Cryopreservation of intact human ovary with its vascular pedicle

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BACKGROUND: The aim of this study was to assess the immediate post-thawing injury to the human ovary that was cryopreserved either as a whole with its vascular pedicle or as ovarian cervical strips. MATERIALS AND METHODS: Bilateral oophorectomy was performed in two women (46 and 44 years old) undergoing vaginal hysterectomy and laparoscopic hysterectomy, respectively. Both women agreed to donate their ovaries for experimental research. In both patients, one of the harvested ovaries was sectioned and cryopreserved (by slow freezing) as ovarian cervical strips. CONCLUSIONS: Cryoperfusion and cryopreservation of entire human ovary can be achieved with the main limitation for this option (Bedaiwy and Falcone, 2004). Although many reproductive-age patients can survive their cancers and lead normal lives, they are at increased risk of impaired reproductive functions. Consequently, fertility preservation is an important quality-of-life issue for them. Fertility preservation strategies were introduced to protect and/or regain impaired reproductive functions. Consequently, fertility preservation is an important quality-of-life issue for them. Fertility preservation strategies were introduced to protect and/or regain impaired reproductive functions.

Key words: apoptosis/Bcl-2/cryopreservation/follicular viability/intact human ovary

Introduction

Although many reproductive-age patients can survive their cancers and lead normal lives, they are at increased risk of impaired reproductive functions. Consequently, fertility preservation is an important quality-of-life issue for them. Fertility preservation strategies were introduced to protect and/or regain impaired reproductive function in patients exposed to cancer chemotherapy and/or radiotherapy. Advances in assisted reproductive technologies (ART), such as ovarian tissue cryopreservation and transplantation, oocyte cryopreservation and novel ovulation induction regimens, have renewed interest in fertility preservation in women scheduled to receive gonadotoxic chemotherapy and/or radiotherapy (Falcone et al., 2004). Of these technologies, ovarian tissue cryopreservation may be a viable option for women who cannot delay treatment to undergo ovarian stimulation to create embryos or obtain oocytes for freezing. In this entirely experimental process, thawed tissue can be implanted after cancer treatment as an autograft to an orthotopic or a heterotopic site. Immature oocytes derived from thawed tissue can be matured in vitro if appropriate protocols are developed in the future. Also, the maturation process of the immature oocytes could be achieved by xenografting in immuno-deficient mice (Gook et al., 2003).

To date, ovulation and creation of a human embryo from heterotopic ovarian transplant have been reported (Oktay et al., 2004). Moreover, two live births from orthotopic ovarian transplants following spontaneous (Donnez et al., 2004) and IVF (Meirong et al., 2005) cycles were reported, following modified cryopreservation and surgical protocols pioneered, in sheep, by Gosden and associates (Gosden et al., 1994; Baird et al., 1999). Despite these advances, ischaemic damage to the tissue and revascularization injury and the theoretical possibility of reintroducing malignant tumour cells remain as the main limitations for this option (Bedaiwy and Falcone, 2004).
The ischaemic damage to ovarian tissues can induce a high rate of follicular loss (Baird et al., 1999; Demirci et al., 2002). Therefore, eliminating this ischaemic damage could maintain both the viability and the functional integrity of the transplant. Ideally, this could be achieved by transplantation of an intact ovary with vascular anastomosis. Transplantation of an intact ovary with its vascular pedicle using microvascular anastomosis was achieved both in murine species (Yin et al., 2003) and in mammals (Jeremias et al., 2002; Bedaiwy et al., 2003). Partial restoration of hormonal functions (Bedaiwy et al., 2003) and pregnancy (Wang et al., 2002) after transplantation of intact cryopreserved–thawed (C–T) ovaries were also reported. Recently, cryopreservation of intact human ovary with its vascular pedicle resulting in high post-thaw survival rates of follicles, small vessels and stromal cells as well as a normal histological structure in all the ovarian components was reported using a slow-freezing protocol. Slow freezing was achieved using a cryofreezing one-degree container (Martínez-Madríd et al., 2004).

The structural homeostasis of tissues is regulated by a delicate balance between cell survival and apoptotic cell death. In tissues, several molecules are involved in the survival (survival molecules such as Bcl-2) or cell death (apoptotic molecules such as p53) processes. Bcl-2 is a membrane-associated protein that resides in the nuclear envelope and mitochondria. It exerts its survival functions by modulating the mitochondrial release of cytochrome c and antagonizing the effects of Bax gene (Bcl-2-associated X). Bcl-2 is expressed in granulosa cells of both fetal and adult ovaries. p53 gene encodes a 53-kDa oncosuppressive nuclear protein that functions to antagonize Bcl-2 effects. In the ovary, p53 protein is expressed in the apoptotic granulosa cells of atretic follicles. In several organs such as the heart, ischaemia is associated with the induction of Bcl-2 and p53 protein expressions. Similarly, the induction of these molecules following ovarian ischaemia may have far-reaching effects on the outcome of the subsequent ovarian transplantation (Hussein, 2005).

Although previous studies examined the long-term effects of cryopreservation–thawing procedure injury on the ovarian cortical strips (Gosden et al., 1994), our understanding of the injury caused to ovarian tissue in the period immediately after thawing is still incomplete. The aims of this study were to: (i) describe a cryopreservation protocol using the programmable freezer allowing freezing and thawing of an intact human ovary with its vascular pedicle; (ii) assess the survival (viability) of the ovarian elements in the C–T tissues using Trypan Blue method; (iii) examine the frozen–thawed ovarian tissues for features of follicular health and atresia using the histological methods; (iv) evaluate the apoptotic changes in the ovarian tissues using combined histological methods and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling (TUNEL) assay, immunoperoxidase method (p53 and Bcl-2 protein alterations, i.e. apoptotic and survival proteins) and (v) examine the status of the blood vessels using both histochemical [periodic acid Schiff (PAS) and Masson’s trichrome] and immunohistochemical (CD34, marker of vascular endothelium) staining methods.

**Materials and methods**

The study group included both control (non-cryopreserved) and cryopreserved human ovaries. Also tissue sections from lymph nodes, squamous cell carcinoma, leukaemia cell lines, normal skin and liver were used as tissue-specific positive controls for Bcl-2, p53, apoptotic cells, PAS and Masson’s trichrome staining.

**Patients in the cryopreservation group**

The Institutional Review Board of The Cleveland Clinic Foundation approved this study. The first patient, aged 46 years, was presented with menorrhagia and symptomatic fibroid uterus, who underwent total vaginal hysterectomy with bilateral salpingo-oophorectomy, McCall culdoplasty and cystoscopy. Intraoperative findings included an 8–10 week-sized uterus and normal-appearing ovaries. The second patient, aged 44 years, was presented with severe premenstrual dysphoric disorders, who underwent laparoscopic bilateral salpingo-oophorectomy. Normal-appearing uterus, tubes and ovaries were detected. Both ovaries and their pedicles, from both patients, were processed immediately as detailed below.

**Patients in the control group**

In the control group, formalin-fixed paraffin-embedded ovarian tissue was obtained from the Archives of the Department of Pathology, Assiut University Hospitals. Four ovarian tissue specimens were obtained from two women, 44 and 47 years old. The first was diagnosed with irregular uterine bleeding due to uterine fibroids and was treated by total abdominal hysterectomy with bilateral salpingo-oophorectomy. The second underwent total vaginal hysterectomy with bilateral salpingo-oophorectomy due to pelvic organ prolapse. The ovaries obtained from both patients appeared normal with a smooth white convoluted surface and firm consistency.

**Cryopreservation**

**Cryopreservation of the intact ovary**

We adopted our previous protocol described in 2003 (Bedaiwy et al., 2003). Briefly, immediately after oophorectomy, one ovary (from each patient) was perfused with heparinized Ringer’s solution followed by perfusion and immersion in a bath containing the cryoprotective mixture which was composed of Leibovitz L-15 medium (Irvine Scientific, Santa Ana, CA, USA), 10% fetal calf serum (FCS) (Irvine Scientific) and 1.5 M dimethylsulphoxide (DMSO) (Sigma, St. Louis, MO, USA). Both the ovarian vessels and excess hilar tissue were dissected, and ovarian ligaments were trimmed. The ovaries were perfused via the ovarian artery with the cryoprotective mixture using Horizon Modular Infusion System (MCGaw, Irvine, CA, USA) to maintain a flow rate at 1.3 ml/min with continuous replenishment of the reservoir. After perfusion, the ovary was bisected and transferred to two cryovials 12.7 × 5.0 mm each. The ovarian cortical strips were cryopreserved human ovaries. Also tissue sections from lymph nodes, squamous cell carcinoma, leukaemia cell lines, normal skin and liver were used as tissue-specific positive controls for Bcl-2, p53, apoptotic cells, PAS and Masson’s trichrome staining.

**Cryopreservation of the ovarian cortical strips**

The other ovary, for each patient, was divided into ovarian cortical strips of 1.0 × 1.0 × 5.0 mm3 each. The ovarian cortical strips were prepared as described by Gosden et al. (1994) and cryopreserved using the previously mentioned protocol but without perfusion.
**Thawing**

**Thawing of the intact ovary**

One week later, the vials were removed from the dewar and held for 1 min at room temperature before plunging in a bath of water at 37°C with gentle shaking. The contents of the vials were quickly emptied into a Petri dish containing Leibovitz L-15 medium supplemented with 10% FCS. The ovaries were washed and immediately perfused with Leibovitz L-15 medium supplemented with 10% FCS using a flow rate of 1.3 ml/min for 20 min. The cryoprotectant was gradually eliminated by pumping Leibovitz L-15 supplemented with 10% FCS into the reservoir (Bedaiwy et al., 2003).

**Thawing of the ovarian cortical strips**

Ovarian cortical strips were thawed using the same procedure as for intact ovary. Then, the strips were washed and held in Leibovitz L-15 containing 10% FCS for 20 min.

**In vitro assessments of the C–T and control ovarian tissues**

**Evaluation of the ovarian follicular viability in the C–T ovarian tissue**

The cryopreserved ovarian tissues were evaluated for follicular viability in terms of plasma membrane function and structural integrity by the Trypan Blue exclusion test. Ovarian fragments were thinly sectioned in Leibovitz L-15 medium supplemented with 1 mg/ml (200 IU/ml) type 1 collagenase (Sigma), incubated at 37°C for 2 h and pipetted every 30 min. Collagenase activity was inhibited by 50% FCS. The suspension was filtered through a 70-μm nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at 400 g for 5 min. The precipitate was diluted with 50 ml of Leibovitz L-15 medium and kept in a water bath at 37°C. Trypan Blue (0.4%, Sigma) was added to the suspension containing the follicles (20 μl), deposited on a glass slide and examined under inverted microscope (×400). For each fragment, 100 small intact follicles were examined while the partially or completely denuded oocytes were excluded.

Both the number of stained cells and the total number of cells were counted. The percentage of viable cells was determined by calculating the percentage of unstained cells.

**Histological evaluation of the ovarian tissues for the features of follicular health and atresia in the C–T and control ovarian tissues.**

The maturing follicle is composed of an oocyte with granulosa layer and lacks reticulum. The hallmark of the healthy follicles include intact oocyte, intact membrana granulosa, lack of necrosis and the presence of few pyknotic nuclei (<5% pyknotic nuclei) in this layer. The atretic follicles are characterized by the presence of attenuated membrana granulosa, loosely attached granulosa cells and increased number of pyknotic nuclei (>5% pyknotic nuclei) (Wood et al., 1997). Initially, the ovarian follicles were sorted into two groups, healthy and atretic, following Wood et al. (1997).

**Histological evaluation of apoptotic changes in the C–T and control ovarian tissues.**

Evaluation of apoptosis was performed using combined histological and TUNEL assays. The results were expressed as mean and SEM. The histological criteria of apoptosis included condensed nuclear fragments, nuclei with marginated chromatin, multiple nuclear fragments, a single condensed nucleus, membrane-bound structures containing variable amounts of chromatin and eosinophilic cytoplasm (Kerr et al., 1972).

**DNA fragmentation assay for the detection of apoptotic cells in the C–T and control ovarian tissues**

To evaluate the apoptotic changes, we examined cellular morphology with the TUNEL assay, using the commercially available QIA33TDT-FragEL™ kit (Oncogen Research Products, Boston, MA, USA) following other groups (Hussein et al., 2006). Positive controls consisted of HL60 promyelocytic leukaemia cells and HL60 cells incubated with 0.5 μg/ml actinomycin D for 19 h to induce apoptosis. Some ovarian specimens were used as negative controls by substituting distilled water for deoxynucleotidyl transferase (Gavrieli et al., 1992; Liu et al., 1995).

Results of the TUNEL assay were evaluated following other groups (Hussein et al., 2006). All slides were examined at ×400 magnification. Ten different areas of each follicular wall were examined, and apoptotic index was determined as mean number of positively stained cells (Hussein et al., 2006).

**Immunohistochemical evaluation of p53, Bcl-2 and CD34 protein expressions in the C–T and control ovarian tissues**

To examine the prosurvival, proapoptotic proteins and the status of the blood vessels, we used immunoperoxidase staining methods and monoclonal antibodies targeting p53, Bcl-2 and CD34 proteins. Briefly, ovary sections mounted on glass slides were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.6% H2O2. Sections were then immersed in the retrieval solution (10 mM sodium citrate buffer, pH 6.0) and subjected to heat-induced antigen retrieval for 20 min (microwave at ~750 W). Non-specific protein binding was blocked with 10 min exposure to 10% normal goat serum. Sections were then incubated with primary antibodies for 1 h at room temperature. The following primary mouse monoclonal antibodies were used: clone DO-7, Catalogue No. M7001 and clone 124, Catalogue No. M0889 for p53 and Bcl-2, respectively (DAKO, Carpinteria, CA, USA). Anti-CD34 monoclonal QBE9d10 (Novoastro, Newcastle, UK) was used to examine the blood vessels. After brief rinsing in phosphate-buffered saline (PBS), a catalyzed signal amplification system (K1500, DAKO) was used according to the manufacturer’s instructions. Two observers independently evaluated the stained slides.

**Positive controls**

Sections from reactive lymphoid hyperplasia (lymph node), p53-positive squamous cell carcinoma and haemangioma were used as positive controls for p53, Bcl-2 and CD34 staining, respectively.

**Negative controls**

Additional sections of the tissues were stained in parallel but with omission of the primary antibody.

**PAS and Masson’s trichrome stains**

PAS stains carbohydrate moieties and basement membrane reticulin and therefore delineates the wall of the blood vessels. Masson’s trichrome stains myofibres, indicating skeletal or smooth muscle differentiation (versus non-muscle fibres). Thus, it stains the smooth muscle of the blood vessels.

**Evaluation of Bcl-2 and p53 immunostaining**

For the evaluation of Bcl-2 and p53 immunoreactivities, several rules were followed: (i) corresponding sections stained by haematoxylin and eosin were examined; (ii) sections were examined to detect the sites of the antibody positivity; (iii) a higher power magnification was used to evaluate the immunostaining; (iv) sections were examined independently by two observers and (v) Bcl-2 and p53 positivity was identified as diffuse brown cytoplasmic and nuclear staining, respectively. The numbers of follicles with positive and negative reactivity were counted in the ovarian tissues (Hussein et al., 2006).
Evaluations of the status of the blood vessels in the C–T and control ovarian tissues

We chose CD34, as it is more sensitive than other markers for the detection of endothelial cells in the ovary. Microvessel density (MVD) was assessed by light microscopy following Weidner et al. (1991). Ovarian tissue was scanned at low magnification (×40 and ×100) to select the areas that showed the most intense vascularization (hot spots). Individual microvessels were counted in three fields at ×200 magnification (×20 objective lens and ×10 ocular lens; 0.7386 mm²/field). The final MVD was the mean value obtained from the counts of the three fields. MVD was expressed as mean (SD) (vessel/mm²). Any immunostained endothelial cells or endothelial cell clusters that were clearly separated from the adjacent microvessels were considered as a single and countable microvessel. Vessel lumens were not a prerequisite for a structure to be defined as a microvessel, and red blood cells were not used to define a vessel lumen (Weidner et al., 1991).

Statistical analysis

Analysis of variance (ANOVA) and Student’s t-tests with a statistical significance of $P < 0.05$ were performed. Calculations were done with the Statistical Package for the Social Sciences for Windows, version 10.0.

Results

Follicular viability in the C–T ovarian tissue

In the 46-year-old patient, the primordial follicular viability was 75 and 81% in C–T intact ovary and C–T ovarian cortical strips, respectively. In the 44-year-old patient, these values were 78 and 83%, respectively. The average value for both intact ovaries (76.5%) was comparable with that for cortical strips (82%). A summary of these data is presented in Table I.

Table I. Morphological analysis of the non-cryopreserved (control), cryopreserved intact ovary and cortical ovarian tissue strips

<table>
<thead>
<tr>
<th>Aspects</th>
<th>Cryopreserved ovarian tissue</th>
<th>Non-cryopreserved ovarian tissue</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ovarian cortical strips</td>
<td>Intact ovary</td>
<td>Cortical strips and intact ovaries</td>
</tr>
<tr>
<td>Follicular viability (unstained cells/total cells (%))</td>
<td>82/100 (82)</td>
<td>76/100 (76.5)</td>
<td>79/100 (79)</td>
</tr>
<tr>
<td>Healthy follicles</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Atretic follicles</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Apoptotic index</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Apoptotic changes</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Healthy follicles</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Bcl-2 protein expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy follicles</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Atretic follicles</td>
<td>Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Status of the blood vessels</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
</tr>
<tr>
<td>Microvessel density (mean ± SEM)</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
</tr>
<tr>
<td>Masson’s trichrome stain</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
<td>Persistent vascularity</td>
</tr>
</tbody>
</table>

The healthy follicles include primordial, primary and secondary follicles. Plasma membrane function and structural integrity were determined by the Trypan Blue exclusion test (viability assay), in which both the number of stained follicles and the total numbers of follicles (100 follicles) were counted. The percentage of viable cells was determined by calculating the percentage of unstained cells. Follicular health and atresia were determined using the histological methods and were reported in descriptive terms. Apoptotic index was evaluated using combined terminal deoxynucleotidy transferase (TdT)-mediated dUTP-digoxigenin nick-end labelling and histological methods where 10 different areas of each follicular wall were examined. The apoptotic index was calculated as mean number of positively stained cells. For the evaluation of Bcl-2 and p53 immunoreactivities, the numbers of follicles with positive and negative reactivity were counted in the ovarian tissues. NA, not applicable; NS, not significant ($P > 0.05$).
control and cryopreserved ovarian tissues, rare weak Bcl-2 expression was seen both in the healthy follicles and in the stromal cells (Figures 3 and 4). p53 expression (nuclear) was virtually absent in the healthy primordial, primary and secondary follicles, germinal epithelium and stromal cells subjected to warm ischaemia (Figures 3 and 4). Bcl-2 immunoreactivity was observed more frequently in the secondary follicles, followed by primary and primordial follicles. In these follicles, Bcl-2 immunoreactivity was restricted to granulosa cells. The theca cells were never immunoreactive regardless of the developmental stage of the follicles (Figures 3 and 4).

**DNA fragmentation assay (TUNEL) in the control and C–T ovarian tissues**

None of the negative controls stained with TUNEL technique showed any immunoreactivity, whereas signal-positive TUNEL staining was observed in the positive controls (Figure 5). In the
control ovarian tissues, granulosa cells with the histologic features of apoptosis were observed in the atretic follicles, either within the central region of membrana granulosa layer or loosely attached with membrana granulosa near its antral surface or in the antral follicular fluid. The healthy (primordial, primary and secondary) follicles were TUNEL negative. TUNEL-positive signals were detected in the atretic follicles. This was evident as punctuated brown staining of the fragmented nuclei of granulosa cells. The positively stained nuclei were observed either in the central layers of the membrana granulosa, at the antral surface or floating in the follicular atrium. DNA fragmentation was absent both in the interstitial cells and in the theca cells of the atretic follicles. Evaluation of apoptosis in the cryopreserved ovaries revealed similar findings: (i) the healthy (primordial, primary and secondary) follicles were TUNEL negative; (ii) TUNEL-positive signals were detected in the atretic follicles. This was evident as punctuated brown staining of the fragmented nuclei of granulosa cells (Figure 5) and (iii) the mean values of apoptotic cells in the intact cryopreserved ovarian tissues were comparable with those in the ovarian cortical strips and control ovarian tissues (1.8 ± 0.1 versus 1.7 ± 0.1 versus 1.8 ± 0.1, respectively). The results are summarized in Table I.

Evaluating CD34 protein expression in the control and C–T ovarian tissues

Endothelial immunostaining with anti-CD34 as well as evaluation of the basement membrane (PAS) and muscle coat (Masson’s trichrome) of ovarian blood vessels demonstrates persistent vascularity in both cryopreserved and control ovarian tissues. Microvessels are heterogeneously distributed within the ovarian tissue. The mean values of ovarian MVD were 5.6 ± 0.9, 5.3 ± 0.8 and 5.3 ± 0.8 for control, cryopreserved intact and cortical ovarian tissues, respectively. A summary of these results is presented in Table I and Figures 6–8.

Discussion

In this investigation, the immediate post-thawing injury to the human ovary (cryopreserved both as a whole with its vascular pedicle and as ovarian cortical strips) was assessed using both histological and immunohistological methods. To achieve our goals, we established a study group formed of four patients. It included both control (non-cryopreserved) and cryopreserved human ovaries. Tissue sections from lymph nodes, squamous cell carcinoma, leukaemia cell lines, normal skin and liver were used as tissue-specific positive controls for the histochemical (PAS and Masson’s trichrome staining) and immunohistochemical (Bcl-2, p53 and apoptotic cells) parameters. Our investigation demonstrated the following findings: (i) the mean values of MVD, follicle counts and Bcl-2 protein expression were comparable between the cryopreserved and the control groups and (ii) no differences were seen between the two groups for the following parameters: healthy and atretic follicle counts, apoptotic index and Bcl-2 protein expression in the atretic follicles.

Ovarian tissue banking is more popular as a fertility preservation option for patients without a partner or those who cannot delay gonadotoxic chemotherapy for the sake of ovarian stimulation to produce oocytes for freezing or embryos for cryopreservation. With the limited long-term survival upon reimplantation with ovarian tissue strips, ovarian transplantation with microvascular anastomosis could help avoid accelerated follicular loss and improve the longevity of the ovarian grafts. This helps to guarantee an immediate revascularization and establishment of the graft function. Although successful in rat and sheep animal models (Wang et al., 2002; Bedaiwy et al., 2003), microvascular anastomosis of intact frozen–thawed ovaries remains technically
challenging. Moreover, cryopreservation of an entire organ with its vascular pedicle is much more complicated than ovarian cortical strips or suspended cell (oocyte) or group of cells (embryo).

In our previous study, we reported a 30% long-term patency rate of the ovarian vessels upon reimplantation of an intact frozen–thawed ovary in merino sheep (Bedaiwy et al., 2003). However, human ovaries are larger and may not be amenable to the same protocol. The technical challenges of the microvascular anastomosis could be circumvented adequately by appropriate training. On the contrary, the main determinant of success would be to develop an appropriate cryopreservation protocol of an entire ovary with its vascular pedicle. Adequate delivery of the cryoprotective agent (CPA) to almost every cell of the ovary is mandatory to guarantee an adequate post-thaw survival. Aiming at adequate CPA permeation to virtually every cellular component of the ovary, we used the Horizon Modular Infusion pump to ensure adequate CPA distribution throughout the ovarian substance. Infusing the CPA slowly through the ovarian artery and allowing it to come out of the ovarian vein ensures adequate use of the ovarian vascular channel as a delivery vehicle. To avoid intracellular ice formation, we used a slow-freezing protocol using the Planer freezer. The use of constant infusion pressure during the freezing and

**Figure 4.** Upper panel: Bcl-2 and p53 protein expressions in both cryopreserved intact ovary and ovarian cortical strips. Bcl-2 protein expression in the primordial (A, ×200), atretic (B, ×400) follicles and the ovarian stroma (C, ×200). The expression appears as golden yellow cytoplasmic staining. Note the lack of p53 protein expression in the primordial (D, ×400), atretic follicles (E, ×400) and the ovarian stroma/Graafian follicle (F, ×400) (arrowheads). Lower panel: Bcl-2 and p53 protein expressions in positive controls. Bcl-2 protein expression in the lymph node with reactive hyperplasia (G, ×200) and p53 protein expression in the cells of squamous cell carcinoma (H, ×400) (arrowheads).
ovarian follicles may be attributed to the presence of apoptosis-related proteins (caspase-3, caspase-8, Bcl-2 and actin) (Zhu et al., 1999) triggering signals and/or degradation of apoptosis-related proteins (caspase-3, caspase-8, Bcl-2 and actin) (Zhu et al., 1999; Schmidt-Mende et al., 2000). At the molecular level, cryopreservation and thawing had no effects on the values of apoptotic cell death and Bcl-2 and p53 protein expressions in both ovarian cortical strips and intact ovaries. A hypothesis to be tested is that cryopreservation and thawing can induce some effects on these values (apoptotic cell death and Bcl-2 and p53 protein expressions) over longer time intervals. This hypothesis stems from the fact that apoptosis is a dynamic process that requires active metabolism and usually takes several hours or even days to be executed (Hussein et al., 2006). These questions are open for future investigations. Apoptosis in the atretic follicles can effectively be present before the cryopreservation, but it does not seem to be increased by the cryopreservation process. Moreover, the presence of apoptotic changes in the atretic follicles supports the link between apoptosis and ovarian follicular atresia.

It is also possible that substances which stimulate apoptosis may have atretogenic effects on the ovarian follicles. The presence of Bcl-2 protein expression in the healthy ovarian follicles concurs with other reports (Tilly, 1996a,b; Felici et al., 1999) and suggests its possible physiologic role in the maintenance of follicular integrity. In support, mice lacking functional Bcl-2 protein possess reduced numbers of primordial follicles relative to their wild-type sisters (Ratts et al., 1995). The lack of p53 protein expression in the healthy follicles versus its presence of atretic ones concurs with previous studies and suggests lack of underlying DNA damage and therefore implies integrity of the genome in the healthy follicles (Hussein, 2005).

During organ transplantation, tissues are subjected to variable duration of ischaemic injury. In the heart, retina, liver and brain, ischaemic injury is associated with apoptosis as well as Bcl-2 and p53 alterations. Namely, the induction of Bcl-2 and p53 can occur as early as 0.5 and 1 h, respectively, following ischaemia (Peralta et al., 2002; Wang et al., 2003). Apoptosis requires active cell metabolism that could not be complete within 30 min and could therefore not alter the status of the follicles within an ovary. A hypothesis to be tested is that the differences observed in this investigation are the result of outright lysis (ischaemia could make cells more prone to lysis). It would be interesting for future investigations to examine this hypothesis.

Our investigation revealed the presence of persistent vascularity and comparable MVD in both control and cryopreserved groups. These findings indicate that our freeze–thawing protocol has no detrimental effects on the integrity of the vascular network of the ovarian tissue. Of note, the status of the ovarian vascular supply is central to dynamic changes occurring during the normal ovarian cycle. Follicular growth and the development of the corpus luteum are dependent on intact vasculature.
The selection of a dominant follicle in monovular species is associated with angiogenesis. Moreover, selected follicles possess a more elaborate microvascular network than other follicles. The vasculature also plays a key role in the delivery of cholesterol to luteal cells for progesterone biosynthesis (Carr et al., 1981; Goede et al., 1998).

Many studies indicate that the ovaries of older women are smaller than those of young women. They are composed
Figure 7. Upper panel: Evaluation of the status of the blood vessels both in cryopreserved intact ovary and in ovarian cortical strips. CD34 protein expression within the ovarian stroma. The ovarian blood vessels show persistent vascularity and moderate density of microvessels. The endothelial cells of the microvessels are stained brown by anti-CD34 antibodies (arrowheads) (×400). Lower panel: Evaluation of the status of the blood vessels both in cryopreserved intact ovary and in ovarian cortical strips. Periodic acid Schiff (PAS) and Masson’s trichrome stain within the ovarian stroma. The ovarian blood vessels show persistent vascularity and moderate density of microvessels. The muscle coat (Masson’s trichrome) and basement membrane (PAS stain) outline the blood vessels (arrowheads). (All pictures are on scale of ×200 except the lower right one, ×400.)
changes have an impact on our results awaits further confirmation. More sensitive to the freezing–thawing process (Couzinet et al., 2001; Longcope, 2001). In this investigation, the study group was formed of older patients knowing in advance the histopathological and follicular implications of ageing on the ovarian structure. Whether the aforementioned age-related changes have an impact on our results awaits further confirmation in younger age group upon ethical approval.

In this study, we assessed injury of the ovarian tissue in the period immediately after thawing. Our investigation provides further evidence that intact human ovary could be cryopreserved using a slow-freezing protocol. We have demonstrated comparable survival rates of follicles and limited molecular alterations between C–T intact ovaries and C–T ovarian cortical strips. The information herein reported is provided for the period immediately after thawing. The changes associated with long-term injury mandates further investigations.

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References


Figure 8. Morphological analysis of the control and cryopreserved intact ovary and cortical ovarian tissue strips. As compared with the control group, the mean values of microvesSEL density, follicle counts and Bcl-2 protein expression tended to be higher in the cryopreserved group; however, the differences were not significant (P > 0.05). No differences were seen between the two groups for the following parameters: healthy and atretic follicle counts, apoptotic index and Bcl-2 protein expression in the atretic follicles.


Tilly JL (1996b) The molecular basis of ovarian cell death during germ cell attrition, follicular atresia, and luteolysis. Front Biosci 1,d1–d11.


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