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ARTICLE

Glucose/lactate metabolism of cryopreserved intact bovine ovaries as a novel quantitative marker to assess tissue cryodamage

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Abstract For some patients, the autotransplantation of a cryopreserved–thawed intact ovary might be the best option to preserve their reproductive potential after fertility-threatening treatment. The best procedure to successfully cryopreserve a human ovary without inflicting a devastating level of cryodamage is to date unknown. To optimize this procedure, this study developed an assay to monitor the extent of cryodamage inflicted on bovine ovarian tissue by different cryopreservation protocols. The assay measures glucose and lactate metabolism of ovarian tissue fragments *in vitro* and determines the extent of cryodamage in cryopreserved ovaries. This study tested the cryoprotective effect of two different routes of administration of the cryoprotectant dimethylsulphoxide (DMSO). The cryoprotective effect was assessed in different tissue layers of the ovary, namely the cortex, the subcortex and the medulla. Submersion of intact ovaries in DMSO prior to freezing–thawing resulted in the complete protection of the glucose/lactate metabolism of the cortex, but not of the inner ovarian mass. Perfusion without simultaneous submersion, resulted in partial protection of cortex, subcortex and medulla, while the combination of submersion and perfusion conveyed the highest level of protection for all three ovarian tissue layers.

KEYWORDS: cell culture, cryopreservation, glucose/lactate metabolism, ovarian transplants, whole ovary, cryodamage

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Introduction

With prospects of survival improving, fertility preservation is becoming increasingly relevant for cancer patients who undergo fertility-threatening treatments, such as chemo- or radiotherapy. Several options to preserve fertility can nowadays be offered (Demeestere et al., 2009; Diedrich et al., 2011; Donnez et al., 2006; Sonmezer and Oktay, 2004; Wallace et al., 2005), including the cryopreservation and autotransplantation of ovarian cortex strips. This approach has already resulted in the birth of a number of healthy offspring (Demeestere et al., 2007; Donnez et al., 2004; Meirow et al., 2005; Silber et al., 2008b and others). Cryopreservation of cortical strips has already become a clinically relevant treatment modality. For a selected group of patients, the cryopreservation of an intact ovary may be an as yet experimental but promising option (Bromer and Patrizio, 2009; Diedrich et al., 2011; Kim, 2010). Transplantation of a fresh intact human ovary is, in fact, technically possible: Silber et al. (2008a) have performed this procedure between identical twin sisters, resulting in healthy offspring.

While the optimal procedure for the cryopreservation of cortical strips has been elucidated (Gook et al., 2000; Kagawa et al., 2009; Newton et al., 1998), cryopreservation of an intact organ is technically more challenging. The structural components of an ovary, its vascular system, the oocytes and the primordial follicles within the ovary, are all crucial for maintaining a functional organ after cryopreservation and thawing. All these different ovarian components may require different cryoprotective protocols, considering the different cell types that constitute them.

To address this problem, this study elaborated on previous work presenting the cryopreservation of intact ovine ovaries, using dimethylsulphoxide (DMSO) as a cryoprotectant (Arav et al., 2010; Bedaiwy et al., 2003; Imhof et al., 2006; Revel et al., 2004). In addition, both Bedaiwy et al. (2006) and Martinez-Madrid et al. (2004, 2007), have made a first attempt at cryopreserving human ovaries, by perfusing them with a 10% DMSO solution. In these studies, cryodamage in the ovarian tissue was assessed by viability tests, electron microscopy and analysis of cells undergoing apoptosis.

This study developed a new assay to measure the degree of cryodamage that the ovarian tissue has sustained that is based on glucose and lactate metabolism. For obvious ethical and practical reasons, human ovaries are not suitable and/or available for the elaborate testing of a large number of different cryoprotective regimes. Therefore, a previously described bovine model system was used (Gerritse et al., 2008, 2010). Bovine ovaries are similar to human ovaries with respect to their size, cycle and the number of developing follicles per cycle. In the current study, the glucose/lactate assay was used in this bovine model system to evaluate the sensitivity to cryodamage of the stromal cell compartment of the ovary. Until now, hardly any attention has been given to this cell type. Stromal cells constitute at least 90% of the ovarian tissue mass and are present in all tissue layers of the ovary. They are therefore imperative for maintaining organ integrity and functionality after freezing–thawing. An additional rationale to focus on stromal cell survival is the observation that stromal cells are vital for optimal follicular development (McLaughlin and McIver, 2009). Finally, the metabolically active stromal cells have been described to be more sensitive to cryodamage than the quiescent primordial oocytes (Kim et al., 2004), emphasizing the need for a cryopreservation protocol that not only efficiently preserves the follicles/oocytes, but the stromal cell compartment as well.

This study investigated the route of administration of the cryoprotectant, namely either submersion of the organ in a DMSO-containing solution or perfusion with DMSO via the vascular pedicle.

Materials and methods

Ovary collection

Intact bovine ovaries were collected at a local abattoir essentially as described previously (Gerritse et al., 2008, 2010). Briefly, ovaries ($n = 6$) used for culturing of fresh cortical, subcortical and medullar biopsies were collected on ice and transferred to the laboratory for further preparation. Ovaries ($n = 30$) used for cryopreservation experiments were perfused on site, with 15 ml Ringers' solution (Baxter, Utrecht, The Netherlands) containing 50 IE/ml heparin (Leopharm, Breda, The Netherlands) and 2.5% methylene blue (Clinical Pharmacy, Nijmegen, The Netherlands) via the vena ovarica. Time between death of the animal and start of the perfusion was kept to a minimum (20–40 min). The efficiency of the perfusion was monitored by the appearance of blue colouration at the ovarian surface. After the perfusion, the ovaries were transported on ice to the laboratory.

Cryopreservation and thawing of intact ovaries

Intact ovaries were cryopreserved basically as described by Martinez-Madrid et al. (2004, 2007) with some modifications. For submersion experiments, the ovaries ($n = 13$) were placed in a bath containing 30 ml Dulbecco's modified Eagle medium (DMEM; PAA laboratories, Pasching, Austria) and 2% fetal calf serum (FCS; Gibco, Breda, The Netherlands) in the presence or absence of 10% DMSO (Sigma–Aldrich, Zwijndrecht, The Netherlands) for 15–180 min. For perfusion experiments ($n = 17$), a blunt-ended needle (23g Olive Tipped Cannula Curved; Aspen Medical, USA) was inserted in to the arteria ovarica and secured using a small clamp (Gerritse et al., 2008). All vessels other than the vena ovarica and the arteria ovarica, were occluded using clamps. Perfusion was performed for 5 or 30 min with a solution of DMEM supplemented with 2% FCS, 2.5% methylene blue and 10% DMSO for 5 min to 120 min using a peristaltic STC-521 syringe pump (Terufusion, Tokyo, Japan) at a flow rate of 2.5 ml/min. During perfusion backflow of the (blue) perfusion fluid from the vein was observed. No leakage of perfusion fluid from the clamped vessels was observed, illustrating the efficiency of the procedure. After the perfusion, the ovaries, with the blunt-ended needle still inserted, were transferred to a sterile 100-ml semen collection container (Deltalab, Barcelona, Spain) containing DMEM and 2% FCS with or without 10% DMSO, which was subsequently placed

into a 5100 Cryo Freezing Container (Nalgene, VWR, Belgium; precooled to 4°C) and left on ice for 15 min. Subsequently the container was transferred to a −80°C freezer overnight to allow for a gradual decrease in temperature, followed by submersion in liquid nitrogen for at least 1 week.

Ovaries were thawed at 60°C for 10 min followed by an incubation at 37°C for 20 min. After thawing the ovary was taken from the container, submerged in 30 ml DMEM and 2% FCS, and reconnected to the peristaltic pump. The cryoprotectant was gradually removed by three additional perfusion steps of 10 min each at room temperature with a flow rate of 2.5 ml/min using DMEM and 2% FCS with 0.25 mol/l sucrose, DMEM and 2% FCS with 0.1 mol/l sucrose, DMEM and 2% FCS without sucrose. After perfusion, ovarian biopsies were prepared and cultured as described below.

Preparation and culturing of ovarian biopsies

From either fresh or cryopreserved—thawed ovaries, end-to-end tissue rods were prepared. In these tissue rods, the boundary between the cortex and medullar tissue could be observed macroscopically. First, the two cortex fragments on either end of the tissue rods were removed. The remaining tissue was subsequently divided into four pieces of equal length of approximately 2—3 mm. The two outer pieces and the two inner pieces were designated subcortex and medulla, respectively (**Figure 1**). Biopsies were transferred to six-well plates (Falcon, Heidelberg, Germany) for culturing. For each ovary, duplicate wells were prepared. Each well contained either three cortical, three subcortical or three medullar biopsies in 5 ml DMEM culture medium supplemented with 10% FCS and 40 μg/ml gentamycin. At day 4 of culture, spent medium was collected and 5 ml fresh medium was added to the wells, followed by an additional culture period of 3 days. Glucose and lactate content of culture supernatants were determined using a standard blood-gas analyser (Chiron Diagnostics, Bayer, Germany). At the end of the culture period, biopsies were weighed and glucose and lactate production and consumption were normalized (and expressed) per milligram of tissue per day.

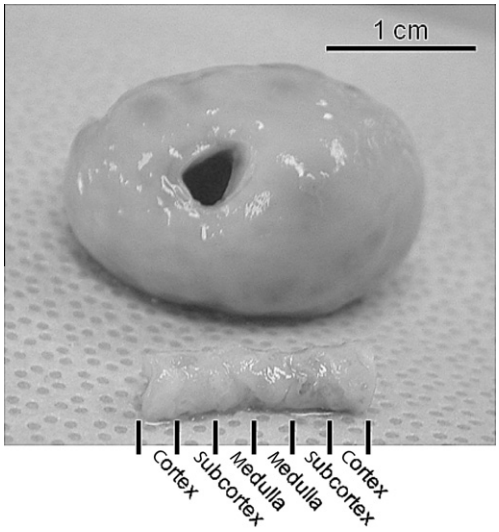

Figure 1 Preparation of ovarian fragments from different tissue layers. Tissue rods (shown at the bottom) were obtained by taking end-to-end punch biopsies from the ovary, with a diameter of 6 mm. Next, tissue rods were divided into two cortical, two subcortical and two medullar fragments of comparable length (2—3 mm).

Monitoring the temperature during cryopreservation

The cryokinetics within an intact ovary in the absence of any cryoprotective measures and in the surrounding fluids during the freezing process were monitored every 3.5 s using a set of microthermometers (Labfacility Temperature Technology, West Sussex, UK) with an accuracy of 0.1°C. Temperature was monitored in the ovarian cortex and medulla, in the container containing the ovary and the DMSO solution, in the passive cooling device and in the freezer. The microthermometers measuring the temperature in the ovary were inserted in the core and just under the surface of the organ, to measure medullar and cortical temperature, respectively (**Figure 2**).

Histochemical staining

Sections of ovaries were fixed overnight in phosphate-buffered 4% formaldehyde and embedded in paraffin. Sections (5 μm) were stained with haematoxylin and eosin. Tissue sections were analysed by conventional light microscopy (×100) and photographed.

Statistical analysis

Data were tested for normality using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA; www.graphpad.com) and found to be normal. Two-sided Student's *t*-test was performed using the same software package.

Results

Cryoprotective effect of submerging intact ovaries in DMSO on the glucose/lactate metabolism of different tissue layers

To investigate the cryoprotective effect of DMSO, intact ovaries were submerged in a 10% DMSO solution, frozen, stored in liquid nitrogen and than thawed. Subsequently, tissue biopsies were taken from the cortex, the subcortex and the medulla. Biopsies were cultured for 1 week. Glucose and lactate concentrations were determined in the spent culture media after 4 days of culture (days 1—4) and again after another 3 days of culture (days 5—7) (**Figure 3**).

The glucose/lactate metabolism of fresh (i.e. non-frozen, non-thawed) tissue (**Figure 3**, condition A) was used as reference value. Glucose consumption in the cortex (**Figure 3**, left panels) increased significantly ($P < 0.0001$) during the second culture period (days 5—7), whereas lactate production did not change during the entire culture period. As shown in **Figure 3** (middle and right panels), the same

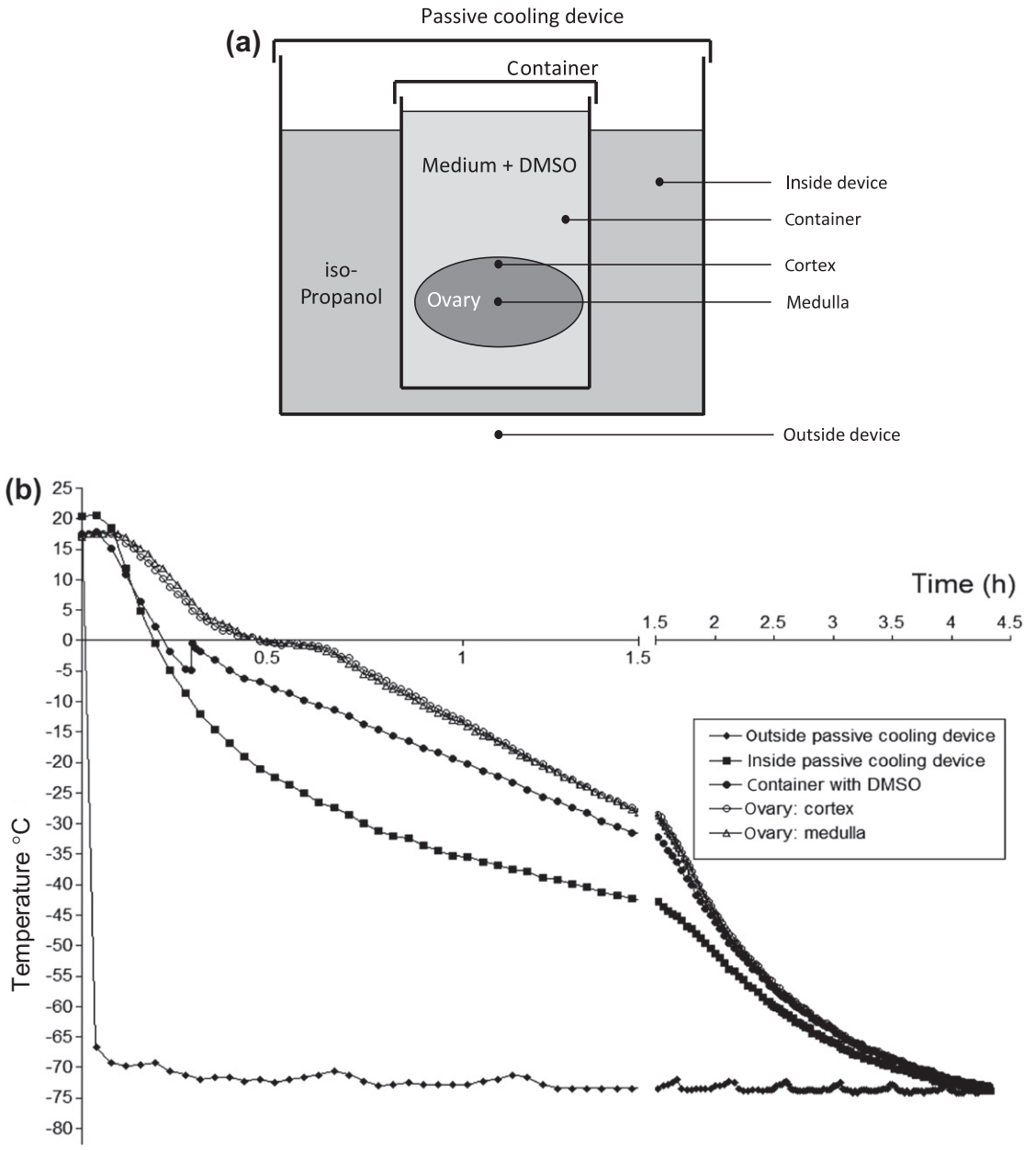

Figure 2 (**a**) Schematic representation of the different temperature monitoring points of an ovary frozen in a passive cooling device. (**b**) Temperature kinetics at the monitoring points indicated in (**a**), shown at 4-min intervals. DMSO = dimethylsulphoxide.

holds true for the fresh subcortex and the medullar tissue ($P = 0.001$ and 0.0003, respectively).

When freezing intact ovaries without any cryoprotective measures (**Figure 3**, condition B), the glucose/lactate metabolism of the cultured tissue fragments decreased, compared with fresh tissue, by more than 80% during days 1—4 and by more than 90% during days 5—7.

To assess the cryoprotective effects of DMSO on ovarian tissue, intact ovaries were submerged in a 10% DMSO solution for different lengths of time (ranging from 15 to 180 min) prior to freezing—thawing and subsequent tissue culture. Submerging the ovary in DMSO for 15 min (**Figure 3**, condition C), resulted for the cortex in a complete protection of glucose/lactate metabolism. Glucose consumption in days 5—7 showed a significant albeit small ($P < 0.05$) decrease, compared with fresh tissue. Levels of glucose/lactate metabolism of the subcortex and the medulla, on the other hand, were not protected at all by the 15-min DMSO submersion. In these tissue layers, glucose consumption as well as lactate production were at levels comparable to those of unprotected ovaries. Increasing the submersion time of the ovaries in DMSO prior to freezing from 15 min to 30, 60, 120 (data not shown) or even 180 min (**Figure 3**, condition D) did not increase protection against cryodamage of the subcortex and the medulla.

The speed of temperature change in the different tissue layers during the freezing procedure was investigated, as variations in this may have contributed to the lack of protection from cryodamage by DMSO in the medulla compared with the cortex. Temperature was monitored at five

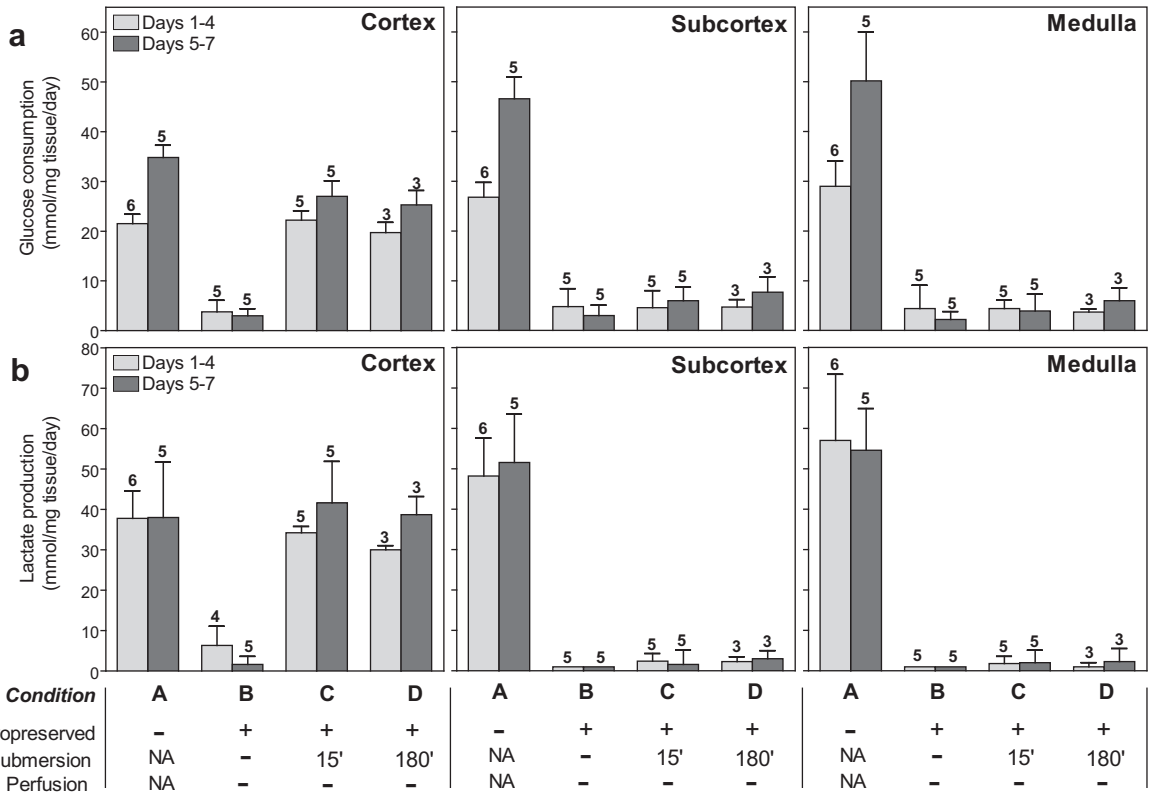

Figure 3 Glucose uptake (**a**) and lactate production (**b**) of cultured ovarian fragments derived from cortex, subcortex and medulla. Glucose/lactate metabolism was determined at two different time points of culture; days 1—4 and days 5—7. Condition A: fresh (non-cryopreserved) tissue; condition B: tissue frozen—thawed without cryoprotective measures; condition C: tissue frozen—thawed after submersion in DMSO for 15 min; condition D: tissue frozen—thawed after submersion in DMSO for 180 min. Values are mean and SD. Numbers above bars represent the number of ovaries analysed per condition. + = treatment applied; — = treatment not applied; NA = not applicable.

different locations (**Figure 2a**) during the freezing process. As expected, temperature outside the cooling device dropped quickly, reaching −70°C after about 5 min (**Figure 2b**). The temperature in the cooling device and in the container declined more slowly. Temperature in the two locations within the ovary dropped at an even slower pace. No significant difference in temperature was observed between the ovarian cortex and the medulla.

Effect of perfusing intact ovaries with DMSO on glucose/lactate metabolism

Intact ovaries were perfused with a 10% DMSO solution containing methylene blue prior to freezing and were submerged in medium with or without DMSO during the perfusion. During a successful perfusion, this study observed macroscopically that the surface of the ovary actually turned blue rapidly (within seconds after start of the perfusion), whereas during failed perfusions no colour change was observed. The latter ovaries were not used for glucose/lactate metabolism analysis. Results are shown in **Figure 4** (conditions E, F and G). As for **Figure 3**, the metabolism of fresh tissue is shown for comparison (condition A). For clarity, data are summarized in **Table 1**.

In contrast to the complete protection of the cortex by submerging the ovary in DMSO, perfusing the ovary for 30 min (i.e. without submersion) did not result in a complete protection of the cortical layer. Instead, only 30—50% of the metabolic activity of fresh tissue remained (**Figure 4**, left panels, condition A versus E). The subcortical and medullar tissue layers, however, clearly benefitted from the perfusion treatment (**Figure 4**, middle and right panels, condition A versus E): in contrast to the submersion experiments where hardly any metabolic activity remained (**Figure 3**), 30—70% of the fresh values were now found.

As both perfusing with DMSO (for the subcortex and medulla), as well as submerging in DMSO (for the cortex), displayed protective effects, the treatments were combined. The effects of 5-min (Martinez-Madrid et al., 2004, 2007) or 30-min perfusion, in combination with submersion were analysed (**Figure 4**, conditions F and G). After combining perfusion and submersion for 5 min, the cortex (left panels) was protected to a level of metabolic activity approaching that of fresh tissue (condition A versus F). It should be noted, however, that a similar level of protection was already achieved by submersion in a DMSO solution alone (**Figure 3**, condition C and D). The same treatment resulted for the subcortex (**Figure 4**, middle panels, condition A versus F) and medulla (left panels) in a level of glucose/lactate metabolism that was not maximal (reaching 25—50% of the fresh values), but clearly increased compared with the level of metabolism obtained by submersion only.

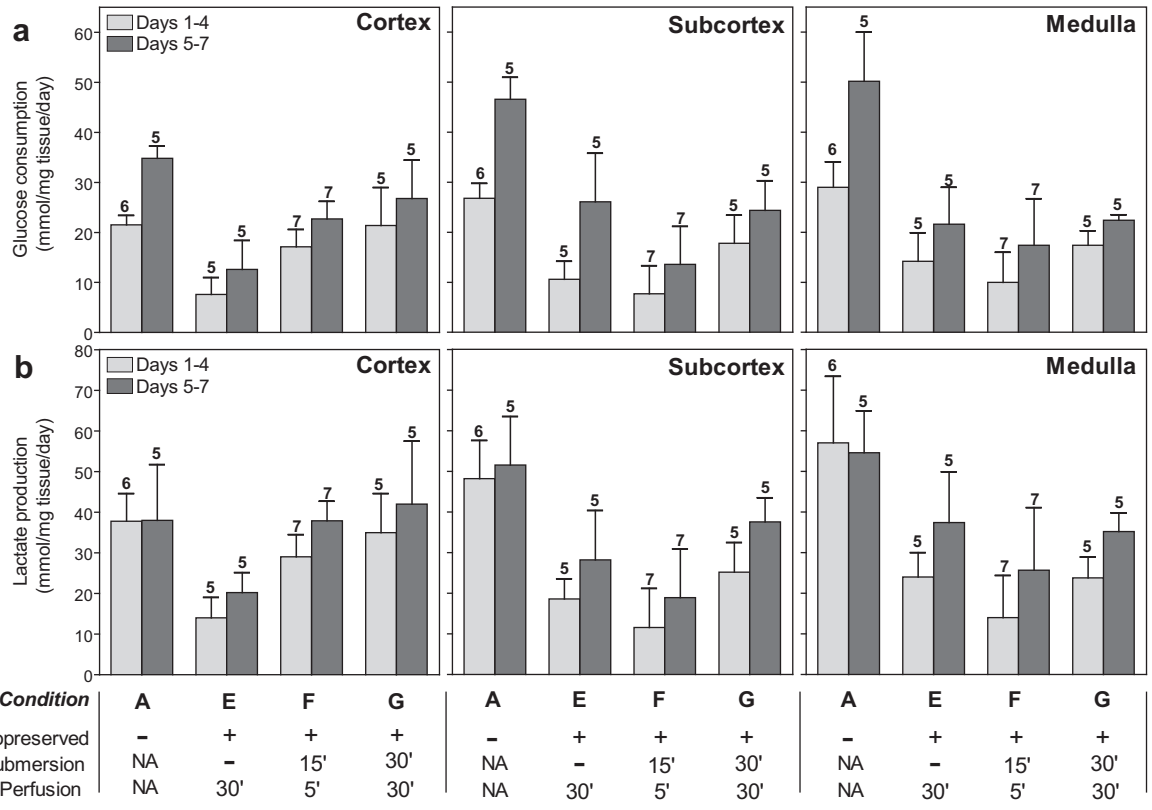

Figure 4 Glucose uptake (**a**) and lactate production (**b**) of cultured ovarian fragments derived from cortex, subcortex and medulla. Glucose/lactate metabolism was determined at two different time points of culture; days 1—4 and days 5—7. Condition A: fresh (non-cryopreserved) tissue; condition E: tissue frozen—thawed after 30 min perfusion, without submersion; condition F: tissue frozen—thawed after submersion in, and perfusion with, DMSO for 5 min; condition G: tissue frozen—thawed after submersion in and perfusion with DMSO for 30 min. Values are mean and SD. Numbers above bars represent the number of ovaries analysed per condition. + = treatment applied; — = treatment not applied; NA = not applicable.

Increasing the combined submersion and perfusion time from 5 to 30 min led to higher glucose/lactate metabolism in both subcortex and medulla, but these levels were still suboptimal (50—70% of the fresh values). Increasing the perfusion time to 60 min, and even 120 min, did not result in an additional protective effect on the ovarian tissue (data not shown).

Histology reflects the metabolic status of cultured ovarian tissue fragments

It is to be expected that cryodamage inflicted on ovarian tissue is not only reflected by the level of metabolic activity of this tissue, but also by its histological appearance. To test this notion, this study performed standard haematoxylin and eosin staining of cultured ovarian tissue derived from the medulla of intact ovaries that were either fresh (non-frozen) or cryopreserved in the presence or absence of DMSO. Unfrozen, non-cultured tissue was also stained for reference. This tissue (**Figure 5A**) showed relatively large, lightly stained nuclei and a regular pattern of extracellular matrix fibres. In the cultured tissues (**Figure 5B—D**), the nuclei were smaller and more intensely stained. In addition, the extracellular matrix that surrounded the stromal cells appeared to be less organized. Culturing unfrozen (fresh) tissue induced the formation of a limited number of pycnotic (i.e. small and dark) nuclei, indicative of a decrease in tissue viability (**Figure 5B**), as can be expected after 7 days of culture. Freezing and thawing the intact ovary before tissue culture clearly increased the number of pycnotic cells, irrespective of the absence (**Figure 5C**) or presence (**Figure 5D**) of DMSO. The apparent decrease in tissue viability is in accordance with the absence of metabolic activity in these medullar tissues, as shown in **Figure 4**.

Discussion

Several studies have dealt with determining the optimal conditions for cryopreserving intact ovaries, using various techniques to assess cellular damage (mainly in follicles/oocytes). Each of these assays has its own advantages and limitations. Results obtained by conventional histology (Arav et al., 2005; Baudot et al., 2007; Bedaiwy et al., 2003; Courbiere et al., 2005, 2006; Martinez-Madrid et al., 2004) and transmission electron microscopy (Camboni et al., 2008; Martinez-Madrid et al., 2007) have in common that they are, in contrast to the glucose/lactate assay, difficult to quantify. The current study found that the number of pycnotic stromal

Table 1 Summary of effects of different cryoprotective treatments on ovarian tissue metabolism.

Cryoprotective treatment	*Condition*[a]	*Protection level*[b] *Cortex*	*Subcortex*	*Medulla*
No protection	B			
Submersion for 15 min	C			
Submersion for 180 min	D			
Perfusion for 30 min	E			
Submersion + 5-min perfusion	F			
Submersion + 30-min perfusion	G			

Protection level (%)

0–20
20–40
40–60
60–80
80–100

[a]The conditions are as those given in **Figures 3** and **4**. B = tissue frozen–thawed without cryoprotective measures; C = tissue frozen–thawed after submersion in DMSO for 15 min; D = tissue frozen–thawed after submersion in DMSO for 180 min; E = tissue frozen–thawed after 30 min perfusion, without submersion; F = tissue frozen–thawed after submersion in, and perfusion with, DMSO for 5 min; G = tissue frozen–thawed after submersion in and perfusion with DMSO for 30 min.
[b]The level of protection obtained with the different cryoprotective treatments was calculated as percentage of mean glucose consumption and lactate production of fresh tissue.

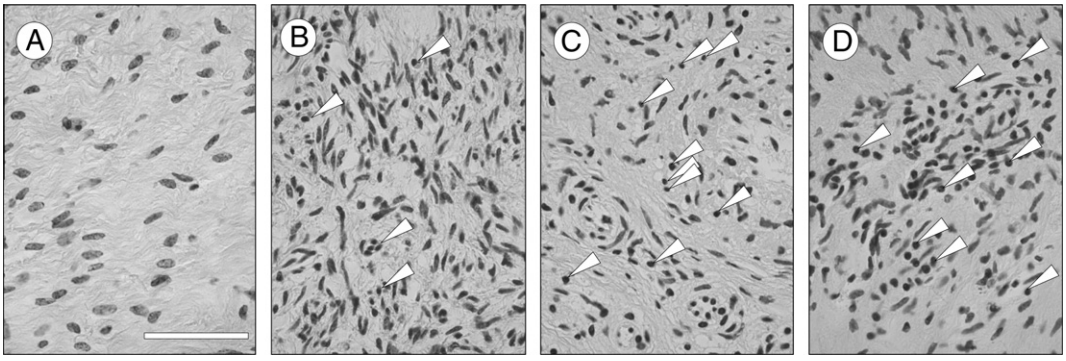

Figure 5 Haematoxylin—eosin-stained ovarian medullar tissue fragments derived from intact ovaries. (**A**) Tissue derived from unfrozen non-cultured ovary. (**B**—**D**) Fragments were cultured for 7 days. (**B**) Tissue derived from fresh (unfrozen) ovary; (**C**) tissue derived from a frozen—thawed ovary in the absence of DMSO; (**D**) tissue derived from a frozen—thawed ovary in the presence of DMSO. Some pycnotic cells are indicated by arrowheads. Bar = 100 μm.

cells was increased in cryodamaged and cultured ovarian tissue. However, quantifying the amount of cryodamage by assessing the number of pycnotic cells is time consuming and often difficult as the pycnotic status of the nuclei is not always evident.

Apoptosis assays (Bedaiwy et al., 2003, 2006; Martinez-Madrid et al., 2007; Xiao et al., 2010) assess programmed cell death at distinct moments in time but may underestimate the actual amount of tissue damage that has been inflicted by the cryopreservation process. The glucose/lactate assay is not limited to determining cryodamage at one particular moment after thawing, but instead measures the accumulated effects of the cryodamage that occurs over a 7-day period. Viability assays (Arav et al., 2005; Baudot et al., 2007; Bedaiwy et al., 2003, 2006; Courbiere et al., 2005, 2006; Imhof et al., 2004; Martinez-Madrid et al., 2004) are predominantly designed for intact (cultured) tissue monolayers rather than tissue sections and are therefore not optimally suited for determining cryodamage in tissue.

In addition to tests that analyse cryodamage based on morphological parameters, several assays measure the production or secretion of specific cell products such as oestradiol (Gerritse et al., 2010; Isachenko et al., 2007), lactate dehydrogenase (LDH; Newton et al., 1998) and cAMP (Wallin et al., 2009). However, oestradiol secretion does not take the stromal compartment of the ovary into account, while the biological variation in oestradiol secretion is too large to obtain significant results (Gerritse et al., 2010). LDH is a large intracellular tetrameric protein that will most likely not be efficiently released into the culture medium and is therefore not suitable for the purposes of this study. cAMP production, finally, could only be measured after stimulating the tissue with the adenylyl cyclase activator forskolin (Wallin et al., 2009), whereas glucose/lactate metabolism is a general marker that is also present in unstimulated tissue.

A third category of assays looks at cell proliferation, for instance via bromodeoxyuridine uptake (Onions et al., 2008). Cell proliferation, however, is unlikely to occur in non-stimulated ovarian tissue, with the possible exception of granulosa cells, and is therefore not suitable.

When searching for the optimal condition(s) to cryopreserve an intact ovary, the study elaborated on the

experimental conditions used in previous studies. Martinez-Madrid et al. (2004, 2007) used a protocol employing a 5-min perfusion with 10% DMSO, in combination with submersion of the ovary in 10% DMSO. The authors described that, after this treatment, a 75% survival of follicles, viable stromal cells and viable blood vessels were observed in the ovarian tissue. Since these data on stromal-cell viability were not quantified, the current study decided to employ a quantitative glucose/lactate assay to analyse the cryoprotective effect of DMSO, for three different ovarian tissue layers separately. Whereas the distinction between the cortex and medulla is both functionally, macroscopically and histologically apparent (Gerritse et al., 2010), the separation made between subcortex and medulla is not. The current study made this division to detect possible differences between the tissue layer immediately beneath the cortex (the subcortex) and the core of the organ (medulla).

The results show that submersion is sufficient for an efficient protection of the cortex. Submersion alone, even after increasing the exposure time to 3 h, did not protect the tissue beyond the cortex. It was found that administration of DMSO via a 5-min perfusion, as described by Martinez-Madrid et al. (2004, 2007), provided some level of protection. Increasing the perfusion time to 30 min, however, clearly improved the protection against cryodamage, confirming the results obtained by Milenkovic et al. (2011).

The contrast between the complete protection of the cortex and the lack of protection of the deeper tissue layers was puzzling. Monitoring the temperature kinetics during the freezing process showed that a (too) slow decrease in the temperature of the deeper tissue layers (resulting in suboptimal freezing conditions and consequently in tissue damage) was not the cause of the lack of protection, as temperature kinetics in the ovarian cortex and the medulla during freezing were practically identical. This suggests that homogenous cooling, i.e. according to Arav and Natan (2009), one of the limiting factors of successful whole-organ freezing, is most probably not a limiting factor in the current freezing protocol. Most likely, DMSO is not able to penetrate the ovarian tissue beyond the cortical layer, irrespective of the incubation time. Analysis of ovarian tissue stained histochemically via the Azan protocol (Gerritse et al., 2010) revealed that the ovarian cortex contains, in contrast to the subcortex and the medulla, large amounts of extracellular matrix components, whereas the number of stromal cells in the cortex is relatively low. As DMSO is likely to progress throughout tissue around rather than through cells, this process will proceed more easily in tissue that contains relatively few cells and is rich in extracellular matrix.

Perfusing the ovary with DMSO for 30 min resulted in a partial (about 40–50%) protection against cryodamage of the subcortical and medullar tissue layers. The cortex, however, was less well protected against cryodamage by perfusion alone compared with submersion (about 30% protection). The fact that the cortex is less well vascularized than the subcortex and the medulla (Delgado-Rosas et al., 2009), may explain why the DMSO did not reach the cortical layer in sufficient amounts to provide optimal protection.

The next logical step was to combine submersion and perfusion. A 5-min perfusion–submersion period resulted in optimal protection of the cortex and some protection of the deeper layers. No additional protective effect was obtained by prolonging the perfusion time beyond 30 min. A further increase in the protection level may require higher DMSO concentrations, and/or other types, or even combinations of cryoprotectants. In this context, however, it should be noted that it is currently unknown what extent of cryodamage is acceptable for maintaining organ integrity and functionality after transplantation. It is conceivable that complete protection is not required and that our current protocol already provides a sufficient level of protection. This notion can only be tested *in vivo*, for instance by transplanting a cryopreserved ovary into an immune-compromised host. Resumption of the physiological function of the ovary, including sufficient circulation, and assessment of follicle development after stimulation with gonadotrophins can then be analysed.

As mentioned previously, this study's assay is aimed at the stromal cell compartment. Therefore, it provides no information on the condition of the other ovarian compartments, including the follicles, the intraovarian vasculature and the vascular pedicle. Damage to the vascular compartment may lead to thrombosis and failure to resume function *in vivo* after reanastomosis (Imhof et al., 2006; Onions et al., 2009). To ensure that a functional organ remains after freezing, thawing and transplantation, all ovarian components are of equal importance, and require optimal protection levels as well. As the aforementioned compartments each consist of many different cell types that may each require different cryoprotective measures, a different optimal cryoprotective protocol may apply to each compartment. This assay may be a valuable tool in delineating and fine-tuning the definitive protocol, which may be a combination, and perhaps even a compromise, of all the separate protocols. Obviously, the vitality of the cell types in other compartments will have to be assessed in parallel.

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