Beneficial effect of directional freezing on *in vitro* viability of cryopreserved sheep whole ovaries and ovarian cortical slices

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**STUDY QUESTION:** Does directional freezing improve the structural and functional integrity of ovarian fragments compared with conventional slow freezing and to whole ovary cryopreservation?

**SUMMARY ANSWER:** Compared with slow freezing, the use of directional freezing significantly improves all structural and functional parameters of ovarian fragments assessed *in vitro* and, overall, whole ovaries were better preserved than ovarian fragments.

**WHAT IS KNOWN ALREADY:** Directional freezing has been developed to provide an alternative way to cryopreserve large biological samples and it is known to improve the structural and functional integrity of whole ovaries. Conventional slow freezing of ovarian fragments is the procedure more widely used in clinical settings but it causes substantial structural damage that limits the functional period after transfer back into the patient.

**STUDY DESIGN, SIZE, DURATION:** We performed a 2 × 2 factorial design experiment on a total of 40 sheep ovaries, divided into four groups (*n* = 10 ovaries per group): (i) directional freezing of whole ovary (DFwo); (ii) directional freezing of ovarian fragments (DFof); (iii) conventional freezing of whole ovary (CFwo); (iv) conventional freezing of ovarian fragments (CFof). An additional eight ovaries were used as fresh controls.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Ewe ovaries were randomly assigned to one of the experimental groups and frozen accordingly. Upon thawing, ovarian tissue was examined morphologically and cultured *in vitro* for 7 days. Samples were analyzed for cell proliferation and apoptosis, for DNA damage and repair activity, and for the presence of a panel of heat shock proteins (HSPs) by immunohistochemistry.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Most studied parameters were significantly improved (*P* < 0.05) in all samples cryopreserved with directional compared with slow freezing. The proportion of primordial follicles, which developed to the primary stage in whole ovaries (53 ± 1.7%) and in ovarian fragments (44 ± 1.8%) cryopreserved with directional freezing, was greater than with slow frozen whole ovaries (6 ± 0.5%, *P* = 0.001) or fragments (32 ± 1.5%, *P* = 0.004). After 7 days of culture, cell proliferation in DFwo (28 ± 0.73%) was the highest of all groups (*P* < 0.05) followed by DFof (23 ± 0.81%), CFof (20 ± 0.79%) and CFwo (9 ± 0.85%). Directional freezing also resulted in a better preservation of the cell capacity to repair DNA damage compared with slow freezing both in whole ovaries and ovarian fragments. Apoptosis and HSP protein levels were significantly increased only in the CFwo group. Direct comparison demonstrated that, overall, DFwo had better parameters than DFof and was no different from the fresh controls.

**LIMITATIONS, REASONS FOR CAUTION:** The study is limited to an *in vitro* evaluation and uses sheep ovaries, which are smaller than human ovaries and therefore may withstand the procedures better.

**WIDER IMPLICATIONS OF THE FINDINGS:** Improved integrity of ovarian morphology may translate to improved outcomes after transplantation. Alternatively, the particularly good preservation of whole ovaries suggests they could provide a source of ovarian follicles for *in vitro* culture in those cases when the presence of malignant cells poses a substantial risk for the patient.

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**Key words:** cryopreservation / directional freezing / whole ovaries / ovarian fragments / follicles
Introduction

A continuous improvement of cancer treatments is steadily increasing the chances of survival in patients of all ages but this positive outcome, at the same time, increases the challenges related to the patients’ quality of life. In young women this is often related to the maintenance of fertility (Reimnuth et al., 2008) since the probability of normal full-term pregnancy is reduced to 30–50% in cancer survivors compared with the corresponding healthy general population (Chung et al., 2013).

In particular, bone marrow transplantation (BMT), a common therapeutic procedure for several hematologic procedures, requires aggressive chemotherapy and or radiotherapy, both of which are primary causes of gonadal dysfunction and failure (Jadoul and Donnez, 2012). The use of alkylation agents associated with abdominal ionizing radiation in the range of 5–20 Gy will render infertility almost 100% of the patients prepared for BMT (Anderson and Wallace, 2013; Donnez et al., 2013; Wallace et al., 2005). The damaging effect will increase exponentially from the age of 25 years and a model can predict with reasonable accuracy the age of menopause based on the radiation doses and the age at the time of treatment (Wallace et al., 2005). The model clearly shows that, irrespective of the parameters applied, the risk of premature ovarian failure is high.

All this brings an increasing demand for suitable treatments for fertility preservation in female cancer patients which are strongly influenced by the type of cancer, the type of treatment and age at the time of treatment. Ovarian stimulation followed by oocyte or embryo banking is a possible choice if it can be performed before initiation of cancer therapies, if the tumor is not hormone sensitive, if the patient is sexually mature and if there is a high risk that metastatic cells may have colonized the ovary (Chung et al., 2013).

Ovarian tissue cryopreservation represents another alternative even if it is still considered to be experimental by the American Society for Reproductive Medicine. Indeed this approach may represent the only option when dealing with pre-pubertal girls or with patients who cannot delay the cancer treatment (Donnez and Dolmans, 2010, 2011; Grynb erg et al., 2012; Silber, 2012; Donnez et al., 2013). It is possible to cryopreserve cortical strips or the entire organ; however, to date, ovarian cryopreservation and subsequent retransplantation in humans has been limited to avascular cortical pieces (Demeestere et al., 2009; Anderson and Wallace, 2011; Grynb erg et al., 2012; Donnez et al., 2013).

Current preference for the clinical use of avascular pieces of ovarian cortex is based on several factors. The smaller size of tissue pieces in comparison with the whole organ facilitates the adequate penetration of the cryoprotectant across the stroma to reach granulosa cells and oocytes (Hovatta, 2005). It also facilitates heat transfer during cooling and warming which typically takes place by convection using slow-programmable freezers (Grynb erg et al., 2012; Donnez et al., 2013), even if vitrification is beginning to be considered as a viable alternative (Bordes et al., 2005; Ker<br>os et al., 2009). However, direct comparisons between slow freezing and vitrification in human have produced conflicting results (Gandolfi et al., 2006; Isachenko et al., 2009, 2010). Transplantation of ovarian fragments either on the exposed medulla of the contralateral ovary (Donnez et al., 2006) or in a peritoneal pocket (Donnez et al., 2012) is technically easier than the vascular anastomosis required for the transplantation of an entire ovary (Kim, 2010). Finally, the cryopreservation of several fragments of the same ovary allows the possibility to repeat the autotransplantation procedure should the transplanted pieces cease to function. At the same time the use of ovarian fragments suffers from some limitations, the main one being the loss of over 50% of the primordial follicles due to tissue ischemia that takes place after transplantation (Van Eyck et al., 2009; Van Eyck et al., 2010). This, together with the damages caused by cryopreservation, leads to a functional lifespan that, depending on the follicle density at the time of the procedure, can vary, on average, from 2 to 5 years (Bromer and Patrizio, 2009; Donnez et al., 2013). In addition, since this procedure is largely devoted to cancer patients, there is the real danger that the ovarian tissue contains malignant cells posing a severe risk to the patient (Meir ow et al., 2008; Dolmans et al., 2010; Greve et al., 2012).

Transplantation of fresh whole ovary has been attempted with success in human (Leporrier et al., 1987; Hilders et al., 2004; Silber et al., 2008) and in several animal species (Bromer and Patrizio, 2009) demonstrating that anastomosis of the ovarian pedicle is difficult but feasible. However, cryopreservation of the entire organ has proved more difficult, largely due to the physical constraints that limit an appropriate heat transfer between the core and the periphery of the organ (Arav and Natan, 2009). In addition, the large volume of the whole organ poses some limitation to the perfusion and diffusion of cryoprotectants (Falcone and Bedawy, 2005; Torre et al., 2013). Both are essential for preventing intravascular ice formation which would irreversibly compromise a rapid and efficient resumption of the blood supply (Pegg, 2010). An efficient protocol for the cryopreservation and re-anastomosis of the entire ovary could potentially reduce ischemic damage. However, data obtained from experiments mostly performed in sheep show some positive results (Grazul-Bilska et al., 2008) and pregnancies (Salle et al., 2002) but also highlight stromal and vascular damage following either slow freezing (Onions et al., 2009; Wallin et al., 2009) or vitrification (Salle et al., 2003; Courbiere et al., 2006).

This has prompted the development of an alternative way to cryopreserve large samples, such as whole ovaries, based on directional cooling (Arav and Natan, 2009).

The technology for directional cooling is based on a series of heat conductive blocks at set temperatures that are arranged in a line along which the sample proceeds at a pre-set velocity. The blocks have different temperatures so that a gradient temperature can be imposed along the track. The samples are frozen are placed inside glass test tubes and moved along the temperature gradient formed by the blocks. The freezing rate is determined by the combination of temperature gradient and speed of the sample along the track (Arav and Natan, 2012). If the velocity of the sample is slower than the speed at which the heat is removed from the center of the sample towards its periphery, heat transfer is quickly removed in the direction opposite to that of the sample movement, thanks to the large mass of the conductive material which forms the blocks. All this results in a uniform cooling rate throughout the sample (Gavish et al., 2008). This method has been used before for the cryopreservation of sheep whole ovaries with good success and maintenance of ovarian function for 6 years (Arav et al., 2005, 2010). However, the number of ovaries cryopreserved with this method is limited and it has never been applied to ovarian fragments.

To move forward the cryopreservation of both avascular ovarian fragments and of whole ovaries must improve (Donnez et al., 2013). Therefore, the aim of the present study was to perform a direct comparison between a convection-based programmable slow-freezer and the multi-thermal-gradient apparatus for the cryopreservation of ovarian
cortical strips and of whole ovaries. Based on our own results (Gandolfi et al., 2006) and those in the literature (Shaw and Trounson, 2002; Arav and Natan, 2009) all experiments were performed on sheep ovaries owing to its similarity with the human ovary.

Materials and Methods

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (Italy).

Sheep female reproductive tracts were collected at the local slaughterhouse and transported to the laboratory in cold (0–4°C) 0.9% saline solution.

Eight ovaries were randomly assigned to fresh control group (CTR) and 10 to each experimental group. The experimental groups included the following: (1) directional freezing of whole ovary (DFwo); (2) directional freezing of cortical tissue (DFot); (3) conventional freezing of whole ovary (CFwo) and (4) conventional freezing of cortical tissue (CFot).

Sample preparation

Upon arrival to the laboratory, ovaries were randomly divided in three groups, i.e. fresh controls, ovaries to be frozen as whole organs or as ovarian fragments. Samples to be frozen as whole organs were perfused via the ovarian artery with Ringer’s solution and 10 IU/l heparin for 10 min, followed by perfusion with cryoprotectant solution containing Leibovitz L-15 medium (Life Technologies, Italy) supplemented with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin and 6.25 ng/ml selenium; Life Technologies), 2 mM glutamine (Life Technologies, Italy), 0.23 mM pyruvate, 2 mM hypoxantine, 1.25 mg/ml bovine serum albumin (BSA), 100 μg/ml penicillin G, 100 μg/ml streptomycin, 200 μIU/ml sheep FSH and 50 ng/ml human recombinant basic fibroblast growth factor (R&D Systems, Italy) (Matos et al., 2007). The medium was replaced every 2 days.

Conventional slow freezing

Conventional slow freezing was performed in a Kryo 560M apparatus (Planer, UK) with the following program: (i) from 4°C to −40°C using a cooling rate of 0.5°C/min (seeding was induced at −7°C), (ii) from −40°C to −100°C at 5°C/min and (iii) immersion in liquid nitrogen (Grazul-Bilska et al., 2008).

Directional freezing

Directional freezing was performed with a Multi-Thermal-Gradient (MTG, IMT Ltd., Ness Ziona, Israel). The three thermal blocks were set at 4°C, −10°C and −70°C, respectively, thereby imposing a temperature gradient around the tubes. Freezing tubes were pushed lengthwise along the thermal gradient and the speed was set at 0.01 mm/s resulting in a cooling rate of 0.3°C/min down to −70°C, after which samples were plunged into liquid nitrogen (Arav et al., 2005, 2010).

Thawing

Samples were thawed by plunging the test tubes into a 68°C water bath for 20 s and then into a 37°C water bath for 2 min. The contents of the tube were quickly emptied into a petri dish containing Leibovitz L-15 medium at 37°C. Whole ovaries were immediately perfused through the ovarian artery with Leibovitz L-15 medium at 37°C supplemented with decreasing sucrose concentrations (0.25, 0.125 and 0 M) at 3 ml/min for a total of 30 min (10 min for each step; Jeremias et al., 2002; Bedaiwy et al., 2003).

Ovarian fragments were washed three times for 10 min each in Leibovitz L-15 supplemented with the same sucrose concentrations (0.25, 0.125 and 0 M). The material was then randomly allocated for further analysis. Each experiment was performed on a minimum of three independent biological replicates and all samples were blinded for analysis.

Follicle culture

The in vitro culture of the thawed and fresh control samples was performed as previously described (Matos et al., 2007). Several ovarian fragments of 2 × 2 × 1 mm were derived from whole ovaries and isolated cortical pieces. Three randomly selected fragments from each ovary were immediately fixed in 10% formaldehyde solution for histological studies (time 0, control). Other randomly selected fragments were placed into 24-well culture dishes (Nuncsted, Italy) and individually cultured in 1 ml of medium for 7 days at 39°C under an atmosphere of 5% CO2 in air. The culture medium was composed of α-Minimum Essential Medium (Life Technologies, Italy) supplemented with ITS (6.25 mg/ml insulin, 6.25 mg/ml transferrin and 6.25 ng/ml selenium; Life Technologies), 2 mM glutamine (Life Technologies, Italy), 0.23 mM pyruvate, 2 mM hypoxantine, 1.25 mg/ml bovine serum albumin (BSA), 100 μg/ml penicillin G, 100 μg/ml streptomycin, 200 μIU/ml sheep FSH and 50 ng/ml human recombinant basic fibroblast growth factor (R&D Systems, Italy) (Matos et al., 2007). The medium was replaced every 2 days.

Morphological analysis

Samples collected before or after cryopreservation and at Days 0 and 7 of follicle culture were fixed with 10% formaldehyde, embedded in paraffin and the sections either stained with hematoxylin and eosin or used for the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay or immunohistochemistry, as described below. Slides were observed under an Eclipse E600 microscope (Nikon, Japan) and pictures were acquired with Nis Elements Software (Version 4.0). For each measure, a minimum of four sections, 200 μm apart, from each sample were examined. Follicles were classified as primordial (one layer of flattened granulosa cells around the oocyte), intermediate (one layer of flattened to cuboidal granulosa cells around the oocyte), primary (one single layer of cuboidal granulosa cells around the oocyte) or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Follicles were further classified as normal when a spherical oocyte with a non-pyknotic nucleus was surrounded by granulosa cells organized in discrete layers, or degenerated when a mis-shapen oocyte, with or without vacuolation, with a pyknotic nucleus was surrounded by disorganized granulosa cells detached from the basement membrane (Silva et al., 2004).

Immunohistochemistry

Sections were boiled for 5 min in antigen unmasking solutions (Vector Laboratories, Italy) and then blocked with a solution of 1% BSA. Non-specific sites were blocked with a solution of phosphate-buffered saline (PBS) containing 5% BSA and 10% non-immune serum (Life Technologies). Samples were incubated overnight at 4°C with the following primary antibodies: rabbit polyclonal antibody specific for the Ki67 marker of cell proliferation (1:50 dilution; Abcam, UK); mouse monoclonal anti-γH2AX, a DNA-damage marker (1:200; Abcam) and rabbit monoclonal anti-RAD51, a DNA-repair marker (1:200; Santa Cruz) (Maffei et al., 2013). Sections were washed three times with PBS and incubated with suitable secondary antibodies (Alexafluor; Life Technologies) for 45 min. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Blinded samples were observed under an Eclipse E600 microscope (Nikon, Japan), pictures were acquired with Nis Elements Software (Version 4.0) and the images were analyzed using ImageJ software as described below.
TUNEL assay
To detect DNA fragmentation, sections were placed on silanized slides (Bio-Optica, Italy) and apoptotic cells were detected by In situ Cell Death Detection Kit, TMR red (Roche, Italy) according to the manufacturer’s instructions. Positive controls were treated with DNase I recombinant (50 U/mL; Roche) in 50 mM Tris–HCl, pH 7.5, 1 mg/ml BSA for 10 min at +25°C before DNA end labeling. For negative controls, TDT was omitted from the reaction mixture. TUNEL-positive cells were calculated as cells per area of ovarian tissue using the image analysis software ImageJ, as detailed below.
Cell proliferation and TUNEL were evaluated on a per mm² basis therefore every cell type present in the examined area was considered.

Image analysis
Pictures were taken with constant exposure parameters in order to be analyzed with the image analysis software ImageJ (http://rsbweb.nih.gov/ij/index.html). Threshold adjustments were applied to generate a black and white image which was analyzed to quantify fluorescent signal. In each case, marker expression was normalized by DAPI fluorescence.

Western blots
Ovarian fragments (~2 mm²) derived from five experimental groups at Days 0 and 7 of culture were homogenized individually, lysed and constitutive proteins were extracted using ReadyPrep™ Protein Extraction Kit (Bio-Rad, Italy). Protein concentration was assessed with DC Protein Assay (Bio-Rad). Aliquots of 50 μg proteins were prepared and resuspended in (1:1) 2× Laemmli sample buffer (4% (w/v) sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 20% (w/v) glycerol, 0.004% bromophenol blue and 0.125 M Tris–HCl (pH 6.8). Samples were loaded and electrophoresed on 8 or 10% SDS-polyacrylamide gels depending on the molecular weight of the target molecule. Proteins were then transferred onto 0.45-m pore size nitrocellulose filters (Life Technologies) according to Towbin et al. (1979) using 0.5 A/cm². Equal sample loading and transfer efficiency were confirmed by staining of the membrane with Ponceau Red. The membrane was probed with the primary antibodies listed in Table I, as described by Pennarossa et al. (2012) and previously validated in sheep. Monoclonal anti-β-actin was used as a loading control (data not shown). Protein bands were visualized by Western Breeze® chemiluminescent kit (Life Technologies, Italy).

Statistical analysis
All experiments were performed on a minimum of three independent biological replicates. Data are reported as mean ± SEM and analyzed using one-way analysis of variance followed by Turkey’s post hoc tests (Statistical Package for the Social Sciences 20, IBM). Differences were considered statistically significant if P < 0.05.

Results
All groups were compared with each other and with a fresh control, shortly after thawing and after 7 days of in vitro culture.

In vitro follicle development
The percentage of follicle growth in each group, after 7 days of culture, is shown in Fig. 1. The proportion of primordial follicles which developed to the primary stage both in whole ovaries (53 ± 1.7%) and in ovarian fragments (44 ± 1.8%) cryopreserved with directional freezing was significantly greater than with conventionally frozen whole ovaries (6 ± 0.5%, P = 0.001) or fragments (32 ± 1.5%, P = 0.004). The percentage of developing follicles in whole ovaries was higher than in ovarian fragments when frozen with directional freezing (P = 0.005) but lower when frozen with conventional freezing (P = 0.001). Finally, whole ovaries frozen with directional freezing had a rate of growing follicles not significantly different from fresh ovaries (55 ± 1.9%, P = 0.106).

Morphological assessment of cryopreservation damage
The percentage of morphologically normal follicles in each group immediately after thawing and cryoprotectant removal is shown in Table II. The percentage of intact follicles was inversely related to the stage of development, in all groups including fresh controls. There were more intact follicles of all developmental stages following directional freezing when compared with conventional freezing. Ovarian fragments showed a higher rate of normal morphology compared with whole ovaries when frozen with conventional freezing but the opposite was observed when whole ovaries and ovarian fragments were preserved with directional freezing. The percentage of morphologically normal follicles in the DFwo group (89.93 ± 4.10%) was not significantly different from the fresh control (92.54 ± 4.36%).

Cell proliferation and apoptosis
Differences in developmental competence of primordial follicles were consistent with differences in cell proliferation rate as examined by the expression of Ki67. The percentage of proliferating cells was comparable among the different groups at Day 0 (Fig. 2A). After 7 days of culture, Ki67-positive cells in fresh tissue (30 ± 0.78%) and in group DFwo (28 ± 0.73%) were the highest of all groups (P < 0.05). The rate of cells expressing Ki67 in group DFof (23 ± 0.81%) was significantly higher than those in group CFof (20 ± 0.79%, P = 0.03) and an average of only 9 ± 0.85% of Ki67-positive cells was found in group CFwo (Fig. 2A).

TUNEL-positive cells were detected both at Days 0 and 7 of culture in all experimental groups but apoptotic levels were not related to cell proliferation. Immediately after thawing and the removal of the cryoprotectant, the number of apoptotic cells per mm² was low and comparable among all groups. The TUNEL assay showed that the number of positive cells after 7 days in culture was comparable for all experimental groups.

Table I List of antibodies used for western blot analysis of proteins extracted from sheep ovaries.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalog number</th>
<th>WB working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSF1</td>
<td>Goat</td>
<td>Santa Cruz Biotech</td>
<td>sc-8061</td>
<td>1:1000</td>
</tr>
<tr>
<td>HSP40</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab69402</td>
<td>1:10,000</td>
</tr>
<tr>
<td>HSP70</td>
<td>Mouse</td>
<td>Abcam</td>
<td>ab5439</td>
<td>1:5000</td>
</tr>
<tr>
<td>HSP90</td>
<td>Mouse</td>
<td>Abcam</td>
<td>ab13492</td>
<td>1:1000</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Mouse</td>
<td>Sigma</td>
<td>a5441</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host species</th>
<th>Company</th>
<th>Catalog number</th>
<th>WB working dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSF1, heat shock transcription factors 1; HSP40, heat shock protein 40; HSP70, heat shock protein 70; HSP90, heat shock protein 90; WB, western blotting.</td>
<td></td>
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</tr>
</tbody>
</table>
ovary induced the activation of proteins involved in stress-response (Fig. 3), indicating that conventional freezing applied to the whole culture, where the protein levels were significantly higher than on Day 0 from the conventionally cryopreserved whole ovaries after 7 days of culture. Changes were only detected in cortical tissue by quantitative analysis of HSP40, HSP70, HSP90 and HSF1 proteins at the end of the warming and dilution/cryoprotectant removal steps and 

The effect on heat stress response after cryopreservation was examined by quantitative analysis of HSP40, HSP70, HSP90 and HSF1 proteins at the end of the warming and dilution/cryoprotectant removal steps and after 7 days of culture. Changes were only detected in cortical tissue from the conventionally cryopreserved whole ovaries after 7 days of culture, where the protein levels were significantly higher than on Day 0 (Fig. 3), indicating that conventional freezing applied to the whole ovary induced the activation of proteins involved in stress-response pathways. There were no differences within or between the other groups (Fig. 3).

Table II Morphologically normal follicles (%)

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>Morphologically normal follicles (%)</th>
<th>Primary</th>
<th>Secondary</th>
<th>All follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicle type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primordial</td>
<td></td>
<td>91.2 ± 4.34 (n = 350)*</td>
<td>85.23 ± 3.95 (n = 300)*</td>
<td>68.62 ± 3.34 (n = 60)*</td>
</tr>
<tr>
<td>Intermediate</td>
<td></td>
<td>80.17 ± 4.30 (n = 150)*</td>
<td>72.91 ± 3.68 (n = 100)*</td>
<td>53.25 ± 3.21 (n = 20)*</td>
</tr>
<tr>
<td>Primary</td>
<td></td>
<td>58.10 ± 3.32 (n = 350)*</td>
<td>43.5 ± 3.21 (n = 300)*</td>
<td>23.4 ± 3.12 (n = 60)*</td>
</tr>
<tr>
<td>Secondary</td>
<td></td>
<td>69.72 ± 3.27 (n = 150)*</td>
<td>64.20 ± 3.34 (n = 100)*</td>
<td>42.70 ± 2.76 (n = 20)*</td>
</tr>
<tr>
<td>All follicles</td>
<td></td>
<td>71.5 ± 4.44 (n = 60)*</td>
<td>73.25 ± 3.21 (n = 20)*</td>
<td>82.24 ± 4.82 (n = 570)*</td>
</tr>
</tbody>
</table>

Percentage (mean ± s.e.m.) of morphologically normal primordial, intermediate, primary and secondary follicles in fresh tissue (CTR) and in experimental groups immediately after thawing. DFwo, directional freezing of whole ovary; DOf, directional freezing of ovarian fragments; CFwo, conventional slow freezing of whole ovary; COf, conventional slow freezing of ovarian fragments.

*aNumber of evaluated follicles.

**Different superscripts in the same column indicate a significant difference amongst experimental groups for the same follicle type as determined by one-way analysis of variance followed by Turkey’s post hoc tests (P < 0.05).}

DNA damage and repair

The expression of γ-H2AX, a marker of DNA damage, and of RAD51, a marker of DNA repair were examined by quantitative analysis of H2AX, HSP70, HSP90 and HSF1 proteins at the end of the warming and dilution/cryoprotectant removal steps and after 7 days of culture. Changes were only detected in cortical tissue from the conventionally cryopreserved whole ovaries after 7 days of culture, where the protein levels were significantly higher than on Day 0 (Fig. 3), indicating that conventional freezing applied to the whole ovary induced the activation of proteins involved in stress-response pathways. There were no differences within or between the other groups (Fig. 3).
**Discussion**

Cryopreservation of ovarian tissue is now a well-established method for preserving female fertility in a series of circumstances when gonadotoxic treatments cannot be delayed or in patients before puberty (Grynberg et al., 2012; Donnez et al., 2013). This can be achieved through the excision and banking of whole organs or of fragments of the cortical region. However, whereas no live births or pregnancies have been achieved following the cryopreservation of whole human ovaries, 24 live babies have been obtained after transplantation of frozen-thawed ovarian fragments (Donnez et al., 2013). This clearly suggests that the use of ovarian fragments is the method of choice even if, at present, the average functional life span of these samples ranges from 2 to 5 years (Bromer and Patrizio, 2009; Donnez et al., 2013). In theory, the cryopreservation of whole ovaries followed by the vascular anastomosis of the ovarian pedicle of the thawed organ should provide a larger follicular reserve and a longer life span of the transplant. However, currently available data do not support this hypothesis. This is largely due to the damage caused by the freezing procedures applied to a sample as large as a human ovary. We have recently performed a direct comparison of sheep ovarian tissue viability using whole ovaries cryopreserved with a conventional programmable slow freezer or a directional freezing apparatus (Maffei et al., 2013). Our results indicated that ovarian structure and function were significantly better preserved by the directional freezer. Therefore, it was of interest to determine whether directional freezing could also improve the viability of ovarian fragments and how these would compare with whole ovaries frozen with the same method.

In the present study, the morphological analysis of ovarian tissue immediately after thawing showed that the use of directional freezing significantly improved the integrity of all follicular structure from primordial to secondary stages. Since it is well known that the major cause of cellular damage is intracellular ice formation (Mazur, 1977; Pegg, 2010), this observation suggests that the controlled direction of the ice front, from one end to the other of the samples, was effective in maintaining a precise cooling rate thereby preventing the formation of intracellular ice. Although the effect was evident in both whole organs and ovarian fragments, it was more efficient in whole organs since the rate of morphologically intact follicles was not significantly different from that recorded in the fresh samples. Functional analysis of in vitro growth of primordial follicles confirmed the same pattern. Directional freezing was beneficial for both entire ovaries and ovarian fragments but DFwo was the only group whose viability was the same as that of fresh tissue. This result is somehow unexpected since cryopreservation efficiency is inversely related to the size of the sample. Large biological samples, in fact, suffer from a long isothermal period caused by the massive release of latent heat during the process of ice formation (Balasubramanian and Coger, 2005; Pegg, 2010). The phenomenon is caused by the energy generated by water molecules when they rejoin to form an ice crystal. Such energy is released in the form of heat that causes a temperature rise of the surrounding structures (Petersen et al., 2006). The heat is normally transferred to the ice crystals that have just been formed because these are made of conductive material. The consequence is a transient thawing followed by refreezing in a sequence that is repeated several times through the thickness of the sample causing severe cell damage (Barratt et al., 1998; Koshimoto and Mazur, 2002). Damage is usually reduced by keeping the ratio of surface to volume as high as possible so that the excessive heat is removed by adjusting the cooling rate to achieve uniform freezing. The thinner the sample the faster the heat, released from its inner parts, is removed minimizing the damage. However the need to have samples as thin as possible is in contrast with the requirement of having a large follicle population upon thawing and it is impossible when using low surface to volume ratio samples like whole ovaries. The large mass of conductive material in the MTG apparatus serves the purpose of quickly removing the latent heat from large samples thereby preventing the thawing and freezing sequence (Gavish et al., 2008).

These considerations largely explain the better results of directional freezing compared with conventional slow freezing described in our experiments but cannot account for the better performance obtained with whole ovaries than with smaller ovarian fragments. We hypothesize that the different ways that the cryoprotectant was administered may have a role. The presence of the ovarian pedicle in entire ovaries enabled an extensive uniform perfusion through the vasculature (Maffei et al., 2013). A study to compare outcomes of conventional to directional freezing after vascular perfusion of cryoprotectants is needed. In this study the ovarian fragments were directly immersed into the cryoprotectant thus relying on diffusion. Our experiments were performed in this way in order to mimic the different way

**Figure 2** Quantitative evaluation of Ki67 and TUNEL at the beginning and the end of in vitro culture. Percentage (mean ± s.e.m) of immunopositive cells for Ki67 (A). Number of TUNEL-positive cells per mm² (B). Values with different indices (a–e) are significantly different (P < 0.05); one-way analysis of variance followed by Tukey’s post hoc tests; n = 2630 ± 340 nuclei counted for each section. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments.
Figure 3  Heat stress response after cryopreservation. Western blots and densitometric analysis of heat shock proteins in sheep ovarian proteins extract from all experimental groups at Days 0 and 7 of culture. Values are expressed as mean ± s.e.m. of three replicates measured in arbitrary units. * P < 0.05 determined with one-way analysis of variance followed by Turkey’s post hoc tests; n = 10 fragments (2 × 2 × 1 mm) for each replicate. HSP40, heat shock protein 40; HSP70, heat shock protein 70; HSP90, heat shock protein 90; HSF1, heat shock transcription factor 1. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments.
Figure 4 Representative pictures of cells positive for gamma H2A histone, member X (γH2AX, marker of double-strand DNA breaks, green) and RAD51 recombinase (RAD51, marker of repair ability, red). Immunostaining was carried out at Days 0 and 7 of in vitro culture. CTR, fresh tissue; DFwo, directional freezing whole ovary; Dfof, directional freezing ovarian fragments; CFwo, conventional freezing whole ovary; Cfof, conventional freezing ovarian fragments. Scale bar = 100 μm.
samples would be treated in a clinical setting. Based upon our findings it will be interesting to study the effect of cryoprotectant administration in more detail.

Consistent with the favorable effect of directional freezing on primordial follicle viability in vitro we observed a higher cell proliferation rate compared with samples that underwent conventional freezing. In an attempt to understand the mechanisms that may have caused this effect we studied known responses to temperature stress, which is inherent to any form of cryopreservation.

One of the best characterized responses to thermal stress is the expression of HSPs (Rupik et al., 2011). HSPs bind to other proteins, termed clients, which are damaged by thermal or other stress conditions and mediate their transport to target organelles for repair or degradation (Vabulas et al., 2010). They have also physiological roles and are constitutively expressed in the absence of stress during many processes including gametogenesis and embryogenesis (Hartl and Hayer-Hartl, 2009).

We examined the expression of HSP70 because it is the most abundant and the most highly conserved (Vabulas et al., 2010). This and other members of the family act through repeated client-chaperone binding and release cycles sustained by ATP hydrolysis and nucleotide exchange reactions. Heat shock responses in vertebrates are regulated at the transcriptional level by the heat shock transcription factor (HSF) family composed of several members, with HSF1 regulating heat shock gene expression in mammals (Fujimoto and Nakai, 2010). When a mammalian cell is exposed to thermal stress, HSF1 molecules present in the cytoplasm as inactive monomers combine to form biologically active trimers. These bind the heat shock response elements (HSEs), which are present upstream of each heat shock gene and activate their transcription (Fujimoto and Nakai, 2010). Our results show that the HSP machinery was significantly stimulated over baseline levels only in the case of conventional freezing of whole ovaries. This is consistent with particularly extensive protein damage in this group since the client-chaperone binding and release cycles are repeated several times until the client has reached its final active conformation, or has entered the proteolytic system (Kampinga and Craig, 2010). The presence of extensive cellular damage in CFwo samples was confirmed by the significant increase in apoptotic cells after 7 days of culture, consistent with the notion that the accumulation of damaged or misfolded proteins, if unresovled, can lead to caspase-8 oligomerization, activation and apoptosis (Pan et al., 2011). However, protein damage is not the only cause of apoptosis as it can also be activated by a failure of DNA repair mechanisms (Batista et al., 2009). In fact the high levels of DNA damage associated with a lack of DNA repair activity observed in CFwo samples is consistent with the high apoptotic rate observed in the same group. This implies that the amount of DNA damage taking place in cells of CFwo ovaries is sufficient to overwhelm their capability for DNA repair and lead to their elimination through apoptosis.

Indeed the analysis of the dynamics of DNA repair mechanisms in our different experimental groups enabled the identification of different levels of cellular damage and was more sensitive than measuring apoptosis, which is an irreversible process (Kale et al., 2012). We have examined the expression of the histone variant γH2AX, the phosphorylated form of H2AX which is formed within minutes of DNA damage (Yuan et al., 2010). The presence of γH2AX at the foci which form at DNA break sites attracts BRCA2 which, in turn, binds to RAD51 and activates DNA repair (Huen and Chen, 2010). In contrast to HSPs, γH2AX and RAD51 are not visible in the nuclei of intact cells; therefore, it was possible to detect their increased expression in our experimental samples. Results were consistent with the pattern of follicular viability since DNA damage was significantly lower in directional freezing samples followed by more intense DNA repair activity suggesting that the DNA damage observed on Day 1 of culture was reversible. In fact, the formation of an Rad51-ssDNA nucleofilament is an essential step that leads to DNA repair (Seeber et al., 2013). The ability of directional freezing to preserve the ability of ovarian tissue to repair DNA damage is particularly relevant for its long-term function after thawing since DNA double-strand break repair efficiency has been identified as an important determinant of oocyte aging in women (Titus et al., 2013).

In conclusion, this study indicated that directional freezing improves the viability of cryopreserved ovarian tissue not only when used with whole organs but also with ovarian fragments. This information provides a way to further improve the efficiency of the cryopreservation procedure. The direct comparison of ovarian fragments and whole organs unexpectedly revealed that the latter show better preservation of early follicles, in many aspects almost identical to those recorded in fresh control samples. However, the persistent technical difficulties linked to the surgical procedures required for retransplantation is likely to still
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limit the use of whole ovaries in clinical settings. On the other hand, the increasing concerns related to the danger of transferring malignant cells with transplanted frozen–thawed ovarian tissue may lead to a re-evaluation of the technique in terms of using whole ovaries as a source of high-quality isolated follicles to be grown in artificial ovaries (Vanacker et al., 2012) or other, yet to be described, methods.

Authors’ roles

S.M. and G.P. performed the research; T.B., A.A. and F.G. designed the research and T.B and F.G wrote the paper.

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Conflict of interest

There are no conflicts of interest to declare.

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