Possible improvements in human ovarian grafting by various host and graft treatments

Or Friedman1,2, Raoul Orvieto3,4, Benjamin Fisch1,5, Carmela Felz1,5, Enrique Freud5,6, Avi Ben-Haroush1,5, and Ronit Abir1,5,*

1Infertility and IVF Unit, Helen Schneider Hospital for Women, Rabin Medical Center, Beilinson Hospital, Petach Tikva 49100, Israel
2Rappaport Faculty of Medicine and Research Institute, Technion-Israel Institute of Technology, Haifa, Israel 3Infertility and IVF Unit, Department of Obstetrics and Gynecology, Brazilai Medical Center, Ashkelon 78278, Israel 4School of Medicine, Ben Gurion University of the Negev, Beer Sheva, Israel 5Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel 6Department of Pediatric Surgery, Schneider Children’s Medical Center of Israel, Rabin Medical Center, Petach Tikvah 49202, Israel

*Correspondence address. Tel: +972-3-9377618; Fax: +972-3-9376618; E-mail: ronita@clalit.org.il

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BACKGROUND: Anticancer treatment poses a high risk of ovarian failure. In many cases cryopreservation of ovarian tissue is the only option for fertility preservation. Although autologous transplantation of cryopreserved–thawed ovarian tissue has resulted in live births, slow graft revascularization and ischemia after transplantation leads to substantial follicular loss. Therefore, methods to improve and hasten graft vascularization are needed. The aim of the study was to examine the benefits of host and graft treatments with melatonin, hyaluronan (HA), vascular endothelial growth factor A (VEGF-A) and vitamin E with regard to the outcome of human ovarian tissue grafting.

METHODS: Five young cancer patients who underwent laparoscopic ovarian surgery for fertility preservation donated ovarian tissue. Thawed ovarian samples were transplanted into immunodeficient mice divided into seven groups: (A) no treatment; (B) host treatment with melatonin before and after grafting; (C) graft incubation with HA-rich biological glue before transplantation; (D) host as in (B), graft as in (C); (E) host as in (B), graft incubation with VEGF-A and vitamin E; (F) graft as in (C) combined with VEGF-A and vitamin E; (G) host as in (B), graft as in (F). Graft survival was assessed by follicle counts, apoptosis assay and immunohistochemical staining for proliferating cell nuclear antigen and VEGF-A expression.

RESULTS: Only grafts implanted in melatonin-treated hosts and grafts incubated with HA-rich biological glue retained their original size. Apoptosis was significantly lower after host treatment with melatonin and graft incubation with HA-rich biological glue plus VEGF-A and vitamin E than in untreated grafts; apoptosis was specifically low in Group G. There were significantly more atretic follicles in the untreated group than in most treated groups.

CONCLUSIONS: The findings suggest that host treatment with melatonin or graft incubation with HA-rich biological glue, especially when combined with VEGF-A and vitamin E improves graft survival. This protocol can be applied and holds promise in ovarian autotransplantation for fertility restoration.

Key words: human ovarian grafting / melatonin / hyaluronan / vascular endothelial growth factor A / vitamin E / fertility restoration

Introduction

Anticancer treatment poses a high risk of ovarian failure in survivors (Feigin et al., 2008). In many cases cryopreservation of ovarian tissue containing immature ovarian follicles is the only option for fertility preservation. So far, the autologous transplantation of cryopreserved–thawed ovarian tissue has resulted in about 15 live births (Feigin et al., 2008; Abir et al., 2010a, 2011; Donnez et al., 2011; Revel et al., 2011). However, too-slow graft revascularization after transplantation can lead to substantial follicular loss due to ischemia (Feigin et al., 2008; Abir et al., 2010a, 2011). Therefore, methods to improve and hasten graft vascularization are needed. For the initial experiments, immunodeficient mice engrafted with ovarian tissue can serve as useful models for examining graft survival (Abir et al., 2009, 2011). Studies should be restricted to treatments with a minimum potential for dangerous side effects in the hosts, and so those that yield successful results can eventually be applied in autotransplantation for fertility restoration in women.

Melatonin and vitamin E are free-radical scavengers with a broad antioxidant activity spectrum and anti-apoptotic function (Sapmaz...
et al., 2003; Reiter et al., 2005; Lissoni, 2007; Molpeceres et al., 2007; Sylvester, 2007; Brigelius-Flohe, 2009). Melatonin prevents both free-radical damage in various diseases including cancer and the induction of the apoptotic mitochondrial pathway by reducing Bcl2 expression and caspase-3 activity (Lissoni, 2007; Molpeceres et al., 2007). Vitamin E in its natural form α-tocopherol regulates apoptotic genes, prevents death receptor ligand-mediated apoptosis and regulates cell cycle, adhesion and growth (Sylvester, 2007; Abir et al., 2011).

Host treatment with melatonin and vitamin E has been shown to improve ovarian graft survival in rats and mice (Nugent et al., 1998; Sapmaz et al., 2003). Moreover, a previous study conducted by our group found that only human ovarian grafts that were incubated before transplantation with vitamin E and vascular endothelial growth factor-A (VEGF-A), the most potent regulator of blood vessel formation, retained their original size (Holmes and Zachary, 2005; Abir et al., 2011).

Hyaluronan (HA), a large glycosaminoglycan composed of repetitive sequences of D-glucuronic acid and N-acetyl glucosamine disaccharides is an important component of the extracellular matrix in the reproductive tract (Saito et al., 2000). Studies have shown that high-molecular-weight HA promotes anti-inflammatory and anti-apoptotic signals in various tissues and cell types including ovarian follicles and preimplantation embryos (Babayan et al., 2008; Bourguignon et al., 2009; Pauloin et al., 2009). HA synthesis was found to be markedly increased in mice at the time of implantation, suggesting its involvement in this process (Carson et al., 1987). Others showed that human embryo transfer medium in the form of HA-rich biological glue was associated with an increase in implantation and pregnancy rates (Friedler et al., 2007; Loutradi et al., 2007; Urman et al., 2008).

Prompted by these observations, we sought to determine if the survival of frozen–thawed human ovaries implanted in immunodeficient mice can be improved by additional host and graft treatments with melatonin, HA, VEGF-A and vitamin E (Abir et al., 2011).

### Materials and Methods

#### Human ovarian material

The study was approved by the local institutional ethics committee. Tissue was obtained from five girls/women aged 6–23 years [mean age ± standard deviation (SD), 16 ± 6 years] during laparoscopic ovarian surgery for fertility cryopreservation before anticancer therapy (Table I) (Abir et al., 2008, 2011; Feigin et al., 2008). Informed consent to donate tissue for the present study was obtained from the patients or the parents of minors. One slice measuring 1–2 mm from every ovarian sample was fixed in Bouin’s solution immediately after ovarian dissection (fresh

#### Table I Transplantation of ovarian tissue from five cancer patients transplanted into 79 immunodeficient mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hosts (number)</th>
<th>Ovarian source</th>
<th>Patient age</th>
<th>Follicles in thawed ungrafted controls</th>
<th>Follicles in recovered grafts</th>
<th>Atretic follicles in recovered grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not treated</td>
<td>5</td>
<td>Patient 1</td>
<td>17</td>
<td>7</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Group A</td>
<td>6</td>
<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td></td>
<td></td>
<td>15</td>
<td>32</td>
<td>10 (31.3%)</td>
</tr>
<tr>
<td>Host</td>
<td>6</td>
<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Group B</td>
<td>5</td>
<td>Patient 3</td>
<td>18</td>
<td>102</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
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<td></td>
<td></td>
<td>110</td>
<td>15</td>
<td>5 (33.3%)</td>
</tr>
<tr>
<td>Biological glue</td>
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<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
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<td>10</td>
<td></td>
<td></td>
<td>21</td>
<td>40</td>
<td>3 (7.5%)</td>
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<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Group D</td>
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<td>Patient 5</td>
<td>15</td>
<td>56</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td></td>
<td></td>
<td>64</td>
<td>28</td>
<td>2 (7.1%)</td>
</tr>
<tr>
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<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Group E</td>
<td>6</td>
<td>Patient 3</td>
<td>18</td>
<td>102</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td></td>
<td></td>
<td>110</td>
<td>5</td>
<td>0 (0%)</td>
</tr>
<tr>
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<td>Patient 2</td>
<td>23</td>
<td>8</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Group F</td>
<td>6</td>
<td>Patient 5</td>
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<td>54</td>
<td>1</td>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
<td>64</td>
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<td>4 (4.9%)</td>
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<tr>
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</tr>
<tr>
<td>Group G</td>
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<td>Patient 2</td>
<td>23</td>
<td>8</td>
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<tr>
<td>Total</td>
<td>12</td>
<td></td>
<td></td>
<td>15</td>
<td>25</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*Percent from total number of grafted follicles in group.

*Significantly higher than Groups C, D, F, G (Groups C, F and G P < 0.0001; Group D P < 0.002).*
ungrafted control) for evaluating pregrafting follicular density following histological preparation (see ‘Histological preparation’). This procedure was done to ensure, on the basis of follicular count results in the fresh ungrafted controls, that only follicle-rich samples would be used for grafting.

Cryopreservation and thawing of ovarian tissue

Cryopreservation was carried out within 1 h of ovarian retrieval. All ovarian samples were cut into slices of 1–2 mm diameter (thickness <1 mm) before freezing (Abir et al., 2011). Our freezing-thawing procedure has been described in detail previously (Abir et al., 2009, 2011). In brief, tissue slices were placed in cryogenic vials (Nalge Nunc International, Delta, Roskilde, Denmark) filled with 1.5 M dimethylsulfoxide (DMSO, Sigma, St Louis, MO, USA). Prior to freezing, the samples were kept on ice for 30 min to establish equilibrium. Samples were frozen slowly in a programmable freezer (Kryo 10; series 10/20, Planer Biomed, Sunbury on Thames, UK), and immediately placed in liquid nitrogen. Thawing was carried out by washouts with decreasing concentration gradients of DMSO (Sigma). One frozen–thawed slice from every ovarian sample (similar in dimensions to the fresh ungrafted control) was fixed in Bouin’s solution immediately after ovarian dissection (thawed ungrafted control) for analysis of the follicular count following histological preparation (described below).

Transplantation into immunodeficient mice

Seventy-nine immunodeficient nu/nu female Balb/C mice aged 10–12 weeks (Harlan, Jerusalem, Israel) were used for the tissue transplantation study (Table I) (Abir et al., 2009, 2011). The distribution of the sources of the grafts (by patient number) for each group is described in Table I. The different treatments and treatment combinations in each of the experimental groups are illustrated in Fig. 1. The mice were divided into the following treatment groups:

(A) No host or graft treatment.
(B) Host treatment with melatonin (240 mg/l, Sigma) dissolved in the drinking water (the highest dissolving concentration) in a light sealed bottle for 2 weeks prior to grafting and throughout the experiment.
(C) Pretransplant incubation of the graft at 37°C for 2 h in HA-rich biological glue (Uterine Transfer Medium, UTM medium, MediCult, Jyllinge, Denmark).
(D) Host treatment as in Group B (melatonin) combined with graft treatment as in Group C (HA-rich biological glue).
(E) Host treatment as in Group B (melatonin) combined with pretransplant incubation of the graft at 37°C for 2 h in alpha minimal essential medium (Biological Industries, Beit Ha’emek, Israel) containing 10% human serum albumin (SAGE, Trumbull, CT, USA) + human recombinant VEGF-A (200 ng/ml, Biological Industries) + vitamin E-oily

Figure 1 Illustration of treatment groups and thawed control tissues. A thawed ungrafted control specimen was fixed immediately for histological evaluation. The remaining thawed samples were divided for transplantation into the seven treatment groups, as follows: (A) No host or graft treatment. (B) Host treatment with melatonin in the drinking water. (C) Pretransplant graft incubation in HA-rich biological glue. (D) Host treatment as in Group B (melatonin) combined with graft treatment as in Group C (HA-rich biological glue). (E) Host treatment as in Group B (melatonin) combined with pretransplant graft incubation in VEGF-A + vitamin E. (F) Graft treatment as in Group C (HA-rich biological glue) + VEGF-A + vitamin E, as in Group E. (G) Host treatment as in Group B (melatonin) combined with graft treatment as in Group F (HA-rich biological glue + VEGF-A + vitamin E). The histological section in the illustration is a thawed ungrafted sample from an 18-year-old girl (Patient 3, as numbered in Table I).
Figure 2 Representative photographs of ungrafted controls and grafted samples. (A) Fresh ungrafted ovarian section from an 18-year-old girl (Patient 3, as numbered in Table I). Note the primordial follicles (arrows) and primary follicle (arrow head). Hematoxylin and eosin, original magnification ×400. (B) Thawed ungrafted ovarian section from a 23-year-old woman (Patient 2, as numbered in Table I). Note the secondary follicle (arrow). Hematoxylin and eosin, original magnification ×400. (C) Section of grafted ovarian sample from a 6-year-old girl (Patient 4, as numbered in Table I) after pretransplantation graft treatment with HA-rich biological glue (Group C). Note the numerous primordial and primary follicles. Hematoxylin and eosin, original magnification ×400. (D) Section of grafted ovarian sample from the same patient as in (B) after host treatment with melatonin and pretransplantation graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). Note the primary follicle (arrow) and the second possible not-mid-section follicle (arrow head). Hematoxylin and eosin, original magnification ×400. (E) Section of grafted ovarian sample from a 17-year-old girl (Patient 1, as numbered in Table I) after host treatment with melatonin and pretransplantation graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). Note the red-brown PCNA staining in the oocyte and the weaker granulosa cell staining (arrow). Original magnification ×400. (F) Section of grafted ovarian sample from the same patient as in (B) (Patient 2, as numbered in Table I) after untreated grafting (Group A). Note the strong brown staining in the stroma cells (arrow), indicating apoptosis. Original magnification ×400. (G) Section of grafted ovarian sample from the same patient as in (B) and (D) after host treatment with melatonin and pretransplantation graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). Note the overall blue staining and lack of brown TUNEL staining, indicating lack of apoptosis. Original magnification ×400. (H) Section of grafted ovarian sample from the same patient as in (B), (D) and (F) after host treatment with melatonin and pretransplantation graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). Note the peripheral red-brown VEGF-A staining pattern in the graft’s border (arrow) and the primordial follicle (arrow head). Original magnification ×100. (I) Section of grafted ovarian sample from the same patient as in (B), (D), (F) and (H) after untreated grafting (Group A). Note the scattered red-brown VEGF-A staining throughout the whole graft. Original magnification ×200. (J) Section of grafted ovarian sample from the same patient as in (B) after untreated grafting (Group A). Note the irregularly shaped atretic follicle (arrow) with its damaged granulosa cell layer and shrunken oocyte. Hematoxylin and eosin, original magnification ×400.
form (400 IU/ml, E-400 natural, SOFT GEL Technologies Inc., Los Angeles, CA, USA) (Abir et al., 2011). (F) Graft treatment as in Group C (HA-rich biological glue) + VEGF-A + vitamin E, as in Group E. (G) Host treatment as in Group B (melatonin) combined with graft treatment as in Group F (HA-rich biological glue + VEGF-A + vitamin E).

The mice were anesthetized as described by us previously (Abir et al., 2009, 2011). We chose the back muscle as a transplantation site because it is not richly vascularized, and we assumed it could mimic ovarian conditions. Moreover, previous studies showed high recovery rates after back muscle transplantation (Soleimani et al., 2010; Abir et al., 2011). After the back muscle was dissected, either treated or untreated ovarian slices (1–2 mm, similar in size to the ungrafted control samples) were pushed inside with delicate forceps and the opening was sutured, as described by us previously (Abir et al., 2011). The mice were euthanized 2 weeks after surgery, and the transplants were removed and fixed in Bouin’s solution.

**Histological preparation**

The fresh and thawed ungrafted control samples and the transplanted ovarian tissue specimens were prepared for paraffin embedding and hematoxylin and eosin staining, as described by us previously (Abir et al., 2010b, 2011). The follicles in all the samples were counted with a computerized image analyzer (analySIS, Soft Imaging System, Digital Solutions for Imaging and Microscopy, System GmbH, Munster, Germany). Atretic follicles were characterized by pyknotic cells, eosinophilia of the ooplasm and clumping of the chromatin material (Gougeon, 1996; Abir et al., 2008, 2009, 2011). Nonatretic follicles were defined as having a normal morphological appearance, with no structural characteristics of atresia. Unstained sections were placed on OptiPlus positively charged microscope slides (BioGenex Laboratories, San Ramon, CA, USA) for evaluation of apoptosis using the terminal deoxynucleotidyl transferase (TdT) (TUNEL) assay and immunohistochemical study of graft VEGF-A expression and follicular proliferating cell nuclear antigen (PCNA), a cell-cycle regulator and useful marker of proliferating cells, such as granulosa cells (Abir et al., 2009, 2010b, 2011).

**TdT assay (TUNEL)**

Apoptosis was evaluated in the thawed control tissues and the grafts (ApopTag, In Situ Detection Kit; Intergen Company, Purchase, NY, USA), as described by us previously (Abir et al., 2008, 2009, 2011). All sections underwent this staining procedure. Staining level was graded on a four-point scale according to intensity and quantity, which were consistently correlated (high staining intensity was related to large numbers of stained cell nuclei and vice versa) (Abir et al., 2011): 0 = no TUNEL staining, 1 = very little TUNEL staining with low intensity, 2 = TUNEL-expressing cells and medium staining intensity, 3 = many TUNEL-expressing cells and high staining intensity. The slides were viewed independently by two of the authors (O.F. and R.A.) (Abir et al., 2011).

**Immunohistochemistry for PCNA and VEGF-A**

The thawed control tissues and the grafts underwent staining, for PCNA and VEGF-A. The procedures have been described by us previously (Abir et al., 2009, 2010b, 2011). Only samples in which follicles were identified in the hematoxylin and eosin sections underwent staining for PCNA and all sections underwent staining for VEGF-A (Abir et al., 2009, 2011). VEGF-A staining was graded on a four-point scale according to intensity and quantity, which were consistently correlated (high staining intensity was related to large numbers of stained cell nuclei and vice versa) (Abir et al., 2011): 0 = no VEGF-A staining, 1 = very little VEGF-A staining with low intensity, 2 = VEGF-A-expressing cells and medium staining intensity, 3 = many VEGF-A-expressing cells and high staining intensity. This scaling system was similar to that described above for apoptosis (see Results regarding differences between VEGF-A and apoptosis staining intensities). The slides were viewed independently by two of the authors (O.F. and R.A.) (Abir et al., 2011).

**Statistical analysis**

Apoptosis and VEGF-A grades (scores) were analyzed with Kruskal–Wallis one-way analysis of variance by Ranks followed by Dunn’s multiple comparison procedure. Follicle number (including atretic follicles) before and after grafting was analyzed by χ² test and Fisher’s exact test for all pairwise groups. Differences were considered statistically significant when P was <0.05. Analyses and graphs were performed using SigmaPlot (version 11.0; Systat, San Jose, CA, USA).

**Results**

Graft recovery varied between 80 and 100% (differences among the groups were insignificant). All grafts were recovered in Groups B (host treatment with melatonin), D (host treatment with melatonin and graft treatment with HA-rich biological glue), F (graft treatment with HA-rich biological glue + VEGF-A + vitamin E) and G (host treatment with melatonin and graft treatment with HA-rich biological glue + VEGF-A + vitamin E). 11/12 grafts were recovered in Group A (no host or graft treatment), 8/10 in Group C (graft treatment with HA-rich biological glue) and 10/12 in Group E (host treatment with melatonin and graft treatment with VEGF-A + vitamin E). Grafts recovered from the groups in which the hosts were treated with melatonin (Groups B and E) or the grafts were treated with HA-rich biological glue (Groups C and F) or both (Groups D and G) retained their original size, and were intact with clear borders. Figure 2 shows representative ovarian samples from the ungrafted controls (Fig. 2A and B) and the different groups (Fig. 2C–J).

Apoptosis levels were significantly higher in the thawed ungrafted controls than in transplanted tissue from Groups B (P < 0.001), D (P < 0.001) and G (P < 0.004), and significantly higher in the absence of host/graft treatment (Group A) than in most of the treated groups (Group B, P < 0.04; Group D, P < 0.001; Group E, P < 0.001; Group F, P < 0.001; Group G, P < 0.001), except Group C (graft treatment with HA-rich biological glue) (Figs 2F and 3). Apoptosis levels were also significantly higher in Group B (host treatment with melatonin, P < 0.04), Group C (graft treatment with HA-rich biological glue, P < 0.02) and Group E (host treatment with melatonin and graft treatment with VEGF-A + vitamin E, P < 0.04) than in Group G (host treatment with melatonin, graft treatment with HA-rich biological glue + VEGF-A + vitamin E) (Fig. 2F). Separate analysis of the data from Patient 2, the only patient whose ovarian samples were allocated to all treatment groups, revealed a similar pattern of significant apoptosis to that illustrated in Fig. 3.

There were no significant differences in VEGF-A expression among the groups (Fig. 4). However, with the exception of Group D (host treatment with melatonin and graft treatment with HA-rich biological glue), a non-significant increase in VEGF-A expression was identified in the treated groups compared with the thawed ungrafted controls and the untreated group (Group A). The increase in VEGF-A level almost
reached significance in Groups E (P < 0.075) and F (P < 0.058), in both of which HA-rich biological glue was combined with VEGF-A + vitamin E graft treatment. Again, separate analysis of the data from Patient 2 revealed a similar VEGF-A pattern to that illustrated in Fig. 4.

Certain patterns of vascularization and VEGF-A expression correlated with specific treatments and the presence of follicles in the recovered grafts (follicular survival). In well-defined intact grafts after pretreatment with HA-rich biological glue (Groups C, D, F and G), VEGF-A staining was strong in the periphery and weak in the center (peripheral staining pattern) (Fig. 2H) and more non-atretic follicles were identified. By contrast, in the absence of pretreatment with HA-rich biological glue (Groups A, B and E), VEGF-A staining was scattered in the whole graft (diffuse staining pattern) (Fig. 2I). These grafts were characterized by more blood vessels in the graft center, unclear borders and fewer follicles. Interestingly, immunohistochemistry for VEGF-A yielded stronger nuclear staining than in situ.
hybridization for apoptosis evaluation. This is consistent with our previous reports of higher staining intensities for immunohistochemistry than in situ hybridization in the identification of other antigens (Abir et al., 2010b).

The recovered grafts showed only a limited number of follicles (Table I; Fig. 2C–E). The number of atretic follicle was significantly higher in the untreated group (Group A) than in treated Groups C, D, F and G (Groups C, F and G, \( P < 0.0001 \); Group D, \( P < 0.002 \)) (Table I; Fig. 2J). Atretic follicles were not identified in the ungrafted-thawed controls, as reported by us previously as well (Abir et al., 2011).

The follicles recovered after transplantation showed weak PCNA staining (Fig. 2E). Grafts implanted with HA-rich biological glue (Groups C, D, F and G) fused more with the surrounding tissue (Fig. 2C–E). This resulted in suboptimal paraffin preparation because the samples became tough during the preparation procedure with folds and creases in the sections. In three grafts including two from Patient 4 implanted in Group C (graft treated with HA-rich biological glue) and one from Patient 1 implanted in Group G (host treatment with melatonin and graft treatment with HA-rich biological glue + VEGF-A + vitamin E), the recovered tissues were folded so extensively that accurate follicular evaluation was difficult. It is noteworthy that, in the two samples from Patient 4 (Group C), there were large numbers of follicles between the folds. Moreover, more follicles were identified in all the recovered grafts of Patient 4 (Table I; Fig. 2C) than in the corresponding fresh ungrafted control sample (photograph not shown). In all the other grafts implanted with HA-rich biological glue, accurate follicular evaluation was possible, despite the folds and creases.

## Discussion

The present study of ovarian graft treatment in a mouse model yielded several important findings. The tissues grafted after host treatment with melatonin and/or graft treatment with HA-rich biological glue before transplantation, retained their original size and had well-defined borders. Apoptosis levels were reduced with all treatments compared with controls, except pretreatment incubation with HA-rich biological glue (Group C). However, the most significant reduction occurred when host treatment with melatonin was combined with graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). Additionally, VEGF-A staining was increased in all treated groups except one, although the differences were not statistically significant. Two patterns of VEGF-A staining were identified: a peripheral pattern which occurred in grafts treated with HA-rich biological glue and was associated with well-defined borders and more non-atretic follicles; and a diffuse VEGF-A staining in the absence of graft treatment with HA-rich biological glue, which was associated with unclear borders and fewer follicles. The proportion of atretic follicles was significantly higher in the untreated group than in the treated groups. The recovery rate after transplantation to the back muscle was high in all transplant groups.

Young age is strongly correlated with high follicular density. Therefore, we chose ovaries from young patients, with large numbers of follicles in the fresh ungrafted control samples (Table I). The low numbers of follicles identified in the recovered grafts could indicate accelerated follicular loss after transplantation. However, there might be an alternative explanation. Follicles are distributed unevenly in the human ovary, and we transplanted relatively small samples. As such, we might have inadvertently transplanted slices with no or only few follicles relative to the fresh and thawed control samples (Schmidt et al., 2003; Abir et al., 2008; Feigin et al., 2008). This possibility seems likely considering that samples from the mice treated with melatonin and/or the HA-rich biological glue-incubated grafts retained their original size after implantation. Furthermore, on analysis of the results from a specific patient (Patient 4), a higher number of follicles was identified in the transplanted samples than in the fresh ungrafted control sample. It is noteworthy that because of the relatively low numbers of recovered follicles, grafted tissue was evaluated by means other than follicular counts and follicular atresia rates.

The availability of human ovarian tissue for research, in particular from young patients, is extremely limited. At the same time, relatively large tissue samples are required for transplantation studies (Abir et al., 2011). Therefore, with the exception of Patient 2, we could not allocate ovarian tissue from each patient to all treatment groups. Moreover, although ovarian tissue from young girls (such as Patient 4) contains numerous follicles, their ovaries are small. We attempted to overcome this problem by using as many as 79 murine hosts, resulting in large numbers of ovarian grafts, and we divided the hosts into seven groups to investigate the effects of all treatment combinations. Similarly, in our previous studies of the expression of growth factors and their receptors in human ovaries (Abir et al., 2010b), we required small ovarian specimens for the histological procedures and large tissue portions for mRNA extraction for reverse-transcription polymerase chain reaction, such that tissue from the same women could not be used in both laboratory procedures. In our experience, only in culture studies that require small ovarian slices can samples from the same patients be used in parallel in different treatment groups (Kedem et al., 2011).

Although the number of atretic follicles was significantly higher in the untreated host/graft group than in most treated groups, it was low in untreated grafts from Patient 2, the only patient whose tissue was allocated to all treatment groups. However, apoptosis levels in the untreated grafts from Patient 2 were significantly higher and followed the same pattern found for the groups as a whole (Fig. 3). Moreover, we reported a relatively higher follicular atresia rate (48.5%) in the untreated group in our previous study (Abir et al., 2011) performed on four untreated samples, including two that were not in the present study. Interestingly, one patient was of similar age to Patient 4, the youngest patient in the present study, but the grafts were devoid of follicles. Moreover, all grafted tissue from the untreated group shrunk after transplantation (Abir et al., 2011). It, therefore, seems highly likely that the inclusion of more untreated samples in the present report would have yielded a higher follicular atresia rate.

The relatively low apoptosis level in the grafts pretreated with HA-rich biological glue is consistent with the anti-apoptotic properties of HA in various cells including ovarian follicles (Babayan et al., 2008; Bourguignon et al., 2009; Pauloin et al., 2009). The good adhesion qualities of HA might have induced swifter revascularization and reduced ischemic graft damage. Accordingly, other studies reported a benefit of HA in embryonic implantation (Friedler et al., 2007; Loutradi et al., 2007; Urman et al., 2008). The graft incubation with...
HA-rich biological glue + VEGF-A + vitamin E (Groups F and G) yielded lower apoptosis rates than other treatments. We speculate that the HA-rich biological glue may have kept the VEGF-A + vitamin E in the pretreated grafts at the transplantation site, thereby increasing their effect. The lowest apoptosis rates were found in Group G, wherein host treatment with melatonin was combined with graft treatment with HA-rich biological glue + VEGF-A + vitamin E. This finding might be explained by the cumulative anti-apoptotic effects of melatonin, HA and vitamin E (Sapmaz et al., 2003; Sylvester, 2007; Bourguignon et al., 2009).

In line with these findings, an earlier study by Sapmaz et al. (2003) reported a reduction in necrosis of rat ovarian grafts in melatonin-treated rat hosts. Both the reduction in apoptosis (the present study) and necrosis (Sapmaz et al., 2003) may be related to melatonin’s anti-apoptotic free-radical scavenger and antioxidant properties (Sapmaz et al., 2003; Reiter et al., 2005; Lissoni, 2007; Molpeceres et al., 2007). Melatonin is present at elevated concentrations in human ovarian follicles and oocytes at various developmental stages; thus, it may have promoted the survival/development of the grafts and follicles (Tamura et al., 2009). An added contribution of our study is the finding that melatonin’s beneficial effects can be achieved by the easy-to-use oral route of administration as opposed to injections (Sapmaz et al., 2003).

The lack of statistical significance of the increase in VEGF-A levels after grafting is in contrast to our previous study (Abir et al., 2011), wherein significantly higher VEGF-A levels were observed in all grafted groups. This difference might be due to the smaller number of hosts per treatment group in the present study. Indeed, VEGF-A levels almost reached significance in two treatment groups, in which as noted above the combination of HA-rich biological glue + VEGF-A + vitamin E might have kept the VEGF-A at the transplant site.

The peripheral VEGF-A staining pattern after graft incubation with HA-rich biological glue as opposed to the diffuse pattern in the absence HA-rich biological glue might be explained by the relatively poor vascularization and limited blood vessels of the intact ovary, which relies mainly on diffusion from surrounding blood vessels (Sapmaz et al., 2003; Soleimani et al., 2010). It is possible that ovarian graft implantation with HA-rich biological glue hastened graft adhesion to the tissue, which in turn hastened revascularization of the immediate area surrounding the graft, while maintaining the internal structure of the graft itself.

As opposed to the other groups VEGF-A staining did not increase when host treatment with melanin was combined with graft treatment with HA-rich biological glue (Group D). Reduced VEGF-A levels after melanin incubation have been noted in other tissues including cancerous tumors (Lissoni, 2007). If melanin was indeed responsible for the reduction in VEGF-A level, its effect was apparently buffered by combining HA-rich biological glue with VEGF-A (Groups E and G).

The weak PCNA follicular staining in this study was probably due to the short transplantation period, as reported by us previously as well (Abir et al., 2011). Although melatonin has been shown to have an anti-proliferative effect in hormone-dependent tumors in mice (Xi et al., 2001), this probably does not explain our finding because the intensity of PCNA staining did not differ between the melanin-treated groups (B, D, E and G) and non-melanin-treated groups (C and F).

The high graft recovery rates in all the transplant groups indicate that most of the tissue samples were properly grafted in the back muscle, as described previously (Soleimani et al., 2010; Abir et al., 2011). The unrecovered samples were probably either absorbed or escaped from the grafting site despite suturing. This finding might also be attributable to specific circumstances; that is, the lack of treatment may account for the single unrecovered graft in Group A; treatment failure to sufficiently reduce apoptosis may account for the two unrecovered grafts in Group C (graft incubated with HA-rich biological glue) and the lack of HA-rich biological glue to enhance VEGF-A maintenance at the graft site may explain the two unrecovered grafts in Group E (host treatment with melatonin and graft treatment with VEGF-A + vitamin E).

In most centers worldwide, ovarian samples from cancer patients are cryostored by slow gradual freezing methods (Feigin et al., 2008), like that used here. Therefore, to improve the success of ovarian implantation, studies should be directed at techniques and potential substances that conform to slow freezing-thawing. The present study is an initial step toward achieving this goal. It identified beneficial effects of combinations of host treatment with melatonin and graft treatment with HA-rich biological glue + VEGF-A + vitamin E (Group G). The second phase of the study should include a longer transplantation period with gonadotrophin stimulation for production of antral follicles from the ovarian grafts. Thereafter, this protocol may be easily modified, with limited or no foreseeable risks, for use in autotransplantation in cancer survivors.

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Authors’ roles

O.F. conducted all the laboratory work, wrote the paper, photographed the pictures, selected the relevant photos, designed all the figures and performed most of the statistical evaluations. R.O. designed the study with R.A. and B.F. performed the operations, took part in writing all versions of the manuscript and edited the manuscript. B.F. designed the study with R.A. and R.O. provided the funding for the study and helped with writing and revising the manuscript. C.F. assisted with the histological studies. E.F. provided the ovarian material, proof read the paper and took part in discussions regarding the results. A.B.-H. provided the ovarian material, proof read the paper and took part in discussions regarding the results. R.A. the principal investigator, designed the study with R.O. and B.F. provided the financial support, assisted in the operations and was the main care taker of the mice, supervised the laboratory studies, photographed the pictures, selected the relevant photos, assisted in writing the paper and edited it in all its revisions and performed a portion of the statistical evaluations.

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Conflict of interest

None declared.

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