

## **ORIGINAL ARTICLE**

### **Variation in follicle health and development in cultured cryopreserved ovarian cortical tissue: a study of ovarian tissue from patients undergoing fertility preservation**

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## **ABSTRACT**

This study investigated how follicle health and development in human ovarian tissue cryopreserved for fertility preservation varied between patients before and after six days of *in vitro* culture. Ovarian tissue from 12 patients (9-25 years) was used. In 3 patients, a 1hr neutral red (NR) incubation was used to identify tissues with viable follicles. Tissues were fixed, sectioned, and follicles staged and graded for health. Inter-patient differences were observed in the non-cultured tissue in the number of both healthy follicles ( $p=0.005$ ) and growing follicles ( $p=0.005$ ). After culture there was significant variation in the number of transitional, primary, and secondary follicles between patients ( $p<0.001$ ). Asymmetric primary follicles with a single complete layer of granulosa cells plus two or more additional partial layers were 5.5 times more likely to be observed in cultured compared to non-cultured tissue ( $p=0.0063$ ). Non-cultured ( $p=0.0125$ ) and cultured ( $p<0.001$ ) tissue selected using NR had more healthy follicles compared to tissue not selected using NR. Non-cultured and cultured tissue selected using NR had more healthy follicles compared to tissue not selected using NR ( $p=0.0125$ ;  $p<0.001$ ). We demonstrate that inter-patient variation exists in the health and development of follicles before and after culture. Culture systems need to be optimized to support cryopreserved ovarian tissue and these findings should prompt researchers to consider patient variation when evaluating culture systems.

**KEYWORDS:** Cryopreservation; ovary; follicle; human; in vitro growth; cancer

## Introduction

Cryopreservation of ovarian cortical tissue has been established as a successful method for preserving fertility in patients for whom traditional approaches, such as storage of mature eggs or embryos, are not possible. This includes pre-pubertal girls and women for whom cancer treatment cannot be delayed (Anderson, Wallace, & Telfer, 2017). Growth of follicles from this tissue *in vitro* is emerging as an alternative method to circumvent some of the limitations associated with transplantation (Nisolle, Casanas-Roux, Qu, Motta, & Donnez, 2000).

The overall goal of *in vitro* follicle growth is to generate systems that support development and maturation of a competent human egg for use by women at risk of premature ovarian insufficiency (De Vos, Smits, & Woodruff, 2014; McLaughlin, Albertini, Wallace, Anderson, & Telfer, 2018; Xiao et al., 2015). For humans, an *in vitro* system capable of generating metaphase II oocytes from fresh cortical tissue has recently been described using a three-step culture method prior to *in vitro* maturation (McLaughlin et al., 2018). However, culture systems aiming to support the initiation of follicle growth and early development have predominantly used fresh tissue from healthy women undergoing gynaecological operations whose biology may differ from those who may benefit from fertility preservation. To date, there is no data regarding the variation in health and development of follicles *in vitro* from tissue cryopreserved for fertility preservation in women undergoing gonadotoxic treatments, despite the high importance of this for developing techniques for fertility restoration.

Follicle distribution in the cortex of human ovaries is extremely heterogeneous. The density of primordial follicles between different pieces of cortex from the same ovary can vary by greater than two orders of magnitude (Schmidt, Byskov, Nyboe Andersen, Muller, & Yding Andersen, 2003). This creates challenges in identifying cortical tissue

fragments with follicles for culture. Neutral Red (NR) is a weak cationic supravital dye that is soluble in water and has been used in cytotoxicity studies as a marker of cell viability (Allison & Young, 1964; Borenfreund & Puerner, 1985). It has been demonstrated to effectively label viable follicles within ovine cortical tissue and has subsequently been used to determine follicular density of fresh human cortical tissue or medulla by incubating the tissue for 4 hours before visualisation (Chambers, Gosden, Yap, & Picton, 2010; Kristensen et al., 2018). It remains to be investigated whether this dye can be incorporated into the workflow of human cortical strip culture without the use of a 4-hour incubation to allow visualisation of follicles in fresh tissue before culture.

The aims of this study were to determine: (i) how the health and developmental stage of follicles differs between women who have cryopreserved cortical tissue for fertility preservation; (ii) whether the *in vitro* developmental capacity of follicles from cryopreserved tissue varies between them; and (iii) whether incubating cortical tissue with NR for a short period of time to identify tissue with viable follicles could improve the number of viable follicles after six days of culture.

## **Materials and methods**

### *Ethical approval*

The use of human tissue was approved by Health Research Authority South Central – Oxford B Research Ethics Committee (REC reference: 14/SC/0041).

### *Ovarian tissue collection*

Cryopreserved ovarian tissue was obtained from twelve patients aged 9 to 25 years. All were undergoing ovarian tissue cryopreservation, as a fertility preservation measure due to malignancy or blood disorder. None had received chemotherapy or radiation treatment

prior to ovarian tissue cryopreservation. As part of the consent process, permission to use tissue in research had been obtained.

#### *Chemicals and consumables*

Leibovitz L-15 medium, McCoy's 5A (modified) HEPES buffered medium, L-glutamine and ascorbic acid were obtained from Thermo Fisher (Paisley, UK). Human serum albumin, ITS liquid media supplement (100x), sucrose, ethylene glycol, sodium pyruvate, NR, Bouin's solution, and Whatman Nucleopore membranes were obtained from Sigma Aldrich (Poole, UK). Recombinant human follicle stimulating hormone (FSH; Gonal-F) was obtained from Merck Serono (Feltham, UK). Corning Costar tissue culture treated 24-well plates were obtained from Scientific Laboratory Supplies (Nottingham, UK).

#### *Cryoprotection and cryopreservation*

Ovarian tissue cryopreservation was performed by the Oxford Cell and Tissue Biobank. Cortical tissue cut into cortical strips (~2 x 1 x 5 mm) were placed into cryovials containing 1 mL of cryoprotectant medium (1.5 M ethylene glycol, 0.1 M sucrose, 10% (v/v) serum substitute supplement in L-15 medium) and incubated for 1 hour at 4°C before being frozen using a controlled rate slow freezer (IceCube 15M; SY-LAB, USA). Following cryopreservation, the vials were stored in vapour phase liquid nitrogen.

#### *Tissue thawing and cortical strip culture*

Cryovials were held at room temperature for approximately 1 minute before being immersed in a 30°C water bath for 3 minutes. Cortical strips were washed through three thawing solutions containing a reversed ethylene glycol gradient (1 M, 0.5 M and 0 M), 0.1 M sucrose and 3 mg/mL human serum albumin (HSA) in Leibovitz L-15 medium for

5 minutes and transferred to a petri dish containing Dissection Medium (3 mg/mL HSA, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine and 2 mM sodium pyruvate in L-15 medium). Tissue was mechanically chopped using the McIlwain tissue chopper, and further cut manually using a scalpel and forceps into ~0.5 x 0.5 x 0.25 mm pieces.

Tissue pieces of uniform size were distributed randomly and evenly between wells of a 24-well plate with excess remnant tissue discarded. A portion of tissue fragments were fixed overnight in Bouin's fixative as a non-cultured control. Tissue was cultured on a polycarbonate membrane (13 mm diameter, 8 µm pore size) floating on 1 mL of culture medium in a 24-well culture plate; culture medium contained McCoy's 5A supplemented with 1 mg/mL HSA, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 10 µg/mL insulin, 5.5 µg/mL transferrin, 5 ng/mL selenium, 50 µg/mL ascorbic acid and 12.5 IU/L recombinant human FSH. Cortical tissue was cultured for 6 days at 37°C under 5% CO<sub>2</sub> in air, with half the medium being replaced with fresh medium every other day. Following the culture period, the tissue pieces were fixed overnight in Bouin's solution and stored in 70% ethanol until they were processed.

#### *Neutral red (NR) visualisation*

To identify fragments containing follicles, after processing cortical tissue with the tissue chopper, fragments from patients E, I, and L (Table 1) were incubated in NR (25 µg/mL in dissection medium) at room temperature for 1 hour while the tissue was cut (as in the standard protocol). Allocation of tissue between groups was performed without a microscope to blind the researcher to the degree of staining and detail of the tissue pieces.

#### *Histological analysis*

The fixed ovarian tissue was dehydrated through a graded series of ethanol (70%, 80%, 95%, 3x100%), cleared in xylene and embedded in paraffin wax at 64°C for three hours. The wax-embedded tissue was serially sectioned at 5 µm and stained with haematoxylin and eosin.

Follicles were staged based on criteria described by Gougeon (1986); primordial (single layer of flattened pre-granulosa cells), transitional (single layer with at least one cuboidal granulosa cell), primary (complete layer of cuboidal granulosa cells) and secondary, two or more complete layers of cuboidal granulosa cells (Figure 1). Where follicles had a single complete layer of cuboidal cells, the number of any additional partial layers of granulosa cells was recorded. Non-growing follicles were those at the primordial stage. Transitional, primary, and secondary follicles were classed as growing. Granulosa cell and oocyte pyknosis, and shrinkage of ooplasm were selected as markers for follicle health based on the existing literature and the ease of assessing health factors in a non-biased manner (Gougeon, 1986). Healthy follicles had a non-pyknotic non-shrunken oocyte with non-pyknotic granulosa cells, degenerating follicles had one of the above factors, while follicles were classified as atretic if they had both an oocyte with a pyknotic nucleus and pyknotic granulosa cells (Figure 2). All tissue sections were analysed for the presence of follicles, and only follicles with a visible nucleolus or a clearly defined nuclear membrane were assessed, to avoid double-counting. A blinded researcher performed follicle counts and assessment; a second blinded researcher confirmed repeatability.

#### *Calculation of follicle density*

To determine the volume of the tissue, the area of every 12th tissue section was measured using ImageJ 1.46r (National Institutes of Health, Bethesda, MD, USA). Follicle density

was determined by dividing the total number of follicles counted in a tissue sample by the tissue volume.

### *Statistical analysis and modelling*

All statistical analyses were performed using R statistical software, version 3.5.0 (R foundation for Statistical Computing, Vienna, Austria). Statistical analysis was performed using Fisher's Exact Test. Tests adjusting for patient variation used the lme4 package in R (Bates et al., 2014). All logistic regressions were offset for tissue volume and included patient as a random effect. Data are presented as mean or as odds ratio with 95% confidence intervals where available, and statistical significance was defined as  $p < 0.05$ . Due to the small number of secondary follicles observed, no statistical analysis was performed on this cohort.

## **Results**

### *Patient characteristics*

Nine of the twelve patients were confirmed as having gone through puberty, the remaining three had not. Of the nine post-pubertal patients, information about menstrual cycle was available for three who were reported as having regular cycles (Table 1). Two patients, where information about their menstrual cycle was unavailable, were taking the oral contraceptive pill. The mean age ( $\pm$ SEM) of patients who had gone through puberty was significantly higher than for those who had not ( $20.56 \pm 1.04$  vs  $12 \pm 2.08$  years, respectively;  $p < 0.01$ ).

There was no difference in the time from tissue procurement to cryopreservation between the pre- and post-pubertal groups. The mean time from completion of surgery to start of cryopreservation was 4 h 13 min  $\pm$  19 min (Table 1).



Follicular density of tissue post-cryopreservation from the 12 patients varied from 18.10 to 448.43 follicles/mm<sup>3</sup> between patient samples (Figure 3), and all cortical strips contained follicles. Despite the small sample of tissue and the known heterogeneity of follicle distribution in the ovary, there was a significant inverse correlation between follicular density and age ( $r = -0.62$ ,  $p < 0.05$ ).

*Number of growing follicles in non-cultured cryopreserved tissue varies between patients*

Follicles were observed in non-cultured tissue from 11 of the 12 patient samples. Non-growing follicles were those classified as primordial based on morphology (Figure 1A), growing follicles were those classified as transitional, primary, or secondary (Figure 1B-D). The number of growing compared to non-growing follicles in uncultured tissue varied significantly based on patient and can be seen in Figure 4A ( $p < 0.001$ ). Non-growing follicles were the dominant population in non-cultured tissue for five patient samples (patients A, B, E, H and I), an equal percentage of growing and non-growing were seen in two patient samples (patients C and D), and the percentage of growing follicles dominated in four patient samples (patients G, J, K and L). Interesting, only growing follicles were observed in non-cultured tissue from patient K however as only three follicles were seen in this tissue (Table 2) it may be due to the small sample size.

*Follicle health in non-cultured cryopreserved tissue varies between patients*

Follicles were graded as being healthy, degenerating or atretic based on the presence or absence of pyknotic granulosa cells, a pyknotic oocyte and shrunken ooplasm (Figure 2). There was significant variation between patient samples in the percentage of healthy follicles in non-cultured tissue (Figure 4B;  $p < 0.001$ ).

In three patient samples, tissue fragments were preferentially selected for non-culture or culture based on visualisation of follicles using the vital dye NR. Tissues from these samples (patients E, I and J) showed a similar percentage of healthy follicles in the primordial and growing follicle populations. This was in contrast to the 9 patient samples in which NR was not used as in 5 of these, the percentage of healthy follicles was greater in the primordial population compared to the growing population (Figure 4C-D).

The probability of a follicle being healthy in non-cultured tissue did not differ between pre- and post-pubertal patients and was unaffected by the time from surgery to cryopreservation.

*Follicle health, but not development is greater in non-cultured tissue selected by neutral red staining*

An example of a follicle with NR staining can be seen in Figure 5A. In non-cultured tissue where NR was used to select tissue fragments as described above, the odds of a follicle being healthy was over 11 times that for follicles from tissue where NR was not used (Figure 5B; OR=11.4, 95% CI (1.7- 77.4); p=0.0125). There was a smaller percentage of growing follicles in tissue selected using NR though this was not significant (Figure 5B).

*Cultured cryopreserved tissue has a dominant population of growing follicles in all patients*

Overall, culture resulted in a significant change in the non-growing and growing follicle populations, with the odds of observing a growing follicle in cultured tissue being 52 times that of non-cultured tissue (OR=52.44, 95% CI (37.05- 76.41), p<0.0001).

The health and development of follicles in tissue from each patient samples are detailed in Figure 6. There was no difference in the percentage of follicles growing

following culture (Figure 6A), however there was variation within the growing population in the proportion of follicles at the transitional, primary, and secondary stages (Figure 6B). Primary follicles were the dominant growing follicles in all patient samples except for patient K and patient B where 50% of follicles were transitional and 50% were primary. The number of secondary follicles observed after six days of culture was low compared to transitional and primary follicles and represented at most 9% of the total follicles in cultured tissue (Figure 6B; Patient C). Between 8 and 80% of follicles were multi-laminar follicles with more than one layer of granulosa cells (Figure 6C). The percentage of growing follicles in tissue after six days of culture was not different between fragments that had been selected based on NR staining and those that had not been selected based on NR (Figure 6F).

#### *Neutral red selected tissue has superior follicle health post-culture*

Despite significant activation and development of follicles in cultured tissue, overall follicle survival in cultured tissue was low. Following six days of culture, all follicles from tissue not selected using NR showed some sign of degeneration (Figure 6E), however this is not surprising as 78.08% of follicles in this cohort showed some sign of degeneration in non-cultured cryopreserved tissue (Figure 5). In tissue selected by NR staining, 18.14% (n=612; 3 patients) of follicles were healthy after six days of culture (Figure 6E). Since 60.39% of follicles were healthy in non-cultured tissue selected using NR (Figure 5), this meant that follicles in these tissues were over 13 times more likely to be healthy in non-cultured compared to cultured tissue (OR= 13.67, 95% CI (10.04-18.91),  $p<0.0001$ ). The odds of a follicle from NR selected tissue being healthy after six days in culture were 26 times that of a follicle from tissue where NR was not used to select fragments for culture (Figure 6E; OR=108.9, 95% CI (12.3-962.8),  $p<0.001$ ).

### *Follicles grown in culture have asymmetric granulosa cell distribution*

In cultured tissue, primary follicles were 3.2 times more likely to have at least one additional partial granulosa cell layer compared to non-cultured primary follicles (OR=3.17, 95% CI (1.83-5.80),  $p<0.001$ ; Figure 7A). Asymmetric follicles, primary follicles with two or more partial layers of granulosa cells and only one complete layer of granulosa cells, were observed in eight of the cultured patient samples (Figure 7C-D). By contrast in non-cultured tissue, only two asymmetric primary follicles were observed, each in fragments of tissue from different patients (Figure 7A). The odds of a primary follicle being asymmetric in cultured tissue was 5.5 times that of non-cultured tissue (OR=5.46, 95% CI (1.40-46.92),  $p=0.0063$ ).

## **Discussion**

The present study is, to our knowledge, the first to evaluate the patient-specific health and development of follicles following *in vitro* culture of cortical tissue cryopreserved for fertility preservation. Furthermore, we describe a protocol for human cortical strip culture utilising NR to identify fragments with viable follicles for culture.

Our results highlight wide variation between patients in the health and developmental stage of follicles in cryopreserved ovarian cortical tissue. Early studies looking at the viability of ovarian cortical tissue cryopreserved in humans focused on success of xenotransplantation studies to demonstrate function without accompanying histological assessment. Variation and lack of clarity in morphological criteria for atresia between studies, as well as differing methods of cryoprotection, has made it challenging to define a baseline for cryopreservation-derived follicle degeneration. Here we describe the morphological criteria assessed, providing images of each variation of follicle health

we observed. Variation in follicle health post-thaw may be tied to follicle health in fresh tissue as despite finding no difference between fresh and frozen-thawed follicles, 27% of follicles in fresh tissue showed multiple signs of atresia; eosinophilia of the ooplasm, contraction and clumping of the chromatin material, and wrinkling of the nuclear membrane (Hovatta et al., 1996).

We found the use of just 1 hr of NR incubation effective and easy to implement within the culture workflow with follicles identified in thin cortical fragments by their red staining. When tissue with viable follicles was selected by NR-staining, a significantly greater number of follicles in non-cultured tissue had a healthy morphology (60% compared to 22%, respectively). Our findings support those from Kristensen et al. (2018) who used a 4 hr NR incubation to demonstrate the specificity of NR for viable follicles in cortical tissue from women undergoing fertility preservation before gonadotoxic treatment. Additionally, our results demonstrate that a shorter NR incubation period is adequate to identify tissue fragments with viable follicles, enhancing the value of such a technique in selecting tissue for culture with the aim of optimising the proportion of healthy follicles.

In several patients the percentage of multi-layered follicles present after six days of culture was equal to or greater than that published by McLaughlin, Kinnell, Anderson, and Telfer, (2014) indicating both methods support equivalent follicle development. Interestingly, a significant proportion of primary follicles in cultured tissue were asymmetric, with two or more partial granulosa cell layers in addition to a single complete layer. This finding is significant as current multi-step *in vitro* protocols involve the excision of secondary follicles based on size following cortical strip culture (McLaughlin et al., 2018; Telfer, McLaughlin, Ding, & Thong, 2008). However, based on size asymmetric follicles would be indistinguishable from non-asymmetric follicles when

using a dissecting, brightfield microscope. It is unclear whether the asymmetry is a result of the physical culture environment or whether it is a result of accelerated growth due to culture conditions. Furthermore, it is important to ascertain the impact of asymmetry on subsequent follicle growth and developmental competence of the enclosed oocytes, particularly since current follicle selection for further development would not differentiate between them and non-asymmetric follicles.

Follicle health declines over the course of culture and there is evidence to suggest that this decline is more marked in cryopreserved tissue (Hovatta, Silye, Abir, Krausz, & Winston, 1997). We assessed if tissue selection using NR-staining would lead to better follicle development after culture. When NR was not used, nearly all follicles showed some sign of degeneration after six days of culture which was unsurprising given that only 22% were healthy in the non-cultured tissue. By contrast from 60% healthy follicles present in NR-selected non-cultured tissue, 18% of follicles retained a healthy morphology. These results are consistent with the 2.5-to-3-fold decrease in the proportion of healthy follicles reported by other groups culturing cryopreserved tissue over a similar timeframe, despite differences in culture systems (Asadi-Azarbaijani et al., 2016; Azarbaijani et al., 2015; Hovatta et al., 1997; Sanfilippo et al., 2013).

Although this analysis was based on a small volume of tissue per patient, an inverse correlation between age and follicle density was observed, confirming and extending that reported by Schmidt et al. (2003) with a sample of tissue from 21 women. The heterogeneity in follicle distribution within the human cortex is well described (Poirot et al., 2002; Qu, Godin, Nisolle, & Donnez, 2000; Schmidt et al., 2003), and has been cited as a strong reason that multiple pieces of ovarian cortex should be replaced upon re-implantation. It is possible that this heterogeneity extends to the response of

follicles within a single ovary to the effects of cryopreservation, culture, or transplantation and the variation between patients reported in this study lends support to this idea.

In conclusion, *in vitro* development of eggs remains an exciting option for fertility restoration, but variation between patients may necessitate a more individual approach to downstream treatment options. We demonstrated here for the first time that there is variation between patients both in the health of non-cultured tissue post-cryopreservation as well as the health and development after six days in culture. The culture system described was effective in initiating follicle activation and growth in this tissue, but optimisation is required to improve the survival of follicles across the culture period. Given the heterogeneity of tissue and clear variation in follicle health post-thaw in these patients, demonstrating that using the non-toxic viability marker NR in the existing culture workflow as described here to select tissue is an important advance in improving the efficiency and effectiveness of *in vitro* follicle development as an alternative method of fertility restoration.

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Table 1. Characteristics of patients diagnosed with malignant disease or blood disorder who underwent ovarian tissue cryopreservation for fertility preservation.

<b>Patient</b>	<b>Age (years)</b>	<b>Post- pubertal</b>	<b>Regular periods</b>	<b>Diagnosis</b>	<b>Prior treatment</b>	<b>Time from surgery to cryopreservation</b>
A	9	No	N/A	Medulloblastoma	None	3 h 16 min
B	11	No	N/A	Sickle cell	None	4 h 19 min
C	16	No	N/A	Osteosarcoma	None	5 h 20 min
D	16	Yes	Unknown	Ewings sarcoma	None	5 h 10 min
E	17	Yes	Yes	Ewings sarcoma	None	2 h 05 min
F	18	Yes	Unknown	Sickle cell	None	3 h 57 min
G	19	Yes	Unknown	Low grade serous adenocarcinoma	None	6 h 15 min
H	22	Yes	Unknown <sup>a</sup>	Burkitt lymphoma stage 1A	None	4 h 18 min
I	22	Yes	Unknown	Atypical teratoid	None	5 h 00 min

				Rhabdoid tumour		
				(Grade IV)		
J	23	Yes	Yes	Breast cancer, grade 3, estrogen positive, non- metastatic	None	3 h 05 min
K	23	Yes	Unknown <sup>a</sup>	Hodgkin lymphoma	None	4 h 20 min
L	25	Yes	Yes	Cervical cancer	None	3 h 32 min

<sup>a</sup>Patient taking the oral contraceptive pill

Table 2. Number of follicles observed in cultured and non-cultured cryopreserved human ovarian tissue.

<b>Patient</b>	<b>Non-cultured</b>	<b>Cultured</b>	<b>Total</b>	<b>Selected using neutral red</b>
A	99	27	126	-
B	63 <sup>b</sup>	85	148	-
C	168 <sup>b</sup>	67 <sup>b</sup>	235	-
D	70	58	128	-
E	1224	489 <sup>b</sup>	1713	Yes
F	0	14	14	-
G	67 <sup>b</sup>	68	135	-
H	13	14 <sup>b</sup>	27	-
I	874	90	964	Yes
J	133 <sup>b</sup>	15 <sup>b</sup>	148	-
K	3 <sup>b</sup>	12	15	-
L	139	33 <sup>b</sup>	172	Yes

<sup>b</sup>Secondary follicles present

## Figure Legends

Figure 1. Developmental staging of human follicles from cryopreserved ovarian tissue from patients diagnosed with malignant disease or blood disorder.

Key: Representative images of follicles in cryopreserved-thawed ovarian tissue. Tissue was fixed in Bouin's, embedded and stained with haematoxylin and eosin. Follicles were staged as: (A) primordial with a single layer of flattened pre-granulosa cells; (B) transitional with a single layer of at least one cuboidal granulosa cell; (C) primary with a complete layer of cuboidal granulosa cells; or (D) secondary with two or more complete layers of cuboidal granulosa cells. Scale bar = 20  $\mu$ m.

Figure 2. Classification of follicle health in human follicles from cryopreserved ovarian tissue from patients diagnosed with malignant disease or blood disorder.

Key: Follicle health was assessed based on the presence or absence of pyknotic granulosa cells, a pyknotic oocyte and a shrunken ooplasm: Images A-C show healthy follicles with healthy granulosa cells, a non-pyknotic and non-shrunken oocyte whereas degenerating follicles had either (D) pyknotic granulosa cells (white arrowhead), (E) a pyknotic oocyte (white arrowhead) with normal ooplasm, or (F) a pyknotic oocyte (white arrowhead) with a shrunken ooplasm (black arrowhead) and normal granulosa cells. Atretic follicles had both pyknotic granulosa cells and a pyknotic oocyte (white arrowheads), without shrunken ooplasm (G), or with shrunken ooplasm (black arrowheads, H-I). Scale bar = 20  $\mu$ m.

Figure 3. The density of follicles in relation to age in cryopreserved ovarian cortical tissue from 12 patients diagnosed with a malignant disease or blood disorder.

Figure 4. Variation between patients in the percentage of growing and healthy follicles in non-cultured cryopreserved human ovarian tissue from patients diagnosed with malignant disease or blood disorder.

Key: Patients are listed by ascending age with the dashed line indicating the split between those who were not post-pubertal (patients A-C) and those who were post-pubertal (patients D-L). Significant variation in the number of growing follicles was observed in non-cultured cryopreserved ovarian cortical tissue from all patients with follicles (A) ( $p < 0.001$ , Fishers Exact Test); no follicles were observed in non-cultured tissue from patient F. The number of healthy (no morphological evidence of degeneration), degenerating (presence of either a pyknotic oocyte, pyknotic granulosa cells, or shrunken ooplasm), and atretic (presence of both pyknotic oocyte and pyknotic granulosa cells) varied significantly between patients (B) ( $p < 0.001$ , Fishers Exact Test). Images (C-D) show the percentage of healthy primordial follicles was greater than that of the growing follicle population in non-cultured tissue, with the exception of those follicles stained with neutral red (NR; patients E, I, and L) which showed a similar percentage of healthy follicles in the primordial and growing follicle populations. NR: neutral red staining used to select tissue.



Figure 5. Use of neutral red facilitates selection of tissue fragments containing viable follicles in cryopreserved ovarian tissue from patients diagnosed with malignant disease or blood disorder.

Key: Cortical tissue incubated in neutral red NR for 1 hr revealed viable follicles stained red shown in image A (white arrowhead). Thawed cryopreserved tissue fragments with follicles identified by NR staining for 1 hour (n=3 patients) contained significantly more healthy follicles compared to tissue where NR was not used (B) (n=9 patients) ( $p<0.02$ , OR=11.4, 95% CI (1.7 – 77.4), logistic regression adjusted for tissue volume with patient as a random effect). There was no difference in the number of growing follicles in non-cultured tissue selected by NR staining compared to tissue where NR was not used. Scale bar = 100  $\mu\text{m}$ .

Figure 6. Variation between patients in percentage of growing, and healthy follicles and the effect of tissue selection using neutral red in cultured cryopreserved ovarian tissue from patients diagnosed with malignant disease or blood disorder.

Key: There was no variation in the overall number of growing follicles in cultured cryopreserved ovarian cortical tissue (A) ( $p=0.289$ , Fishers Exact Test), however within the growing population of follicles in cultured tissue (B), the relative number of transitional, primary, and secondary follicles varied between patients ( $p<0.001$ , Fishers Exact Test), as did the percentage of multi-layered follicles, multi-laminar follicles with more than one layer of granulosa cells (C). There was significant variation in the number of healthy, degenerating, and atretic follicles between patients (D) ( $p<0.001$ , Fishers

Exact Test). Tissue selection using neutral red had a significant impact on the proportion of healthy follicles present (E) ( $p < 0.001$ , OR=108.9, 95% CI (12.3-962.8), logistic regression adjusted for tissue volume with patient as a random effect) but did not impact the number of growing follicles (F) ( $p > 0.05$ , Fishers Exact Test). NR: neutral red staining used to select tissue.

Figure 7 Asymmetric granulosa cell layers were more common in primary follicles in cultured cryopreserved tissue from patients diagnosed with malignant disease or blood disorder.

Key: Asymmetric primary follicles with variable numbers of partial granulosa cell layers were observed in non-cultured and cultured tissue. Primary follicles were significantly more likely to be asymmetric (those with a single complete layer of granulosa cells and two or more partial layers) with culture (A) (OR=1117.3, 95% CI (250.2-4990.7),  $p < 0.0001$ , logistic regression adjusted for tissue volume with patient as a random effect). A follicle with a single complete granulosa cell layer and one partial layer is shown in image B. Asymmetric primary follicles with a single complete granulosa cell layer and two or three partial layers are shown in images C and D, respectively. Scale bar = 20  $\mu\text{m}$ .