Optimal perfusion of an intact ovary as a prerequisite for successful ovarian cryopreservation

R. Gerritse1,3, C.C.M. Beerendonk2, M.S.L. Tijink2, A. Heetkamp2, J.A.M. Kremer2, D.D.M. Braat2 and J.R. Westphal2

1Streekziekenhuis Koningin Beatrix, Department of Obstetrics and Gynecology, Beatrixpark 1, 7101 BN Winterswijk, The Netherlands; 2Radboud University Nijmegen Medical Centre, Department of Obstetrics and Gynecology, Nijmegen, The Netherlands 3Correspondence address. Tel: +31-543-544444; Fax: +31-543-522395; E-mail: r.gerritse@skbwinterswijk.nl

BACKGROUND: Cryopreservation and subsequent reimplantation of intact ovaries from cancer patients, offers potentially the best prognosis for restoring fertility after sterilizing cancer treatment. We used bovine ovaries as a model system to explore the perfusion procedure that is required for cryopreservation of intact ovaries.

METHODS: The arteria ovarica was cannuled, and ovaries were flushed with Indian ink for 5 min. RESULTS: Successful perfusion of blood vessels was immediately visible macroscopically by a grey to black discoloration of the ovary and was confirmed microscopically, by examining tissue sections. There was no correlation between the time interval from removal of the ovary to the start of the perfusion, and success of perfusion. We determined the percentage of Indian ink-perfused vessels and scored blood vessels in four different size classes. The percentage of perfused vessels increased with an increase in vessel size. In a limited set of preliminary experiments with human ovaries, comparable results were obtained. CONCLUSIONS: Our results show that bovine ovaries are a suitable and adequate model system for optimizing the cryopreservation of human ovaries. As bovine are at least of comparable size to human ovaries, we expect that our results can be extrapolated to the human situation.

Keywords: fertility preservation; ovary; cryopreservation; vascular perfusion; animal model

Introduction

Over recent years, improvements in the therapy of cancer have lead to significant increases in long-term survival rates. However, aggressive chemotherapy and radiotherapy can severely deplete the follicular reserve in women, often compromising ovarian function (Wallace et al., 2005).

Removal, cryopreservation and subsequent reimplantation of ovarian tissue strips after cancer treatment have been successfully used to re-establish female fertility (Donnez et al., 2004, 2006; Meirov et al., 2005). When ovarian tissue is used for cryopreservation, numerous immature oocytes can be cryopreserved. Immature oocytes are small, relatively quiescent and lack a zona pellucida and cortical granules, which makes them, in theory, more tolerant to freezing and thawing than mature oocytes (Kim, 2006). A major problem in ovarian tissue transplantation, however, is follicular loss due to ischaemic reperfusion injury, as assessed in both humans (Oktay and Karlikaya, 2000; Nisolle et al., 2000) and sheep (Baird et al., 1999; Kim et al., 2004).

Cryopreservation of an intact ovary (with vascular pedicle) for fertility preservation in cancer patients has potentially the best prognosis for restoring fertility after sterilizing cancer treatment. However, successful freezing and thawing of intact ovaries represents an immense technical challenge. Freezing of intact ovaries has been investigated in ovine (Bedaiwy et al., 2003; Revel et al., 2004; Courbiere et al., 2006; Imhof et al., 2006) and porcine (Imhof et al., 2004) animal models. These studies yielded some promising results, with regard to the number of surviving follicles as well as follicular function. One spontaneous pregnancy resulting in a live birth has been reported in sheep (Imhof et al., 2006). Recently, perfusion and subsequent cryopreservation of human ovaries was described for the first time (Martinez-Madrid et al., 2004; Jadoul et al., 2007).

Our objective is to optimize the technique of cryopreservation of intact ovaries to make it an efficacious and safe procedure. As ovine ovaries are much smaller than human ovaries, and porcine ovaries, in contrast to human ovaries, have multiple follicles maturing each month, we chose bovine ovaries as our model system. Vessel perfusion is crucial for infusing the ovary with a cryoprotectant agent as well as for reperfusion of the ovary after thawing. In the present study, we tested vessel perfusion in bovine ovaries, and also in preliminary studies in human ovaries.
Materials and Methods

Determining ovarian volume

Bovine (n = 30), ovine (n = 31) and porcine (n = 28) ovaries from freshly slaughtered animals were obtained from the local abattoir and submerged in a vessel filled with water. The increase in volume of the content of the vessel was determined and was taken to represent the volume of the ovary. Data from 28 human ovaries were taken from Munn et al. (1986).

Perfusion of bovine ovaries

Ovaries (n = 66) were obtained from on average 6 years old, freshly slaughtered cows at a local abattoir. The phase of the ovary was determined by macroscopical observation of absence (follicular phase) or presence (luteal phase) of a fresh (yellow/orange coloured) corpus luteum, which was always clearly visible after dissection of the ovary after perfusion, and the extracorporeal presence (follicular phase) or absence (luteal phase) of cervical mucus. As we had no influence on the choice of the animals to be slaughtered, we decided to perfuse all ovaries we received, independent from the phase they were in. The arteria ovarica in the vascular pedicle was prepared; the vessel (inner diameter 0.8–1.2 mm) was transected 10 cm under the ovarian hilus. Next, a canule (18 GA 1.77, 1.3 × 35 mm Venflon) was inserted into the vessel and secured with sutures. The canule was connected with an Original perfusion Hiose (Braun, Melsungen, Germany) to a peristaltic STC-521 Seringe pump (Terufusion, Tokyo, Japan). Perfusion rate was set at 2.5 ml/min as described by Martinez-Madrid et al. (2004). Vessels branching of from the A. ovarica that became visible during perfusion with Indian ink were ligated during the procedure. After perfusion, ovaries were fixed in buffered formalin for subsequent histological examination.

The ovaries (n = 66) were perfused with a solution of 25% Indian ink/167 IU heparin in 0.9% NaCl for 5 min. The elapsed time between the death of the cow and start of the perfusion varied between 30 and 240 min, with 30 min being the shortest interval that was practically possible. A perfusion was considered to be successful if Indian ink was seen to perfuse the ovary macroscopically (black discoloration of tissue and follicles), the peristaltic pump was functional, and injection of Indian ink was visible in the venous plexus of the artery.

Immunohistochemical staining of blood vessels

As no antibodies against bovine endothelial cells were available, a panel of antibodies directed at different vascular components (human CD34, Factor VIII, laminin and collagen IV) was tested to determine the optimal staining for highlighting bovine blood vessels. After fixation, sections of ovaries were embedded in paraffin. Briefly, 4 μm sections were deparaffinized with xylol for 10 min, and endogenous peroxidase activity was blocked by incubation for 30 min in 10% H2O2 diluted in phosphate-buffered saline (PBS) solution. Next, sections were washed in PBS, incubated for 10 min with protease 14 (Sigma, St Louis, USA) at 37°C for 10 min to unmask the antigens and epitopes. After washing in PBS, sections were incubated at room temperature with 2% normal goat serum (anti-factor VIII and anti-laminin) or normal horse serum (anti-collagen IV and anti-CD34) (Vector, Burlingame, USA) diluted in PBS for 10 min. Subsequently, sections were incubated overnight at 4°C with polyclonal rabbit-anti-human von Willebrand factor VIII antibody (CLB, Amsterdam, the Netherlands) diluted 1:600 in PBS with 1% bovine serum albumin (BSA), polyclonal goat-anti-laminin antibody (Dako, Glostrup, Denmark, dilution 1:4000), horse-anti-mouse collagen IV antibody (Sigma-Aldrich, St Louis, USA), or monoclonal mouse-anti-human CD34 antibody (Labvision/Neomarkers, Fremont, USA, diluted 1:750). After washing in PBS for 15 min, sections were incubated for 30 min with either (depending on the primary antibody) anti-rabbit IgG, (diluted 1:200 in PBS/1% BSA), anti-goat (diluted 1:200), anti-horse (diluted 1:200) or anti-mouse IgG (1:200) all conjugated to orio genese, respectively (Vector). After washing in PBS for 15 min, sections were incubated at room temperature for 45 min with peroxidase-conjugated avidin–biotin complex (Vectastain ABC-kit, Vector, Burlingame, USA) diluted 1:50 in PBS/1% BSA. Finally, sections were incubated with AEC (SKY Tek) 1:50 diluted in AEC Buffer (SKY Tek, Logan, USA), counterstained for 1 min with hematoxylin, dehydrated with Imsol (Klinipath, Duiven, the Netherlands) and mounted with permount (Fisher, NJ, USA). Tissue sections were analysed by conventional light microscopy.

Determination of percentage of perfused vessels

Numbers of perfused and non-perfused (as determined by the presence and absence of Indian ink in the vessel lumina, respectively) blood vessels were counted in 11 ovaries, which were randomly selected from the 34 successful perfusions we performed, making sure to include different time intervals between death of the animal and start of the perfusion in the series that was histologically analysed. We counted the vessels in five fields per section of stromal tissue, and five fields of perifollicular tissue, at a magnification of ×100 (arterioles and venules, medium sized and large vessels, see Fig. 2), or ×400 (capillaries). Mean perfused vessel number per high-powered field was calculated for the vessel classes mentioned above, and mean vessel numbers were statistically analysed using Mann–Whitney U-test.

Perfusion and staining of human ovaries

Human ovaries were obtained from a 42-year-old woman and from a 38-year-old woman, who had bilateral ovariectomies as a consequence of the presence of a hormone susceptible breast tumour, and of being a carrier of a BRCA-1 gene mutation, respectively. Approval for the procedure was obtained from the local ethical committee, provided the procedure would not interfere with the necessary diagnostic histology. The vascular pedicle was prepared and an 18 GA 1.77, 1.3 × 35 mm canule (Venflon) was inserted in the arteria ovarica. The ovaries were perfused for 5 min with an Indian ink/heparin solution as described for the bovine ovaries. After perfusion, ovaries were fixed, and an anti-CD34 immunohistochemical staining to highlight blood vessels was performed as described for bovine ovaries.

Results

Choice of bovine ovaries as a model system

We decided that the species providing the ovaries for our model system had to encompass the following properties: monthly rather than yearly ovulation and a maximum of two maturing follicles each month. Sheep ovulate only once or twice a year, whereas in porcine ovaries, multiple follicles mature each month. In addition, we decided that the ovaries of the test species should be at least as large, or larger, as human ovaries. Ovarian volumes from different species were determined or derived from literature (Fig. 1). With a volume of 6.5 ± 2.9 cm3 (mean ± SD) (Munn et al., 1986) human ovaries are comparable in volume to porcine (7.3 ± 2.2 cm3) ovaries, whereas bovine and ovine ovaries are considerably larger (14.3 ± 5.7 cm3), and smaller (1.0 ± 0.4 cm3), respectively. Taking together these considerations, we decided to select bovine ovaries as our model system for subsequent experiments.
Determination of optimal conditions for macroscopically successful perfusion

In clinical practice, perfusion of the ovary to be cryopreserved will not always be feasible within a short period after removal. Therefore, we varied the time between death of the cow, i.e. stopping of ovarian circulation, and start of the perfusion procedure with the Indian ink/heparin solution. We considered perfusion to be successful when we observed complete blackening of the tissue after perfusing the ovary for 5 min (Fig. 2). Already 3 min after start of the perfusion, the afferent arterioles started to blacken, clearly demonstrating that a vascular plexus rather than a limited number of arteries is responsible for the ovarian blood supply. A slight darkening of the ovarian tissue was observed as well (Fig. 2b). Five minutes after start of the perfusion, the blackening of both the vascular plexus and the ovarian tissue was more distinct (Fig. 2c). After dissection, the grey to black appearance of the ovarian tissue indicated complete perfusion of the ovary (Fig. 2d).

Table I shows the results of the perfusion procedure at different time points after stopping the ovarian circulation. Perfusion was successful most of the time (80%) when the perfusion was started soon after stopping the ovarian circulation (up to 60 min). To our surprise, however, perfusion was still feasible 2 and even 4 h after removal of the ovary. Crucial for successful perfusion after more than 60 min, however, was insertion of the canule in the afferent vessel (arteria ovarica), as soon as possible after obtaining the ovary. When data obtained at different time points were pooled, the

**Figure 1:** Ovarian volumes of different species
Volumes of bovine, porcine and ovine ovaries were determined experimentally. Each solid circle represents an individual measurement, and mean volumes ± SD are indicated. In the right hand column, volumes of human ovaries as derived from literature (Munn et al. 1986) are presented, depicted as mean ± SD. Open squares indicate the range of the measurements, representing the minimum and maximum volume

**Figure 2:** Perfusion of bovine ovary with an Indian ink/heparin solution
(a) At start of perfusion; (b) 3 min after start of perfusion; (c) 5 min after start of perfusion; (d) after perfusion: ovary after transversal dissection. Note extensive blackening of the tissue after perfusion. F, follicle
successful perfusion rate was significantly higher with ovaries in the follicular phase (27 out 34) than with ovaries in the luteal phase (7 out of 32, \( P < 0.01 \)).

Optimal perfusion of the ovary with cryoprotectant may require a perfusion period of more than 5 min. Therefore, we examined the effect of increasing the perfusion time with Indian ink/heparin to 15 or 30 min. After 15 min, we observed an increased pressure in the tissue, as indicated by shutdown of the peristaltic pump and swelling of the tissue. Vascular leakage and tissue damage was indicated by release of Indian ink throughout the tissue. Our results indicated that no additional perfusion occurred when the perfusion period exceeded 5 min.

### Immunohistochemical determination of vessel perfusion in bovine ovary

To further analyse the results of the perfusion experiments with Indian ink, we performed immunohistochemical analysis of tissue sections. Briefly, endothelial cells in tissue sections of perfused ovaries were stained with anti-endothelial cell antibodies to highlight all blood vessels. Next, we scored the total number of stained blood vessels, and determined the percentage of blood vessels containing Indian ink, for different size classes of vessels.

We tested a panel of antibodies directed against different vascular components. Only the anti-factor VIII antibody resulted in satisfactory staining of blood vessels and was therefore used for further analysis (data not shown). Fig. 3 shows a tissue section of perfused ovary, stained with anti-Factor VIII. Our data show that the Indian ink was contained within the blood vessels and was not observed in the surrounding tissue.

We scored blood vessels in four different size classes, ranging from capillaries to very large afferent and efferent vessels, both in the cortex (perifollicular vessels) and in the medulla (stromal vessels). The percentage of perfused vessels increased, with an increase in vessel size (Fig. 4). As indicated in Fig. 4, this increase was statistically significant in a number of instances. Whereas in the medulla (Fig. 4a) only 16% of the capillaries was perfused, this percentage increased to 37, 65 and 62% in arterioles and venules, medium sized vessels and large vessels, respectively. In the cortex (Fig. 4b), percentages of perfused vessels were 15, 32, 63 and 62%, respectively. There was no statistical difference in the percentage of perfused vessels in the ovarian medulla compared with vessels in the cortex (data not shown).

### Perfusion of human ovaries with Indian ink

We perfused two human ovaries with Indian ink to investigate whether we could extrapolate our bovine data to the human situation.

Although cannulation of the afferent vessel was more difficult than with bovine ovaria, we were able to successfully perfuse one of the ovaries of each woman (the other ovary was used for diagnostic purposes). Tissue sections of the successfully perfused ovary were stained with anti-CD34 antibody to highlight blood vessels (Fig. 5). Analogous to our bovine data, we observed the presence of Indian ink mainly in the larger vessels, whereas capillaries hardly ever contained ink. Again, tissue damage as a result of the perfusion process was not observed, as indicated by the absence of Indian ink in the stromal tissue.

### Discussion

To date, IVF procedures before cancer therapy to produce embryos that can be transferred in a later stage of life can be employed to preserve fertility in women with cancer who are subjected to a fertility threatening therapeutic intervention. Recently, cryopreservation followed by transplantation of cortical ovarian strips has been employed to establish pregnancies in cured cancer patients. Both options are limited, since the number of embryos that can be produced, as well as the life expectancy of reimplanted cortical strips, is restricted. Obviously, the fact that generally a number of ovarian strips are cryopreserved implies that several attempts at fertility restoration can be undertaken. In addition, a stable relationship
between the patient and her partner must have been established for IVF to be an option. Cryopreservation and subsequent reimplantation of total ovaries may therefore in selected cases be an alternative option for fertility preservation, as all options are re-established by this procedure. If successful, revascularization of the retransplanted ovary not only results in prolonged survival of the graft, but, as result of decreased ischaemia in the ovarian tissue, leads to an increase in the number of surviving follicles. Of course, as with transplantation of cortical strips, extreme caution should be taken to prevent reintroduction of the tumour.

Transplantation of total ovaries that had been cryopreserved leading to a pregnancy and live birth in sheep has been reported by Imhof et al. (2006). This finding indicates that an analogous therapy in humans is, at least theoretically, feasible, although one should bear in mind that ovine ovaries are much smaller than human ovaries (this paper), and will therefore be much easier to successfully freeze and thaw. The same authors showed that perfusion of porcine ovaries with DMSO as a cryoprotective agent, resulted in improved protection from damage induced by the cryopreservation process, compared with only submerging the ovary in the cryoprotective solution (Imhof et al., 2004). Extrapolating on this finding, we expect that perfusion of bovine and human ovaries with cryoprotective agent will be more effective in supplying protection, as already shown by Martinez-Madrid et al. (2004). In addition, perfusing the ovary via its afferent vessel may increase the survival and condition of the ovarian blood vessels which are crucial for successful reperfusion after transplantation (Martinez-Madrid et al., 2007).

In our Indian ink perfusion studies, we observed that especially the larger vessels were well perfused, whereas the smaller vessels and the capillaries, via which extravasation of the cryoprotective fluids will occur most prominently, were less well perfused. On the other hand, the relatively smaller molecular weights of cryoprotective agents such as DMSO and sucrose imply that they would be less subject to molecular filtering mechanisms by endothelial cells and basal membranes. We therefore assume that these will also enter the tissue from larger vessels, although we have no experimental data to support this assumption. In addition, even if 16% of capillaries and 37% of arterioles are perfused, this may still result in sufficient dispersion of the cryoprotective agent. We are currently performing perfusion studies with fluorescently labelled molecules to assess the tissue saturation with the cryoprotective perfusion fluid. As an alternative, one may consider injecting the ovarian tissue with cryoprotective agents in order to ensure a more complete saturation of the tissue. However, as this procedure may result in tissue damage, this is, in our opinion, not the option of choice.

Martinez-Madrid et al. (2007) have described the follicular survival in cryopreserved and subsequently thawed complete
human ovaries that were perfused with, and frozen in, a solution containing DMSO as a cryoprotective agent. Remarkably, 75% of follicles survived the freeze/thaw cycle, indicating that the crucial structures in the ovary are more robust than one would expect. Conceivably, more follicles are lost due to ischaemic conditions after retransplantation of ovarian tissue than by the process of cryopreservation. Proof of concept, however, will be the assessment of follicular survival after the transplantation and the in vivo reperfusion and revascularization of the ovary. In addition, the long-term toxic effects of remnants of the cryoprotective agent in which the ovary has been frozen, which may remain in the tissue even after flushing with saline, will have to be evaluated.

To date, no data have been published on the optimal freezing and thawing procedure for intact human ovaries. In this study, we have performed experiments that may be indicative for properly performing the initial stage (perfusion) of the procedure for freezing intact ovaries, using animal ovaries as a model system. For a suitable model system, (i) the ovaries should be at least comparable in size with human ovaries; (ii) only one or two follicles should mature each cycle as the maturation of multiple follicles greatly influences the ovarian volume and therefore the outcome of the freezing process and (iii) the animal of choice should have a monthly cycle. We considered bovine, porcine and ovine ovaries as possible candidates for our model system. Volume measurements indicated that whereas porcine ovaries are comparable in size to human ovaries, bovine ovaries are considerably larger and ovine ovaries are much smaller. However, in pigs multiple follicles mature each cycle, possibly resulting in a different architecture and/or vasculature of the ovaries compared with ovaries in which only one or two follicles mature each month. Therefore, we selected bovine ovaries as our model system. The larger size of the bovine ovaries may actually be an advantage in extrapolating our results to the human situation. When we succeed in successful cryopreservation of large bovine ovaries, the chances are that attempts at cryopreservation of the relatively smaller human ovaries will be successful as well.

We assessed several parameters that determine the efficacy of the perfusion process. Applying pressure on the vasculature of the ovarian tissue by the perfusion pump may result in vessel rupture and tissue damage. Our histological data have shown that after 5 min of perfusion, the Indian ink was completely contained within the blood vessels, and no tissue damage was visible.

Obviously, the time between removal of the ovary, and start of the perfusion process and the cryopreservation should be minimized as much as possible, to prevent ischaemic damage to the ovary. We have shown that successful perfusion is possible as long as 4 h after removal of the ovary. After 4 h, perfusion was no longer possible, probably due to intravascular coagulation. In addition, we have shown that a perfusion time of 5 min suffices to perfuse the majority of the larger vessels. It should be noted, however, that we could not exclude the possibility that we underestimated the total number of blood vessels. In human tissues, it is well established that the anti-factor VIII antibody does not stain all blood vessels, and the same phenomenon may be occurring in our bovine ovaries. As a consequence, the percentage of perfused vessels we observed may in reality be lower. Our preliminary data indicate that perfusion via this protocol results in a satisfactory perfusion of human ovaries as well.

We observed a statistically significant ($P < 0.01$) higher success rate of the perfusion process in ovaries in the follicular phase compared with ovaries in the luteal phase. This may be explained by the fact that, at least in humans, ovaries in the follicular phase are better vascularized than ovaries in the luteal phase (Jokubkienė et al., 2006).

Usually perfusion prior to cryopreservation of tissue is performed at temperatures between 0 and 4°C, whereas we performed our experiments at about 12–15°C. In addition, the perfusion characteristics from DMSO, sucrose or other cryoprotective agents may differ from that of the Indian ink solution we employed. In future experiments, we intend to address these matters.

In conclusion, our study has provided the first experimental data in determining the first stage of the optimal procedure to cryopreserve intact human ovaries. In a next set of experiments, we intend to investigate the diffusion time of small molecular weight molecules such as cryoprotectants into the tissue after perfusion and to determine whether a longer perfusion time may increase the saturation of the tissue with the cryoprotectant. In addition, we intend to study the effects of different types of cryoprotective agents on the functional survival of the ovarian tissue.

Acknowledgements
The authors wish to thank Cathy Maass, Jozé Verbeet and Emiel Lindeman for their expert technical assistance.

Funding
The De Leeuw-Huiden Foundation and Mr. A. Joldersma (Bc.S.) are greatly acknowledged for financially supporting this study.

References


Submitted on June 4, 2007; resubmitted on September 27, 2007; accepted on October 18, 2007