isolated follicles. However, mechanical isolation was not different from low (0.7 Wünsch units/mL) enzyme for these metrics, or for expression of key genes indicative of follicular cell functions. Further, differences in follicle growth/survival were not apparent until 7+ days in culture. Thus, ovarian follicle isolation strategies influence developmental potential in culture, and extended culture will be required to identify optimal methods for fertility preservation efforts.

Key Words: ▶ *Felis catus* ▶ ovarian follicle ▶ antrum ▶ theca cell ▶ *in vitro* culture ▶ liberase blendzyme

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Introduction

The ovarian follicle is a complex unit consisting of vascularized theca cell layers, a basement membrane or basal lamina, and granulosa cells surrounding a centralized oocyte. Supporting the development and functionality of these cell types over prolonged culture to produce a competent oocyte is the goal of *in vitro* folliculogenesis systems. Such an *in vitro* system would be invaluable to both human fertility preservation, and endangered mammal genome rescue efforts (Comizzoli *et al.* 2010, Smitz *et al.* 2010). However, supporting folliculogenesis *in vitro* is particularly challenging in large mammalian species with prolonged folliculogenesis and large preovulatory follicle sizes. Thus far, follicles are typically isolated from ovarian tissues at the preantral/early antral stage to promote further growth *in vitro*. For example, a multi-step culture method starting with ovarian tissue culture followed by isolated follicle culture was recently utilized to produce

metaphase II oocytes from human follicles (McLaughlin *et al.* 2018). As increased follicle size has been shown to improve oocyte developmental competence in a variety of species (Bagg *et al.* 2004, Songsasen & Wildt 2005, Han *et al.* 2006, Rosen *et al.* 2008), the ability to isolate functional follicular units from surrounding tissue is key. Yet, methods to perform follicle isolation carry the risk of damaging the follicular unit, particularly theca cells, their precursors, and the follicle's basement membrane, which in turn adversely affects normal hormonal function of the follicle and its future developmental capacity (Young & McNeilly 2010).

Two methods are primarily utilized to isolate ovarian follicles from surrounding tissue. First is mechanical isolation, either via micro-dissection from the cortical tissue or by pressing samples through cell-dissociating sieves (Jewgenow & Göritz 1995, Nagashima *et al.* 2019). In the cat, the latter method has been utilized for the recovery of preantral stage follicles (Jewgenow & Göritz

1995) but the primary disadvantage of the sieve technique is that later-stage follicles are too large to pass through the mesh intact. Micro-dissection involves the isolation of individual follicles from surrounding tissue using needles and/or scalpel blades. This method allows for the isolation of early antral and antral stages but is a time-consuming and labor intensive process, which is highly dependent on the skill of the user. Nevertheless, this method has also been utilized for the isolation of antral stage (0.5–2 mm diameter) domestic cat ovarian follicles (Rojo *et al.* 2015) as well as preantral follicles from human (McLaughlin *et al.* 2018) and domestic dog (Nagashima *et al.* 2019) ovarian cortices.

The second method for follicle isolation is by enzymatic digestion of the surrounding cortical tissue. Enzymatic isolation of ovarian follicles has been performed in several species, including the mouse (Eppig & Schroeder 1989), hamster (Roy & Greenwald 1985), pig (Hirao *et al.* 1994, Fattahi *et al.* 2020), cow (Figueiredo *et al.* 1993), dog (Durrant *et al.* 1998, Nagashima *et al.* 2017), and human (Roy & Treacy 1993, Lierman *et al.* 2015). A significant disadvantage of this method is that proteolytic enzymes such as collagenase or DNase have damaging effects on the basement membrane and theca layers of the developing follicles (Demeestere *et al.* 2002). Enzymes typically used for isolation include collagenase alone (types I, IV, or crude) or a blend of collagenase I and DNase. These traditional collagenase preparations can contain variable endotoxin levels, which have been proposed to have detrimental influences on the structural integrity of isolated follicles (Fattahi *et al.* 2020). The use of Liberase, a purified enzyme blend, has been investigated and compared with collagenase type IA for the isolation of human primordial and primary follicles (Dolmans *et al.* 2006) in which Liberase-isolated follicles displayed superior morphology compared with those isolated with collagenase. Evaluation of a variety of enzymatic isolation protocols on human follicle quality at different stages of maturation found that Liberase had fewer damaging effects on the structural integrity of the follicle than collagenase (Lierman *et al.* 2015).

While these previously published studies assess follicle quality immediately after isolation and after 7 and 10 days *in vitro* (Dolmans *et al.* 2006, Vanacker *et al.* 2011, Lierman *et al.* 2015, Fattahi *et al.* 2020), they are limited to the evaluation of preantral stage follicles and rarely specifically evaluate the effects of isolation strategy in longer-term culture. Specifically, we were interested in identifying ovarian follicle isolation strategies that allow for the maintenance of theca cell function during

longer-term culture and in a non-rodent mammalian model. For this, we utilized the domestic cat (*Felis catus*), which serves as an excellent model for both human and endangered felid physiology (Menotti-Raymond & O'Brien 2008, Rojo *et al.* 2015). The first objective of this study was to optimize an enzymatic isolation protocol by which early antral and antral stage domestic cat follicles can be recovered with minimal damage to the basement membrane and theca cell layers. The second objective was to examine the influence of enzymatic digestion vs mechanical isolation on follicle survival, growth, antral cavity development, and the expression of genes that are indicative of follicular cell functions.

Materials and methods

All chemicals were purchased from Sigma–Aldrich unless otherwise stated.

Source of ovaries

Ovaries were obtained by routine ovariohysterectomy performed at local veterinary clinics and transported to the laboratory in L-15 medium containing 30 µg/mL penicillin G and streptomycin sulfate, and 8.8 µg/mL ascorbic acid on ice. Tissues were processed within 6 h of excision. Slices of cortical tissue were dissected from the surface of each ovary and cut into 2 mm² pieces. All the recovered tissue from each cat was then divided evenly among treatment groups for follicle isolation.

Ovarian follicle isolation

Follicles were isolated either via enzymatic digestion with Liberase blendzyme or mechanically. All isolation methods were carried out in a 'Base medium', consisting of minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50 U/mL penicillin G, 50 µg/mL streptomycin sulfate, 0.1 mg/mL ascorbic acid, and 3 mg/mL BSA. Liberase TM (medium thermolysin concentration) was used at 0.7 Wünsch units/mL (0.7 L) (previously utilized for domestic dog ovarian follicle isolation (Nagashima *et al.* 2017) and equivalent to 0.135 mg/mL) and 1.4 Wünsch units/mL (1.4L). The Liberase TM enzyme blend contains the neutral protease thermolysin, which disrupts the extracellular matrix of the tissue and it has been shown to maintain normal morphology in isolated ovarian follicles better than collagenase (Dolmans *et al.* 2006, Lierman *et al.* 2015). Ovarian tissue

fragments (2 mm²) were digested at 38°C for 60, 90, or 120 min (60 and 120 min only in preliminary study) along with tissue disruption by vortexing (5 s) at low speed every 15 mins. The reaction was discontinued by the 1:1 addition of base medium with 20% fetal bovine serum (FBS) and transferred to a petri dish for the collection of isolated follicles. The digested tissue was teased apart with needles to achieve complete isolation of the follicles after which follicles were collected via WireTrol (Drummond Scientific Co., Broomall, PA) and transferred to a fresh warm base medium until encapsulation. For mechanical isolation (Mech), follicles were isolated from the surrounding cortical tissue by using a scalpel and a 20-gauge needle under a stereomicroscope. Follicles were then also transferred via WireTrol to a fresh warm base medium until encapsulation. A randomly selected subset ($n=3-5$) of freshly isolated follicles from each treatment group were preserved in 200 μ L of RNAlater stabilization solution (Fisher Scientific, Waltham, MA) at 4°C for 1 h and then stored at -20°C for subsequent gene expression evaluation via RT-PCR.

Encapsulation and incubation of isolated follicles

A 0.3% alginate hydrogel (Pronova sodium alginate, Novamatrix) was prepared by dissolving the alginate in warm PBS without calcium or magnesium. Follicles were briefly washed in the alginate solution, then drawn up in 4 μ L alginate and pipetted into a 140 mM NaCl, 50 mM CaCl₂ solution (1 mL in a Nunc 4-well dish) and allowed to crosslink for 2 mins before being transferred to a fresh base medium (Xu *et al.* 2009). Follicles were incubated in 100 μ L of 'culture medium' consisting of base medium with 0.42 μ g/mL insulin, 0.38 μ g/mL transferrin, and 0.5 ng/mL selenium, and supplemented with 1 μ g/mL FSH and 100 ng/mL EGF in 5% CO₂ in air at 38.5°C. Every 48 h, 40 μ L of the incubation medium was manually exchanged with fresh medium.

Using a Leitz DM-IL inverted microscope (Research Instrument, Falmouth, Cornwall, UK) with a heated stage and equipped with a Nikon D5500 camera, follicles were imaged on days 1 (Day 0=onset of culture) and 4 (Study #1) as well as days 7 and 14 (Study #2). Follicle diameters were measured using ImageJ image processing software (US National Institutes of Health, Bethesda, MD). To account for varying follicle shapes (e.g. oblong), two measurements of each follicle were made at the onset and end of the incubation, including the widest length, and the diameter perpendicular to it. The average of these two measurements was calculated for each follicle

and reported as diameter. Diameter assessments made at the onset of culture were used to determine follicle stage with preantral stage follicles at ≤ 205 μ m (excluded in the current study), early antral follicles >205 to ≤ 340 μ m, and antral stage follicles > 340 μ m diameter (Reynaud *et al.* 2009).

Liberase blendzyme optimization

Ovaries from four cats (aged 3–9 months) were used to identify the optimal digestion incubation period for subsequent experiments. Ovarian cortical tissue from individual cats was divided evenly among 0.7L and 1.4L digestion protocols for 60, 90, or 120 min in a 2×3 design). Post-digestion, follicles displaying good morphology (apparently intact basement membrane and homogenously dark centralized oocyte, n total=197) were counted.

Isolated follicle culture

Ovaries from ten cats (aged 6–14 months) were used to evaluate the effects of Liberase TM vs mechanical isolation via micro-dissection on follicle quality and viability in long-term *in vitro* culture. A 90-min digestion period was selected for enzymatic follicle isolation based on results from preliminary experimentation. A total of 339 follicles (including preantral stage, which were subsequently excluded from culture to focus on stages with developed theca layers) were collected. Early antral ($n_{\text{Mech}}=10$, $n_{0.7\text{L}}=41$, $n_{1.4\text{L}}=41$) and antral stage ($n_{\text{Mech}}=49$, $n_{0.7\text{L}}=78$, $n_{1.4\text{L}}=85$) follicles were encapsulated in 0.3% alginate and cultured individually in a 96-well plate with 100 μ L culture medium in 5% CO₂ in air at 38.5°C for 14 days. Every 48 h, 40 μ L of culture medium from each well was manually exchanged with fresh medium. Diameter evaluations were made on days 1, 3, 7, and 14 at which point damage to basement membranes (granulosa cell or oocyte extrusion) was also evaluated. On day 14, intact follicles (combining early antral and antral stages) were removed from alginate beads using two 20-gauge needles, and preserved together by isolation group in 200 μ L of RNAlater stabilization solution as earlier described for subsequent gene expression evaluation via RT-PCR ($n=20-39$ follicles/treatment, two to three replicates per treatment). For follicle morphology and apoptosis evaluations (below), follicles from an additional four cats, aged 2–12 months (EA: $n_{\text{Mech}}=5$, $n_{0.7\text{L}}=6$, and $n_{1.4\text{L}}=6$; Antral: $n_{\text{Mech}}=15$, $n_{0.7\text{L}}=13$, and $n_{1.4\text{L}}=8$) were isolated, encapsulated, and cultured for 14 days. At the end of

in vitro incubation, surviving and intact follicles were grouped by treatment and individual animals and fixed in 4% PFA until histological evaluation.

Histological evaluations

Alginate-encapsulated, fixed cultured follicles ($n_{\text{Mech}} = 19$, $n_{0.7\text{L}} = 16$, and $n_{1.4\text{L}} = 13$) were embedded in paraffin, cut into 6 μm thick sections and mounted on glass slides. For morphological analyses, on average, a total of 13 sections representing approximately 300 μm depth were processed for hematoxylin and eosin (H+E) staining, as previously described (Nagashima *et al.* 2019), for each follicle and treatment groups. This was achieved by evaluating the first eight sections (i.e. 48 μm depth) and then two sections of every ten subsequent slices. Following H+E staining, slides were assessed under light microscopy (Olympus B \times 40) for antral cavity status and putative theca cell layer presence. In H+E stained sections containing follicular oocytes with visible nucleoli, oocyte diameter was measured by averaging measurements of the oocyte at its widest diameter and its perpendicular diameter.

For apoptosis evaluation, two sections of every ten slices (also cumulatively representing ~ 300 μm depth) were assessed for indirect terminal deoxynucleotidyl transferase dUTP nick end label using ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Sigma–Aldrich S7110), as per manufacturer's instructions. Positive control slides were incubated with DNase for 15 min at 37°C following deparaffinization and rehydration, and negative controls were incubated in PBS in lieu of anti-digoxigenin antibody. All slides were counterstained with 1 $\mu\text{g}/\text{mL}$ DAPI (Invitrogen S33025) in Prolong Anti-fade mountant (Thermo Fisher P36980) and imaged under fluorescent microscopy (EVOS FL Auto 2, Thermo Fisher). Area of ApopTag- positive staining was normalized to area of DAPI-positive staining per image (as a proxy quantitation for proportion of TUNEL-positive cells) in ImageJ (v 1.52p, from imagej.nih.gov/ij). For each cat ($n = 4$ individuals), 3–6 follicles were evaluated per treatment

group, with the exception of one cat for which only one 1.4L follicle survived until day 14. Results are reported as average \pm s.e.m. across individual cats for each treatment group.

RNA isolation and RT-PCR

Freshly isolated and post-14 days cultured follicles were grouped together according to isolation method and culture status and RNA extracted via RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's protocol followed by treatment with RapidOut DNA removal kit (Thermo-Scientific) to eliminate genomic DNA contamination. Total extracted RNA was quantified using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo-Scientific).

cDNA was synthesized from 100 ng mRNA using a Transcriptor High Fidelity cDNA synthesis kit (Roche), according to the manufacturer's instructions. Expression of the following genes was evaluated: β -actin (Thongkittidilok *et al.* 2018), a reference gene), aromatase (CYP19A1 (Thongkittidilok *et al.* 2018), a measure of steroidogenesis in granulosa cells), growth differentiation factor 9 (GDF9 (Chansaenroj *et al.* 2019), oocyte-derived factor necessary for theca cell layer development (Dong *et al.* 1996)), luteinizing hormone receptor (LHR, a marker for theca cell function), STAR protein (Thongkittidilok *et al.* 2018) a regulator of cholesterol transfer for theca cell steroidogenesis), and vascular endothelial growth factor (VEGFA, a potential marker of theca cell presence and functionality). Primer details are included in Table 1. Reactions were performed according to the following parameters: 95°C for 10 min preincubation, followed by 50 cycles amplification at 95°C 30 s, 60°C for 10 s, 72°C for 10 s, and melting at 95°C for 10 s, 65°C for 1 min, and 97°C for 1 s. All reactions were performed in duplicate using a LightCycler® 96 (Roche). Primer efficiency was assessed for each gene by serially diluting cDNA and used for analysis via the Pfaffl method (Pfaffl 2001).

Table 1 Primer details.

Genes	Primer sequence		Accession number	Product length, bp
	Forward	Reverse		
ACTB	ATCCACGAGACCACCTTC	CACCGTGTAGCGTAGAG	AB051104.1	75 (Thongkittidilok <i>et al.</i> 2018)
CYP19A1	CAATCTGCTGCTCACTG	CCATGCAATAGCCAGGAC	GU306147.1	84 (Thongkittidilok <i>et al.</i> 2018)
GDF9	CATCCGTGGACCTGCTATTT	CCAGGTTGCACACACATTT	NM_001165900.1	129 (Chansaenroj <i>et al.</i> 2019)
LHR	GTCATGCCTTCAACGGGA	GGATGGACTCCAGCCCAT	XM_023251671.1	165
STAR	ATGGAAGCGATGGGAGAG	CAACTCGTGGGTGATGAC	NM_001246196	90 (Thongkittidilok <i>et al.</i> 2018)
VEGFA	CAGATGGAGAGCACAACC	ATACTCGATCTCATCAGGGT	XM_023253550.1	122

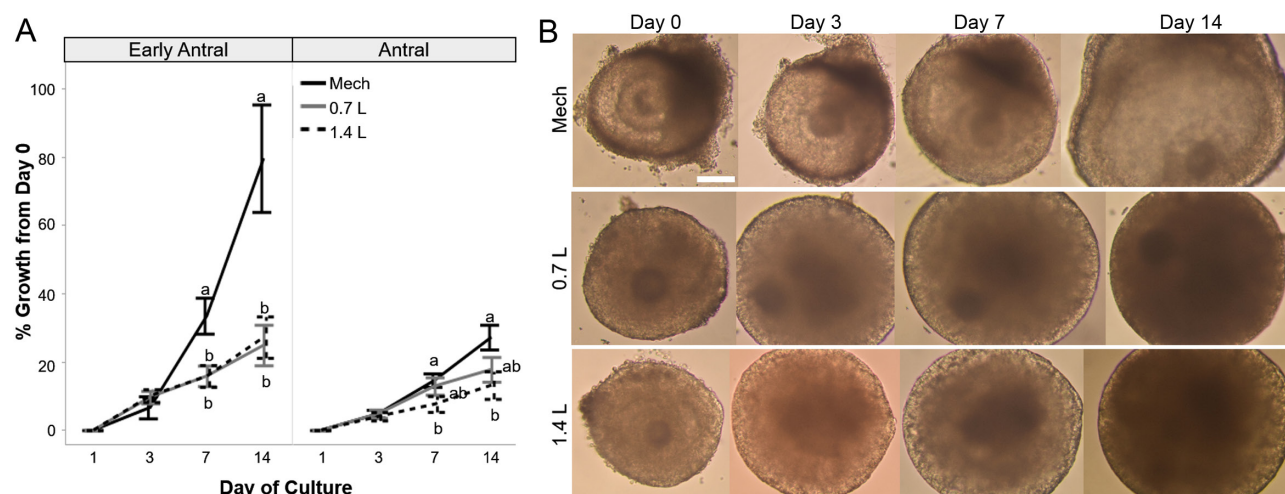


Figure 1 Growth over 14 days alginate-encapsulated *in vitro* culture of (A) early antral and antral stage follicles isolated mechanically or via 0.7L or 1.4L digestion, with letters indicating differences among isolation groups for each stage on a given day of culture ($P < 0.05$), and (B) representative images of size-matched follicles isolated via the three methods. White bar = 100 μm.

Statistical evaluations

Data analyses were performed with JMP Pro 12 software (SAS Institute Inc., Cary, NC, USA). Follicle growth was evaluated via standard least squares with the day of culture, isolation technique and follicle stage as well as the interaction between isolation technique and stage, as fixed effects, and individual cat as a random variable. *Post hoc* evaluations of growth on each day of culture were performed via Wilcoxon non-parametric test with significance set at $P < 0.05$. Evaluations of basement membrane integrity, antral cavity status, putative theca cell presence, and TUNEL staining between treatment groups after culturing for 14 days were also performed via Wilcoxon non-parametric test. Survival over the culture period was evaluated with Cox proportional hazards test with isolation strategy and follicle size and their interactions.

Results

In the preliminary evaluation of enzymatic digestion time (60, 90, vs 120 min) with both 0.7 and 1.4 Wünsch units/mL of Liberase blendzyme, more differences were observed between individual cats than between treatment groups in terms of follicle yield (Supplementary Fig. 1, see section on [supplementary materials](#) given at the end of this article). Thus, a digestion period of 90 min was selected to promote the isolation of early antral and antral stage follicles while allowing sufficient time for mechanical follicle isolation to be completed in parallel.

Subsequently, 0.7 and 1.4 Wünsch units/mL Liberase blendzyme were compared with mechanical dissection. Both early antral and antral stage follicles isolated via mechanical dissection experienced significantly higher growth than the enzymatically isolated counterparts, an effect that reached statistical significance in the latter half of culture (Fig. 1A). There was no difference between enzyme concentrations. For example, mechanically isolated early antral stage follicles increased in diameter $79.6 \pm 15.7\%$ (mean \pm S.E.M., from an average 290.5 ± 12.4 μm initial diameter to 513.5 ± 52.1 μm final diameter) by day 14, compared with $24.9 \pm 5.9\%$ (282.5 ± 5.8 to 343.8 ± 22.0 μm) and $27.2 \pm 6.0\%$ (284.9 ± 5.5 to 369.1 ± 20.7 μm) for 0.7L and 1.4L-isolated follicles, respectively ($P < 0.05$). Similarly, Mech-isolated antral follicles experienced a change in diameter of $27.2 \pm 3.6\%$ (438.9 ± 8.7 to 613.6 ± 17.7 μm) compared with $17.8 \pm 3.6\%$ (420.5 ± 6.0 to 499.2 ± 11.7 μm) for 0.7L follicles and $13.2 \pm 4.0\%$ (419.2 ± 6.5 to 481.1 ± 17.4 μm) for 1.4L follicles ($P < 0.05$). It should be noted that while ‘excess’ stromal cells were present on most follicles at the onset of culture, more were co-isolated with mechanical dissection compared with enzymatic methods (Fig. 1B).

A loss of basement membrane integrity in 40–60% of enzymatically isolated follicles was observable over the course of *in vitro* culture (Fig. 2A). While the loss of basement membrane integrity also occurred in mechanically isolated follicles, rates were significantly reduced for antral stage follicles. As a result, mechanically isolated antral follicles experienced significantly improved survival rates over 2-week culture ($P < 0.05$, Fig. 2B).

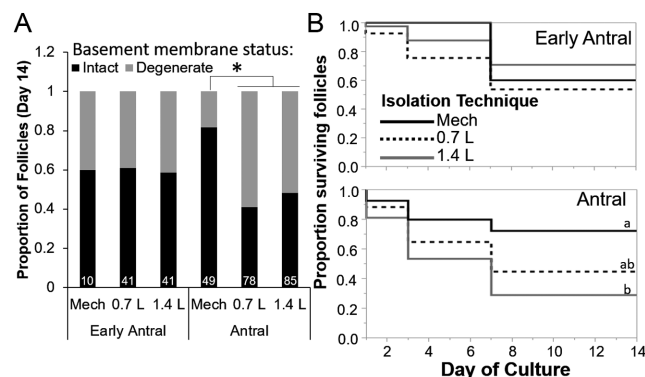


Figure 2 Early antral and antral stage domestic cat follicle survival *in vitro* after Mech isolation compared with 0.7L and 1.4L with (A) proportion of intact basement membranes on day 14 of culture, with asterisk (*) indicating differences among isolation groups for each stage and total follicles per group in white text, and (B) Kaplan-Meier survival, with letters indicating differences among isolation groups for each stage. $P < 0.05$.

In histological evaluations, a large portion of mechanically isolated follicles demonstrated antral cavities ($63.8 \pm 9.0\%$) and presence of cells with theca-like morphology ($65.0 \pm 12.6\%$). In contrast, follicles isolated via enzymatic digestion in 1.4L displayed the reduced presence of these characteristics ($P < 0.05$, Fig. 3) – with follicles from only one cat maintaining either following culture. While H+E sections containing oocyte nucleoli

were only identified in 20 follicles, and the measured values at nucleoli-containing slices did not necessarily represent the largest diameters, only one oocyte with a pyknotic nucleus was observed and average diameters were 77.7 ± 3.6 , 77.8 ± 4.8 , and 85.2 ± 5.5 μm for 0.7L, 1.4L, and Mech follicles, respectively ($P > 0.05$). Indirect-TUNEL staining of follicles following *in vitro* culture (Fig. 4A, controls in Supplementary Fig. 2) revealed a higher proportion of cells in Mech-isolated follicles with positive signal (normalized to DAPI, Fig. 4B). Apoptotic cells were observed at low levels, primarily in the granulosa cell layer in all treatment groups (Fig. 4A). Mech and 0.7L-isolated follicles also displayed evidence of apoptosis in theca cell layers (Fig. 4A and C).

Early antral and small antral follicles were pooled for gene expression evaluations. Fresh follicles collected immediately following isolation in either Mech, 0.7L, or 1.4L were compared with follicles isolated with each method and subsequently cultured for 14 days. Gene expression results were compared with fresh, mechanically isolated follicles. No significant differences in expression were observed among treatment groups for *CYP19A*, *GDF9*, *LHR*, or *VEGFA* (Fig. 5). Cultured follicles, regardless of isolation strategy, displayed reduced *STAR* expression compared with fresh/uncultured Mech- and

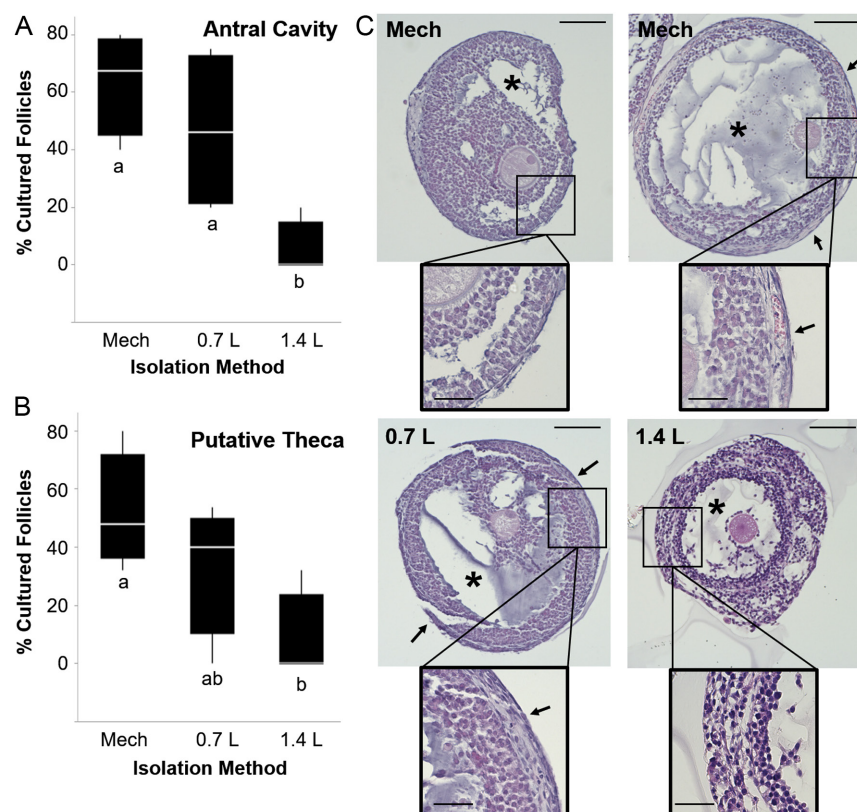


Figure 3 Cat follicles with antral cavities and/or putative theca cell layers after 14 days of *in vitro* culture, with percent of follicles (Mech, $n = 19$; 0.7L, $n = 16$, and 1.4L $n = 13$) displaying (A) antral cavities, or (B) putative theca cell layers, with letters indicating differences among isolation methods ($P < 0.05$), and (C) representative H+E images of Mech- vs 0.7L and 1.4L-isolated follicles, with * denoting antral cavities and black arrows marking putative theca layers. Scale bar = 100 μm .

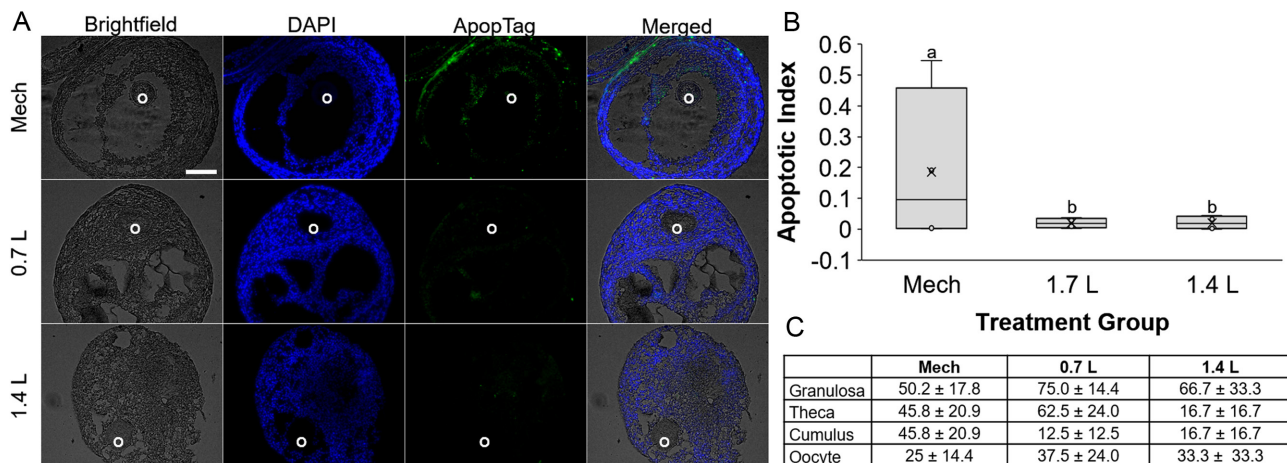


Figure 4 Apoptosis in domestic cat ovarian follicles isolated mechanically (Mech) or with 0.7 or 1.4 Wünsch units/mL Liberase blendzyme (0.7L, 1.4L) following 14 days *in vitro* culture with (A) representative brightfield, DAPI (blue), TUNEL (ApopTag, green), and merged images, (B) apoptotic index, where letters indicate statistically significant ($P < 0.05$) differences among treatment groups, and (C) percentage (\pm s.e.m.) of follicles from each treatment group with indirect TUNEL signal in granulosa, theca, or cumulus cells, or the oocyte.

0.7L-isolated controls. Uncultured follicles isolated in 1.4L also displayed reduced *STAR* expression compared with uncultured, Mech- and 0.7L-isolated controls ($P < 0.05$).

Discussion

In this study, we evaluated the impact of various isolation strategies for domestic cat early antral and antral stage ovarian follicles on *in vitro* survival, growth, and gene expression. We found that (1) mechanical isolation resulted in improved follicle growth and survival for early antral and antral stage follicles, respectively, when compared

with enzymatic methods, (2) differences in isolation strategy on *in vitro* follicle survival and development were only observable after >7 days culture, and (3) both mechanical and low-concentration enzymatic isolation appear to sustain theca cell layers for subsequent culture.

Previous studies have utilized either mechanical or enzymatic methods to isolate varying stage cat ovarian follicles. Yet, direct comparisons of the efficacy and impact of follicle isolation methods on *in vitro* growth and survival have been limited. In this study, we compared mechanical dissection vs enzymatic digestion with Liberase blendzyme TM at low (0.7 Wünsch units/mL) or high (1.4 Wünsch units/mL) concentrations. Our observation of the appearance of significant deviations in follicle growth and survival trajectories based on isolation strategy only after 7+ days of culture is notable. Namely, though fewer early antral stage follicles were collected via mechanical dissection compared to enzymatic in the 90 min time frame, Mech-isolated early antral follicles displayed the most robust growth *in vitro*. While several studies have evaluated various enzymatic isolation techniques for ovarian follicles, to our knowledge the longest culture duration applied to evaluate isolation methodology have been 7 (Vanacker *et al.* 2011) and 10 days (Fattahi *et al.* 2020). In the former study, similar protocols were applied to human ovarian biopsies for primordial and primary follicle isolation; however, owing to the stage, follicles were group-cultured, so following the individual fate of follicles over the culture period was not possible. In the latter study, porcine preantral follicles were isolated using mechanical (hand blender or mincing with a scalpel) and enzymatic (collagenase or Liberase DH) methods

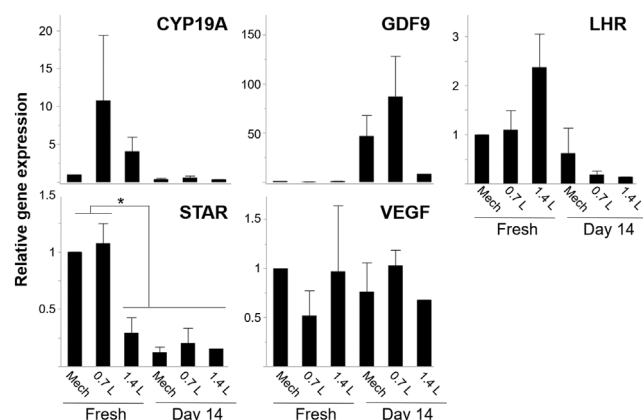


Figure 5 Gene expression relative to fresh, mechanically isolated domestic cat ovarian follicles, normalized to β -actin after isolation (Fresh), or following 14 day *in vitro* culture (Day 14) for each isolation method. Asterisk (*) represents statistically significant differences among groups ($P < 0.05$).

(Fattahi *et al.* 2020). Few intact follicles were collected via the mechanical methods utilized in that study, and therefore subsequent cultures were not performed on mechanically isolated follicles. Nevertheless, the authors similarly demonstrated a large disparity in survival following 10 days culture between follicles isolated with the two other strategies – collagenase (~28% survival) and Liberase DH (~58% survival) (Fattahi *et al.* 2020). Though these previous studies focused on preantral stage follicles in contrast with the current study, our results emphasize that both survival and growth potential of follicles are impacted by isolation method. These findings emphasize the need for prolonged (>7 days) post-isolation assessment for full evaluation of isolation techniques.

Various Liberase blends, dosages, and exposure times have been applied to the isolation of ovarian follicles in a variety of species (Dolmans *et al.* 2006, Lierman *et al.* 2015, Nagashima *et al.* 2017). For the Liberase TM utilized in the current study, concentrations typically range from 0.2–1.3 Wünsch units/mL with incubation periods ranging from 12–80 min (Dolmans *et al.* 2006, Kristensen *et al.* 2011, Schmidt *et al.* 2018, Buckenmeyer *et al.* 2020) for the isolation of preantral follicles. Specifically, 1.3 Wünsch units/mL concentration of Liberase TM has previously been applied to the isolation of murine primordial follicles over a 12 min incubation period (Buckenmeyer *et al.* 2020), but for human preantral follicles a range of 0.20–0.25 Wünsch units/mL with 70–80 min incubation is typically utilized (Dolmans *et al.* 2006, Kristensen *et al.* 2011, Schmidt *et al.* 2018). Recently, 0.5 mg/mL Liberase DH (equivalent to 3 Wünsch units/mL but with lower neutral protease activity than TM) for 70 min was used in the isolation of preantral porcine follicles (Fattahi *et al.* 2020). Although the 1.4 Wünsch units/mL for 90 min condition used in the present study was within the range used in previous research, follicles isolated via 1.4L rarely maintained/developed antral cavities during the *in vitro* incubation. This was likely due to damage to the theca cell layer during isolation, which was indicated in both the histological evaluations and STAR expression level reduction following isolation. However, follicles recovered by incubating ovarian tissue with a lower concentration (0.7 Wünsch units/mL) of Liberase grew and sustained the antral cavity and theca-like cells even after 14 days. The findings observed in the present study for the cat were similar to those reported previously by our laboratory with pre- and early- antral stage domestic dog follicles (Nagashima *et al.* 2017). In that study, 0.7 Wünsch units/mL of Liberase TM supported the isolation

of early antral stage follicles with the capacity to grow and produce steroids for at least 14 days *in vitro*.

The influence of excess co-isolated stromal cells on follicle growth cannot be discounted. These cells were not observed to proliferate disproportionately *in vitro* (Figs 1B and 3C), so did not contribute directly to the increased percent growth reached by mechanically isolated follicles in culture. Rather, we hypothesize that they supported follicle development. In the mouse, preantral follicles co-cultured with murine fibroblasts had improved survival and growth in 12 days *in vitro* culture (Kim *et al.* 2013). Ovarian stromal cells, identified as primarily theca cells and macrophages, also support primary and early secondary murine follicle growth and survival (Tingen *et al.* 2011). In a large mammalian example, buffalo preantral follicles had better survival and development in ovarian mesenchymal cell co-culture compared with controls, although co-culture with isolated cumulus cells was determined to have the best influence (Ramesh *et al.* 2008). In these studies, it was suggested that cytokines and growth factors produced by the stromal cells or involved in their communication with the ovarian follicles are likely responsible for the beneficial effects. As such, it is possible that the presence of additional stromal cells, less prevalent in enzymatically isolated follicles, conferred a similar advantage in the current study with the mechanically isolated follicles.

Our finding of higher percentages of TUNEL-positive cells in mechanically vs enzymatically isolated follicles following 14 days culture was surprising. Looking more closely at the localization, over half of the cultured follicles, regardless of treatment group, displayed low levels of indirect-TUNEL staining in granulosa cells. However, as noted by others, the observation of apoptotic granulosa cells in antral follicles can also be a part of the normal developmental process (Van Wezel *et al.* 1999, Regan *et al.* 2018). As previously noted, 1.4L-isolated follicles rarely displayed evidence of theca cell layers or antral cavities after 14 days culture, therefore little to no staining was localized to theca or cumulus cells in that group. In contrast, the relatively high positive signal in Mech-isolated follicles after 14 days culture can be attributed to these areas. In the cow, stimulation of theca cells with luteinizing hormone (LH) has been demonstrated to promote insulin-like growth factor expression, which in turn protects granulosa cells from apoptosis and supports follicle survival (Hattori *et al.* 2018). In our current culture system, we did not supplement LH during incubation. Though chronic LH supplementation *in vitro* has been

demonstrated to downregulate follicle responsiveness to FSH in the rat (Orisaka *et al.* 2013), sequential (e.g. added only in the latter half of culture) or intermittent (i.e. timed pulsatile addition) supplementation of LH may better support theca cell presence/function during extended culture *in vitro*. As oocyte developmental competence improves with advancing follicle size/stages, we propose that additional modifications to the culture system for these early antral and antral stage follicles should be explored for future genome rescue efforts.

It was promising to observe that, overall, gene expression was not significantly impacted by follicle isolation strategy. Based on what is known about the follicular localization of *CYP19A1* (primarily granulosa cells (Duggavathi *et al.* 2006, Echternkamp *et al.* 2012)), *GDF9* (primarily oocyte (Aaltonen *et al.* 1999, Paradis *et al.* 2009)) and *VEGFA* (both theca and granulosa cells (Yamamoto *et al.* 1997)) in other species, it follows that they were not overly influenced by isolation technique. Conversely, *STAR* is primarily localized to theca cells in murine and human follicles (Kiriakidou *et al.* 1996, Ronen-Fuhrmann *et al.* 1998). Reduced *STAR* in fresh, 1.4L-isolated follicles compared with Mech and 0.7L further indicates theca cell loss as a result of the more intensive enzymatic isolation strategy. *LHR* immunolocalizes to theca cells of domestic cat follicles of the early antral stage on but also in granulosa cells of large (>800 µm diameter) antral follicles and ovarian stromal cells (Saint-Dizier *et al.* 2007). LH stimulates *STAR* expression (Clark *et al.* 1995) and subsequently the production of androgens (Short 1962) to support the steroidogenic activity needed for advancement of folliculogenesis. Thus, the observed lower *STAR* expression in cultured follicles also supports the need for LH supplementation in the culture of these early antral and antral stage domestic cat follicles mentioned earlier.

In conclusion, we have determined that both mechanical micro-dissection and enzymatic digestion with 0.7 Wünsch units/mL Liberase blendzyme allow for the isolation of developmentally functional, advanced stage domestic cat ovarian follicles. Still, mechanically isolated early antral and antral follicles display improved growth and survival, respectively, during extended *in vitro* culture, potentially owing to the presence of an intact theca layer and supportive stromal cells. Oocyte morphology and size following culture were comparable to what has been reported for early antral stage cat follicles; however, subsequent studies should also evaluate the developmental competence of oocytes from these cultured follicles. Further, improved

long-term culture conditions to maintain theca cell presence and/or steroidogenic activity will be beneficial toward the objective of improving the production of meiotically competent oocytes from isolated domestic cat follicles *in vitro*.

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Supplementary materials

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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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Author contribution statement

J B N and A M H designed the study and performed the experiments, J B N, A M H and N S analyzed the results and prepared the manuscript.

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