BACKGROUND: Chemo- or radiotherapy can induce premature ovarian failure (POF), and ovarian tissue cryopreservation and transplantation may be proposed to restore ovarian function. Our aim was to evaluate the quality of oocytes and embryos derived from frozen-thawed transplanted ovarian tissue.

MATERIALS AND METHODS: Women were 21–28 years old at tissue cryopreservation. Nine women suffering POF following chemotherapy with or without radiotherapy underwent orthotopic ovarian tissue transplantation. After 12 months of spontaneous cycles without pregnancy, oocyte retrieval was performed in four patients during mildly stimulated or spontaneous cycles. ICSI was performed in all cases, with embryo transfer on day 3. Light and electron microscopy was used to study oocytes and embryos.

RESULTS: Signs of ovarian function restoration (estradiol peak, decreased FSH, follicular development) began 16–26 weeks after reimplantation. Twenty-one oocyte retrieval attempts were made. At least one oocyte was collected in 15 cases, giving an empty follicle rate per retrieval of 29% (6/21). Sixteen oocytes were recovered, of which 6 were abnormal or immature (38%) and 10 (62%) were in metaphase II (MII). Three MII oocytes failed to fertilize, two showed abnormal fertilization and five normal MII oocytes successfully fertilized with subsequent normal embryo development (Grade 2), yielding an embryo transfer rate of 24% per retrieval. No pregnancy occurred.

CONCLUSIONS: IVF in women with orthotopically grafted frozen-thawed ovarian tissue involves a higher risk of empty follicles, abnormal or immature oocytes, and low embryo transfer rates.

Key words: IVF / orthotopic autotransplantation / ovarian tissue cryopreservation / fertility preservation / chemotherapy

Introduction

In 2008, around 692,000 women in the USA were diagnosed with some form of invasive cancer. Approximately 8%, or 55,000, of these women were under the age of 40 years (American Cancer Society, 2001; Jemal et al., 2008).

Gonadal dysfunction is a common consequence of cytotoxic chemotherapy or radiotherapy. Before initiating such potentially sterilizing treatments, three non-exclusive methods can be proposed to preserve female fertility: ovarian tissue cryopreservation, oocyte cryopreservation and embryo cryopreservation. For those who have a steady partner, IVF with embryo cryopreservation should be considered. However, ovarian stimulation is time consuming and, in most cancer patients, chemotherapy cannot be delayed. In these cases, as well as in prepubertal girls, cryopreservation of ovarian tissue is currently proposed to preserve fertility.

To date, transplantation of frozen-thawed ovarian tissue has yielded six live births worldwide. All these pregnancies were obtained after orthotopic transplantation, either after natural conception (Donnez et al., 2004; Demeestere et al., 2007; Silber et al., 2008) or IVF (Meirow et al., 2005; Andersen et al., 2008).

Little is known about the quality of oocytes after transplantation of cryopreserved ovarian tissue.

In 2005, Meirow et al. reported a live birth after orthotopic autotransplantation of cryopreserved ovarian tissue in a patient with premature ovarian failure after chemotherapy. Nine months after orthotopic transplantation, following a modified natural cycle, a single mature oocyte was retrieved and inseminated; 2 days later, a
4-cell embryo was transferred and the patient became pregnant. Andersen et al. (2008) published a series of six women who underwent orthotopic autotransplantation of cryopreserved ovarian tissue. Two women became pregnant after oocyte retrieval during natural cycles. Both delivered a healthy baby.

After heterotopic transplantation of frozen-thawed ovarian tissue (usually under the skin of the abdomen or arm), IVF is mandatory. Oktay et al. (2004) obtained one 4-cell embryo and Kim et al. (2008) 4 poorly developed embryos after heterotopic transplantation, but no live births have yet been reported after heterotopic transplantation of cryopreserved ovarian tissue.

The aim of this study was to evaluate the quality of oocytes and embryos after oocyte retrieval in natural or modified natural cycles in patients with orthotopically transplanted frozen-thawed ovarian tissue.

**Materials and Methods**

**Patients and grafting**

In our department, ovarian tissue cryopreservation has been performed since 1996. So far, 330 women have had their ovarian tissue cryopreserved before chemotherapy with or without radiotherapy and nine have undergone reimplantation (for review and patient details, see Donnez et al., 2006, 2008), with all of them recovering ovarian function. In four patients, IVF was proposed after 12 months of spontaneous menstrual cycles without pregnancy, despite normal sperm parameters.

The characteristics of patients undergoing cryopreserved ovarian tissue transplantation and oocyte retrieval are described in Table I. They were all between 21 and 28 years of age at the time of cryopreservation, but two patients had already received first-line chemotherapy. Note that three patients underwent bone marrow transplantation and another took cyclophosphamide daily for 5 years. One patient also received total body irradiation (TBI).

The amount of ovarian cortical tissue cryopreserved ranged from 10 cortical strips (c.10 x 4 x 1 mm) to a whole ovary. The slow freezing cryopreservation protocol used and rapid-thawing procedures are described in Donnez et al. (2008). Ovarian tissue fragments were grafted orthotopically onto the native ovary after the cortex of that ovary had been removed, according to a previously described technique (Donnez et al., 2006; 2008). The amount of grafted tissue was less than half of the total cryobanked tissue, which represented a total surface of 160 to 400 mm² per graft. The first signs of ovarian function restoration [estradiol (E₂) peak, decrease in FSH, ultrasound showing follicular development] occurred between 16 and 26 weeks after reimplantation. There were no signs of disease recurrence in any patients with malignant disease, and restoration of ovarian endocrine function was observed in all cases (Donnez et al., 2008).

**Stimulation cycles**

In total, 21 attempts at oocyte retrieval were carried out. Of these 21 attempts, 13 were made during modified natural cycles with mild stimulation with recombinant FSH or hMG, and GnRH antagonists. The daily stimulation dose of FSH was 150 IU. When the leading follicle reached a mean diameter of 12 mm, daily administration of 0.25 mg of GnRH antagonist (Cetrorelix, Serono, London, UK) was initiated up to the day before ovulation trigger. Ovulation was triggered by 10 000 IU of hCG when the leading follicle was at least 15 mm in size. Oocyte retrieval was performed 35 h later. An ovum aspiration single lumen needle with a tap (CCD Laboratories, Paris, France) was inserted into a needle guide attached to an endovaginal ultrasonic probe. The needle was connected to a suction pump (pressure maintained at 100–120 mmHg) and fluid from the follicle was aspirated. If no oocytes were directly retrieved, the follicle was then flushed two to six times with flushing medium (Medicult, Jyllinge, Denmark). Oocyte retrieval was always performed by the same consultant (CP).

Eight attempts at oocyte retrieval were made during unstimulated cycles, either with a spontaneous LH surge followed by oocyte retrieval less than 24 h later, or with hCG administration. Oocyte insemination was always achieved by ICSI. Oocyte recovery, fertilization and embryo culture were performed according to the protocols published by our team (Van Langendonckt et al., 2001).

**Morphological evaluation of oocytes and embryos**

Oocyte maturation was assessed by evaluating the presence of a polar body (PB), and oocyte quality was determined according to classic parameters such as cytoplasm homogeneity, perivitelline space (PVS) size and content, and zona pellucida integrity. Fertilization was considered morphologically normal when two pronuclei (PN) were observed, and abnormal when only one pronucleus

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**Table I Ovarian tissue cryopreservation and oocyte retrieval: patient characteristics**

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Age at OTC (years)</th>
<th>Chemo before OTC</th>
<th>Treatment after OTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Drepanocytosis</td>
<td>21</td>
<td>None</td>
</tr>
<tr>
<td>Patient 2</td>
<td>HL</td>
<td>23</td>
<td>First-line ABVD</td>
</tr>
<tr>
<td>Patient 3</td>
<td>NHL</td>
<td>28</td>
<td>First-line ACVB</td>
</tr>
<tr>
<td>Patient 4</td>
<td>Wegener’s G.</td>
<td>22</td>
<td>None</td>
</tr>
</tbody>
</table>

OTC: ovarian tissue cryopreservation; Chemo: chemotherapy; HL: hodgkin’s lymphoma; NHL: non-Hodgkin’s lymphoma; Wegener’s G.: wegener’s granulomatosis; ABVD: adriamycin, bleomycin, vinblastine, dacarbazine; ACVB: doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone; BMT: bone marrow transplantation; MOPP-BEAM: mustine, oncovin, procarbazine, prednisone–carmustine, etoposide, cytarabine, melphalan; TBI: total body irradiation.
or more than two PN were encountered. An absence of PN and the presence of only one PB constituted fertilization failure. Zygotes were cultured up to day 3 in G1 medium (Vitrolife, Kungsbacka, Sweden). On day 3, embryos were graded according to previously described criteria (Van Langendonckt et al., 2001), evaluating the number of blastomeres, the presence of fragments, the symmetry of the cells and the aspect of the cytoplasm. The embryos were graded from 1 to 4, with 1 denoting excellent and 4 denoting poor.

**Ultrastructural analysis**

Two metaphase I (M1) oocytes from patient 1 that were unsuitable for ICSI were processed for ultrastructural analysis.

Oocytes were fixed in 1.5% glutaraldehyde (TAAB, UK) in phosphate-buffered saline (PBS) solution. After fixation for 2–5 days at 4°C, the samples were rinsed in PBS, post-fixed with 1% osmium tetroxide (Agar Scientific, Stansted, UK) in PBS, and rinsed again in PBS. Oocytes were then embedded individually under a stereomicroscope in small blocks (width: 5 mm; height: 1 mm) of 1% agar (Dolmans et al., 2006), dehydrated through ascending series of ethanol, immersed in propylene oxide (solvent substitution) and embedded in Epon 812. Embedded samples were serially sectioned using a Reichert-Jung Ultracut E ultramicrotome. Semithin sections (1 μm thick) were stained with toluidine blue, examined by light microscopy (LM) (Zeiss Axioskop, Germany) and photographed using a digital camera (Leica, DFC230). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids and contrasted with saturated uranyl acetate followed by lead citrate. Sections were examined and photographed using a Zeiss EM109 electron microscope at 80 kV (Nottola et al., 2008).

By LM and transmission electron microscopy (TEM), we evaluated the following oocyte parameters: shape and dimensions, zona pellucida, width and content of the PVS, oolemma integrity, ooplasm characteristics, including the distribution and quality of ooplasmic organelles, and the presence of abnormal vacuoles.

**Results**

In all nine cases, restoration of ovarian function was observed after transplantation. It took between 16 and 26 weeks after transplantation before a rise in E2 and a drop in FSH concentrations were observed (for details, see review by Donnez et al., 2006).

Results on the quality of oocytes and embryos derived from orthotopically transplanted frozen-thawed ovarian tissue are presented in Table II.

**Stimulation cycles**

In total, 21 oocyte retrieval attempts were made. Thirteen were carried out during modified natural cycles adding recombinant FSH, GnRH antagonists and hCG, and eight during spontaneous cycles, four of which showed a spontaneous LH surge.

In 6 out of 21 oocyte retrievals, no oocytes were retrieved from aspirated follicles (3 natural cycles and 3 stimulated cycles), giving an empty follicle rate of 29% per retrieval. In 14 cases, one oocyte was retrieved, and in 1 case, two oocytes. Among the 16 recovered oocytes, 6 were classified abnormal, ruling out ICSI. Ten oocytes (62%) were in metaphase II (MII) and ICSI was performed. Among these 10 MII oocytes, 3 failed to fertilize and 2 showed abnormal fertilization. The remaining five normally fertilized oocytes developed into cleavage-stage embryos. Five embryos were therefore transferred after 3 days of culture. Thus, 24% (5/21) of cycles resulted in embryo transfer. Unfortunately, none of these embryos implanted.

**Morphological evaluation of oocytes and embryos**

Six oocytes were classified abnormal and were not used. Two of them were M1-stage oocytes (both from patient 1), one of which exhibited a granular PVS (Fig 1a). These M1 oocytes did not mature in vitro and were fixed for ultrastructural analysis. Three oocytes of unknown maturation status showed lysis at different stages, presenting as a partially preserved cytoplasm (Fig 1b) or empty zona pellucida (Fig 1c,d,e). The last abnormal oocyte was a 2-cell embryo-like structure (Fig 1f).

Ten MII oocytes were obtained. Their morphology appeared variable (Fig 2a–f), but all these oocytes were used for ICSI. Six out of 10 MII oocytes had a totally normal structure (Fig 2c–f), while 4 exhibited an altered cytoplasm: 2 were irregular, dark, vacuolar and granular (Fig 2a), 1 was ovoid (Fig 2b), and 1 showed two large vacuoles.

After ICSI, half of the mature oocytes were found to have fertilized (5/10), two showed abnormal fertilization with one pronucleus and three failed to fertilize.

Five embryos developed, all from the normally structured MII oocytes, except one that developed from a vacuolar MII oocyte. On day 3, they were at the following stages: a 7-cell, 10-cell, morula and two 12-cell embryos. All the embryos were of intermediate quality (Grade 2), with less than 20% fragments, but most of them showed cells of different sizes (Fig 3a,b,d) or poor cell adhesion (Fig 3a,d).

**Ultrastructural analysis**

Two immature oocytes were fixed in glutaraldehyde for microscopic evaluation. One was lost during processing and the other was analysed.

By LM, the oocyte appeared regularly rounded, 110 μm in diameter, surrounded by a continuous zona pellucida, with a slight degree of vacuolization of the cytoplasm and a dark inclusion in the central part of the cell (Fig 4a). At the periphery of the ooplasm, chromosomes were seen condensed in clumps. The PVS looked abnormally enlarged (>20 μm). No first PB was observed in the PVS.

By TEM, zona pellucida texture showed increased density in its inner part, but appeared looser in its outer areas (Fig 4b). In the PVS and zona pellucida, follicular cell projections were observed (Fig 4a,b). The oolemma was found to be continuous and long microvilli were seen projecting from the oolemma surface into the PVS in the areas where the zona pellucida was in close contact with the oolemma (Fig 4b,c). By contrast, only a few microvilli were detected where the PVS was enlarged (Fig 4d). A few rounded and dense cortical granules (CGs) ranging in diameter from 250 to 350 nm were found in the ooplasm and scattered just beneath the oolemma (Fig 4e).

Ooplasmic organelles were abundant and quite uniformly distributed. At higher magnification, the organelles mainly consisted of rounded mitochondria with a few peripheral or transversal cristae (Fig. 4d). Mitochondria were seen either dispersed in the ooplasm...
<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Chemo before OTC</th>
<th>Stimulation protocol</th>
<th>Foll size (mm) day − 2</th>
<th>E2 (pg/ml) day − 2</th>
<th>LH (mIU/ml) day − 2</th>
<th>Oocyte number</th>
<th>Oocyte maturation/aspect</th>
<th>Fertilization</th>
<th>Day 3 embryos stage/score</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>P1</td>
<td>No</td>
<td>1: hMG + antag + hCG</td>
<td>19</td>
<td>269</td>
<td>15</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2: hMG + antag + hCG</td>
<td>17</td>
<td>174</td>
<td>4.4</td>
<td>1</td>
<td>M I</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3: rec FSH + antag + hCG</td>
<td>20 + 15</td>
<td>103</td>
<td>5.6</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4: rec FSH + antag + hCG</td>
<td>17</td>
<td>58</td>
<td>42.5</td>
<td>1</td>
<td>One oocyte: lysis (empty zona pellucida)</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5: spontaneous</td>
<td>17 (day of OPU)</td>
<td>89</td>
<td>86.5</td>
<td>1</td>
<td>One oocyte: lysis (abnormal)</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6: spontaneous</td>
<td>17 (day of OPU)</td>
<td>53</td>
<td>64.7</td>
<td>1</td>
<td>M I: granular PVS</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7: spontaneous</td>
<td>19 (day of OPU)</td>
<td>62</td>
<td>62.8</td>
<td>1</td>
<td>M II, brown, vacuolar, granular</td>
<td>Abnormal</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>Hodgkin's lymphoma</td>
<td>Yes</td>
<td>1: spontaneous</td>
<td>17</td>
<td>276</td>
<td>132</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2: spontaneous + hCG</td>
<td>21 + 16</td>
<td>502</td>
<td>15</td>
<td>1</td>
<td>Empty zona pellucida and extruded cytoplasm</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3: spontaneous + hCG</td>
<td>22 + 19 + 16</td>
<td>395</td>
<td>29.8</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4: hMG + antag + hCG</td>
<td>18</td>
<td>69</td>
<td>14.7</td>
<td>1</td>
<td>2-cell embryo-like structure</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5: spontaneous + hCG</td>
<td>24</td>
<td>576</td>
<td>30</td>
<td>1</td>
<td>M II, ovoid</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6: hMG + antag + hCG</td>
<td>16</td>
<td>169</td>
<td>4.8</td>
<td>1</td>
<td>M II</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Yes</td>
<td>1: hMG + antag + hCG</td>
<td>17 + 10</td>
<td>167</td>
<td>13.7</td>
<td>0</td>
<td>M II</td>
<td>Yes</td>
<td>7-cell, grade 2</td>
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<tr>
<td></td>
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<td>2: hMG + antag + hCG</td>
<td>18</td>
<td>164</td>
<td>10.2</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3: hMG + antag + hCG</td>
<td>18</td>
<td>314</td>
<td>8</td>
<td>1</td>
<td>M II</td>
<td>Yes</td>
<td>10-cell, grade 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4: hMG + antag + hCG</td>
<td>16</td>
<td>108</td>
<td>7.6</td>
<td>1</td>
<td>M II</td>
<td>Yes</td>
<td>morula, grade 2</td>
</tr>
<tr>
<td>P4</td>
<td>Wegener’s granulomatosis</td>
<td>No</td>
<td>1: spontaneous + hCG</td>
<td>16 + 14</td>
<td>122</td>
<td>16</td>
<td>0</td>
<td>No</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2: hMG + hCG</td>
<td>16</td>
<td>98</td>
<td>19.2</td>
<td>1</td>
<td>M II, brown, vacuolar, granular</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3: hMG + antag + hCG</td>
<td>15</td>
<td>77</td>
<td>11.2</td>
<td>1</td>
<td>MII, 2 vacuoles</td>
<td>Yes</td>
<td>12-cell, grade 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4: hMG + antag + hCG</td>
<td>17 + 15</td>
<td>241</td>
<td>6.0</td>
<td>2</td>
<td>2 MII</td>
<td>1 normal + 1 abnormal</td>
<td>12-cell, grade 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21 cycles</td>
<td>&gt;= 15 mm (15–27)</td>
<td>58–576</td>
<td>6 empty foll/21 cycles</td>
<td>6 'abnormal' oocytes/16 oocytes</td>
<td>3 no fertiliz'/10 MII oocytes</td>
<td>5 embryos</td>
<td></td>
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</table>

Abbreviations: antag: GnRH antagonists; PVS: perivitelline space; rec: recombinant; M I: metaphase I; M II: metaphase II; E2: estradiol.
Abnormal oocytes

Figure 1 Abnormal oocytes: (A) Oocyte from patient 1, cycle 6: immature oocyte at the intermediate stage between germinal vesicle and metaphase I (MI) with a granular perivitteline space (PVS) and a large vacuole. (B) Necrotic oocyte with a double zona pellucida, shown by arrow (patient 1, cycle 5). (C) Empty zona pellucida with no ooplasm visible inside (patient 1, cycle 4). (D,E) Empty zona pellucida (D) with its extruded cytoplasm (E) (patient 2, cycle 2). (F) 2-cell embryo-like structure (patient 2, cycle 4).

Metaphase II oocytes

Figure 2 Metaphase II oocytes: (A,B) Two poor quality MII oocytes. Oocyte from patient 1, cycle 7, is irregular, dark, vacuolar and granular and presents a granular PVS (A). Oocyte from patient 2, cycle 5, is ovoid (B). (C–F) Four MII oocytes showing a normal structure: oocytes from patient 2, cycle 6 (C), patient 3, cycle 3 (d), patient 3, cycle 4 (E) and patient 4, cycle 4 (F).
or in association with rounded smooth endoplasmic reticulum (SER) vesicles and with SER tubules, forming mitochondrion-vesicle complexes and mitochondrion-SER (M-SER) aggregates respectively. M-SER aggregates were generally small (2–5 μm) or medium-sized (8 μm), except one shown by LM to be a central dark inclusion (Fig 4a): by TEM, the latter corresponded to a large aggregate of SER microtubules surrounded by several mitochondria. Some degree of vacuolization was seen, the vacuoles varying in diameter from 0.1 to 2 μm (Fig 4b,c,d).

The nuclear envelope was not visible, but the nuclear material still had a spheroid configuration and looked paler and more homogeneous than the ooplasm (germinal vesicle breakdown) (Fig 4a,c). Condensed chromosomes were mainly organized in pairs and were seen in association with microtubules forming the meiotic spindle (Fig 4c).

**Discussion**

Here we report the largest series investigating the quality of oocytes and embryos after transplantation of frozen-thawed ovarian tissue. In the literature, only a few reports describe oocyte retrieval with a view to fertilization in patients undergoing autotransplantation of frozen-thawed ovarian tissue to orthotopic or heterotopic sites.

In case of orthotopic grafts, Meirow *et al.* (2007a) described 4 cycles of oocyte retrieval in one patient. Three cycles did not yield any oocytes but, during the fourth, a mature oocyte was obtained that developed into a 4-cell embryo, leading to a live birth. Andersen *et al.* (2008) reported 6 patients whose ovarian tissue was grafted to an orthotopic site. Twenty-eight cycles were carried out, 25 oocytes were recovered, and 11 embryo transfers were performed. Two patients delivered a healthy baby, and one miscarriage and one biochemical pregnancy were also reported. In this series, however, the number of empty follicles and oocyte and embryo quality were not mentioned.

In case of heterotopic ovarian grafts, IVF is mandatory. Oktay *et al.* reported their results of oocyte retrieval in 2 patients with implantation beneath the skin of the abdomen. In the first patient, 20 oocytes were retrieved during 8 percutaneous oocyte retrievals and only one 4-cell embryo developed but failed to implant (Oktay *et al.*, 2004). The second patient underwent one oocyte retrieval yielding one MII oocyte that did not fertilize (Oktay *et al.*, 2008). Kim *et al.* (2008) reported a 27-month follow-up of 2 cancer patients after heterotopic transplantation between the rectus muscle and fascia. Six oocytes were retrieved from the grafts and 4 embryos developed, but we have no information about the total number of oocyte retrievals, the empty follicle rate or the quality of the oocytes or embryos.

In a comparison of heterotopic and orthotopic sites, it should be pointed out that the rate of MII oocytes obtained after oocyte retrieval from heterotopic grafts, is significantly lower than that from orthotopic transplants.

Finally, in a recent review of results from published papers on ovarian tissue transplantation, Bedaiwy *et al.* (2008) reported the reproductive outcome after ovarian tissue grafting, but no data on oocyte or embryo quality.
Empty follicle rate and oocyte quality

We evaluated the empty follicle rate and the quality of oocytes and embryos after oocyte retrieval in patients with orthotopically transplanted ovarian tissue.

In our series, the empty follicle rate per oocyte retrieval was 29% (6/21). In the general population, the incidence of empty follicle syndrome in IVF cycles has been estimated to range from 0.6% to 7% (Stevenson and Lashen, 2008).

In the present study, 16 oocytes were recovered; 10 (62%) were in MII and 6 (38%) were classified abnormal, either failing to mature (n = 2) or showing severe abnormalities, ruling out ICSI (n = 4). Three of the abnormal oocytes showed lysis at different stages. In another study of 60 infertile patients with polycystic ovaries undergoing ovulation induction for ICSI, the mean number of immature oocytes (germinal vesicles) and degenerated oocytes not suitable for injection was calculated to be between 11.76 and 17.49%, according to treatment group (Papaleo et al., 2008).

The percentage of abnormal oocytes (immature or degenerated) is thus much higher (38%) in frozen-thawed transplanted tissue than in the general population undergoing ICSI.

How can we explain the high rate of empty follicles and oocyte anomalies in grafted tissue compared with the normal population?

(1) Women with transplanted frozen-thawed ovarian tissue have low ovarian reserves and elevated FSH concentrations, frequently failing to show oocytes in aspirated follicles. A possible cause of empty follicle syndrome is dysfunctional folliculogenesis, whereby early oocyte atresia occurs with an apparently normal hormonal response (Tsuiki et al., 1988). This dysfunctional

Figure 4 Germinal vesicle breakdown in an immature oocyte, unsuitable for ICSI. (A) Early MI oocyte surrounded by a thin and continuous zona pellucida and an abnormally large PVS, containing some material. Chromosomes are condensed at the periphery of the oocyte (white arrow). The ooplasm is slightly vacuolated and shows a large granular inclusion in its central part (black arrow). (B) The oocyte surface shows microvilli projecting into the PVS. Several mitochondria (dark spots) and vacuoles are seen in the ooplasm. Note the high density of the inner part of the zona pellucida. A follicular cell is attached to the zona pellucida (white asterisk). (C) The nuclear envelope is not visible, but the nuclear material presents a spheroid configuration. Condensed chromosomes (Cr) appear mostly arranged in pairs and microtubules are evident inside the nuclear area (white arrowhead). Scattered cortical granules are observed just beneath the oolemma (arrows). (D) Most of the mitochondria (M) appear dense, round or oval with a few peripheral or transverse cristae. Note the absence of oocyte microvilli in this area. V: vacuoles. Scale bar: 20 μm (A); 4 μm (B); 3.5 μm (C); 1 μm (D).
Asynchrony between granulosa cell maturation and oocyte maturation described in human ovarian xenotransplants (Nottola et al., 2008) could be responsible for the absence of oocytes upon follicle aspiration, with granulosa cells often found to be mature (regarding size, $E_2$ concentration), while oocytes are immature. Indeed, premature follicular cell activation was observed in xenografts (Nisolle et al., 2000; Oktay et al., 2000; Dolmans et al., 2007) and may result from stimulation of follicular growth by ischemia and oxidative stress after transplantation or result from the removal of some inhibitory mechanisms that normally operate in an intact ovary (Baird et al., 2004).

The oocyte itself could be damaged by freezing, thawing and transplantation, leading to a higher rate of empty follicles or oocyte alterations, while granulosa cells may be more resistant (Camboni et al., 2008). Indeed, it should be pointed out that granulosa cell function was maintained, as demonstrated by the high $E_2$ levels during the follicular phase and high progesterone levels in the mid-luteal phase. It therefore appears that granulosa cells and oocytes could either be in asynchrony during follicular maturation, or react in different ways to the freezing-thawing-grafting procedure. Indeed, the main difference between these 2 compartments is that the oocyte consists of a single large cell and granulosa cells are multiple. While some granulosa cell loss can be compensated for by mitosis of the surviving granulosa cells, this is impossible in the case of oocyte damage.

Can TEM analysis corroborate the hypothesis of cryopreservation-induced damage?

Even if the ultrastructural alterations described in our study are also encountered in MI oocytes during IVF in the general population (Sathananthan et al., 1993; El Shafie et al., 2000), we cannot exclude the possibility that they are induced by cryopreservation and transplantation procedures. Cytoplasm vacuolization is the non-specific feature most commonly observed in degenerative cells (Zamboni et al., 1972) and it has been described in human oocytes after different cryopreservation protocols (Ghetler et al., 2006; Nottola et al., 2007). Vacuolization, a reduced number of microvilli and increased PVS volume are signs of atresia and appear to be related to unsuccessful fertilization of human oocytes during IVF (Motta et al., 1988). These data underline the importance of communication between the oocyte and granulosa cells for normal follicle development (Camboni et al., 2008).

We observed a smaller number of CGs that appeared to be scattered in the sub-plasmalemmal area instead of arranged in a continuous layer (Zamboni et al., 1972), associated with high density of the inner part of the zona pellucida. Several authors have identified, by TEM, an abnormal reduction in CG number in the cytoplasm of mature human oocytes after different cryopreservation protocols (Schalkoff et al., 1989; Ghetler et al., 2006; Nottola et al., 2007). The decrease in CGs is due to premature release of CG contents, with consequent hardening of the inner part of the zona pellucida.

Thus, the oocyte shows ultrastructural alterations that may be attributed to the cryopreservation procedure, but we cannot exclude transplantation itself as a cause of these changes.

Fertilization rate

After ICSI, 5 out of 10 mature oocytes fertilized, 2 showed abnormal fertilization and 3 failed to fertilize. This fertilization rate (50%) is lower than in the general population, where the overall fertilization rate after ICSI was 70% in the study by Liu et al. (1995) and 68% in our series of patients treated during 2007 (personal data).

Here, three out of 10 oocytes failed to fertilize, giving a fertilization failure rate of 33%. In a study of 2732 ICSI cycles for male infertility, Liu et al. (1995) reported a total fertilization failure rate of 3%. It is known that total fertilization failure after ICSI is associated with sperm abnormalities or a low number and poor quality of oocytes (Liu et al., 1995). In our study, the sperm characteristics were normal and thus only poor quality oocytes could have been responsible for the higher rate of fertilization failure observed.

How can we explain the high fertilization failure rate?

Failure to fertilize and properly form PN following assisted reproduction technologies is likely to be linked to the inherent qualities of oocytes, and therefore oocyte maturation. Some oocyte deficiencies recently identified by Swain and Pool (2008) may possibly explain fertilization failure encountered after ICSI with oocytes retrieved from cryopreserved autotransplanted tissue.

In our study, anomalies of the zona pellucida, which appeared enlarged, double or fractured, and a decrease in the number of microvilli may go some way to explaining the fertilization failure observed. Indeed, microvilli distribution appears to be a dynamic process reflective of maturation status and quality of the female gamete (Suzuki et al., 1981; Sezen and Cincik, 2003; Cecconi et al., 2006). We also noted increased vacuolization that may well be associated with a low fertilization rate, as suggested by Rienzi et al. (2008).

Another explanation for the high rate of fertilization failure is inadequate or abnormal Ca$^{++}$ oscillation. In our study, ultrastructural analysis showed the presence of rounded SER vesicles forming aggregates with mitochondria. It has been reported that the presence of SER clusters in oocytes is associated with fertilization failure (Otsuki et al., 2004), as malfunctioning endoplasmic reticulum or mitochondria can cause abnormal Ca$^{++}$ oscillations. Ca$^{++}$-dependent signalling pathways regulate most processes associated with completion of oocyte maturation and fertilization events. Inadequate or inappropriate oocyte cytoplasmic maturation can result in abnormal fertilization due to insufficient protein synthesis or aberrant signalling pathways (Swain and Pool, 2008).

In our study, 2 of the 10 MII oocytes showed abnormal fertilization, with formation of only one PN. Failed sperm head decondensation or premature sperm chromatin condensation can both lead to abnormal fertilization with a single female PN (Swain and Pool, 2008). Sperm decondensation and formation of the male PN require sufficient levels of oocyte glutathione, which increase during oocyte maturation (Sutovsky and Schatten, 1997). The ability of oocytes to process sperm components and remodel paternal chromatin is therefore dependent upon their maturation status.

Thus, fertilization failure as well as abnormal fertilization can both be the result of oocyte activation abnormalities.

Embryo quality

Five embryos developed in our series. On day 3, they were at the following stages: 7-cell, 10-cell, morula and 2 × 12-cell embryos.
considered to be in the normal range. All these embryos were of intermediate quality (Grade 2 according to the classification of Van Langendonckt et al., 2001), with less than 20% fragments, but most of them showed cells of different sizes.

Twenty-four per cent (5/21) of our oocyte retrievals resulted in embryo transfer, but no pregnancy occurred.

**How can we explain the absence of implantation of these embryos?** Although only five embryos developed and were transferred, we may question why none of them implanted.

The prerequisite for a healthy embryo is a healthy oocyte. Poor oocyte quality could be the first reason for poor implantation, though these embryos reached normal cleavage stages for day 3 embryos.

Another equally important factor for embryo implantation is the condition of the endometrium. The implantation window is the period of maximal uterine receptivity, which lasts no more than 2 days. Previous chemotherapeutic treatment on endometrium or radiotherapy can damage blood vessels and thereby potentially impair endometrial receptivity. Cytokine expression alterations induced by prior chemotherapy or radiotherapy may also interfere with implantation potential (Fitzgerald et al., 2008). In our study, one patient underwent TBI and all four patients received high doses of chemotherapy. Although pregnancies are reported in young patients treated for cancer, a direct negative effect of chemotherapeutic treatment on endometrium cannot be excluded.

On the other hand, embryos derived from frozen-thawed-grafted ovarian tissue may show impaired signalling pathways.

No pregnancies were obtained after transfer of 5 embryos in our series. However, despite the possibility of impaired oocyte quality in frozen-thawed transplanted tissue, Andersen et al. (2008) reported two healthy births.

If oocytes are to be collected from ovarian grafts for the purposes of IVF, it will be important to maximize the graft yield of normal mature oocytes (Yang et al., 2006). Although six live births have been published after orthotopic transplantation of cryopreserved ovarian tissue, our study demonstrates that the quality of oocytes is not optimal, probably owing to a combination of different factors. Indeed, women with transplanted ovarian tissue are similar to poor responders and some inhibitory mechanisms, such as inhibin or anti-Mullerian hormone, are diminished in grafted tissue. Further investigation is required to determine why oocytes that develop in cryopreserved ovarian grafts show reduced developmental competence.

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