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Bovine Ovarian Cortex Tissue Culture

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Cover Letter

University of Nebraska Lincoln

INSTITUTE OF AGRICULTURE AND NATURAL RESOURCES
DEPARTMENT OF ANIMAL SCIENCE

May 15, 2020

Editors of *JoVE*,

Please find the enclosed manuscript from my laboratory entitled "**Bovine Ovarian Cortex Tissue Culture.**" This manuscript describes how to conduct bovine ovarian cortex cultures and shows data from both histology (changes in follicle progression by follicle staging, altered fibrosis and oxidative stress) as well as measurement of daily and pooled ovarian cortex media steroids and metabolites and cytokines.

There are no copyrights, etc. for any figures or data in the manuscripts. We have data collected from individual animals (steroid metabolite and cytokines) to demonstrate to readers how much you can obtain from individual wells of 4 pieces of ovarian cortex per animal. These data also give the reader an idea of the variation between animals. Daily concentrations of androstenedione were also presented for several animals to show the daily measures that may be obtained.

We appreciate your consideration of this manuscript for publication in *JoVE*.

Sincerely Yours,

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TITLE:

Bovine Ovarian Cortex Tissue Culture

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KEYWORDS:

bovine ovarian cortex, hormones, follicles, tissue culture, culture medium, steroids, cytokines

SUMMARY:

In vitro culture of bovine ovarian cortex and the effect of nutritional Stair-step diet on ovarian microenvironment is presented. Ovarian cortex pieces were cultured for seven days and steroids, cytokines, and follicle stages were evaluated. The Stair-Step diet treatment had increased steroidogenesis resulting in follicle progression in culture.

ABSTRACT:

Follicle development from the primordial to antral stage is a dynamic process within the ovarian cortex, which includes endocrine and paracrine factors from somatic cells and cumulus cell-oocyte communication. Little is known about the ovarian microenvironment and how the cytokines and steroids produced in the surrounding milieu affect follicle progression or arrest. In vitro culture of ovarian cortex enables follicles to develop in a normalized environment that remains supported by adjacent stroma. Our objective was to determine the effect of nutritional Stair-Step diet on the ovarian microenvironment (follicle development, steroid, and cytokine production) through in vitro culture of bovine ovarian cortex. To accomplish this, ovarian cortical pieces were removed from heifers undergoing two different nutritionally developed schemes prior to puberty: Control (traditional nutrition development) and Stair-Step (feeding and restriction during development) that were cut into approximately 0.5–1 mm^3 pieces. These pieces were subsequently passed through a series of washes and positioned on a tissue culture insert that is set into a well containing Waymouth's culture medium. Ovarian cortex was cultured

for 7 days with daily culture media changes. Histological sectioning was performed to determine follicle stage changes before and after the culture to determine effects of nutrition and impact of culture without additional treatment. Cortex culture medium was pooled over days to measure steroids, steroid metabolites, and cytokines. There were tendencies for increased steroid hormones in ovarian microenvironment that allowed for follicle progression in the Stair-Step versus Control ovarian cortex cultures. The ovarian cortex culture technique allows for a better understanding of the ovarian microenvironment, and how alterations in endocrine secretion may affect follicle progression and growth from both in vivo and in vitro treatments. This culture method may also prove beneficial for testing potential therapeutics that may improve follicle progression in women to promote fertility.

INTRODUCTION:

The ovarian cortex represents the outer layer of the ovary where follicle development occurs[1]. Primordial follicles, initially arrested in development, will be activated to become primary, secondary, and then antral or tertiary follicles based on paracrine and gonadotropin inputs[1–4]. To better understand physiological processes within the ovary, tissue culture can be used as an in vitro model, thereby allowing for a controlled environment to conduct experiments. Many studies have utilized ovarian tissue culture for research in assisted reproductive technology, fertility preservation, and ovarian cancer[5,6,7]. Ovarian tissue culture has also served as a model for investigating reproductive toxins that damage the ovarian health and the etiology of reproductive disorders such as Polycystic Ovary Syndrome (PCOS)[8–11]. Thus, this culture system is applicable to a wide array of specialties.

In rodents, whole fetal or perinatal gonads have been used in reproductive biology experiments[12–15]. However, gonads from larger domestic livestock cannot be cultured as whole organs due to their large size and potential degeneration. Therefore, bovine, and non-human primate ovarian cortex is cut into smaller pieces[16–18]. Many studies have cultured small ovarian cortex pieces to study various growth factor(s) in primordial follicle initiation in domestic livestock and non-human primates[1,17–19]. The use of ovarian cortex culture has also demonstrated primordial follicle initiation in the absence of serum for bovine and primate cortical pieces cultured for 7 days[20]. Yang and Fortune in 2006 treated fetal ovarian cortex culture medium with a range of testosterone doses over 10 days and observed that the 10^{-7} M concentration of testosterone increased follicle recruitment, survival, and increased progression of early stage follicles[19]. In 2007, using ovarian cortex cultures from bovine fetuses (5–8 months of gestation), Yang and Fortune reported a role for Vascular Endothelial Growth Factor A (VEGFA) in the primary to secondary follicle transition[21]. Furthermore, our laboratory has utilized ovarian cortex cultures to demonstrate how VEGFA isoforms (angiogenic, antiangiogenic, and a combination) may regulate different signal transduction pathways through the Kinase domain receptor (KDR), which is the main signal transduction receptor that VEGFA binds[16]. This information allowed for a better understanding of how different VEGFA isoforms affect signaling pathways to elicit follicle progression or arrest. Taken together, culturing of ovarian cortex pieces in vitro with different steroids or growth factors can be a valuable assay to determine effects on mechanisms regulating folliculogenesis. Similarly, animals that are developed on different nutritional regimes may have altered ovarian microenvironments, which may promote or inhibit folliculogenesis affecting

female reproductive maturity. Thus, our goal in the current manuscript is to report the bovine cortex culture technique and determine whether there are differences in ovarian microenvironments after in vitro culture of bovine cortex from heifers fed either Control or Stair-Step diets collected at 13 months of age as described previously[16].

Therefore, our next step was to determine the ovarian microenvironment in these heifers that were developed with different nutritional diets. We evaluated ovarian cortex from heifers fed with either a Stair-Step or Control diet. Controls heifers were offered a maintenance diet of 97.9 g/kg$^{0.75}$ for 84 days. The Stair-Step diet was initiated at 8 months containing a restricted fed diet of 67.4 g/kg$^{0.75}$ for 84 days. After the first 84 days, while Control heifers continued to receive 97.9 g/kg$^{0.75}$, the Stair-Step beef heifers were offered 118.9 g/kg$^{0.75}$ for another 68 days, after which they were ovariectomized at 13 months of age[16] for studying changes in follicular stages and morphology before and after culture. We also assayed for differences in steroids, steroid metabolites, chemokines, and cytokines secreted into cortex media. Steroids and other metabolites were measured to determine if there were any direct effects from treatments conducted in vivo and/or in vitro on tissue viability and productivity. Changes in the ovarian microenvironment prior to and after culture provided a snapshot of the endocrine milieu and folliculogenesis prior to culture and how culture or treatment during culture affects follicle progression or arrest.

Ovaries were collected after ovariectomies were performed at the U.S. Meat Animal Research Center (USMARC) according to their IACUC procedures from Control and Stair-Step heifers at 13 months of age[16], cleaned with sterile phosphate buffer saline (PBS) washes with 0.1% antibiotic to remove blood and other contaminants, trimmed excess tissue, and transported to the University of Nebraska-Lincoln (UNL) Reproductive Physiology laboratory UNL at 37°C[23]. At UNL, ovarian cortex pieces were cut into small square pieces (~0.5–1 mm^3; **Figure 1**) and cultured for 7 days (**Figure 2**). Histology was conducted on the cortex culture slides prior to and after culture to determine follicles stages[16,24] (**Figure 3** and **Figure 4**), and extracellular matrix proteins that may indicate fibrosis (Picro-Sirus Red, PSR; **Figure 5**). This allowed determination of effect of in vivo nutritional regimes on follicle stages and allowed comparison of 7 days of ovarian cortex on follicle stages and follicle progression. Throughout the culture, the medium was collected and changed daily (approximately 70% of media was collected each day; 250 µL/well) so that either daily hormones/cytokines/chemokines can be assessed or pooled over days to obtain average concentrations. Steroids such as androstenedione (A4) and estrogen (E2) can be pooled over 3 days and assessed through radioimmunoassay (RIA; **Figure 6**) and pooled over 4 days per animal and assayed via High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)[24,25] (**Table 1**). Cytokine arrays were utilized to assess cytokine and chemokine concentrations in ovarian cortex culture medium[26] (**Table 2**). Real-time polymerase chain reaction (RT-PCR) assay plates were conducted to determine gene expression for specific signal transduction pathways as demonstrated previously[16]. All of the steroid, cytokine, follicle stage and histological markers provide a snapshot of the ovarian microenvironment and clues as to the ability of that microenvironment to promote “normal” or “abnormal” folliculogenesis.

PROTOCOL:

The ovaries were obtained from U. S. Meat Animal Research Center[16]. As stated previously[16], all procedures were approved by the U.S. Meat Animal Research Center (USMARC) Animal Care and Use Committee in accordance with the guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching. The ovaries were brought to the University of Nebraska-Lincoln Reproductive Laboratory where they were processed and cultured.

1. Preparation of required media

1.1. Waymouth MB 752/1 medium

1.1.1. Fill a 1 L tissue culture bottle with 900 mL of sterile water. While the water is gently stirring on a stir plate, gradually add the powdered medium. Once the powdered medium is dissolved, add 2.24 g of sodium bicarbonate followed by 1.25 g of bovine serum albumin (BSA). Use a pH meter and adjust the pH to 7.25–7.35. Add additional sterile water to bring the final volume to 1 L.

1.1.2. Move to a biological safety cabinet and add penicillin-streptomycin sulfate at a concentration of 0.1% v/v of the medium. Filter the medium with a 0.22 µm pore 33.2 cm^2 500 mL bottle top filter.

1.1.3. Pour off the filtered medium into several 50 mL conical tubes. Add 0.5 mL of Insulin-Transferrin-Selenium per 50 mL of aliquoted medium.

1.1.4. Wrap the conical tubes and stock bottle of the medium in aluminum foil and store at 4 °C. This medium is light sensitive.

NOTE: Waymouth medium can be stored for up to 1 month.

1.2. Leibovitz's L-15 (LB-15) medium

NOTE: LB-15 medium is used to clean tissue in preparation for culture.

1.2.1. Fill a 1 L tissue culture bottle with 900 mL of sterile water. While the sterile water is gently stirring on a stir plate, gradually add the prepared powdered medium. Use a pH meter and adjust the pH to 7.25–7.35. Add additional sterile water to bring the final volume to 1 L.

1.2.2. Move to biological safety cabinet. Make 1 L of LB-15 with 0.1% antibiotic (see **Table of Materials**). Filter the medium into two 500 mL tissue culture bottles using a 0.22 µm pore 33.2 cm^2 500 mL bottle top filter. Wrap bottles in aluminum foil as LB-15 medium is light-sensitive and store at 4 °C.

NOTE: LB-15 medium can be stored for up to 1 month.

1.3. Phosphate Buffered Saline (PBS)

1.3.1. Make PBS in the lab or purchase sterile PBS without calcium or magnesium (**Table of Materials**). To make PBS in the lab, begin with 800 mL of distilled water and add 8 g of sodium chloride (NaCl) to it. Then, add 0.2 g of potassium chloride (KCl), 1.44 g of sodium phosphate dibasic (Na_2HPO_4), and 0.24 g of potassium phosphate dibasic (KH_2PO_4). Adjust pH to ~7.4 and adjust total volume to 1 L. Sterilize the solution by autoclaving.

1.3.2. Make 1 L PBS with 0.1% antibiotic (see **Table of Materials**) while in a biological safety cabinet.

2. Ovarian cortical culture protocol

NOTE: Ovaries were obtained from spring born USMARC heifers at 13 months of age. Ovaries were rinsed thoroughly, and all blood and other fluid were removed with PBS containing antibiotic (0.1%) and transported at 37 °C[23] to University of Nebraska-Lincoln Reproduction Laboratory UNL (1.5 h away). (For comments on temperature of ovaries during transport please see **Discussion**.)

2.1. Prepare the ovarian tissue on a clean bench (**Figure 1**).

2.1.1. Disinfect the clean bench with 70% ethanol. Place a fresh absorbent pad on the benchtop. Ensure that the clean bench blower is turned on half an hour prior to dissection along with UV light to sterilize anything in the clean bench, including absorbent pad and make sure appropriate PPE is used.

2.1.2. Arrange the Petri dishes (60 x 15 mm) for tissue washes. Three Petri dishes are required for PBS wash, three for PBS with antibiotic, and three for LB-15 washes. An additional LB-15-containing Petri dish with accompanying lid will be utilized for final placement of pieces after washes.

2.1.3. Fill each Petri dish with approximately 10 mL of appropriate fluids, either PBS or LB-15.

[Place **Figure 1** here]

2.2. Remove the prepared Waymouth and LB-15 medium from the refrigerator and warm to room temperature.

2.3. Autoclave all tools to ensure sterilization prior to use.

2.4. Maintain ovaries at 37 °C until ovarian cortex is ready to be collected.

2.5. Using forceps with serrated jaws, pick up the ovary and thoroughly wash in the first PBS-filled Petri dish. Transfer the ovary to the second PBS wash and thoroughly cleanse once more.

NOTE: The ovary will stay in the second PBS wash while ovarian cortical strips are removed.

2.6. Using serrated jaw forceps, secure the ovary and slice in half. At this time, the ovarian cortex will cut away from the medulla. Using a ruler, make sure that no more than 1–2 mm of depth of surface of ovary is removed away from the medulla[16]. Remove transverse sections of the ovarian cortex from medulla, cut 3–4 thin strips of ovarian cortex (**Figure 2**) with a scalpel (#11 scalpel blade; #3 handle), and place the strips in the third PBS-filled Petri dish.

NOTE: At this time, additional ovarian cortical tissue can be collected for RNA extraction or fixed and collected for histology of initial non-cultured cortex pieces. When removing strips of ovarian cortex, avoid areas with visible antral follicles or corpora lutea. In addition, avoid collecting medullary tissue. The histology of the medulla is very different as shown previously[16]. If the ovarian cortex is not cut to more than a 1–2 mm depth, then the medulla should not be obtained. Distinct histology allows for landmarks between the cortex and the medulla.

2.7. Cut the ovarian cortex strips in the third PBS wash into small, square pieces (~0.5–1 mm^3) with a #21 scalpel blade. Use a ruler underneath the Petri dishes to ensure the pieces are of similar size and thickness to make consistent ovarian cortex pieces. Use forceps to secure the strips while cutting the pieces with a scalpel.

NOTE: The number of tissue pieces cut is dependent on the experiment. Four pieces of ovarian cortex is the minimum amount of tissue necessary for culture. Other methods for ensuring appropriate length and depth include using special slicers[26] or precut plastic pieces as templates [27].

2.8. Wash ovarian cortical pieces through all three PBS with antibiotic-filled Petri dishes. Use a curved tip forceps to move pieces between washes.

2.9. Move cortex pieces through the series of LB-15 washes and place in final LB-15-filled Petri dish. Label the lid with animal ID and ovary side (left or right).

NOTE: Fully submerge the ovarian cortex pieces in each wash for thorough cleaning.

2.10. Collect four ovarian cortex pieces per ovary and fix for day zero histology. Additional pieces can also be flash frozen for RNA. The remaining tissue pieces will be used for culture. Wipe down dissecting tools with 70% ethanol after each tissue collection.

2.11. Prepare a biological safety cabinet for final tissue wash and culture preparation. Sanitize supplies with 70% ethanol before placing in the biological safety cabinet. Use the aseptic technique when working in the biological safety cabinet.

2.12. Move all ovarian cortex intended for culture to the biological safety cabinet and wash once more in an LB-15-filled Petri dish.

2.13. In a 24-well tissue culture plate, pipette 350 µL of Waymouth medium per well.

2.14. Place uncoated culture well inserts into each well using forceps. Ensure that no bubbles are formed under the base of the insert as this would result in the tissue drying out. The medium must be touching the inserts to allow for the medium to be absorbed up and surround the ovarian cortex pieces.

2.15. Carefully position four ovarian cortex pieces onto the mesh of each insert (**Figure 2**). The forceps can puncture the mesh if the tissue pieces are not delicately placed. The tissue pieces should not be touching each other or the side of the insert.

2.16. Incubate the tissue at 37 °C with 5% CO_2[16].

NOTE: Others have used 38.8 °C[28]. However, no difference has been observed in the integrity of the tissue nor in the ability of follicles to progress in 37 °C tissue nor have others[39,30]. Thus, at this point any of these temperatures should be conducive to experiment success. Others have used 400 µL of medium. Either amount is fine as long as one is consistent, and the tissue is partially submerged allowing for adequate surface tension to allow for hydration of tissue (media surrounding ovarian cortex pieces). Fill empty wells with 500 µL of sterile water to help reduce evaporation from other wells.

Place **Figure 2.** (Insert and plate)

3. Media collection

3.1. Change ovarian cortex culture medium daily for 7 days. Medium changes should be as close to 24 h apart as possible to prevent large pH and color changes in medium. Warm Waymouth medium to 37 °C prior to medium change. Approximately 250 µL is obtained from each well each day (about 70% of initial culture medium).

3.2. During medium changes, use forceps to gently lift the insert out of the well. Collect the cultured Waymouth medium in 0.5 mL tubes (approximately 250 µL /day). Set the insert back in well and add 350 µL of fresh culture medium by dispensing the medium between the side of the insert and well.

NOTE: Change most of the media daily to obtain enough media to measure all the steroids, cytokines, and chemokines necessary to determine ovarian microenvironment. Also, daily medium changes are important to prevent large pH changes (indicated by color change) in the medium. Drops of medium were retained surrounding the ovarian cortex pieces to ensure the pieces remained wet. No problems were observed with cultured tissue due to changing 70% of the media.

3.3. Store the collected medium from tissue culture at -20 °C.

4. Imaging and downstream processing

4.1. After 7 days of culture at 37 °C with 5% CO_2, image the ovarian cortex pieces using a dissection microscope with an attached camera and a computer imaging software program.

NOTE: A dark room is usually best for achieving the best picture quality for imaging.

4.2. After imaging, fix two ovarian cortex pieces per well in Bouins for histology and flash freeze two ovarian cortex pieces in liquid nitrogen to obtain RNA for cDNA. Repeat this step for all the wells with tissue. Collect the medium from day 7 and store at -20 °C.

4.3. Let the ovarian cortex pieces remain immersed in Bouins (picric acid 750 mL, glacial acetic acid 50 mL, and 37%–40% formalin 250 mL) for approximately 1.5 h before being washed with 70% ethanol three times. The tissue will remain in 70% ethanol and be cleared daily until the solution is no longer yellow.

NOTE: Fixatives other than Bouins as well as paraformaldehyde can be used. In this experiement Bouins is used as it is the fixative to achieve optimal morphology. If more tissue is required for other analysis, additional wells of media and pieces of ovarian cortex can be obtained from each animal.

REPRESENTATIVE RESULTS:

This bovine cortex culture procedure can be used to determine a wide variety of hormone, cytokine, and histology data from small pieces of the ovary. Staining, such as hematoxylin and eosin (H&E), can be used to determine ovarian morphology through follicle staging[16,23,31] (**Figure 3**). Briefly, follicles were classified as primordial, which is an oocyte surrounded by a single layer of squamous pre-granulosa cells (0); transitional follicle or early primary, which is an oocyte surrounded by mostly squamous pre-granulosa cells and some cuboidal granulosa cells (1); primary follicle, which is an oocyte surrounded by 1–1.5 layers of cuboidal granulosa cells (2); secondary follicle, which is an oocyte surrounded by two or more cuboidal granulosa cells (3); antral follicle, which is no larger than 1 mm in diameter and surrounded by two or more layers of granulosa cells containing a distinct antrum (4)[16,23] (**Figure 3**). Follicle staging can be conducted on ovarian cortex fixed prior to and following culture to assess folliculogenesis (**Figure 4**). We took three images per slide from three different slides stained with H&E. Then, the follicles were staged and counted by three individuals and averaged to determine the number of follicles at each stage[16,23]. The area of the field of view for an image (three per slide) at 400x magnification is 0.4mm^2. Thus, 30% of the area of the ovarian cortex pieces were counted to determine follicle stages. The initial follicle number (before culture) (**Figure 4A**) is used to normalize the follicles counted after culture (**Figure 4B**).

Additionally, differences in morphology as determined by collagen deposition (Picro Sirus Red staining) can indicate fibrosis in ovarian cortex from Stair Step or Control heifers (**Figure 5**). Daily collection of culture medium can be pooled over 3 days to assess varied steroid hormone production by RIA (using 200 µL medium sample per animal; **Figure 6**) or steroid metabolites

using high performance liquid chromatography mass spectrometry (HPLC-MS; 220 µL medium sample pooled over 4 days per animal; **Table 1**) and cytokine production (**Table 2**). Therefore, several replicates of one animal may be required to ensure enough cortex medium to perform all the desired assays.

Because Stair-Step heifers have increased primordial follicles at the beginning of the culture we expected these to progress in culture and obtain greater number of secondary follicles, which we observed in the results. Also, due to increase in secondary follicles, we would expect greater concentrations of steroids. We did see tendency for increases in androgens, glucocorticoid metabolites, and progesterone metabolites, which would support this in the current manuscript. Our lab has also evaluated effects of different VEGFA isoforms on follicle progression, steroidogenesis, and activation of different signal transduction molecules in the KDR (also known as Vascular Endothelial Growth Factor Receptor 2; VEGFR2) using signal transduction array plates[16]. To reduce animal variation, we use 4–6 animals per treatment and for other experiments depending on power analysis and variability we have used as many as 11.

Repeatability of results from bovine ovarian cortex culture is most affected by contamination of ovarian cortex pieces. Additionally, negative, or subpar results can occur if the medium is not changed regularly within a 24-h period. The medium when originally added is pink in color, but when the medium is collected, and the color appears to be orange or bright yellow this could indicate a change in pH that could be detrimental to the tissue. Also, tissue pieces that are cut too large might develop degeneration in the middle that would not be observed until tissue sectioning for histology. This degeneration will limit the use of the tissue for analysis. The data presented in this paper has been analyzed using nonparametric tests and a general linear model analysis in a statistical software program. The number of primordial, primary, secondary, and antral follicles per section before and after culture were analyzed using a generalized linear mixed model. Significance was determined at $P < 0.05$ and a tendency was reported at $0.14 \leq P \geq 0.05$.

FIGURE AND TABLE LEGENDS:

Figure 1: Layout of plates for washing the ovary and cortex pieces in the clean bench. (**A**) PBS used for washing the ovary as sections of the cortex are removed. (**B**) PBS with antibiotic washes that cortex pieces are moved through. (**C**) Ovarian cortex pieces are washed four times in LB-15 before moving to the biosafety cabinet for final wash in LB-15.

Figure 2: Ovarian cortex pieces and culture plate. (**A**) An ovarian strip being cut from the cortex of the ovary. (**B**) Ruler and cortex piece shown side by side. (**C**) Four cortex pieces (~0.5–1 mm^3) resting on the insert in the culture medium in the plate. (**D**) Lifting the insert to collect the culture medium from the well. Collect and replace all the culture medium daily (250 µL) to maintain proper pH. Approximately 250 µL is obtained from each well each day (about 70% of the initial culture medium).

Figure 3: Hematoxylin and Eosin staining for follicle staging of ovarian cortex. Different stages of follicles are indicated by arrows. (**A**) Primordial follicles (stage 0); (**B**) Early primary follicles (stage 1); (**C**) Primary follicles (stage 2); (**D**) Secondary follicle (stage 3); (**E**) Antral follicle (stage

4). Area of the field of view for an image at 400x magnification is 0.4 mm^2. We count 30% of the area of the ovarian cortex pieces to determine the follicle stages.

Figure 4: Average number of follicles at different follicular stages in Control (n=6) and Stair-Step (n=6) heifers. (**A**) Before culture Primordial P = 0.001, Early Primary P = 0.12, Primary P = 0.31, Secondary P = 0.22. (**B**) After 7 days of culture, Primordial P = 0.37, Early Primary P = 0.84, Primary P = 0.69, Secondary P = 0.02. Error bars are representative of SEM.

Figure 5: Collagen (Picro Sirius Red; PSR) staining in ovarian cortex. PSR in (**A**) Control and Stair-Step heifers from Day 0 and Day 7. (**B**) Graph comparing the average area of PSR-positive staining per ovarian cortex field (pixels/µm2) between Control (n = 4) and Stair-Step (n = 4) heifers. Error bars are representative of SEM. Area of the field of view for an image at 400x magnification is 0.4mm^2.

Figure 6: Concentrations of A4 and E2. (**A**) Concentration of A4 and (**B**) concentration of E2 pooled over 3 days of culture in ovarian cortex media of Control and Stair-Step heifers as measured by RIA's. n = 4 for each group. Error bars are representative of SEM.

Table 1: Steroid and steroid metabolites measured in ovarian cortex culture medium from one well for each animal pooled over 4 days of culture. Data presented with mean ± SEM. Blue indicates P < 0.1 and has a tendency to be different.

Table 2: Cytokine and chemokines measured in ovarian cortex culture medium from one well for each animal pooled over 4 days of culture. Data presented with mean ± SEM. Blue indicates P < 0.1–0.14 and has a tendency to be different.

DISCUSSION:

The benefit of in vitro ovarian cortex culture, as described in this manuscript, is that the follicles develop in a normalized environment with adjacent stroma surrounding the follicles. The somatic cells and oocyte remain intact, and there is appropriate cell-to-cell communication as an in vivo model. Our laboratory has found that a 7-day culture system provides representative folliculogenesis and steroidogenesis data for the treatment of the ovarian cortex. Other ovarian tissue culture protocols have either relatively short culture periods 1–6 days[7,32] or long culture periods of 10–15 days[5,6,10]. However, we have observed that culturing for greater than 7 days leads to tissue degradation, reduced steroidogenesis, and potentially an increased likelihood of contamination (data not shown). Culturing ovarian cortex following ovariectomy also provides direct insight into the ovarian microenvironment within that particular animal. This is of interest to our research laboratory as we have identified changes in follicular development after nutritional regimes were imposed after weaning and leading to puberty in heifers[16].

Follicle development from the primordial to antral stage is a dynamic process within the ovarian cortex, which includes endocrine and paracrine factors from somatic cells and cumulus cell-oocyte communication[33]. Tissue culture, such as ovarian cortex culture, offers a controlled environment to investigate the mechanistic role of these factors and the endocrine milieu in the

ovarian microenvironment. Ovaries can be collected from animals that have gone through an in vivo treatment or are genetically altered to determine effects on follicle progression.

Ovaries can also be collected from a local abattoir (1 h away) and transported at 37 °C in a thermos containing PBS with antibiotic. If transport is longer (e.g., overnight), the ovaries are shipped or transported on ice[22]. Similar results have been observed whether the transport of ovaries happens on ice overnight or at 37 °C with short-term transport in a thermos. The 37 °C with a thermos transport allows for harvest of oocytes from this tissue for in vitro maturation (IVM) or in vitro fertilization (IVF)[34]. Other studies have found that transporting tissue at temperatures between 2–8 °C have also been used for fertility preservation in reproductive tissues[35–37]. Yet other studies have used ovaries transported at 34–37 °C and on ice and have not observed differences in bovine tissue culture[38].

If ovarian cortex culture tissue does not look healthy after culture, this could be due to the pieces of ovarian cortex being too large. A critical step in the protocol is to ensure that the ovarian cortex pieces are no larger than 0.5–1 mm^3. We utilize a ruler to measure the pieces and use a scale within the dissecting microscope to determine size of the ovarian cortex pieces (**Figure 2C**). Others utilize specific equipment (see **Table of Materials**) for uniform thickness and length/width, respectively[26]. If these pieces of equipment are not available, then plastic squares can be used to as a template to obtain uniform thickness and ensure similar size pieces[27].

To ensure cut pieces that are only from the cortex and do not have medulla after washing the ovary we place it in the 60 mm dish and cut in half. The cortex and medulla are very different histologically as seen previously in Abedal-Majed, 2020[16]. Each half of the ovary is filleted with the blade to ensure that only cortex is removed from the ovary and the medulla remains. If individuals are just starting to perfect this cutting technique, they can also use neutral red to closely see the histology of each half of the ovary[39]. This will allow for development of landmarks as their cutting technique, improves. Furthermore, they can use instruments that can cut uniform thickness (see **Table of Materials**) as stated above[26,27].

Ovarian cortex medium should be light pink. We have observed that the pH of the ovarian cortex culture medium changes quickly in some animals, thus, the majority (70%) of the ovarian cortex medium should be changed every 24 h to promote culture health. Previous papers have discussed changing half of the medium daily[40]. Our reason for changing a majority of the media in the current manuscript was to promote the health of the ovarian cortex cultures. Drops surrounding the ovarian cortex pieces remain and there were no negative effects of medium change on the culture. Furthermore, this allowed us to analyze more hormones, cytokines, and chemokines for each animal to generate insight into the ovarian microenvironment.

This tissue culture protocol offers several advantages. Culturing ovarian cortex allows follicles to develop in an environment that is similar to in vivo. Follicles remain supported by the surrounding stroma and communication between somatic cells and cumulus cell-oocyte continues. The usage of culture well inserts enables the ovarian cortex to rest upon the culture medium without being submerged, thereby preventing the tissue from binding to the plastic base of the well plate.

Another advantage is the culture window. The 7-day culture window described in the protocol provides representative hormone and growth factor data. In addition, folliculogenesis continues to progress in this in vitro environment as we have counted fewer early-staged follicles (primordial) and more late-staged follicles (secondary, antral) after 7 days of culture[16]. Previous ovarian tissue culture protocols have utilized relatively short (1–6 days[7,32]) or long (10–15 days[5,6,10]) culture periods. Shorter culture windows have been used to investigate primordial follicle activation in fetal bovine ovarian cortex[41]. In non-human primates, a 20-day culture period was used to evaluate the ability of the primate primordial follicles to survive and initiate growth in vitro in serum-free medium[18]. However, we have observed increased tissue degradation when culturing ovarian bovine cortex for longer than 7 days in serum-free medium. We have also determined in analysis of daily samples that steroid concentrations are decreased after 4 days of culture (data not shown). A longer culture window can also increase the possibility of contamination in ovarian cortex cultures. Therefore, major effects can be measured by 7 days of culture[15] and time of culture may be dependent on the animal model and scientific question addressed.

While several advantages of this bovine ovarian cortex protocol exist, there are a few limitations. One limitation is the quantity of ovarian cortical tissue and culture medium volume collected during ovarian cortical culture. The small size of the ovarian cortex pieces allows for a limited number of tissue sections to be used for staining purposes (H&E, immunofluorescence, etc.). To perform RT-PCR, a minimum of four cortex pieces are needed[16]. Furthermore, the low volume of culture medium collected may limit the amount of analyses conducted. To combat these limitations, we suggest culturing several replicates per ovary to provide an adequate supply of culture medium and tissue for histology, assays, and PCR. Quite often we culture several pieces of each ovary from one cow/heifer to obtain more tissue for further analysis and to obtain increased culture medium to measure cortex secretion of hormones/cytokines/chemokines. Preantral follicles are more diffuse in older cow ovaries than younger females (heifers). Thus, a potential limitation is obtaining preantral follicles on all ovarian cortex pieces that are cultured. Several ways to mitigate this limitation is to obtain more ovarian cortex pieces and to culture additional wells for each animal or to use neutral red to visualize follicles and ensure that all cortex pieces contain early preantral follicles. A third limitation of the ovarian cortex culture is that any contamination of the culture system makes the media and cortex pieces unusable for analysis. Thus, several ways to maintain a sterile environment is to filter the medium used in the culture. Prior to processing and cutting ovarian cortex pieces make sure that the clean bench has been sterilized with 70% ethanol, and the air flow has been running for at least 30 min prior to dissections. Also, if using an absorbent pad to enable easier clean-up (**Figure 1**), ensure that the UV light has been turned on for 30 min prior to placing any tissue in the clean bench to sterilize the pad and clean bench. Finally, all media changes should be conducted in a biosafety cabinet with adequate airflow, sterile instruments, and changing pipet tips to ensure only sterile tips are introduced into the medium to be placed in wells for ovarian cortex culture.

Application of this technique will aid in understanding the ovarian microenvironment and may start to unravel mechanisms involved in female reproductive disorders that involve altered follicular development. For example, the etiology of polycystic ovary syndrome (PCOS) and

aspects of premature ovarian failure (POF) remain unclear. Since cows are mono-ovulatory, they make an excellent model to understand factors that affect follicle progression and arrest in other mono-ovulatory species (e.g., humans and non-human primates). Furthermore, this ovarian cortex culture method may also prove beneficial for testing potential therapeutics that may improve follicle-mediated disorders resulting in infertility in women.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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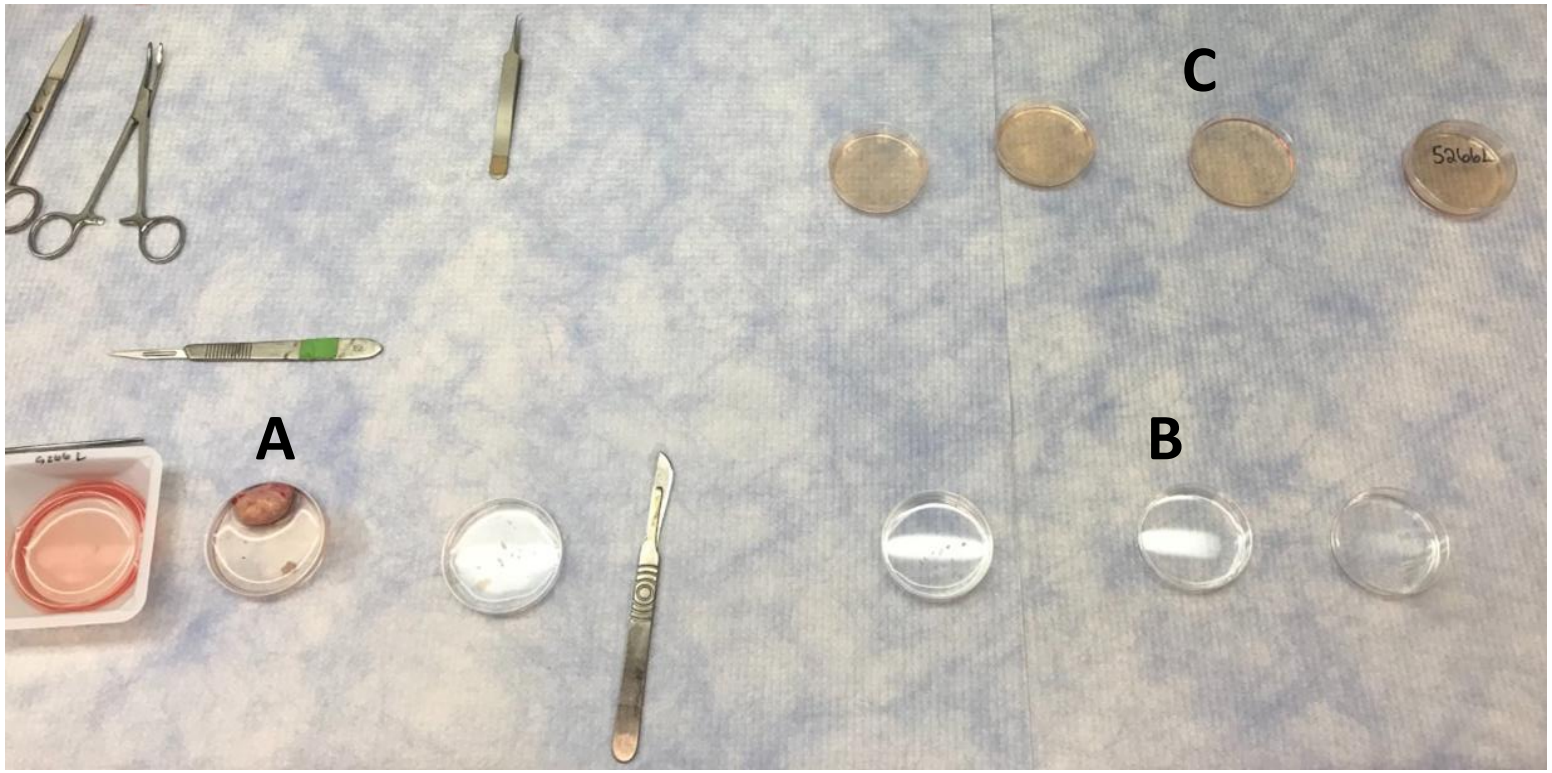

Figure 2

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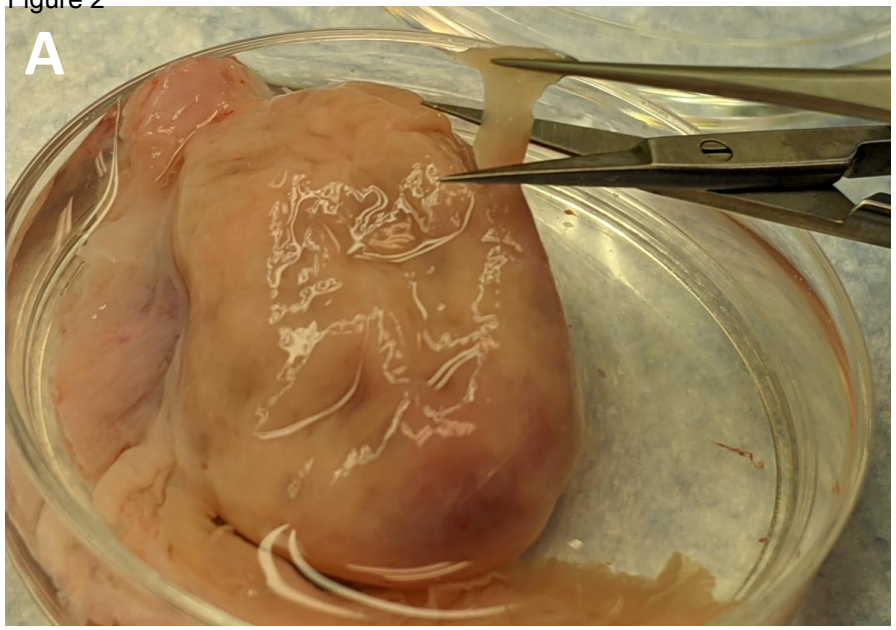

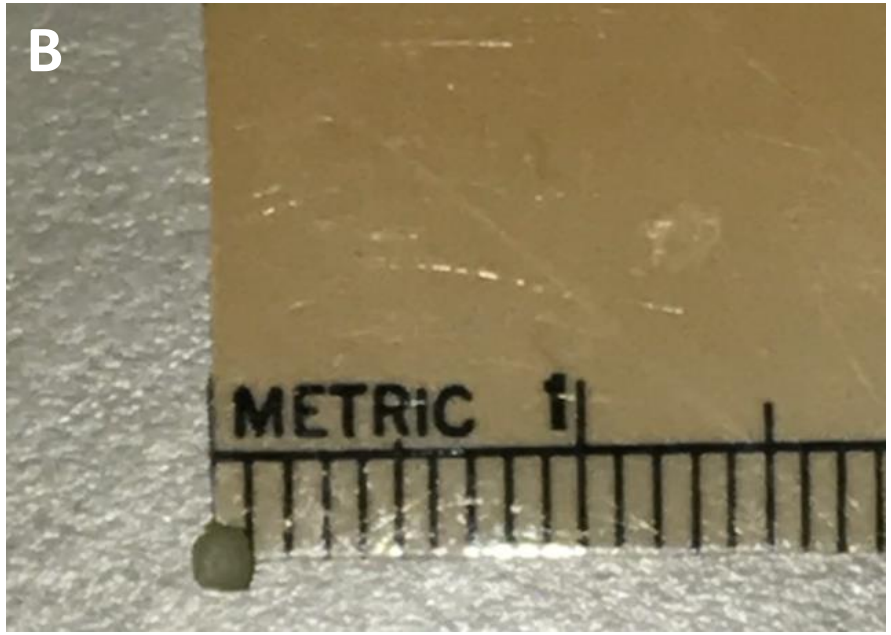

Figure 3

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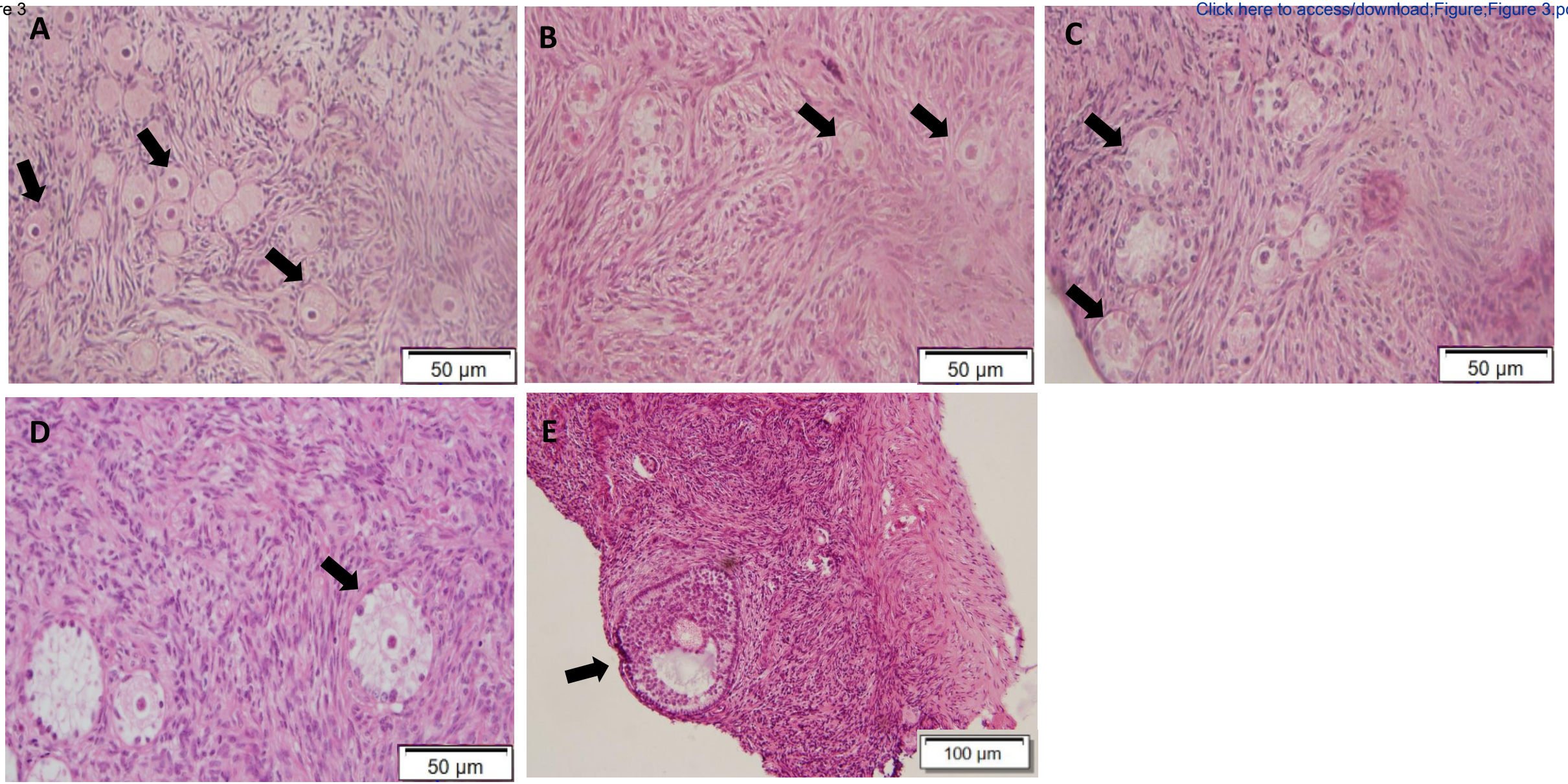

Figure 4

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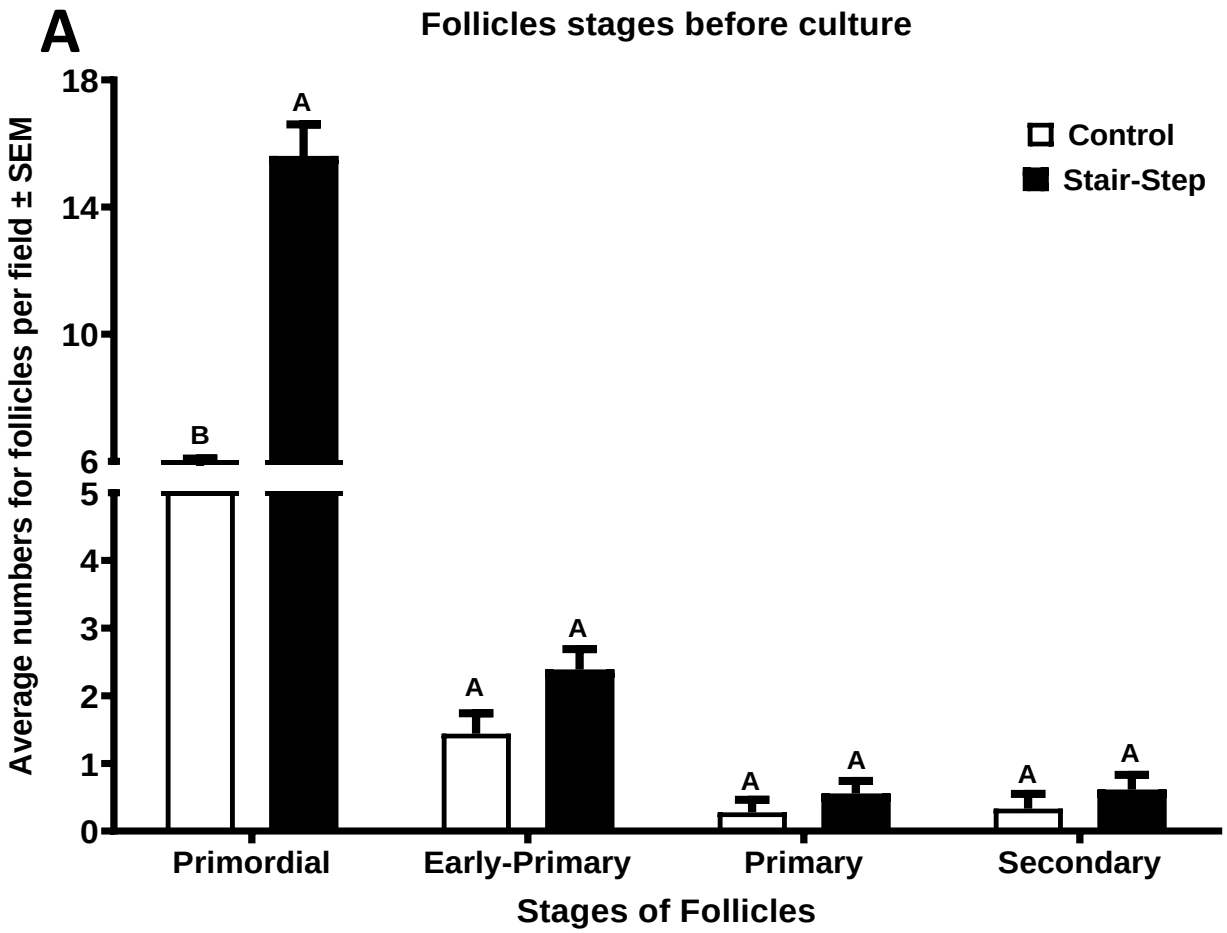

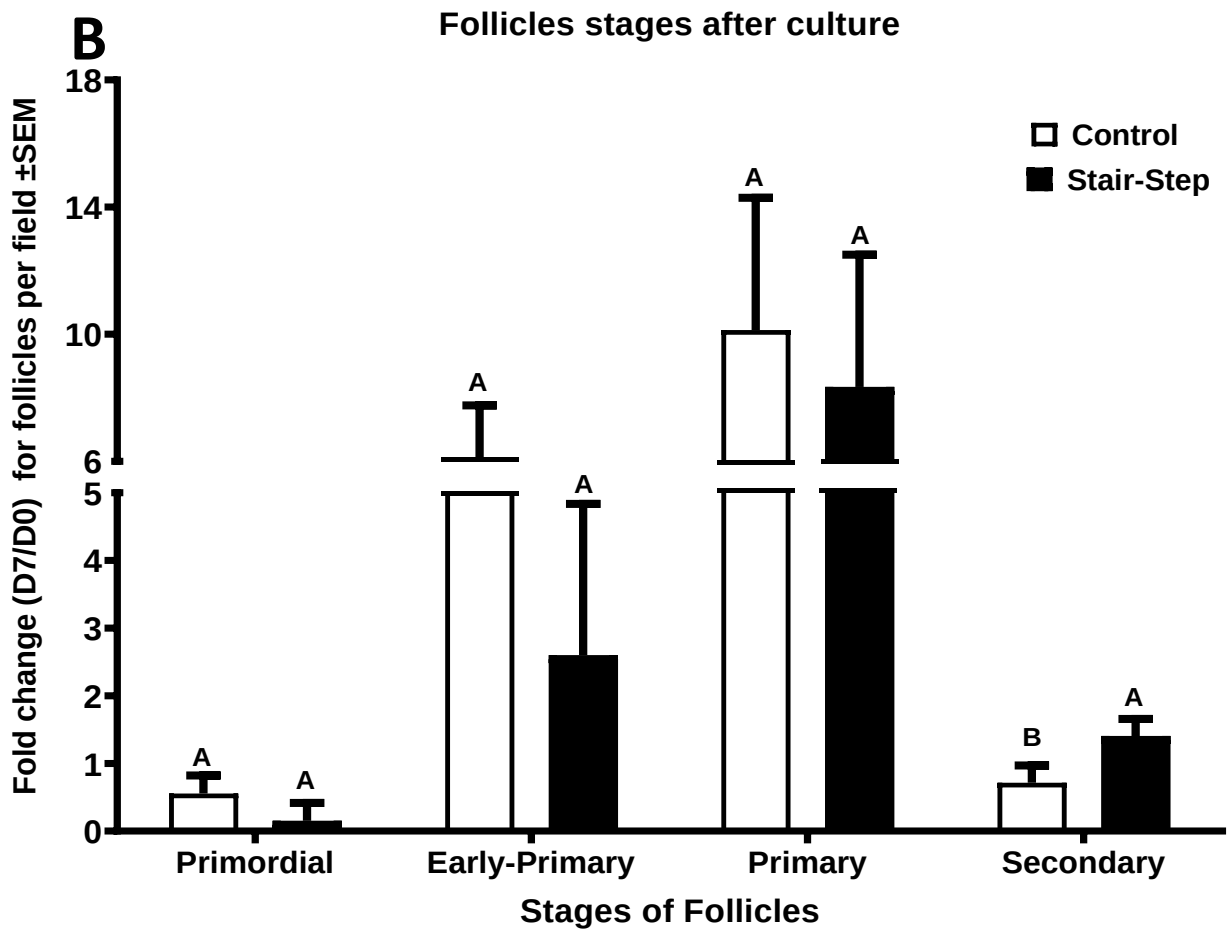

Figure 5

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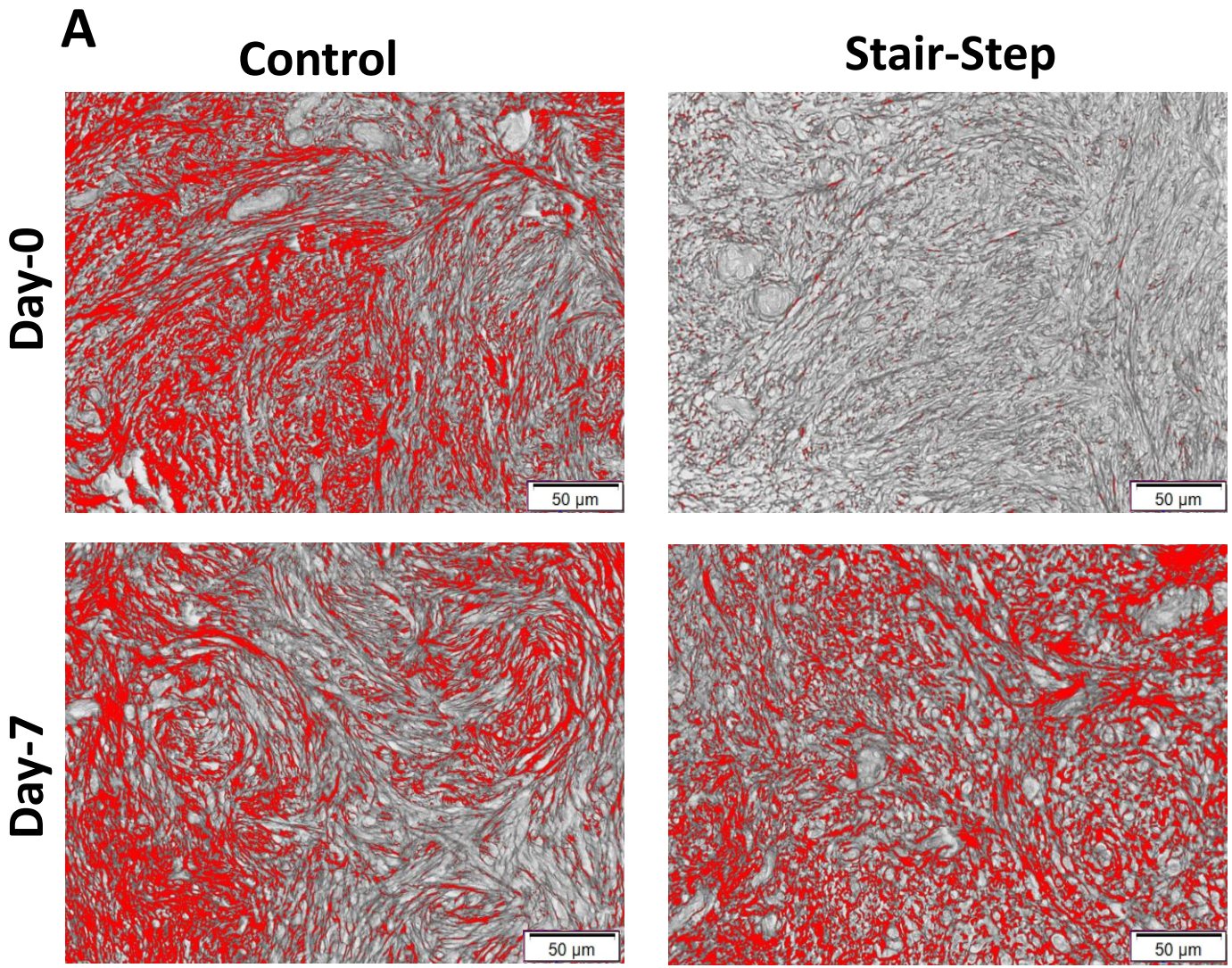

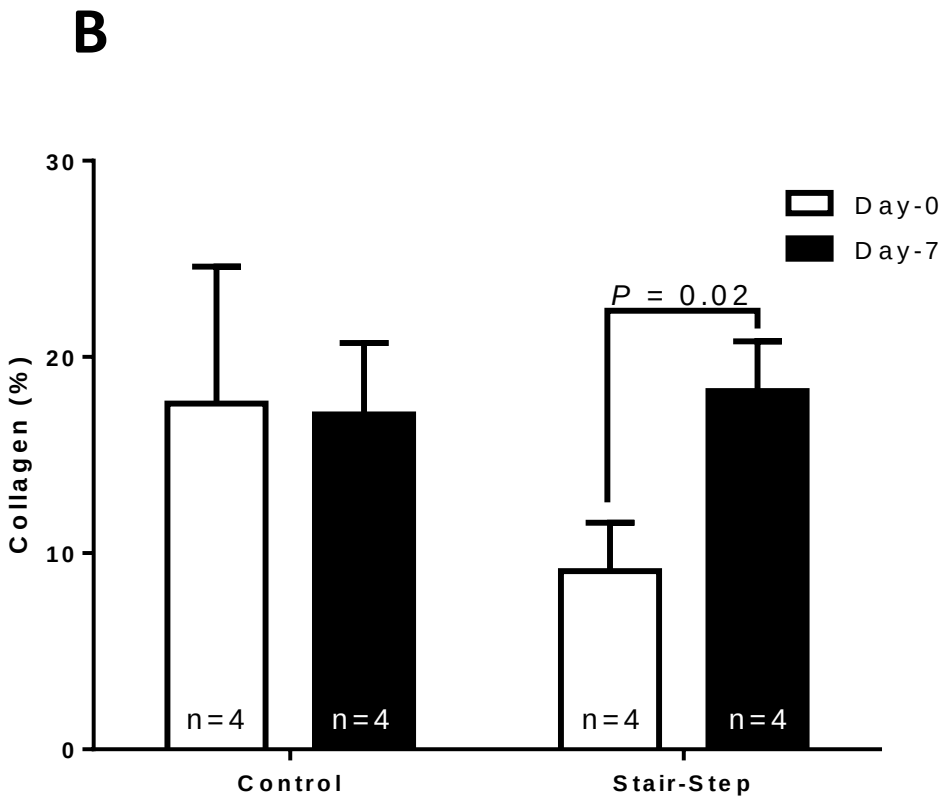

Figure 6

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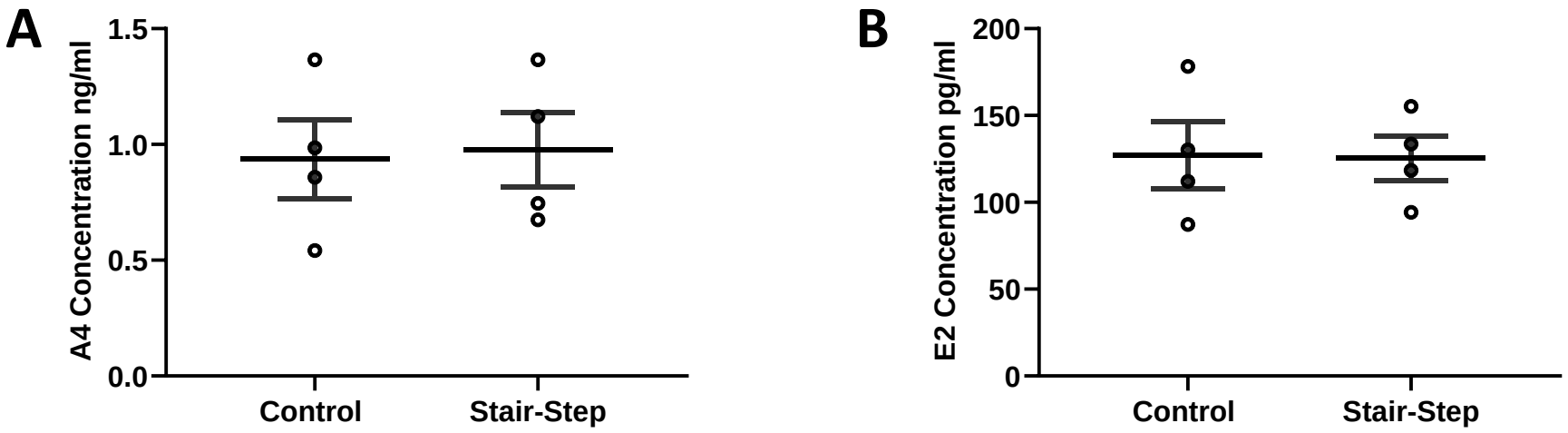

Click here to access/download;Table;Table 1. Hormones StairStep.xlsx

Table 1.

Hormones	Control		Stair-Step		P-Value
	n	4		4	
ng/mL	Mean	SEM ±	Mean	SEM ±	
DOC	0.33	0.28	0.72	0.48	0.15
INN	0.61	0.60	4.93	3.99	0.08
CORT	0.003	0.003	0.01	0.01	0.85
17OHP	0.59	0.53	1.88	0.99	0.08
A4	1.46	1.43	5.74	3.27	0.08
AN	0.15	0.09	0.54	0.32	0.08
DHEAS	4.50	2.60	7.59	0.85	0.56
E2	0.05	0.05	0.13	0.08	0.32
P4	5.05	2.78	6.52	1.66	0.39
T	0.33	0.33	1.33	0.96	0.14
DHT	0.07	0.02	0.01	0.01	0.09

DOC - 11-Deoxycorticosterone, INN - 11-Deoxycortisol, CORT – Corticosterone, 17OHP – 17-Hydroxyprogesterone, A4- Androstenedione, AN – Androsterone, DHEAS – dehydroepiandrosterone sulfate, E2 - Estradiol, P4 – Progesterone, T – Testosterone, DHT – Dihydrotestosterone

Click here to access/download;Table;Table 2. Cytokines StairStep.xlsx

Table 2.

Cytokines	Control		Stair-Step		P-Value
n	4		4		
pg/mL	Mean	SEM ±	Mean	SEM ±	
ANG1	27.29	11.71	63.66	25.06	0.39
CD40L	4527.01	2986.34	3537.59	3537.59	0.74
DCN	1307.68	320.87	996.54	282.96	0.77
INFβ	0.36	0.36	0.002	0.002	0.85
IL18	0.00	0.00	1832.89	1265.33	0.13
LIF	97.45	88.12	337.58	231.25	0.54
RANTES	698.06	322.59	1254.13	811.19	0.56
INFγ	4.49	1.37	3.68	0.65	0.77
IL13	501.21	285.07	810.81	159.67	0.25
IL21	24.38	9.51	18.52	6.17	0.56
IL1F5	5.07	3.85	5.40	1.70	0.56
TNFα	61.45	35.00	44.91	13.41	0.77

ANG1 – Angiopoietin 1, CD40L – CD40 Ligand, DCN – Decorin, IFNβ – Interferon Beta 1, IL18 – Interleukin-18, LIF – Leukemia inhibitory factory, RANTES – Regulated on Activation, Normal T Cell Expressed and Secreted, IFNγ – Interferon Gamma, IL13 – Interleukin 13, IL21 – Interleukin 21, IL1F5 – Interleukin 1 family member 5, TNFα – Tumor Necrosis Factor alpha

Click here to access/download;Table of Materials;JoVE_Table of Materials 9.2.20.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#11 Scapel Blade	Swann-Morton	303	Scaple Blade
#21 Scapel Blade	Swann-Morton	307	Scaple Blade
500mL Bottle Top Filter	Corning	430514	Bottle Top Filter 0.22 µm pore for filtering medium
AbsoluteIDQ Sterol17 Assay	Biocrates	Sterol17 Kit	Samples are sent off to Biocrates and steroid panels are run and results are returned
Androstenedione Double Antibody RIA Kit	MPBio	7109202	RIA to determine androstenedione from culture medium
Belgium A4 Assay Kit	DIA Source	KIP0451	RIA to determine androstenedione from culture medium
Bovine Cytokine Array Q3	RayBiotech	QAB-CYT-3-1	Cytokine kit to determine cytokines from culture medium
cellSens Software Standard 1.3	Olympus	7790	Imaging Software
Insulin-Transferrin-Selenium-X	Gibco ThermoFisher Scientific	5150056	Addative to the culture medium
Leibovitz's L-15 Medium	Gibco ThermoFisher Scientific	4130039	Used for tissue washing on clean bench, and in the biosafety cabniet
Microscope	Olympus	SZX16	Disection microscope used for imaging tissue culture pieces
Microscope Camera	Olympus	DP71	Microscope cameraused for imaging tissue culture pieces
Millicell Cell Culture Inserts 0.4µm, 12,mm Diameter	Millipore Sigma	PICM01250	Inserts that allow the tissue to rest against the medium without being submerged in it
Multiwell 24 well plate	Falcon	353047	Plate used to hold meduim, inserts, and tissues
Petri dish 60 x 15 mm	Falcon	351007	Petri dish used for washing steps prior to culture
Phosphate-Buffered Saline (PBS 1X)	Corning	21-040-CV	Used for tissue washing
SAS Version 9.3	SAS Institute	9.3 TS1M2	Statistical analysis software
Thomas Stadie-Riggs Tissue Slicer	Thomas Scientific	6727C10	Tissue slicer for preperation of thin uniform sections of fresh tissue
Waymouth MB 752/1 Medium	Sigma-Aldrich	W1625	Medium used for tissue cultures

Rebuttal Letter

Click here to access/download;Rebuttal Letter;Reviewers Comments With Notes 12-04-20.docx

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.
2. Please address all the specific comments marked in the manuscript.
3. Once done, please ensure that the highlight is no more than 3 pages including headings and spacings.
4. Please address reviewers' comments as well.

Reviewers' comments:

Reviewer #2:

the authors addressed all my critiques.

Reviewer #3:

Manuscript Summary:

This manuscript by Sutton et al. describes a technique for isolating bovine ovarian cortex and performing in vitro culture for seven days. This is a very useful technique that enables the study of folliculogenesis and steroidogenesis and it enables the investigation and discovery of factors that regulate these processes. The current version of the manuscript has been greatly improved. A few important questions and concerns remain, however. My comments below are intended to further improve the quality of this manuscript.

Major Concern:

Unfortunately, the description of how the cortex is separated from the medulla does not seem clear. I suggest adding a figure showing the plane of cut of the ovary (what the authors explain as cutting in half) and "filleting" the ovary. It seems that either a sagittal or a transversal cut in half would leave each half containing cortex and medulla. If the "filleting" means transversal cuts in each half, medulla would potentially remain attached. The paragraph detailing step 2.6 is somewhat clear but needs to be revised (please see my specific comments above); the paragraph extracted from the discussion is confusing and does not add clarity to this step, which is so critical to the entire paper. Here is that passage: "To ensure cut pieces that are only from the cortex and do not have medulla after washing the ovary we place it in the 60 mm dish and cut in half. The cortex and medulla are very different histologically as seen previously in Abedal-Majed, 2020. Each half of the ovary is filleted with the blade to ensure that only cortex is removed from the ovary and the medulla remains. If individuals are just starting to perfect this cutting technique, they can also use neutral red to more closely see the histology of each half of the ovary. This will allow for development of landmarks as their cutting technique improves. Furthermore, they can use instruments that can cut uniform thickness (see table of Materials) as stated above"

Please note: if this step is shown in detail in the video that will accompany this paper, please disregard this comment. My only intention is to ensure that readers clearly see how this is accomplished so the technique can be replicated.

- We have added a figure to show the filleting technique (Figure 2A). This will also be shown on the video.

Minor Concerns:
Use of Picro Sirius Red stain: this stain is used for evaluation of connective tissue - specifically, collagens. In my opinion, it would be more accurate to state the results as "% collagen" instead of "% fibrosis". Same in the caption of Figure 5.

-The change has been made so that PSR now says % Collagen or describes collagen instead of fibrosis (lines 346, 408).

I would like to offer my opinion for the author's evaluation: my understanding of fibrosis as a medical term is that it is associated with pathology, which would indicate loss of tissue function. The pathological process of fibrosis includes abnormal deposition of collagen, but in many instances would be associated with other changes as well. Given that the authors are only assessing collagen deposition with the picro Sirius staining, I think that it is inaccurate to conclude that fibrosis is developing in this case. It is accurate to conclude, however, that there is accumulation of collagen in the tissue, indicating that a process of fibrosis might be 1) happening in the stair-step heifers; and 2) developing as a result of in vitro culture. In line 343, I suggest replacing "quantify fibrosis" by "indicate fibrosis".

-The change has been made so line 347 now says indicate fibrosis.

Early primary follicles: this is something I commented in my first review. I understand the authors think it's more appropriate to maintain this nomenclature; therefore I would like to suggest adding "transitional" in parenthesis after the term "early primary". Otherwise, please add a reference and a short description of what early primary means.
-A brief description of follicle staging has been added to lines 332-338.

General question about processing of the spent media before hormonal evaluation. Was the medium centrifuged to separate any particles before freezing (maybe the supernatant was collected and frozen), or was the medium immediately frozen without separation?

-Media is collected and then frozen immediately

Lines 74-77: The 2006 paper by Yang and Fortune describes experiments using fetal bovine ovary, similarly to the 2007 paper. I recommend stating the use of fetal ovaries in line 74 instead of line 77 - the way it is written, it may be interpreted as only the second paper was done in fetal tissue.

-Fetal has been added to this sentence to help clarify the meaning (line 75).

Line 88: I suggest adding a sentence to describe what the stair-step regimen is. Readers can go to the citation for more details, but if they wish to continue reading this manuscript and have a full understanding of the results, a brief explanation of that treatment would go a long way.

Line 96-101- description of the Stair-Step heifer protocol was added.

Line 127: the sentence about A4 and E2 measurement is confusing "Steroids (androstenedione; A4) and estrogen (E2) were can be pooled over 3 days and assessed through radioimmunoassay…"

-This sentence has been altered slightly to help improve clarity (lines 123-124).

Line 130" Cytokine Quantibody Array seems to be referring to a specific product (by Ray Biotech if I'm not mistaken). The manufacturer of the array should be included in that sentence.

-The "Quatntibody" has been removed from this sentence as it is a brand/specific product. We were asked to remove all brand names and include them in the table of materials (Line 126).

Line 147: I recommend switching the order to state IVM before IVF - just to be consistent with the sequence of events.

-This change has been made so that IVM is first (Line 454-455).

Line 162: I am not aware of an antimicrobial called penstreptomycin sulfate. This may be a commercial name. I would recommend replacing by penicillin/streptomycin sulfate or something similar. Also in this sentence, it may be useful to add the units to the percentage (0.1% v/v or 0.1% w/v).

Line 152- we added Penstreptomycin sulfate 0.1% v/v

Line 227: "Using a ruler, make sure that no more than 1-2 µM of depth of surface of ovary is removed away from medulla". The unit in this sentence is micromolar, which is not a unit of size. Please correct. One to two micrometers is a very small measurement to be accurate without magnification. In figure 2, the width of the strip is 10 mm. I understand that in section 2.6 the authors are describing the depth thickness, not the width, but I'm just trying to make the point that 1-2 µm (if that's what the authors mean) is 5-10,000 x smaller than 10 mm. In the caption of Figure 3 it is stated that the strips are 0.5 - 1 mm3 in size. Please reword section 2.6 to improve clarity.

-This change has been made so that it now says mm (lines 226, 235).

The use of the terms "ovarian strips" and "ovarian pieces" seems interchangeable and it is a little bit confusing at times. I recommend choosing one and keeping it throughout the manuscript.

-Clarification has been added to this so that it should say pieces throughout the manuscript.

Line 258: "for future (or subsequent) RNA extraction". Stating "flash frozen for RNA" seems vague.

Line 231: We have changed this to read- additional ovarian cortical tissue can be collected for RNA extraction

Line 292: Color changes in the medium are the indication that the pH has changed. I recommend stating

that more clearly - for example, "to prevent large pH changes (indicated by color changes) in the medium".

-Clarification has been added to this so that it should read better about color change and pH (line 302).

Line 312: 4.1. At this point what's being imaged is the cortical strips, correct?

-Clarification has been added to this so that it describes that images should be taken of the pieces (line 309)

Line 387 (Figure 2 caption): the authors state in the methods that approximately 70% of the medium is harvested and exchanged for fresh medium every 24 hours. For consistency, I recommend stating the same here or changing the methods to state that 100% of the medium is removed each time.

-This change has been made in the figure caption for Figure 2, so that it does that 70% of the media is recovered (Line 394-395)

Line 500: please add reference to the use of neutral red to attempt to find follicles within the cortical strip. This will help guide readers to that article and its methods.

-A reference has been included. (Line 471-473)

Reviewer #5:
Manuscript Summary:
The manuscript has been provided in good order and compared the pros and cons of laboratory method in accordance with its potential applications. Please check the pdf file for minor revisions.

-Revisions from the PDF have been made in the track changes version of this paper (Line 26, lines 226, 235, and line 252).

Reviewer #6:
Manuscript Summary:
The authors should indicate the goal of the study in the abstract in addition to the introduction (lines 88-91).
lines 37-39 we added the goals of the study to the abstract.

The authors should mention the main results achieved in this study in the abstract.

Line 48-50 we addressed the main results in the abstract
Major Concerns:
Introduction
Lines 93-104: This part could be included in the discussion.

Moved to discussion first paragraph (Lines 430-441)

Lines 115-131: This part could be included in the protocol section.
This provides an overview of the technique conducted in this manuscript. It was moved out of the protocol section during the last review. We request that the editors decide where to move it because it is not apparent to us where this should go in the protocol –again since it is an overview.

Representative Results
This section should include the summary of the data achieved, instead of detailed methodology or comments about the author expectations.
We have results in lines 355-359.

Discussion
Lines 430-438: This part should be removed, as it includes repetitive information about the protocol.
We have removed this.

Minor Concerns:
"Bouin's fixative" should be detailed.
The authors should detail their "follicle count strategy" (reference: DOI: 10.1186/s13048-016-0222-2).

-A brief description of follicle staging has been added to lines 332-338

The recipe for Bouins fixative is included Lines 318-319

bio Sutton

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bio Springman

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bio Cupp

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