Assessment of tissue injury in cryopreserved ovarian tissue

Ovarian tissue banking is a developing technique aimed to preserve fertility in women at risk for premature ovarian failure (1–3). It entails cryopreservation of ovarian tissue with the option of autografting. Reports on the autografting of cryopreserved ovarian cortex segments describe the resumption of steroid production (4), follicle development (5, 6), and (in sheep) model successful pregnancies (7, 8). However, tissue loss due to procedural steps is expected to have detrimental effects on the reproductive potential of frozen-thawed ovarian grafts. We conducted a study in a porcine model to investigate how freezing and thawing affects ovarian tissue and whether warm ischemia time before the cryopreservation process has any added detrimental effects.

Bilateral oophorectomy was performed in eight adult, nonpregnant sows at the Cleveland Clinic Foundation Biological Resources Unit in accordance with the clinic’s standard operating procedures. The animals were cared for according to the standards of the U.S. Public Health Policy of the Humane Care and Use of Laboratory Animals and the study was approved by the institutional review board. The harvested ovaries were divided into five segments and subjected to assigned time intervals of warm ischemia at 21°C (1, 5, 10, 20, and 30 minutes). At each interval one part was fixed for histologic study (freshly fixed group) and the other section was cryopreserved (cryopreserved group). For all samples a 1-mm thick cortex layer was obtained. To compare the effects of tissue size on the outcome, within the cryopreservation group one sample measuring 1–2 × 1 × 1 mm² and a section measuring 5 × 1 × 1 mm² were taken and compared. In the freshly fixed group, all samples measured 1–2 × 1 × 1 mm² and were used for reference.

Porcine ovarian cortex fragments measuring 1–2 × 1 × 1 mm² and 5 × 1 × 1 mm² were transferred into cryovials (Nalgene; New York, NY) containing 1 mL of Leibovitz L-15 medium (Sigma Chemical Co. Ltd.; St Louis, MO). 10% calf serum, and 1.5 M DMSO (dimethylsulfoxide) (Sigma) and kept on ice for 30 minutes. The cryovials were placed into cryocans and cryosleeves and kept in a freezer at −20°C for 30 minutes. Subsequently they were placed into a receptacle with the vials on top and inserted into a SM43 liquid nitrogen vapor tank for an additional 30 minutes. Then they were flipped by submerging the specimens into liquid nitrogen for 3-week storage at −196°C. The specimens were then removed and held at room temperature for 1 minute. The tissue was thawed in a water bath within 2 minutes. The thawed tissue was transferred to Petri dishes for three washes with Leibovitz L-15 medium and subsequently fixed in Bouin’s solution, paraffin-embedded, and stained with hematoxylin and eosin for histological evaluation.

The tissue was assessed by primordial follicle count per high magnification field, presence of primary and secondary follicles, and signs of autolysis. Repeated measures analysis of variance (ANOVA) were used to simultaneously examine the role of method of preservation, time, and left vs. right side with follicle number, while controlling for multiple use of each animal. From the ANOVA tests, examination of significance at each time point was performed with Student’s t-test. The sample size had 90% power to detect an overall difference of two or more follicles between the cryopreserved and fresh methods. P<.05 was considered statistically significant.

In the cryopreserved group, the follicles displayed some degree of dyshesion and disorganization; however, overall changes did not amount to substantial autolysis or cell injury. As compared to the freshly fixed group, in cryopreserved samples the number of primordial follicles per high magnification field was significantly reduced (4.9 ± 5.3 vs. 7.2 ± 5.4, P=.03). However, when the cryopreserved samples were stratified according to specimen size, there was no statistically significant difference between the follicle count in the larger tissue sections (5 × 1 × 1 mm³) and the freshly fixed group (9.3 ± 6.5 vs. 7.2 ± 5.4, P=.87, Fig. 1). The follicle count in the 1 × 1 × 1 mm³ cryopreserved group displayed significantly fewer follicles per high magnification field than the larger samples (2.1 ± 2.4 vs. 9.3 ± 6.5, P=.002). Changes in the percentage of primary and secondary follicles were not statistically significant. Warm ischemia time had no additional effect on any of the described changes in either group (see Fig. 1).

Ovarian tissue banking and autografting of ovarian cortex segments are promising treatment options for premature ovarian failure (9). Ovarian cortex tissue has to be grafted to allow the follicles to mature in vivo. Grafting procedures include autografts, xenografts, and allografts in heterotopic and orthotopic locations (10–14). To ensure long-term function of the grafts, sources of tissue injury during each procedural step must
be identified and minimized. Although the resumption of steroid production and follicle development have been described in frozen-thawed ovarian grafts (4, 6), tissue damage is significant and jeopardizes in particular long-term fecundity.

Frozen-thawed ovaries have given rise to pregnancies in several reports on multiple species documenting the potential of cryopreservation and autografting procedures; however, even in such successful cases the tissue loss is substantial. Human trials have also demonstrated significant tissue loss and poor oocyte quality with no reported pregnancy. We found that exposure to warm ischemia for up to 30 minutes was tolerable. The freezing and thawing methods applied in this study did not add any significant damage to the tissue, and our data indicated that preserving larger pieces (5 mm²) is preferable. Investigating follicle counts and function of fresh ovarian grafts compared to frozen-thawed heterotopic grafts in ewes, Aubard et al. (5) concluded that the key factor for follicle survival and resumption of ovulation was the postgrafting ischemia time.

Thus, the future of ovarian transplantation will depend on minimizing postgrafting ischemia time. Recent reports from studies on mice ovaries also confirmed that with shorter the ischemia times more tissue survives (15, 16). This can be most effectively achieved by transplanting ovarian segments with preserved vasculature and immediate revascularization by vascular anastomosis (17). The cryopreservation of larger ovarian segments requires an adapted protocol that is based on cryoperfusion rather than passive diffusion (18, 19). These techniques are developing and may offer more effective ways for long-term tissue preservation in the future.

In summary, although optimizing the procedural steps involved in cryopreservation and thawing of ovarian tissue will improve the tissue survival rate, procedures before grafting, including cryopreservation, account for relatively little tissue injury in ovarian grafts.

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