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Ovarian tissue health after laparoscopic unilateral oophorectomy: A porcine model for establishing optimized fertility preservation techniques in children

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ABSTRACT

Background: The only pre-treatment fertility preservation option for prepubertal girls, who are at risk for infertility due to their diagnosis or treatment, is surgical removal of ovarian tissue for cryopreservation (OTC). We investigated ovarian tissue health following isolation with an ultrasonic advanced energy device (UAED), that has a previously reported thermal spread of ≤2 cm.
Methods: The ovaries of eight Yucatan minipigs were isolated by laparoscopy (1) close dissection with the UAED located up to 2 mm away from the ovarian capsule, (2) far dissection with the UAED located >2 cm away, or by (3) laparotomy for control ovaries using cold scissors. Ovarian cortex tissues were cultured for 4 days to assess tissue health.
Results: Ovarian cortex tissue isolated using a UAED produced an altered metabolic ratio in both the far and close dissection compared to control ($p < 0.001$). There was an increase in folliculogenesis in the control samples over samples isolated with far and close dissection ($p < 0.0001$), and a reduction in estradiol production in experimental groups ($p < 0.0001$).
Conclusions: This model defines differences in ovarian tissue health among different isolation techniques. Ongoing work will further define the standard of care surgical technique for OTC.

Because of advancements in pediatric cancer treatment, 38 of the 43 children in the US diagnosed with cancer each day will survive their disease, leading to increasing populations of survivors of childhood and adolescent cancers around the world [1]. Many of the life-saving treatment regimens that these children receive are highly gonadotoxic and can render adult survivors infertile [2–4]. Currently, oocyte and embryo cryopreservation are non-experimental options for post-pubertal females who want to preserve their fertility prior to starting cancer treatment. The only pre-treatment option to preserve fertility for prepubertal girls or for those in whom egg banking is not feasible is surgical removal of ovarian tissue for cryopreservation (OTC) [5]. For these patients, future auto-transplantation of thawed cortical tissue can re-establish fertility and reproductive hormone function [6, 7].

Ovarian tissue auto-transplant has resulted in over 130 reported live-births in women whose tissue was cryopreserved when they were postpubertal and 2 live-births in women who underwent OTC pre-menarche [7, 8]. However, the current reported success rate of auto-transplantation is 28% with restoration of hormone production for up to 12 years (average of 2–5 years) [6, 7]. In order to improve upon the results of tissue function after restoration, each step of the OTC process needs investigation, from surgical removal of the tissue, to processing techniques, to reimplantation.

To date, there has been no consistent standard operative approach, technique, or equipment used for ovarian tissue removal for OTC in women or girls [9]. Additionally, the surgical techniques used for ovary removal in adult women, including use of staplers for hemi-oophorectomy, cannot be applied to ovary removal in infants and prepubertal children given their inherent differences in body habitus and adnexal anatomy. In a meta-analysis of surgical techniques used worldwide for ovarian tissue removal for OTC, the techniques with the highest rate of major complications, including hemorrhage requiring a blood transfusion, were those which involved cutting across the ovarian cortex compared to unilateral oophorectomy [9]. In particular, the small size of the prepubertal pediatric ovary with the large primordial follicle reserve situated at the cortical surface, coupled with the reduced working space in the pediatric pelvis, make the surgical technique for ovary

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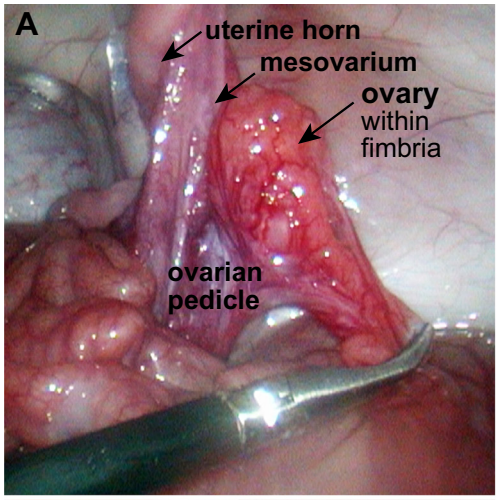
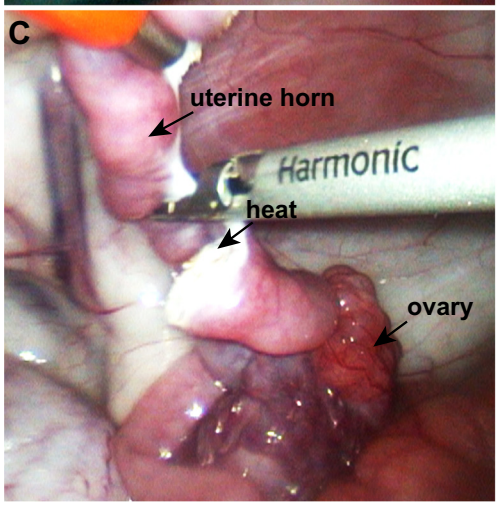
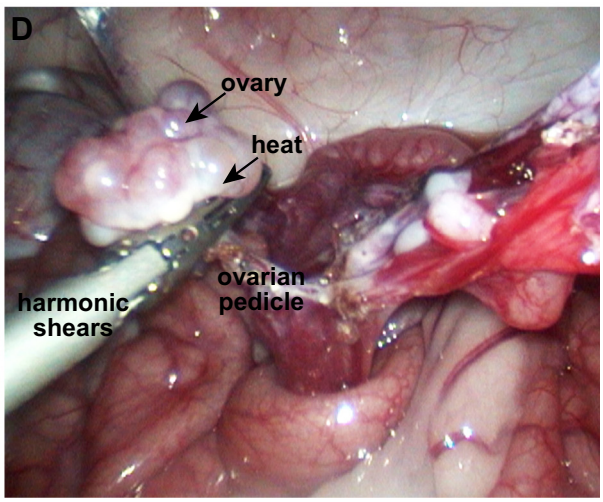


B

Animal ID	Ovary	Experimental Group	Cultured Samples, n
SNS 1615	Right	Far Dissection	5
	Left	Control	8
SNS 1616	Right	Far Dissection	6
	Left	Close Dissection	8
SNS 1623	Right	Close Dissection	10
	Left	Control	7
SNS 1624	Right	Far Dissection	8
	Left	Control	7
SNS 2101	Right	Far Dissection	12
	Left	Close Dissection	16
SNS 2107	Right	Close Dissection	10
	Left	Control	11
SNS 2112	Right	Far Dissection	10
	Left	Control	7
SNS 2247	Right	Far Dissection	12
	Left	Close Dissection	16





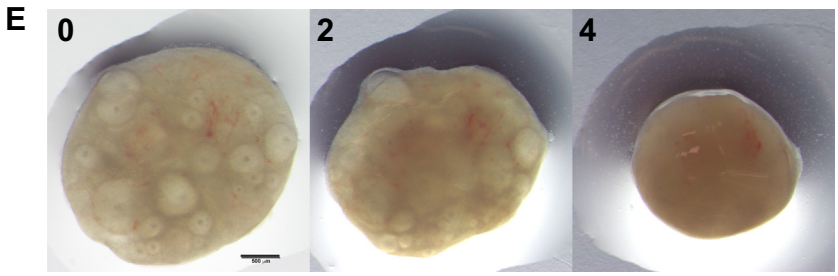


Fig. 1. Yucatan minipigs intra-operative photos and procedures. (A) The laparoscopic intra-operative photos show that the minipig ovary is wrapped underneath the oviduct fimbria and adjacent to the uterine horn. The ovarian vessels are within the ovarian pedicle and the ovary is anchored in place by the mesovarium, the broad ligament that suspends the ovary from the uterine horn. (B) Ovaries from each animal were randomly assigned into the control, close dissection and far dissection experimental groups. Each ovary was then processed into biopsy punches which were cultured to monitor the effects of the procurement technique on the overall tissue health. The number of pieces ranged from 5–16 per ovary. (C) The far dissection technique required dividing the uterine horn to remain at least 2 cm away from the ovarian capsule. (D) The close dissection technique involved removing the oviduct fimbria from around the ovary and dividing the ovarian pedicle as close to the ovary as possible to save the adnexal structures. (E) Representative image of ovarian biopsy sample cultured for 0, 2 and 4 days. Scale bar, 500 μm.

removal of prime importance in order to avoid inadvertent injury to the ovary. The laparoscopic surgical approach is preferred for small incisions with short recovery and precise hemostasis, which should not impact the start of definitive medical therapy for the patient [10, 11].

Many pediatric surgeons and gynecologists perform unilateral laparoscopic oophorectomy utilizing an ultrasonic advanced energy device (UAED) to divide the mesovarium and the ovarian artery, due to a narrowly reported range of thermal spread and excellent hemostasis for larger vessels, such as the ovarian artery [15]. There have been no major surgical complications using this technique [9, 14]. Our group sought to design an experimental Yucatan mini-pig animal model in order to replicate laparoscopic oophorectomy using an UAED to evaluate ovarian tissue health after removal. The reproductive anatomy of the peripubertal mini-pigs approximates the size of the adnexal and pelvic anatomy in a prepubertal human female. Mini-pigs, like many mammals, have oviducts instead of fallopian tubes and uterine horns instead of a single uterus. However, the mini-pig uterine horns lie in close proximity to the ovary, similar to the fallopian tube in human patients (Fig. 1A). Additionally, both have a limited operative working space in the pelvis, a small volume ovary, and a narrow mesovarium between the uterine horn/fallopian tube and ovary. The overall goal of this work is to determine the safety of use of UAED for oophorectomy

in order to maximize ovarian tissue health for OTC in children, as a first step to defining standard of care surgical technique.

1. Methods

1.1. Animal care

Animal use was performed under a Northwestern University (NU) Animal Care and Use Committee (ACUC)-approved protocol. Surgery was performed on 19- to 21-week-old Yucatan mini-pigs (S & S Farms, Michigan, USA). Pigs were anaesthetized using isoflurane by the NU Center for Comparative Medicine veterinary staff via endotracheal intubation. Pulse oximetry, electrocardiography, respiratory rate, and rectal temperature were monitored throughout the entirety of the procedure. Intraoperative photos and videos were captured using a Tele Pack X (Karl Storz-Endoskope, Germany).

The mini-pigs were housed in conventional facilities with food given *ad libitum* and refilled twice per day for 4–7 days prior to surgery. Two mini-pigs underwent surgery each day over the course of eight days and performed between 900 and 1300 CST. Mini-pigs were ordered four at a time and experimental variables were batched together (Fig. 1B). The animals were given a euthanizing dose of pentobarbital (Euthasol®, Virbac, France) following the surgeries. The experimental groups are detailed below and each ovary was randomly assigned to an experimental or control group.

1.2. Surgery – laparoscopic oophorectomy

Pigs were positioned supine on the operating table. Skin was prepared using an alcohol prep followed by betadine scrub and betadine paint in outward circular fashion. The abdomen and pelvis were draped using a standard laparotomy drape. An infra-umbilical incision was made using a 15-blade scalpel and tissue dissected using a hemostat and monopolar electrocautery. Access to the peritoneal cavity was obtained using a Veress needle (Step™ Short Insufflation/Access Needle, 14-Gauge, Covidien USA). The abdomen was insufflated with carbon dioxide between 10–15 mmHg. A 12-mm laparoscopic trocar (VersaOne™ Optical Trocar, Standard Length Fixation, 12-mm, Covidien) was inserted at the umbilicus. A 5-mm 30-degree laparoscope (Hopkins® Forward-Oblique Telescope 30-degrees, 5-mm, Karl Storz-Endoskope) was then used to inspect the abdomen and pelvis. Two additional 5-mm laparoscopic working ports (VersaOne™ Optical Trocar, Standard Length Fixation, 5-mm, Covidien) were placed in the left lower quadrant and suprapubic positions. Two 5-mm blunt graspers (Grasping Forceps, 5 mm, Karl Storz-Endoskope) were used to expose and inspect the uterus and bilateral uterine horns.

1.2.1. Far dissection

The ovary was exposed and the uterine horn was measured at least 2 cm from the ovarian hilum using a standard open-jawed instrument (Fig. 1C, Video 1). The uterine horn was grasped at this distance and then the mesovarium was divided using a harmonic scalpel (HARMONIC ACE®+7 Laparoscopic 5mm Diameter Shears, 36 cm Length with Advanced Hemostasis, Ethicon US, LLC), at least 2 cm away from the ovarian capsule, leaving a wide rim of tissue (settings of min: 3, max: 5 on Ethicon Endo-surgery Generator (EES) GEN11, Ethicon). The vascular pedicle, containing the ovarian artery, was divided last. The ovary and surrounding tissue was then pulled into the 12-mm trocar and extracted through the umbilical fascial incision. The ovary was then placed in DMEM/F-12 (Gibco, USA, 11330057).

1.3. Close dissection

The ovary was exposed, and the uterine horn grasped just proximal to the ovarian hilum (Fig. 1D, Video 2). The mesovarium was then dissected using a UAED, harmonic scalpel (HARMONIC ACE®+7 Laparoscopic 5mm Diameter Shears, 36cm Length with Advanced Hemostasis, Ethicon) within 2 mm of the ovarian capsule with the same settings as above and the vascular pedicle was divided last. The ovary was then pulled through the umbilical fascial incision and placed in DMEM/F-12 (Gibco, 11330057).

1.4. Surgery – laparotomy

1.4.1. Control: cold scissors sharp dissection

A low, transverse incision (Pfannenstiel) was made using a 15-blade scalpel. Layers of the anterior abdominal wall were divided and abdominal access obtained using monopolar electrocautery. The uterus and uterine horns were exposed and inspected. The ovary was then exposed by following the uterine horn distally. Curved hemostatic clamps were placed at least 2 cm away from the ovary and tissue divided sharply using a tenotomy scissor and ovary removed. The ovary was then placed in DMEM/F-12 (Gibco, 11330057). The vascular pedicle and remaining tissue were ligated (PERMA-HAND* Silk, braided suture, 3-0, Ethicon) and clamps removed.

1.5. Tissue processing

The ischemic time (time from division of the ovarian arterial blood supply to placement in processing media) was recorded and standardized across all groups (1.25 ± 0.39 minutes). Ovarian tissue was processed and cultured in a similar way to what has previously been reported with human ovaries [16, 17]. Ovaries were bisected through the hilum in a sagittal plane; one half placed into Modified Davidson's Fixative (MDF, Electron Microscopy Sciences, 64133-50). The other half of the ovary was sliced using a Stadie-Riggs Tissue Slicer (Thomas Scientific, USA) to isolate the cortical region. The 0.5 mm slice of ovarian cortex was further processed into uniform pieces using a 3 mm biopsy punch. The tissue was then washed in DMEM-F12 with L-glutamine and 15mM HEPES (Caisson Labs, DFL13) supplemented with 2× Gibco Antibiotic-Antimycotic (Anti-Anti, Thermo Fisher, USA, 15240062) at 37 °C in 5% CO_2 for 1 hour. Tissue was then washed for 5–10 minutes in a 1× red blood cell lysis buffer (Miltenyi Biotec, Germany, 130094183) at room temperature. Tissue was then washed 3 times for 5 minutes each in Hank's Balanced Salt Solution (HBSS, Corning, USA, 21023CM).

1.6. Ovarian spheroid culture

The cortical region, which is isolated and saved in current fertility preservation protocols, contains clusters of primordial follicles in both mini-pigs and humans and growing follicles in mini-pigs. Biopsy punches of cortical tissues were cultured on transwells and analyzed over the 4-day culture. Cortex biopsy punches were placed surface epithelium side up and cultured at 37 °C in 5% CO_2 on Millicell transwell inserts (Millipore, USA, PICM01250) from 0 to 4 days in Minimum Essential Medium supplemented with GlutaMAX (αMEM, Gibco 32561102), 3 mg/ml bovine serum albumin (BSA, Fisher Scientific, USA, BP671), 1 mg/ml bovine fetuin (Sigma-Aldrich, USA, F3385), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Corning, MT25800CR), and 0.1 mIU/ml FSH (Abcam, UK, ab51888). The entire volume of growth media (400 μl) was replaced every 48 hours. Spent media was stored at −20 °C and analyzed for estradiol, glucose, and lactate. Biopsy punches were imaged after plating and at each media change using a dissecting scope (Olympus, Japan, SZX10) with camera (Olympus, DP22) and software (Olympus Cellsens Entry, v1.14). Selected biopsy punches were also fixed at each media change in 4 % paraformaldehyde (diluted from 16 %, Electron Microscopy Sciences, USA, 15710).

1.7. Hormone and metabolic assays

Estradiol (Calbiotech, USA, ES180S), glucose (Abcam, ab65333), and lactate (Abcam, ab65331) were detected in the media using colorimetric ELISA kits on a Biotek Synergy Neo2 plate-reader. Estradiol was tested in media from n = 15–48 samples per group and plotted as a percent of the control cultures. Glucose consumption and lactate production was performed on n = 16–52 per group. Standard curves and interpolated results were calculated using Prism (GraphPad, USA, version 7.0a 2016).

1.8. Histological analysis

Fixed tissue was processed using an automated tissue processor (Leica, Germany) and embedded in paraffin.

To identify proliferative cells within the culture, the percent of cells that stained positive for Ki67 were identified over total cells counted in histological sections from day 0 (D0), D2 and D4 cultured tissue samples (n = 3–5 sections per sample, 20–50 sections per group) using an automated stainer (Ventana, USA, Benchmark Ultra). An antibody against Ki67 (1:200; Abcam, ab16667) was used and visualized with Discovery Chromomap DAB Kit (Roche Diagnostics, Switzerland, 760-159). Ki67 were identified over total cells counted in histological sections from day 0 (D0), D2 and D4 cultured tissue samples. The same procedure was performed for two apoptotic markers cleaved Caspase 3 (Cell Signaling Technologies, Danvers, MA, USA, 9661S) and cleaved PARP (Cell Signaling Technology, 9548S). Additional apoptosis analysis was performed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) on tissue from the culture (3–4 sections per sample, n = 16–51 per experimental group, DeadEnd Fluorometric TUNEL System assay, Promega, USA, G3250). Tissue sections were counterstained with propidium iodide solution (false-colored blue in Figures, Sigma Aldrich, P4864). The stained sections were imaged and stitched to create an image of the entire area of tissue (Keyence, Japan, BZ-X700), then analyzed using BZ-X Analyzer, Hybrid Cell Count software (Keyence). The percent of cells that stained positive for TUNEL were identified over total cells counted in histological sections from D0, D2 and D4 cultured tissue samples.

1.9. Follicle counting

Serial sections were cut at 5 μm and every fifth section, or approximately every 25 μm, was stained with hematoxylin and eosin (H&E) using a Leica Autostainer XL (Leica Microsystems, Germany). They were imaged and stitched to create an image of the entire area of tissue (Keyence, BZ-X700). Coded images were provided to a blinded, skilled researcher. Follicles were counted and surface area calculated using Adobe Photoshop (Adobe, USA, Creative Cloud Suite 2015; 4–28, average 12, sections were scored each for n = 4–9 samples per group, per day). Follicles that have oocytes in the center were counted. Primordial follicles have no more than one layer of squamous granulosa cells and were often found in clusters. Primary follicles were identified as having a complete layer of granulosa cells where most are cuboidal. Secondary follicles have two or more layers of cuboidal granulosa cells. Antral follicles were identified by having antral spaces within the granulosa cell layers. Each follicle stage was normalized to 100 % and shown relative to D0 numbers for their respective experimental group.

1.10. Statistical analysis

All data sets were analyzed using IBM SPSS Statistics, USA (version 25.0.0.0) and expressed as the average and standard errors of the mean. Statistical significance and comparison of means was performed using ANOVA with Tukey's multiple comparisons test with $\alpha = 0.05$. GraphPad Prism (version 7.0a 2016) software was used to generate figures.

2. Results

2.1. Gross anatomy and ovarian tissue health

Eight mini-pigs underwent surgery and were included in this study and produced 5 control ovaries, 6 far dissection, 5 close dissection ovaries (Fig. 1A).

The flat cortical tissue biopsy from the piglet oophorectomy remodels into a spheroid that supports follicles, like what was previously demonstrated in human tissue (Fig. 1E) [16]. The culture system was utilized to compare the effects of the 3 procurement techniques on ovarian tissue health. Overall, there were no differences between surgical technique for procurement in gross observations of biopsy punches and how they formed spheroids in culture.

There was a decrease in proliferation in cultured tissue that was procured using close and far dissection techniques at different points in the culture. The proliferative cells, as identified by Ki67 staining (Table 1, Fig. 2C), were predominantly localized to granulosa cells of growing follicles with fewer positively stained stromal cells. Additionally, there were more positive cells toward the edges of the biopsy punch (~200–500 μm) than the center for all experimental groups. The remodeling ovarian cortical tissue has an edge that stains positively and may be remodeling ovarian surface epithelium on D2–4. There was up to 15.2 % more proliferative cells in control tissues at D0 and D2 than in experimental groups. Additionally, the far dissection group contained 10.9 % more proliferative cells at D0 than the close dissection group. There was a reduction in proliferation over the course of the culture across all experimental groups.

Apoptotic cells, as identified by cleaved Caspase 3, cleaved PARP and TUNEL staining, were localized to the center of each biopsy punch in 34 % of sections, with more staining identified around tissue folding that occurs during the remodeling process. Some punches (15%) had distinct follicles marked by positively stained granulosa cells. Positive edge staining (<500 μm from edge) of a biopsy punch was seen more often on D2–4 (Table 1, Fig. 2B). The culture at the air-liquid interface, used here, induces ovarian cortical tissue into spheroids and requires remodeling of the tissue overall. This is apparent by the proliferation of cells more localized around the perimeter and more apoptotic cells localized to invaginated folds within the center of the tissue as it develops into as spheroid. There was little or no difference in apoptosis between the groups (Table 1).

Table 1
Histological analysis of proliferation and apoptosis of ovarian spheroids cultured over 4 days after procurement through control, far or close dissection techniques. Means ± standard error. a, significantly different than control; b, significantly different than close.

Groups	0 days			2 days			4 days		
	Control	Far	Close	Control	Far	Close	Control	Far	Close
Proliferation (%)									
Ki67	28.23 ± 2.49	23.94 ± 5.60, b	13.01 ± 2.68, a	14.92 ± 3.84	6.19 ± 0.96, a	8.03 ± 1.04	8.68 ± 2.05	7.95 ± 1.52	5.55 ± 1.03
Apoptosis (%)									
c-Casp3	8.18 ± 1.10	12.68 ± 2.68	13.94 ± 1.74	13.33 ± 2.93	10.58 ± 2.19	7.55 ± 1.56	27.84 ± 31.95	31.95 ± 2.60	24.98 ± 3.60
c-PARP	62.36 ± 12.75	75.00 ± 3.63	68.37 ± 4.14	67.00 ± 6.66	61.50 ± 4.82	73.85 ± 2.99	59.22 ± 3.88	40.93 ± 5.97	48.27 ± 7.47
TUNEL	13.55 ± 7.90	2.15 ± 1.27	18.51 ± 6.87	29.41 ± 5.39	23.92 ± 7.20	27.35 ± 5.70	52.66 ± 5.01	32.72 ± 3.99, a	43.42 ± 4.74

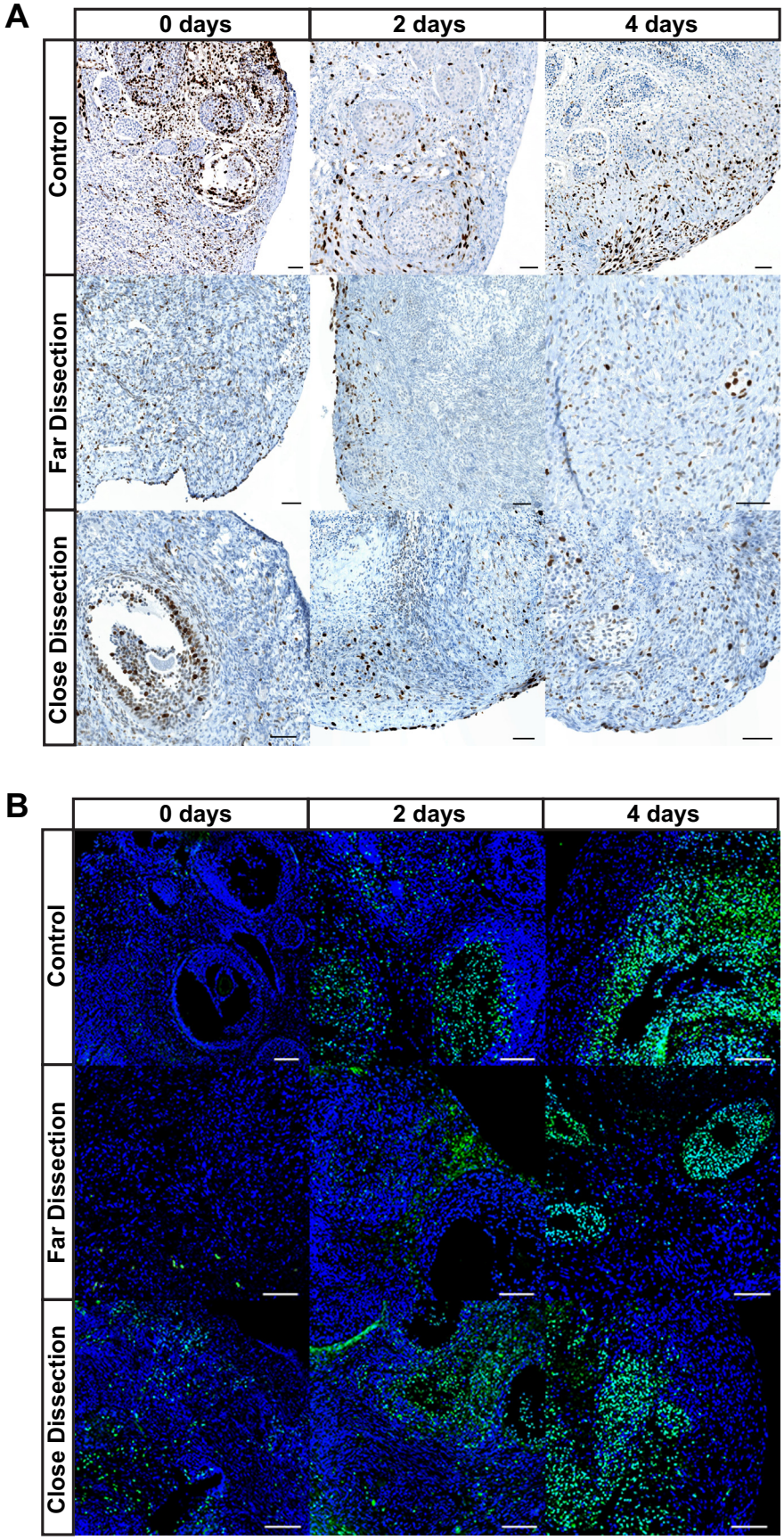


Fig. 2. Effects of proliferation or apoptosis by ovarian procurement technique. Representative images of (A) Ki67, a marker of proliferation (brown), or (B) TUNEL, a marker of apoptosis (green), in ovarian tissue cultured for 0, 2 or 4 days. The tissue incubated in nuclear stains hematoxylin (A, blue) or propidium iodide (B, blue). Scale bar, (C), 50 μm, (D) 100 μm.

2.2. Hormone and metabolic output

More estradiol was produced by control cultures (100%) over far dissection (52.6 %) and close dissection (22.5%, $p < 0.001$) groups on D2 (Fig. 3A). More estradiol was also produced by control cultures (100%) over far dissection (47.3%) and close dissection (41.6%, $p < 0.0001$) groups on D4. The far dissection culture produced more estradiol than the close dissection culture, although the difference in estradiol production did not reach statistical significance between the two groups.

To further understand the health of the tissue, we investigated markers of metabolism, glucose consumption (Fig. 3B) and lactate production (Fig. 3C). There was a 6.7 % reduction in glucose consumed by the far dissection group compared to the controls on D2 and a 0.4 % reduction on D4. There was a more significant reduction in glucose consumed by the close dissection group than the controls on D2 (19.5 %) and D4 (11.9 %). Significant differences in D2 lactate production were observed between the control and close dissection groups on D2 and D4 and the far and close dissection groups on D2 and the control and far groups on D4. Likewise, the metabolic ratio of these samples was affected in the tissue collected through far and close dissection methods when compared to the controls, indicating less metabolic activity in the tissue procured by the experimental methods over the control tissue.

2.3. Follicle counts and stages

Follicles from primordial (Fig. 4A), transitional, primary (Fig. 4B), secondary (Fig. 4C) and antral (Fig. 4D) stages were identified (Fig. 4E) to determine the effects of the procurement technique on follicular health and folliculogenesis. The number of follicles per initial 3 mm biopsy punch at D0 varied; however, the percentages of primordial, transitional, primary, secondary and antral follicles were not different at D0 across groups (Fig. 4E). Because of this, D2 and D4 follicle counts were normalized to D0 follicle numbers of the same group. Under FSH stimulation that was present in all cultures, there was an increase in the percent of primary and antral follicles in the control group at D2 and D4 and there were 793.75 % and 790.42 % more follicles in the control culture over far and close dissection culture at D2 and 251.02 and 128.84 % more follicles on D4 ($p < 0.001$). There was no significant difference between follicle percentages in far and close dissection groups on D2 ($p > 0.999$) and D4 ($p = 0.814$).

3. Discussion

Surgical dissection with an energy source close to the ovarian capsule may be expected to show evidence of energy transfer and possible tissue damage. Even a small amount of tissue damage could dramatically affect the quality of the ovarian tissue removed for cryopreservation in a pediatric patient with a small volume ovary. We set out to study this through use of a mini-pig model of laparoscopic oophorectomy using an UAED for tissue dissection and hemostasis. Technical specification for the Harmonic ACE™ UAED describes an average thermal spread of 1 mm with a range of 0.1–2.0 mm that was defined by the area of denatured adventitial collagen [15]. The ovarian cortical tissue procured using close and far dissection techniques was cultured to identify differences in gross histology, overall tissue health, follicle health and folliculogenesis in comparison to tissue isolated using laparotomy and cold sharp scissors.

The currently reported low pregnancy rate in adult women following ovarian tissue auto-transplant after OTC and limited lifespan of the tissue may be attributed to any one of several steps along the path from surgical removal of the tissue, to processing the cortical tissue, to the auto-transplantation procedure [6, 7]. Here we sought to examine surgical removal of ovarian tissue using a UAED as one step along this path. To date, there is no standard surgical procedure in place for the procurement of ovarian tissue for future use in restoring fertility and hormone function [9]. It is particularly important to establish a procedure that considers the pediatric patient, who has no alternative method for preserving fertility prior to gonadotoxic treatments, and who presents both unique anatomic challenges, given the small volume of the pediatric ovary and unique opportunities, given the abundant primordial follicle reserve. Our institution routinely performs laparoscopic unilateral oophorectomy for OTC using a UAED without surgical complications and without delay to the start of definitive medical therapy [14].

We understand the limitations of this culture assay when compared to a transplantation assay for analyzing the effects of the surgical technique for tissue removal on ovarian tissue health, since a functional assay would be preferred over gross histological examination of tissue at one point in time immediately following removal. However, we chose to establish this *in vitro* assay system to assess the estradiol

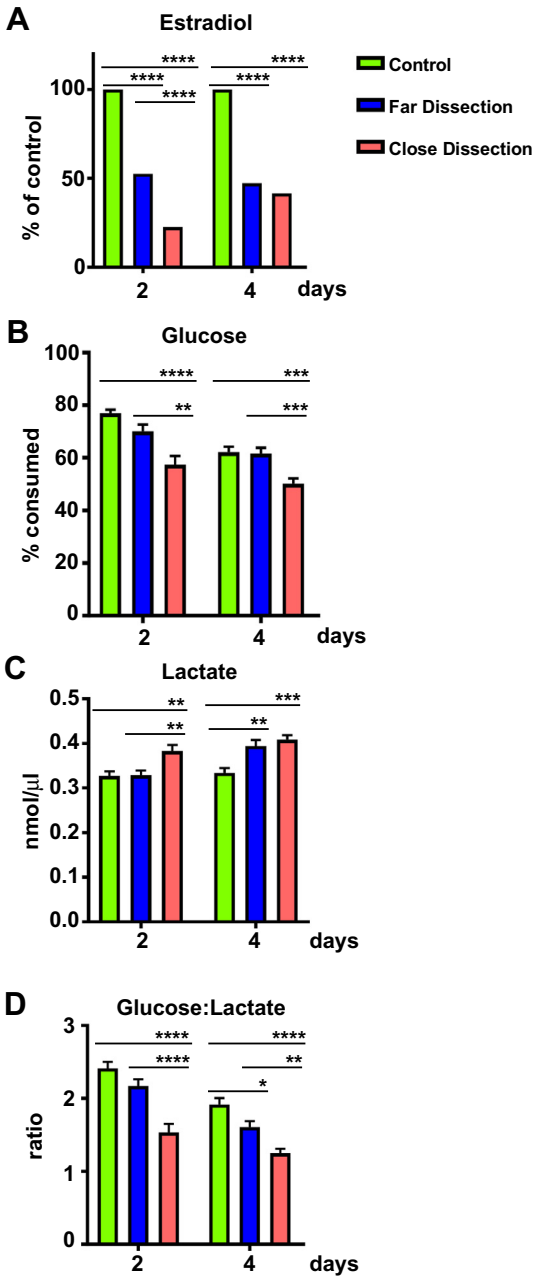


Fig. 3. Overall ovarian tissue health. (A-D) Estradiol, glucose and lactate were measured in the media of ovarian cultures over time. Bars represent the mean and error bars represent standard error of the mean. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$.

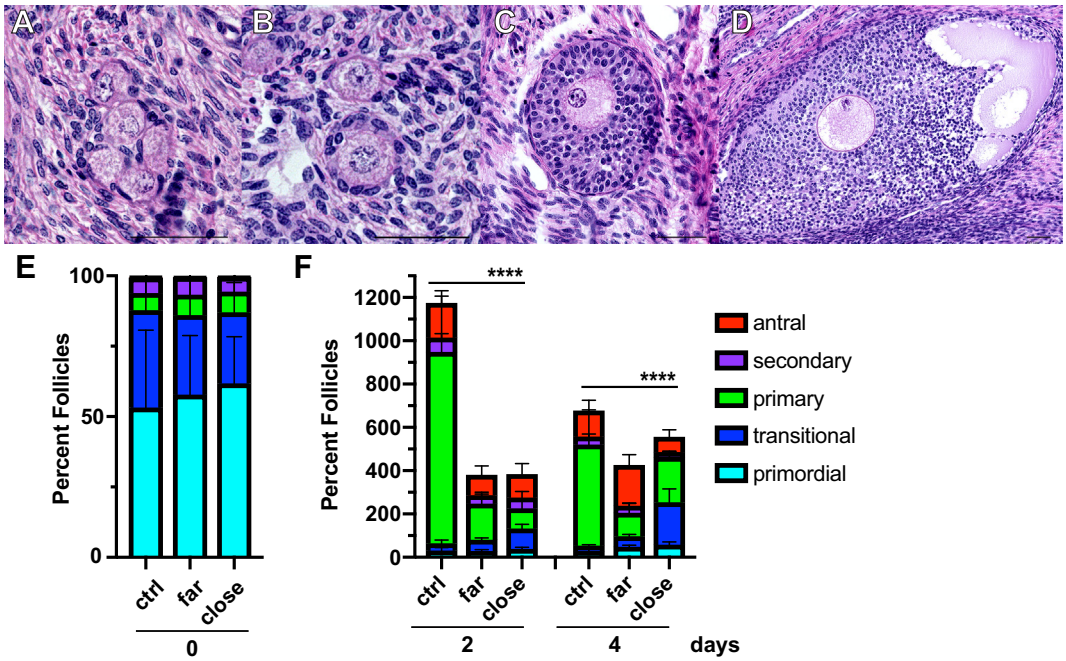


Fig. 4. Folliculogenesis in culture following ovarian procurement technique. (A-D) Representative images of hematoxylin and eosin stained primordial, primary, secondary and antral follicles identified within cultures. (E-F) The percentage of follicles within each stage at D0 (E) or following 2–4 days of culture (F) with ovarian tissue procured using control, far or close dissection procurement techniques. Scale bar, 50 μm. Bars represent the mean and error bars represent standard error of the mean. ****, $p < 0.001$.

output, folliculogenesis and overall health of the cortical tissue over time. Overall, there were no differences between surgical techniques in gross observations of biopsy punches and how they formed spheroids in culture. Our results demonstrate a significant decrease in proliferation in cultured tissue that was procured using close and far dissection techniques at different points in the culture. Surprisingly, the opposing mechanism of tissue remodeling, apoptosis, was not different between the experimental groups as identified by cleaved caspase 3, cleaved PARP and DNA fragmentation assays.

Estradiol is produced by viable growing follicles that are activated by follicle stimulating hormone (FSH) in the media. More estradiol was produced by the control and far dissection groups, compared to close dissection, indicating more viable growing follicles in the control and far dissection groups on D2 and D4. However, the results also revealed a significant reduction in estradiol production in both experimental groups compared to the control, inferring a reduced ability of the ovarian cortical tissue removed by UAED to support follicles compared to control sharp dissection.

Glucose is the predominant energy source provided in the growth media. Less glucose was consumed by the cultured ovarian tissue procured by close dissection technique at D2 and D4, and more lactate was produced at both time points, indicating anaerobic metabolism of the tissue. The ratio of glucose consumed to lactate produced was significantly lower for the close dissection group than the control or far dissection groups, which implies that the tissue health would be better in the control and far dissection groups compared to close dissection. However, there still remains a statistical difference in ratio of glucose consumed to lactate produced between the control and far dissection groups, which demonstrates negative metabolic effects on the ovarian tissue from energy transfer even with far dissection.

While the number of follicles varied within each sample, there was a greater percent increase in follicles in the FSH stimulated control cultures versus the two experimental groups. At D2 and D4, there were more follicles present in the far dissection culture versus the close dissection culture, although the difference did not reach statistical significance between the two cultures compared to control.

We chose a laparotomy procedure with cold scissors as our control procurement method, since this would reflect no energy transfer injury of any kind. While it is possible that the differences observed between our control and experimental groups could be indications of damage incurred through removing an ovary through a port versus and open incision, our hypothesis that the UAED has an effect is supported by the additional differences between far and close dissection experimental groups in some assays. While an open procedure with cold scissors may reduce the risk of any damage to the ovarian tissue upon procurement, it is not the best option for a pediatric patient where the laparoscopic approach minimizes complications and does not delay definitive medical therapy. The laparoscopic approach typically necessitates the use of an energy source for division and hemostasis of the mesovarium. Many surgeons use UAED or electrocautery for hemostasis in a variety of laparoscopic procedures [18, 19]. However, we recognize the limitations of this current study and, based on the findings here, we plan to perform additional experiments to delineate the optimal surgical technique without using a heat source or with another heat source such as bipolar cautery. Additionally, in future studies, we plan to re-implant the procured tissue to evaluate fertility and hormone restoration as these are the ultimate goals of the oophorectomy procedure. Nevertheless, our results demonstrate the effects of energy damage to the ovarian tissue from UAED in both close and far dissection, with increased injury in the close dissection group. For surgeons worldwide, who perform routine hemi-oophorectomy for ovarian tissue removal, we caution that the energy source applied directly to the ovary during that operation causes damage to both the tissue removed and tissue left in-situ [9]. As a result of this translational research, our surgical team has adjusted our current surgical technique in pediatric patients undergoing laparoscopic unilateral oophorectomy for ovarian tissue cryopreservation, to maintain a 2-cm distance from the ovarian capsule when UAED is needed. Ongoing work examining other techniques, such as use of bipolar cautery and endoloops, will further define the standard of care surgical technique to provide the best quality ovarian tissue for preservation and future restoration of fertility and hormone function.

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Author Contributions

Each author made significant contributions to this study including: study conception and design (EER, KSC, MML), acquisition of data (EER, KSC, KAE, MML), analysis and interpretation of data (EER, KSC, KAE, MML), drafting of manuscript (EER, KSC, MML), critical revision (EER, KSC, KAE, MML).

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