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Laboratory Aspects of Establishing a Fertility Preservation Service

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Introduction

For those new to fertility preservation (FP) or thinking of establishing a service, I'm writing this from a laboratory point of view with the aim of providing food for thought and, with the benefit of hindsight, highlighting pitfalls that may be encountered. Thanks to ISFP biennial meetings, ASFP and a number of highly experienced groups, there are numerous hands-on workshops available which can provide an introduction to handling and cryopreservation methodology for ovarian tissue. To build on the knowledge gained from these, practice with human ovarian tissue is obviously preferable but, in many situations only animal ovaries are available, and my suggestion would be to use fresh sheep ovaries.

Freezing method

There have now been a number of births obtained using each of the slow freezing methods adopted by the large groups performing FP [1-7] but there is no evidence to suggest that one method results in better outcomes than the others. However, to achieve consistent reproducible success with a method, whichever one you choose, it is important to follow the published methodology. A good theoretical knowledge of cryopreservation is needed before altering parameters that will affect the cryoprotectant permeability associated with a method [8]. Attention to cryoprotectant concentrations, temperature during dehydration and duration of dehydration together with freezing rate and seeding are all critical, as are similar considerations during thawing of tissue.

Vitrification of ovarian tissue has resulted in live births [9] but to achieve vitrification (no ice formation) tissue pieces must be very thin (0.5-0.8 mm across the whole cortex) and this is technically challenging to achieve. In addition, it will be challenging to transplant these thin pieces back to a patient.

Quality assurance of the method is important to establish before embarking on FP using patient tissue, and neutral red visualisation of live follicle numbers [10] before and after thawing is an easy and quick method for validating methodology.

Aspects related to consent

Provision of information regarding legal storage limits and obtaining consent related to a patient's wishes in the event of death can have implications for the laboratory. Our data suggests that approximately 10% of our patients are deceased and, without clear direction on what is to be done with their tissue, the laboratory may have to continue storage indefinitely in the absence of a legal framework. Also, with only < 10% of patients returning to have frozen tissue transplanted [11, 12], tissue may remain in storage for extended periods. It is also worthy noting that many young women who freeze tissue are in a transient phase of their lives, so periodic follow up and contact is essential in helping to update contact information and allowing discussion around continuing storage for future use, or potentially discarding or donating tissue to research. Resolution of issues relating to fate of tissue may also help to alleviate some of the pressure on storage tank capacity.

Although a FP service may have little advance warning of cases, clear referral pathways that include laboratory input will facilitate preparation of consumables and staff for cases and also ensure assignment of samples for subsequent testing (see later) during tissue preparation.

Regulatory and safety aspects related to laboratory procedures

Many of the clinics undertaking ovarian tissue cryopreservation are based in Europe and are required to adhere to the EU Tissues and Cells Directive EC/2004/23. Although not mandatory outside of the EU, the basic principles of the directive provide sensible recommendations for treatment of biological material destined for transplantation after storage; an accredited facility, traceability of identification, quality management, standard operating procedures, minimising microbial contamination, trained staff and monitoring of critical equipment. The implications of some of the above with specific regard to ovarian tissue warrant further discussion. Leibowitz L15 is the medium used to process ovarian tissue by many groups. Although the product is of cell culture quality, this only establishes sterility and that the included reagents are not cytotoxic. The product is not, however, approved as a medical device and, therefore, should not be used in a clinical procedure. Media and cryoprotectant

solutions used in ART procedures are often registered as medical devices and, for this reason, we use an ART medium (human tubal fluid medium buffered with HEPES) for the handling and preparation of ovarian tissue and an embryo freezing kit containing 1.5M PROH + 0.1M sucrose for tissue cryopreservation. The other cryoprotectant solutions used for ovarian tissue freezing are not available commercially and are made in house, where quality of reagents is paramount and a system of quality assurance should be established [13].

Included in the EU tissue directive is a requirement to minimise microbial contamination, and reduce staff exposure to tissue, indicating that all processing should be in a biological class II hazard cabinet. Although this is particularly important in the context of samples containing HIV and hepatitis, it is also relevant in cases of urgent ovarian tissue storage where this status is unlikely to be known and also in cases where the tissue has the potential to contain malignant cells. During the final storage, cross contamination should be eliminated by storage in vapour phase or in a secondary sealed system. It is important to note that all cryogenic vials stored under liquid nitrogen allow seepage of liquid nitrogen and therefore require a heat-sealed plastic sleeve covering.

Storage Facility

Births from ovarian tissue stored for ≥ 9 years and subsequently grafted in patients without ovaries [14, 15] have clearly established that, when stored at the appropriate temperature, tissue viability can be maintained for extended periods of time. It is also clear from this data and the nature of the patients storing ovarian tissue, that the duration of storage is often likely to exceed a decade and, in some cases, two decades before thawing for use. Therefore, it is paramount to ensure maintenance of storage temperature, either through automatic filling or manual measurement and filling, together with temperature monitoring and alert alarm systems. Recent catastrophic failures of storage tanks emphasise the importance of monitoring, alarm testing and the documentation of a disaster plan. The highest risk of failure appears to be associated with smaller tanks and will continue to occur, causing distress for many patients and enormous legal ramifications, unless a quick response backup system is routinely available. Our storage system is a vapour tank (liquid nitrogen in the bottom 14cm with samples suspended above this level) with dual independent alarms monitoring temperature and liquid nitrogen depth and patched into the hospital response system with a hierarchical responders list. If the tank has ruptured, our backup is to remove racks containing

boxes with vials and quickly place in a -80°C freezer in a room next door. We have performed a mock version of this catastrophic event and can empty the vapour tank containing over 1000 patient tissue samples in 20 minutes. It is also important to note that our tank is situated in a large open laboratory with an oxygen monitor, again patched into the hospital emergency response system. This is in contrast to many ART laboratories, where tanks are stored in a small locked room, and where a small tank full of liquid nitrogen rupturing would result in the room being unsafe to enter for a number of hours, by which time all samples would have been destroyed. We also have a handheld oxygen monitor which allows us to test if an area is safe to enter. In anticipation of a potential catastrophic failure, all ART laboratories should have a vacant tank, filled with liquid nitrogen ready for transferring patient material from a rupturing tank, available at all times.

Aspects related to testing of tissue

Although the risk of ovarian tissue being contaminated with cells from some malignancies may be low [16, 17], histological testing should be performed on all patient tissue at time of freezing. This routine evaluation has detected malignancy in cases thought to be of low risk [18-20]. Assigning some tissue at the time of freezing for potential future testing prior to undertaking transplantation is useful. A small amount of medulla, which is normally discarded, can be frozen for subsequent testing before transplantation to give another level of reassurance to the patient. Although there can be no absolute guarantee that the remainder of frozen tissue is free of malignant contamination, it is important to adopt this risk minimisation strategy. This medullar tissue, and a small amount of cortex frozen for testing, will also eliminate the need to thaw vials with large amounts of precious clinically usable cortex for testing, particularly as technology progresses and future developments in testing may be accessible. Although we have been snap freezing small fragments of medulla for SARS-CoV-2 testing, when an appropriate test is available, for all FP patients since April 2020, we are also potentially able to thaw the medulla from those frozen at the start of the pandemic before we were aware of the impact of the SARS-CoV-2 virus. Snap freezing small fragments of medulla from all leukemia patients for subsequent PCR testing also allows us to benefit from future developments in molecular testing.

Transport of tissue

In some countries a centralized processing and storage facility in which expertise, standard operating procedures and appropriate backup systems are available [1, 21, 22] has been established to facilitate many of the above requirements and this can also accrue benefits in procuring charity or government funding [23]. However, this model requires transport of freshly harvested tissue under appropriate conditions, often for significant periods of time, prior to cryopreservation.

Ovarian tissue is frequently transported for 4-5 hrs at 4°C [24] and has been transported in some cases for up to 28 hrs [22, 25]. The consequences of exposure to these conditions are largely unknown for human tissue although there have been pregnancies and births after orthotopic grafting of transported tissue for 4-5 hrs [24, 26] and 20 hrs [12, 27]. In the latter case healthy follicles were observed on the peritoneally grafted tissue at the caesarean delivery and sampling of this tissue revealed numerous healthy follicles at all stages [28]. Similarly, healthy follicles at all stages were observed in human cryopreserved ovarian tissue which had been transported for 4 hours at 4°C and subsequently grafted into immunodeficient mice (xenografted) [29]. Some of the technical issues related to cold transport are covered in more detail [8].

Conclusion

As stated initially, good FP laboratory training platforms are available including hands-on training workshops, which I believe are essential for those in the laboratory about to embark on establishing a FP service. Often however, due to time constraints at some workshops, many of the above logistic aspects are not covered despite their importance in being able to think through the entire process and plan accordingly. Hopefully, I have shown that this technology cannot be performed as an ad hoc service whereby we merely put tissue in an ART tank with embryos and hope for the best. Our patients and medical staff expect an appropriate laboratory with good documentation, quality control and sufficient knowledge of the critical aspects associated with the procedures involved to ensure a functionally competent service that delivers optimal outcomes.

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