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THE EFFECT OF THAWING PROTOCOLS ON FOLLICLE CONSERVATION IN HUMAN OVARIAN TISSUE CRYOPRESERVATION

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Abstract

BACKGROUND: Ovarian tissue cryopreservation has the potential to improve fertility preservation for a growing number of patients undergoing sterilising therapy, particularly where oocyte or embryo cryopreservation is not suitable. However, its success is limited by significant follicular apoptosis upon thawing, and there is wide variation in thawing protocols used with little evidence of efficacy. **OBJECTIVE:** To determine the best warming rates to maintain tissue viability. **MATERIALS AND METHODS:** Ovarian tissue biopsies from 11 patients were taken with informed consent and divided into four pieces, which were allocated to either fresh assessment or to one of several freeze-thaw protocols. Cryopreservation was undertaken using a Stirling cycle cryo-cooler and cryopreserved samples were exposed to different warming protocols. Tissue conservation was then assessed using a marker, neutral red, to identify viable follicles. **RESULTS:** The results showed greatest follicle conservation rates in fresh samples, followed by those thawed using a rapid thawing protocol (Protocol 1). Tissue thawed using an ultra fast protocol (Protocol 2) and slow warming (Protocol 3) resulted in greater follicle loss. **CONCLUSION:** These preliminary results indicate thawing conditions significantly affect follicle conservation in cryopreserved human ovarian tissue.

Keywords: fertility preservation, cryopreservation, thawing protocols, human ovarian tissue, and follicle conservation

INTRODUCTION

According to the most recent data published by Cancer Research UK (2), 374,400 new cases of cancer were diagnosed in women within Europe between 2009 and 2010. Of these ~12% were in women under 50 years old. Although, progress in cancer therapies has improved the long-term survival of many cancer patients, aggressive cytotoxic therapy, which includes chemotherapy and/or radiotherapy, whilst curative, may cause premature ovarian failure (POF). For women of this particular age group premature ovarian failure and infertility is one of the most common adverse effects (10).

At diagnosis, these patients may be presented with the option of having an emergency cycle of ovarian stimulation followed by oocyte or embryo freezing to

allow assisted conception after successful cancer treatment. However, this option invariably delays cancer therapy. Furthermore is not an appropriate method for pre-pubescent patients or those with hormone-sensitive tumours as they are unable to have any hormonal stimulation required for oocyte retrieval (13).

A further problem is that oocyte freezing has low rates of success as the cell has relatively high water content and as such is particularly prone to cryoinjury. Embryo freezing is much more successful and is an established technique. However if the patient does not have a conception partner at diagnosis then she must accept the use of donor sperm to create embryos in order to improve her chances of successful conception at a later date.

As there is no way to predict ovarian failure induced by chemo/radiotherapy, cryopreservation of the ovarian tissue prior to cytotoxic treatment could be advantageous. By dissecting the thin layer of cortex from the underlying medulla, follicles within the ovarian tissue can be cryopreserved for future use. Unlike freezing individual oocytes or embryos, ovarian tissue cryopreservation facilitates a more efficient way of preserving thousands of immature follicles at one time (14). This not only increases the number of preserved oocytes, but also does not delay the start of therapeutic treatment, and eliminates the need for ovarian stimulation. Compared to mature oocytes, primordial follicles are more resistant to cryoinjury, probably because of their smaller size, slower metabolic rates and absence of the zona pellucid (23).

It has been nearly 60 years since Parrott's work on successful cryopreservation of mouse ovarian grafts using glycerol and slow cooling was published (20). In 1993, Carroll and Gosden (3) showed that mouse primordial follicles had the ability to remain fertile following enzymatic isolation, cryopreservation, storage at -196˚C in liquid nitrogen and retransplantation; using dimethylsulphoxide (DMSO) as a cryoprotective agent. Gosden et al (11) further explored this field with additional experiments, which have become extremely important in this area of research. His group reported the restoration of oestrous cycles and fertility to oophorectomised sheep following ovarian tissue cryopreservation, thawing and subsequent autografting to the ovarian bursa. Sheep were chosen for their ovaries likeness to humans in terms of size and tissue structure.

These experimental models laid the foundations for the first attempts at human pregnancies achieved using ovarian tissue cryopreservation and transplantation in cancer patients. In 2004 healthy live births from frozen–thawed human ovarian tissue were claimed in lymphoma survivors who underwent orthotopic transplantation (8). In 2005, Meirow and his colleagues harvested ovarian tissue from a patient prior to undergoing high-dose chemotherapy for Hodgkin's lymphoma (16, 17). Thawed ovarian tissue was transplanted orthotopically, and the patient conceived following the transfer of a four-cell embryo using *in vitro* fertilization. This success of this pregnancy demonstrated that ovarian tissue cryopreservation and transplantation could not only aid in achieving conception but provide hormonal support which also may have been lost during cytotoxic treatment.

However, in 2008 meta-analysis identified that although the technique was having some success it was limited. In 46 women who underwent ovarian cryopreservation and transplantation, resumption of ovarian function was established with a median time of 120 days. Among them, four women had recurrent ovarian failure within 6 months. In 25 women who sought pregnancy, eight women had nine pregnancies at 12 months, giving a cumulative pregnancy rate of 37% (1). Most recently worldwide, the number of live births as a result of frozen ovarian tissue transplantation had risen to 60 of which two

were born using vitrification as a technique for cryopreservation (7).

Thus while the results for ovarian tissue cryopreservation are promising, the technique remains experimental with relatively low numbers of live births in comparison to number of transplantations occurring. There are many factors likely to be responsible for the relatively low success rates with this technique, both experimentally in terms of tissue conservation post-thaw and clinically in terms of live birth rate as a result of cryopreserved ovarian tissue autografting. An optimal protocol for cryopreservation and thawing is still not widely agreed upon, and the viability of tissue following this technique warrants further investigation. In particular, the warming profiles for cryopreserved ovarian tissues have not been evaluated. Whilst rapid warming rates have been widely applied in cryopreservation (9), early studies on embryo cryopreservation suggested that slow warming was effective (25). More recently Seki & Mazur (2010) demonstrated the dominance of the impact of warming rate in cryopreservation of oocytes. However, this freezing was done using vitrification, which does differ greatly to the tissue slow cooling used in this research study (22).

The aim of this study was therefore to investigate different warming profiles for cryopreserved human ovarian tissues. In addition, the application of a Stirling motor cryo-cooler for controlled slow cooling of human ovarian tissue was investigated. This technology is electrically powered and negates the need for any cryogen such as liquid nitrogen during cryopreservation (Asymptote Ltd, Cambridge UK). Stirling motors operate by cyclic compression and expansion of a gas at different temperatures, converting heat energy to work. However, this system can operate in reverse; by adding work in, cooling to low temperatures can be achieved. This equipment has already been shown to be effective for cryopreservation of suspensions of stem cells, sperm, embryos and hepatocytes (1, 7, 9) and potentially has wider applications in controlled atmosphere environments such as operating theatres where ovarian tissues may be accessed.

MATERIALS AND METHODS

Permissions and ethical approval for research access to human ovarian tissues

Ethical approval for this study was granted by the Fulham Research Ethics Committee on 16th May 2012. Reference Number 12/LO/0418. Furthermore, the Research and Development Department of the Royal Free Hospital allowed the study to be carried out on NHS premises on 1st July 2012, reference number 8559.

Subject group and consent

The tissue biopsies for this particular study were taken from 11 women undergoing elective caesarean section at the Royal Free Hospital. Recruiting patients from a cohort scheduled to undergo cesarean section had been carried out in other studies investigating ovarian tissue in Leeds, UK Stockholm, Sweden (4, 12).

The women selected were aged between 18 and 35, showing no signs of coagulopathy or malignancy, and having the capability to consent. Women that fulfilled these criteria were identified by the midwifery staff when they attended the Day Assessment Unit two days prior to their operation date. At this point, the research teams were given an opportunity to inform and counsel the women regarding participation in the study. On the day of their operation, these ladies were once more counseled, and informed consent was obtained.

Ovarian tissue collection

Two cortical biopsies from each woman were taken during the caesarean section following closure and hemostasis of the uterine incision, in order to prevent any excessive blood loss. The biopsies were taken using a 6 mm diameter Stiefel Punch Biopsy tool, which separated the required tissue from the loose medullary tissue

underneath. This all ensured uniform size of the biopsy.

The tissue biopsies were immediately placed into Leibovitz-L15 medium (Life Technologies, Invitrogen Ltd, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies, Invitrogen Ltd, Paisley, UK) and 0.1 mol L^{-1} sucrose solution (Sigma-Aldrich company Ltd, Gillingham, Dorset, UK) at 4°C and subsequently transported to the laboratory on ice, where the two cylindrical biopsies were cut in half using a scalpel, leaving four separate tissue pieces. Three pieces from each patient were cryopreserved for the research study and where possible one piece was processed fresh to act as control. Given certain logistical constraints it was not always feasible to conduct an assay on fresh tissue.

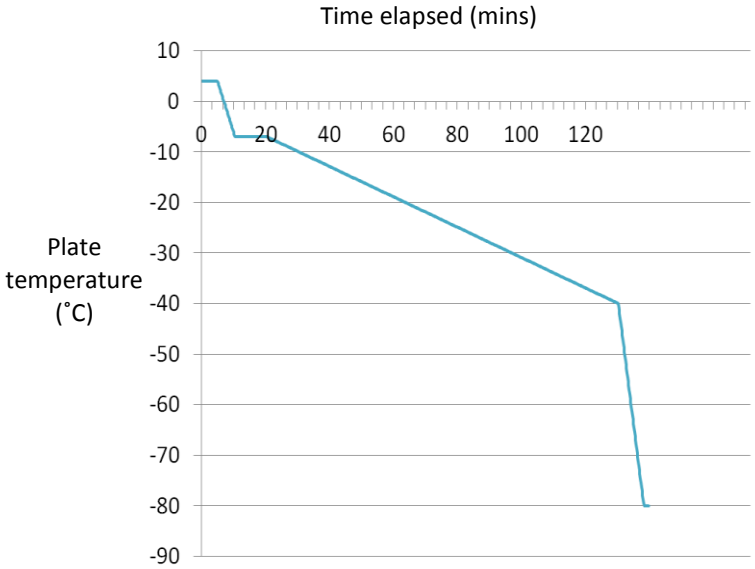
Ovarian tissue freezing

Cryopreservation and rewarming of all ovarian tissues was performed in the Cell, Tissue and Organ Cryopreservation Unit of Molecular Biology Laboratory, UCL. Each patient's sample was processed and cryopreserved when received.

Freezing solution consisted of Leibovitz L-15 medium supplemented with 1.5 mol L^{-1} DMSO (Sigma-Aldrich company Ltd, Gillingham, Dorset UK) as cryoprotectant (CPA), 10% (v/v) FBS and 0.1 mol L^{-1} sucrose, which was prepared and stored at 4°C. Each cryovial (Corning B.V. Life Science, Amsterdam, Netherlands) was preloaded with IceStart autonucleation beads (Asymptote, Cambridge, UK). One ml of the freezing solution was added to each cryovial and they were stored at 4°C. Each ovarian fragment was transferred into one cryovial and held at 4°C for 10 min for CPA permeation. The Asymptote EF600 controlled rate freezer (Asymptote, Cambridge, UK) was used to perform the freezing protocol. The freezing protocol was performed at the following cooling rates: from 4°C to -7°C at 2°C min^{-1}, maintained at -7°C for 10 min for seeding, then cooling to - 40°C at 0.3°C min^{-1} and to -80°C with the rate of -5°C min^{-1} (18). The cryovials, were later plunged into liquid nitrogen at -196°C, and stored (for a minimum of 14 d or longer) until they were rewarmed for assessment (18). This cooling profile can be seen in Figure 1.

Preparation of cortical slices and visualization using neutral red staining

Fresh control tissues or those previously cryopreserved were sectioned using a Leica VT1000S microtome with vibrating blade (Vibratome) to produce tissue slices that were approximately between 80 µm and 100



Temperature Range	Cooling rate
4 to -7˚C	2˚C/min
Hold for 10 mins for seeding	
-7 to -40˚C	0.3˚C/min
-40 to -80˚C	5˚C/min
Transfer to LN_2	

Figure 1. Cooling profile for cryopreservation of ovarian tissue. From Newton et al (18).

µm thick and 1 x 1 mm in size. Not all slices were exactly uniform. Each ovarian biopsy was secured to the cutting stage with cyanoacrylate glue, and the stage immersed in a solution bath containing HEPES-modified minimum essential medium (H-MEM) supplemented with 0.1% (w/v) bovine serum albumin fraction V (BSA) and 1% (v/v) penicillin G/streptomycin sulphate. The solution bath was surrounded by ice to maintain the sectioning solution at 0°C. Initial work was performed with pieces of animal liver and kidney tissue (purchased from retail outlets) as comparable tissue-consistency models for human ovarian tissue. Using this model, the settings of the vibratome were optimised for cutting tissue of this consistency at 0.24 mm s^{-1} cutting speed, at a blade vibration frequency of 60 to 70 Hz. These settings offered the best quality slices most rapidly.

After slicing the human ovarian tissue samples, slices were placed into sterile four-well culture plates, with each well containing 500 µL of serum-free culture medium with dissolved neutral red (NR). The culture medium consisted of alpha-minimum essential medium (α-MEM) supplemented with Glutamax, 0.1% (w/v) BSA, 1% (v/v) penicillin G/streptomycin sulphate and 0.1mol L^{-1} sucrose solution. The NR dye was diluted in the culture solution to a final concentration of 50 µg mL^{-1}. The four-well plates were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 h. After incubation, the tissue pieces were washed in fresh culture medium (without NR) three times and then observed using dark-field microscopy. The number of red structures in each tissue slice was then counted and each slice photographed.

Thawing protocols

Ovarian tissue fragments were kept frozen between 14 and 76 d prior to thawing using different protocols. One piece of tissue from each biopsy was thawed following the three protocols:

Protocol 1 (rapid-thawing) - has been previously reported by Newton et al (18). Three DMSO wash solutions were prepared in stepwise dilution, containing Lebovitz L-15 medium supplemented with DMSO in 1, 0.5 and 0 mol L^{-1}, 10% (v/v) FBS and 0.1 mol L^{-1} sucrose stored at 4°C. The vials of cryopreserved ovarian biopsies were rewarmed in air at room temperature for 120 s, followed by immersion in a water bath at 37°C for a further 120 s (total time 4 min) until visible ice had disappeared (average warming rate of about +50°C min^{-1}). Following thawing, the ovarian tissue was washed by agitating in the sequential dilutions of DMSO solution for 2 min in each solution, to remove any excess CPA. The tissues were then sectioned using the Vibratome as described above.

Protocol 2 (ultra-fast thawing) - has been previously described by Martinez-Madrid (15) when they cryopreserved whole ovaries. This is a very rapid-thawing protocol which consists of taking the cryovials out of liquid nitrogen and plunging immediately into a waterbath at 60°C until the solid phase has completely melted. This takes approximately 80 to 100 s (total time 1.5 min) with an average warming rate of about +120°C min^{-1}. The tissue was considered thawed when visible ice crystals had disappeared. Again, the stepwise dilution process was applied. After thawing, the tissue was washed in Leibovitz-L15 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.1 mol L^{-1} sucrose solution and decreasing concentrations of DMSO (1 M, 0.5 M, 0 M) to expel the cryoprotective agent from the tissue and culture medium. The tissue was then sectioned in the manner described above.

Protocol 3 (slow warming) - was attempted by reversing the slow cooling profile on the Asymptote EF600. The machine was reprogrammed to initiate passive warming at a rate of approximately +1°C min^{-1}. When the reserve cycle was complete, the tissues were considered to be thawed.

Statistical analysis

Data evaluation was carried out using

Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistic 21, IBM Corporation, Armonk, NY, USA). A *P* value < 0.05 was considered statistically significant. The comparisons between fresh and cryopreserved xenotransplanted tissue were carried out using a Wilcoxon's signed ranks test, a non- parametric test used to compare our matched samples.

RESULTS

Follicle counts and neutral red staining of cortical ovarian tissue slices

A total of 164 cryopreserved and fresh slices were analysed from the biopsies. There were no fresh comparison biopsies in five cases, which were due to logistical reasons with the assay. In these instances, all four pieces of the tissue biopsy were cryopreserved.

The mean number of NR follicles per slice was highest for the fresh tissue (7.6 per slice, n=6) as expected, but there was reasonable conservation of follicles in the cryopreserved group thawed using Protocol 1 with a mean follicle count of 4.0 (n=11). Protocol 2 resulted in larger follicle losses with a mean follicle count of 1.9 per slice following this thawing protocol (n=11).

Contrastingly, Protocol 3 showed an extremely low conservation rate with a mean attrition rate of 98.85%. The staining of these follicles is questionable as very few of the NR follicles out of a total of all follicles present expressed the red colour. As such the data for this protocol is not included in Table 1.

Table 1. Mean follicle conservation rates in tissue thawed using the two protocols.

Thawing protocol	Mean follicle conservation (%)	Mean follicle attrition (%)
1	27	73
2	13	87

Using Wilcoxon's signed ranks test the data show a significant decrease in mean follicle count between fresh tissue and cryopreserved tissue thawed using Protocol 1 and that thawed using Protocol 2 ($p<0.05$, n=6.) When the mean follicle counts in tissue thawed by the two protocols are compared, the difference is highly significant with Protocol 2 being the less successful ($p<0.01$, n=11).

Follicle conservation rates

The data in Table 1 shows that there is a greater mean follicle conservation rate in tissue thawed with Protocol 1 (27%) compared to that thawed with Protocol 2 (13%). These percentages were calculated by comparison with paired counts in the fresh tissues when these were available. The fresh biopsies were sliced at the time of collection, whilst the cryopresrevd biopsies were sliced after thawing. Of the cortical slices obtained, 97.1% of the fresh slices stained positive for neutral red in follicles. In some experiments fresh counts were not possible because of small size of the initial biopsies. Overall follicle conservation was found in 112 out of 164 tissue slices from cryopreserved tissue (68.3%). A visual example of NR positive follicles in a fresh tissue slice is shown in Figure 2.

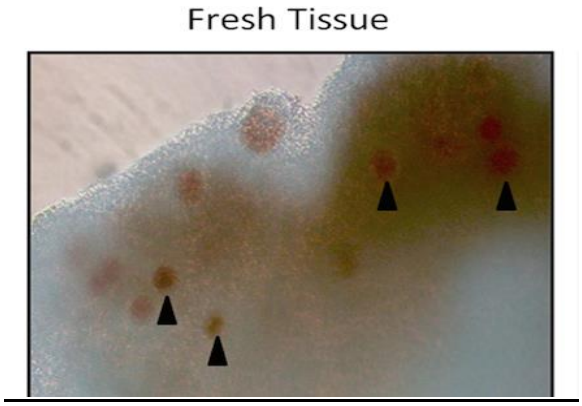


Figure 2: Follicles stained with NR in situ in fresh cortical ovarian slices.

DISCUSSION

The potential for human ovarian tissue cryopreservation as a method of fertility preservation in young women about to embark on treatment for malignancy, is highly significant in light of recent improvements in cancer survival rates (5). Cytotoxic treatments cause depletion to the ovarian follicle reserve rendering patients sterile or inducing early menopause (2, 24). For those women who may still wish to conceive, restoring their reproductive capabilities after treatment is often one of their greatest concerns.

Our experiments indicate that ovarian tissue cryopreservation and transplantation is a successful technique, with numbers of follicles identified in the majority of tissue slices prepared from the biopsies. The NR staining test has been applied to ovarian slices before (4, 6) and relies on uptake of stain and accumulation within lysozomes by viable follicles, with non-viable follicles showing absence of staining (4). Additionally, we have utilised an experimental protocol for the in-situ identification of viable follicles, which has been used only once previously, and have demonstrated follicle-staining rates comparable to those obtained by the original authors (4).

The presence of follicles in the tissue stained by the NR dye correlated to the predicted number of follicles found in the histological data, thereby validating NR as a legitimate method to detect viable follicles within slices of ovarian tissue (4).

The Stirling motor cryo-cooler used in the cryopreservation work (EF600) functioned well with the slow cooling profiles applied for ovarian tissues. There have been a number of studies using these profiles, with similar slow cooling rates (19). For human tissues, the majority of live births have resulted from slow cooling cryopreservation (21). Whilst vitrification as a technique for ovarian tissue cryo-banking has been more recently studied in some animal models, few human live births have been reported. The ability to use the electrically powered Stirling motor cryo-coolers may be more convenient in operating theatre suites where biopsies are being harvested, thereby possibly improving the logistics of cryopreservation.

The importance of warming profiles has not often been studied in human ovarian tissue cryopreservation and very little is published on the topic. The data provided here suggests that moderately fast warming rates (Protocol 1) resulted in higher maintenance of viable follicles compared to ultra-fast rates (Protocol 2), where warming rates were calculated according to the shortest time taken for all ice crystals to disappear.

Rapid warming in general has become accepted as beneficial to avoid prolonged exposure of the tissues to high salt and CPA concentrations as the ice matrix melts, whilst minimizing the potential for any ice nuclei to rearrange into intracellular ice. However, there is also the concern that differential thermal profiles caused across tissues during fast warming by the thermal – mechanical properties of the materials at very low temperatures may cause stress and cracking events (21), which can also be injurious. This may be one reason why the ultra-fast Protocol 2 was less successful.

In summary this study shows that thawing conditions significantly affect follicle conservation in cryopreserved human ovarian tissue and there is a great need for an optimized protocol to ensure conservation of follicles.

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