Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue

Tomer Israely1,3, Nava Nevo1, Alon Harmelin2, Michal Neeman1 and Alex Tsafriri1

1Department of Biological Regulation and 2Department of Veterinary Resources, The Weizmann Institute of Science, Rehovot, Israel
3To whom correspondence should be addressed at: Department of Biological Regulation, The Weizmann Institute of Science, Rehovot 76100, Israel. E-mail: alex.tsafriri@weizmann.ac.il

BACKGROUND: Anti-cancer therapies frequently lead to ovarian damage and impaired fertility. To preserve fertility, cryopreservation and subsequent transplantation of the ovaries have been suggested. One of the challenges in ovarian graft transplantation is overcoming the initial ischaemic damage that depletes a significant fraction of the oocyte pool. METHODS AND RESULTS: Follicular survival in ovarian grafts was examined by magnetic resonance imaging (MRI) and fluorescence microscopy in a model system in which rat ovaries were transplanted into nude mice. Transplantation into angiogenic granulation tissue created during wound healing shortened the ischaemic period by 24 h and significantly increased the pool of healthy primordial follicles and the perfused area of the transplanted grafts. Functional blood vessels were detected within the grafts as early as 2 days after transplantation. Gain of function was demonstrated both by growth of the grafts and by the hormonal influence on the host uteri. CONCLUSION: Implantation of ovarian grafts into an angiogenic granulation tissue improved graft vascularization and follicular survival. This procedure/treatment may be used for reducing the ischaemic damage in ovarian transplants, thus prolonging graft functionality and increasing the yield of oocytes that can be easily recovered for fertilization.

Key words: animal model/granulation tissue/ischaemia–reperfusion injury/oocyte pool/ovarian transplantation

Introduction

Ectopic transplantation of cryopreserved ovarian tissue fragments has been suggested as an option for preservation of fertility in women who suffer from premature ovarian failure following aggressive chemo- or radiation therapy (Kim et al., 2001). In contrast to transplantation of large organs, in which reanastomosis of blood vessels is achieved surgically, transplantation of small ovarian fragments depends on the growth of new blood vessels for restoring adequate perfusion. One of the urgent challenges in the development of reproducible and reliable procedures for ovarian transplantation is to find ways to accelerate graft revascularization. It has been shown that more follicles die from ischaemia during transplantation than from freeze–thaw injury during cryopreservation (Newton et al., 1996; Aubard et al., 1999). While there is consensus regarding the extensive follicular damage occurring during the period in which the graft is disconnected from the blood circulation (Aubard et al., 1999; Baird et al., 1999; Oktay et al., 2000; Liu et al., 2002), there is scarce spatial and temporal information regarding the changes occurring during these early post-transplantation stages (hours–days). Thus, the first aim of this work was to investigate the changes in blood supply and follicle survival throughout this critical time window. The second aim was to investigate whether the ischaemic period can be shortened by experimental intervention. The third aim was to examine the impact of such intervention on the recovery of grafts, long-term follicular development and systemic endocrine recovery of the ovariectomized mice.

Autotransplantation of juvenile rat ovaries to ectopic sites showed first stages of revascularization, as detected by corrosion casts, within 48 h after transplantation (Dissen et al., 1994). Examination of the graft itself in mice after injection of the Evan’s Blue dye revealed initiation of perfusion from the third day after transplantation (Nugent et al., 1998). It was previously shown that ovarian grafts regained functionality after the initial damage (Kim et al., 2002; Liu et al., 2002). Therefore, we hypothesized that shortening the ischaemic time period by implanting the graft into angiogenic granulation tissue would reduce the damage and enlarge the surviving follicle pool, thus extending the graft functionality.

Surgical wounds are characterized by an intensive and rapid angiogenic activity, peaking at 2–4 days after injury. This process
involves deposition of a fibrin clot, secretion of growth factors fibroblast growth factor (FGF), TGFβ and vascular endothelial growth factor (VEGF) (Nissen et al., 1998; Li et al., 2003) and invasion of endothelial cells (Nissen et al., 1998). The newly formed blood vessels are an important component of early granulation tissue, delivering nutrients, inflammatory cells and oxygen to the wound site (Lingen, 2001). The beneficial angiogenic effect of a surgical wound for tissue interaction was demonstrated by Barash et al. (2003), showing that embryo implantation in the uterus was significantly improved when it was deposited into a pre-prepared injury site. Moreover, we showed that tumour angiogenesis and tumour growth are accelerated at the site of surgical wounds (Abramovitch et al., 1998). Likewise, in the first live birth reported to be obtained after orthotopic transplantation of cryopreserved human ovarian tissue, Donnez et al. (2004) induced angiogenesis and neovascularization in the area of transplantation by creating a peritoneal window 7 days before reimplantation. Another important aspect of surgical operations and reperfusion of organ transplants is the damage of tissue by oxygen-derived free radicals associated with ischaemia–reperfusion injury and increased lipid peroxidation (Jassem and Heaton, 2004). For example, reperfusion of ischaemic kidney transplant resulted in functional and structural damage (Kadkhodaee et al., 2004). The protective effect of vitamin E on survival of follicles in ovarian grafts was previously reported (Nugent et al., 1998).

We reported previously that ovarian grafts transplanted into intact tissue were vascularized by day 6 after transplantation, while at earlier days (1–3 days), the grafts remained mostly avascular. The damage in these transplants was maximal in the centre and was reduced in its edges forming contact with the host tissue (Israely et al., 2004). In the study reported here, we evaluated a procedure for transplantation of ovarian grafts into granulation tissue that promoted early graft vascularization and resulted in improved graft perfusion and follicle survival. The increase in the surviving follicle pool is expected to extend the period of ovarian graft activity and the success of oocyte retrieval.

Materials and methods

Ovarian retrieval and transplantation

All animal experiments were approved by the Institutional Animal Care and Use Committee.

Donor rat ovarian fragments

Immature 15-day-old Wistar rats were sacrificed using CO2 and subsequent cervical dislocation. The ovaries were collected and cleaned of fat at room temperature in L-15 Leibovitz medium (GibcoBRL, Invitrogen Corporation, Paisley Scotland, UK) supplemented with 0.1% bovine serum albumin (BSA) and antibiotics (penicillin/streptomycin; 100 IU/ml). Ovaries were halved and transplanted within 20–30 min after collection. Three half ovaries were not transplanted and they served as a control of time 0.

Recipient mice

CD-1 female nude mice, 6–10 weeks old, were ovariectomized (OVX) a week before transplantation. Half rat ovaries were transplanted into mice anaesthetized with intraperitoneal ketamine (75 mg/kg; Ketaset, Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine (3 mg/kg; XYL-M, VMD, Arendonk, Belgium). The graft was inserted through a 1 cm full-thickness dermal incision and through a cut along the gluteus superficialis muscle fibres of the hind limb and superficially attached to the muscle beneath, facing the skin. This implantation site allowed improved follicular conservation, detection and access for oocyte retrieval, as we reported previously (Israely et al., 2004). The graft was sutured to the muscle with 8/0 surgical thread, while most of it was left out of the muscle. The incision was sealed with sutures or Super Glue Gel (ethyl-2-cyanoacrylate, Loctite, Cleveland, OH, USA).

Transplantation into wound-healing granulation tissue

An approximately 5-mm incision along the muscle fibres was made by using fine surgical scissors. The wound was closed by 6/0 sutures. Four days after the incision, the wound was exposed, and the sutures and any excessive granulation tissue were removed. The graft was placed inside the wound and sutured.

All transplantations were performed under a surgical microscope (OPMI pico, Carl Zeiss, Oberkochen, Germany), while the animals were placed on a heated surface to maintain core body temperature (37°C).

Ovarian grafts were transplanted in 60 mice (31 in a 4-day wound and 29 in intact mice), and the grafts were monitored for a period of 1, 2, 3, 6, 26, 45 and 53 days after transplantation (Table I).

Examination of uteri

Histological evaluation of the uteri was performed 28 days after transplantation in nine additional mice (eight with a graft and one without).

In vivo measurements of grafts by magnetic resonance imaging

Magnetic resonance imaging (MRI) experiments were performed on a horizontal 4.7 T Bruker Biospec spectrometer (Bruker BioSpin, Ettlingen, Germany) by using an actively radio-frequency decoupled 1.5-cm surface coil embedded in a Perspex board and a birdcage transmission coil.

Anaesthetized mice at the indicated time points after transplantation were placed supine, with the graft located above the centre of the surface coil. The mice were immobilized using adhesive tape and were covered with a paper blanket to reduce temperature drop during the measurement.

Gradient echo anatomical images were acquired from intramuscular grafts at 8 (n = 4), 16 (n = 3), 28 (n = 12), 29 (n = 8) and 33 days (n = 5) post-transplantation.

Table I. Summary of ovarian transplantations examined on the indicated days

<table>
<thead>
<tr>
<th>Days after transplantation</th>
<th>Wound</th>
<th>Number of animals</th>
<th>Total</th>
<th>Contrast agents (CAs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>–</td>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>–</td>
<td>5</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>9</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>–</td>
<td>3</td>
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<tr>
<td>45</td>
<td>+</td>
<td>1</td>
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<tr>
<td>53</td>
<td>+</td>
<td>2</td>
<td></td>
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</tr>
</tbody>
</table>

*Number of animals for which their perfused area was examined quantitatively by the combination of two CAs.
after transplantation to evaluate the graft size. The grafts were scanned such that the maximal cross-section was obtained. The cross-sectional area of the grafts was measured using ParaVision software (Bruker BioSpin).

**Quantification of perfusion by blood markers**

BSA-based macromolecular MRI and histology contrast material, biotin–BSA–GdDTPA, and BSA labelled with rhodamine (BSA-ROX) were prepared as reported previously (Dafni et al., 2002, 2003). Biotin–BSA–GdDTPA and BSA-ROX were injected through a tail vein catheter as a bolus [12.4 mg/mouse in 0.2 ml of phosphate-buffered saline (PBS) and 2 mg/mouse in 0.2 ml of PBS, respectively]. The BSA-ROX was administered 20 min after the biotin–BSA–GdDTPA. Animals were sacrificed 1 min after the BSA-ROX administration by anaesthesia overdose, and the grafts were retrieved for histological analysis. Biotin–BSA–GdDTPA served as an indicator of vascular functionality and vascular permeability, while the BSA-ROX served as an indicator of vascular functionality. These two markers were also used for the quantification of the perfused area of the grafts that were transplanted into a wound granulation tissue and into the intact control animals for 2 and 3 days. In Table I, the number of mice evaluated by the two markers is indicated. Two to three representative slides from each graft were stained for these blood markers and were photographed at ×10 magnification. The perfused fraction of graft was calculated by NIH Image.

**Histology**

Samples were placed overnight in Carnoy fixative solution (6:3:1; ethanol:chloroform:acetic acid), transferred into 70% ethanol and stored at room temperature until processing. Fixed tissues were embedded in paraffin blocks, and the whole ovary was sectioned serially at 4 μm thickness. Three sequential sections were put on each slide. Every second or third slide was stained with haematoxylin and eosin (H&E), while other representative slides were used for specialized stains. The paraffin-embedded unstained sections were deparaffinized with xylene for 5 min followed by sequential ethanol hydration and double-distilled water. Sections were then washed with PBS for 5 min and were blocked by overnight incubation in 1% BSA in PBS at 4°C. The sections were stained with monoclonal anti-α-smooth muscle actin (αSMA; Sigma; stain for pericytes and vascular smooth muscle cells), conjugated to alkaline phosphatase and visualized with Fast Red (Sigma, St. Louis, MO). The slides were counterstained with Mayer’s haematoxylin solution. Alternatively, biotin–BSA–GdDTPA was stained with avidin–FITC conjugate (Sigma). The fluorescence of the intravascular marker, BSA-ROX, was preserved in the tissue and was observed directly after deparaffinization. The slides were counterstained with Hoechst reagent (Nuclear staining; Molecular Probes, Eugene, OR, USA; 1:1000 in PBS for 2 min) and sealed with antifade reagent (SlowFade, Molecular Probes).

Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–bioitin nick-end labelling (TUNEL; ApopTag Plus Peroxidase in situ Apoptosis Detection Kit; Chemicon, Temecula, CA, USA), according to the manufacturer’s instructions. Briefly, after deparaffinization, hydrated sections were incubated in a humidified chamber with 0.5% Triton X-100 (Sigma) at room temperature until processing. Fixed tissues were embedded in paraffin blocks, and the whole ovary was sectioned serially at 4 μm thickness. All the grafts were examined and evaluated blindly by the same observer.

**Oocyte aspiration and in vitro maturation**

Grafs transplanted into a 4-day wound were examined for the capability of oocytes to resume meiosis in vitro and extrude the first polar body [in vitro maturation (IVM)]. Oocytes harvested from one of the grafts (45 days after transplantation) were examined without subsequent IVM, and oocytes harvested from two other grafts (53 days after transplantation) were examined for subsequent IVM. The grafts were transferred into L-15 medium supplemented with 5% fetal calf serum and antibiotics (penicillin/streptomycin; 100 IU/ml) and were punctured.
using 25G needle under the binocular. Oocytes retrieved from two of the grafts 53 days after transplantation were transferred into the same medium and matured spontaneously upon overnight incubation in a humidified chamber at 37°C. The grafts were examined under inverted microscope (Eclipse TE2000, Nikon) prior to the dissection and the retrieval of oocytes.

Statistical analysis
The data were analysed by SAS Software (Cary, NC, USA). Two-way analysis of variance (ANOVA) was used for comparison of the primordial follicle data in the wound versus the intact control 1, 2, 3 and 6 days after transplantation. Arcsin transformation was applied to the square root of the primordial follicle proportions before ANOVA. Multiple comparisons between means were performed using Fisher's least significant differences (LSD), when significant differences were found in the two-way ANOVA. Student’s t-test (unpaired, two-tailed) was used to compare the perfused areas of the groups transplanted for 2 and 3 days. All data are expressed as mean ± SEM. A P value of <0.05 was considered statistically significant.

Results
Effect of transplantation into granulation tissue on revascularization of ovarian grafts
Delayed graft vascularization is one of the primary factors associated with follicular damage. This early ischaemic period (day 3) was characterized by apoptotic death of primordial follicles and perivascular endothelial cells as shown by TUNEL staining. The nuclei of degenerated oocytes became condensed and in the H&E lost the normal purple stain of the healthy oocytes (Figure 1A and B). These degenerated oocytes and the perivascular endothelial cells stained brown by TUNEL (Figure 1C). However, detection of intact and functional blood vessels at later stages (28d; Figure 1E–H) suggested post-transplantation recovery of vascularization. Apoptotic cells were detected in degenerating corpora lutea (CL) and atretic follicles (Figure 1F). Graft perfusion and vascular permeability were examined using two intravenously injected macromolecular markers. The first marker, biotin–BSA–GdDTPA, circulated for a relatively long period (20 min) with subsequent extravasation of the marker out of permeable blood vessels, whereas the second marker, BSA–ROX, was injected immediately prior to sacrifice and thus remained intravascular (Figure 1H). To confirm the positive cell staining by TUNEL, we used the apoptotic marker caspase-3, which in its cleaved, active form serves as an indicator for apoptotic death. Time 0 (non-transplanted) controls were negatively stained both by the TUNEL and by the caspase-3 (Figure 2B–E). In the grafts transplanted for 3 days into the intact control animals, both atretic granulosa cells in developing follicles and degenerating oocytes in primordial follicles were detected by TUNEL (Figure 2F–H and K–M). This positive stain was confined to the granulosa cells of the developing follicles when the caspase-3 marker was used (Figure 2I and J and N and O). The TUNEL-positive oocytes, which were not stained for active caspase-3, are, most probably, at late stages of apoptosis. Caspase-3 activity is associated with the initial stages of apoptosis.

To accelerate vascularization of the graft, the ovaries were transplanted into granulation tissue at the peak of neovascularization, inflicted by surgical injury 4 days earlier (Table I). Typical multinucleated giant myocytes and blood vessel invasion, detected adjacent to the grafts, indicated regeneration of muscle fibres 5 days after the surgical injury (Figure 3A and B). Intravenously injected Biotin–BSA–GdDTPA contrast agent (CA) was detected in the wound tissue surrounding the graft, but not inside the graft, indicating lack of perfusion in all the grafts on day 1 (Figure 3C and D). Two days after transplantation, tissue damage was confined to the graft centre. Beginning of blood penetration to the outer boundaries of the grafts was detected mainly in the ovaries transplanted into the wound site (Figure 3E–G). The perfused area in this time point was significantly higher in the grafts transplanted into the wound site compared to the intact control group (11.4 ± 5 versus 2.8 ± 2%, respectively; P = 0.02). These significant differences in the perfused area continued to grow also 3 days after transplantation (Figure 4A–H). The grafts transplanted into the wound site were already 66.1 ± 14% perfused, while only 15.6 ± 5% of the area of the grafts transplanted in the intact control was perfused (P = 0.0004). The ischaemic unperfused areas (Figure 4C and F) colocalized with degenerated/necrotic follicles stained positive by TUNEL, while the perfused areas were colocalized with intact healthy follicles (Figures 3F and G and 4C–E) not stained by TUNEL. Invading fibroblasts were detected within several of the grafts transplanted into the wound site (Figure 4I and J).

Vascular hyperpermeability in the muscle surrounding the graft was evident from extravasation of biotin–BSA–GdDTPA 2 and 3 days after transplantation (Figures 3C and 4A). Transplantation into the granulation tissue led to a shift of hyperpermeability, from the muscle surrounding the graft, into the grafts, and functional blood vessels were detected inside the vascularized grafts (Figure 4C; arrowheads). Overall, the perfusion of grafts transplanted in the rich angiogenic granulation tissue was accelerated compared with these grafts transplanted in the intact mice.

Improved graft protection of primordial follicles
Preconditioning of the transplantation site by surgical injury resulted in improved graft perfusion and survival of follicles. To quantify this effect, follicles were counted and classified according to their size and integrity. In the non-transplanted ovaries (time-0 control; n = 3), the number of healthy primordial follicles counted serially throughout the entire tissue (one of nine sections) was 39.2/mm² (Table II). Preparation of the implantation site by surgical wounding resulted in an increase in the percentage of healthy primordial follicles out of the total pool of primordial follicles on each of the days examined (P = 0.03, two-way ANOVA). The combined overall proportion of healthy primordial follicles (days 1–6 examined) increased from 52.3% in the grafts transplanted into intact control groups (n = 26 mice) to 67.8% in the wound site (n = 23 mice). The proportion of healthy primordial follicles was significantly higher in the grafts examined after 1 and 6 days compared with the grafts on days 2 and 3 whether transplanted into the wound or not (P < 0.05). A major effect of the wound was protection of the primordial follicles, reflected by the lower number of...
Figure 1. Vascular and follicular damage and recovery after ovarian graft transplantation. (A–D) Graft 3 days after transplantation into intact control mice. (A, B) Haematoxylin and eosin (H&E) stain of an area with degenerating (arrows) and healthy primordial follicles (arrowheads). B is an enlargement of the box from A in which an intact primordial follicle and a degenerated primordial follicle are marked. (C) Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick-end labelling (TUNEL) staining of sequential sections of the same area. Degenerated primordial follicles (arrows) and perivascular endothelial cells (arrowheads) stain in brown. (D) Negative control TUNEL staining of a sequential section of C. (E–H) Intact and functional blood vessels observed 28 days after transplantation (arrows), using H&E stain (E), TUNEL staining (F), smooth muscle cells and pericytes stained positive (G; α-smooth muscle actin; red stain) and vascular functionality (H). Degenerating corpus luteum and atretic follicle are marked with arrowheads (E, F). Functional blood vessels were demonstrated by intravenous administration of biotin–BSA–GdDTPA, injected 20 min prior to sacrifice (indicating vascular permeability; green), and BSA-ROX, injected 1 min prior to sacrifice (intravascular stain; red); nuclear staining (Hoechst; blue). Bars: 100 μm (A, E–H), 10 μm (B) and 50 μm (C, D).
degenerated follicles (Table II). Corresponding to the increase in the proportion of healthy primordial follicles, there was a significant decrease in degenerated follicles in the wound treatment as compared with the intact control ($P = 0.04$, two-way ANOVA). One day after transplantation, there were 52.2 degenerated primordial follicles in the intact controls, and their number was reduced to only 16.0 follicles in the grafts transplanted into the wound. The number of degenerated primordial follicles increased significantly 2 and 3 days after transplantation in both groups whether transplanted into the wound or not ($P < 0.05$ compared to the grafts transplanted for 1 and 6 days). The protective effect of the wound was expressed by a 13.7 and 11% increase in the percentage of healthy primordial follicles on days 2 and 3 after transplantation, respectively (Table II). Six days after transplantation, the percentage of healthy primordial follicles was higher in the treated group (89.6% per graft; Figure 5A and B) and decreased to 79.5% per graft in the intact control group (Table II), indicating improved follicular protection. Overall, a decrease in the total number of primordial follicles was detected after 6 days in both groups. This reduction can be attributed to the growth of follicles, causing an increase in graft volume (Table II) and clearance of degenerated follicles. Thus, the improved perfusion of the grafts transplanted into the wound site resulted in an increase in the proportion of the healthy primordial follicles.

**Improved protection and development of growing follicles**

In addition to the primordial follicles, developing pre-antral and antral follicles were detected within the grafts (Figures 3A and F, 4E and I and 5K). In non-transplanted ovaries of control 15-day-old rats nearly all the follicles were healthy (time-0 control, Table III), whereas in the grafts that were transplanted for 1–3 days most of the developing follicles degenerated. This degeneration correlated with follicular size. Within a day after transplantation, the proportion of healthy pre-antral follicles was reduced when transplanted into the wound site to 30.2% and into the intact controls to 17.3%. No antral follicles survived in any of these groups 1 day after transplantation. The damage to the antral follicles was so extensive that even remnants of degenerating follicles were only rarely detected. This could be attributed to a total destruction and disappearance of the oocytes or collapse of the follicle due to loss of the antrum. A progressive reduction (up to day 3 after transplantation) in the number of pre-antral follicles was detected both in the grafts transplanted into the wound site and in the grafts transplanted into the intact control (Table III). The antral follicles reappeared only 6 days after transplantation when on the average 4.0 ± 1.7 antral follicles were detected in the grafts transplanted into the wound site and 1.4 ± 0.7 were detected in the intact control ($P = 0.22$).

Most of the grafts were well perfused by 6 days after transplantation (Figure 5). Follicular development to pre-ovulatory stages was seen in one of the five grafts transplanted into the intact control group (Figure 5C–F). Clearance of degenerated follicles of all stages was observed. Damaged areas within the grafts transplanted into the intact control group were confirmed by TUNEL staining (Figure 5G and H). In contrast, large pre-ovulatory follicles were detected in four of the five grafts transplanted into the wound site (Figure 5I–L), and only one graft showed no developing antral follicles. The supportive environment provided by the wound was manifested by both the
number of pre-ovulatory follicles and the enlarged volume of the graft. The average volume of the four grafts in the group transplanted into the wound site that contained pre-ovulatory follicles was $8.0 \pm 2.8 \text{ mm}^3$ compared to the group of grafts transplanted into the intact control group in which the volume was $6.1 \pm 0.8 \text{ mm}^3$.

**Regain of ovarian function in the grafts**

Twenty-six days after transplantation, CL were present in 5/5 of the grafts transplanted into the wound site and in 2/3 of the grafts transplanted into intact control mice. Follicular development and the formation of the CL were manifested by the increase in graft size, as determined by MRI and histology (Figure 6). The recovery of graft functionality was expressed not only by follicle development and CL formation, but also by the endocrine influence on the mouse uterus, which also regained functionality. As expected, the uteri of ovariectomized mice in the absence of an ovarian graft were atrophic, while the uteri of the mice with the grafts showed various stages of the normal estrus cycle (Figure 7).

Follicles, intact oocytes at different stages of maturation and degenerated oocytes were retrieved from three grafts. Oocyte aspiration from a graft 45 days after transplantation into a wound site yielded six viable oocytes of which four showed GV, one was at the germinal vesicle breakdown (GVB) stage and one with
a polar body (Figure 8A and B). From two grafts transplanted for 53 days, 14 viable oocytes were retrieved at the GV stage and after IVM overnight, eight underwent GVB and additional two extruded a polar body (Figure 8C–G), that is 10/14 (71%) matured spontaneously. Full functionality of the grafts was shown by follicular development up to the pre-ovulatory stage and formation of CL. The endocrine activity of these follicles and CL was evident by the uterine response in ovariectomized mice. Oocytes were easily collected and appeared to be suitable for IVF after IVM.

Figure 4. Effect of granulation tissue on ovarian grafts (day 3 after transplantation). (A, F–H) Graft 3 days after transplantation into an intact control mouse. (B–E) Graft 3 days after transplantation into the wound. (A, B) Low-magnification fluorescence microscopy showing the extravasation of biotin–BSA–GdDTPA from leaky vessels (green; contrast material was injected 20 min prior to sacrifice). Note the shift of vascular hyperpermeability and extravasation of biotin–BSA–GdDTPA from the muscle surrounding the grafts in the intact control (A) towards the ovary grafted into the granulation tissue (B). (C, F) High-magnification images of B and A, respectively, showing functional blood vessels stained with BSA-ROX (injected 1 min prior to sacrifice; red) and Hoechst (blue, nuclear staining). Functional blood vessels are marked with arrowheads in the muscle around the graft (F) and inside the graft (C). Necrotic regions with degenerated follicles are marked by asterisks and stain positively by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL) (D, G; brown stain). Note the correlation between the unperfused ischaemic area in C and F (asterisks), the positive TUNEL stain and the degenerated follicles in the corresponding haematoxylin and eosin stain (E and H, respectively). (I, J) Invading fibroblasts (arrows) in a 3-day graft transplanted into wound site (J magnification of the box in I). Bars: 500 μm (A, B), 100 μm (C–I) and 10 μm (J).

Table II. Morphometric data of 0-time (non-transplanted) and ovarian fragments 1, 2, 3 and 6 days after transplantation

<table>
<thead>
<tr>
<th>Days after transplantation</th>
<th>Wound</th>
<th>n</th>
<th>Primordial follicles</th>
<th>Ovary or graft volume (mm³ ± SE)</th>
<th>Total primordial follicles/mm³</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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<td>215.8 ± 52</td>
<td>99.6 ± 0.1</td>
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*Healthy/(healthy + degenerated) × 100. Calculated for individual animals then given as a mean.
*bP = 0.03 in two-way analysis of variance with wound groups (+) versus intact control groups (−).
*cOvary or graft volume = Scan area (mm²) × number of slides × 9 or 6 × 0.004 (mm³).
Discussion

The major objective of ovarian transplantation is to preserve fertility by minimizing loss of primordial follicles and oocytes and optimizing follicular development. Transplanted fragments of the ovaries are prone to ischaemic damage during the early period after transplantation. Thus, reducing the duration of ischaemia is expected to reduce follicular degeneration and thus enlarge the surviving pool of follicles and improve the probability for subsequent successful retrieval of oocytes. In the study reported here, we demonstrated progress towards optimization of the ovarian graft transplantation procedure. The procedure suggested is aimed to improve access for oocyte retrieval and minimize oocyte loss by accelerating graft revascularization.

Two common sites were previously utilized for ovarian transplantation—the kidney capsule (Gosden et al., 1994; Candy et al., 1995; Newton et al., 1996; Oktay et al., 1998) and subcutaneous tissue (Weissman et al., 1999; Nisolle et al., 2000; Van den Broecke et al., 2001). We reported improved follicular survival when the xenograft was transplanted entirely into a skeletal muscle (Israely et al., 2004). Preferably, the location of the graft should allow safe and convenient transplantation to reduce the time in which the graft is out of the body (Liu et al., 2002) and ensure access for oocyte detection and aspiration. The location should also allow rapid revascularization to minimize ischaemia. Although subcutaneous transplantation provides a convenient location, it has a relatively poor blood supply and angiogenic capacity. On the other hand, the kidney capsule and intramuscular transplantation provide a rich angiogenic environment, but recovery of follicles and oocyte retrieval require more invasive procedures. In the study reported here, we propose the use of superficial muscle transplantation, which allows simple and rapid graft transplantation, adequate blood supply, convenient in vivo tracking and easy access for oocyte retrieval. This site was shown to allow a relatively simple procedure to improve vascularization and preservation of the graft.

Creation of a site which is rich in new developing blood vessels can be potentially achieved by two ways. The first one is the use of exogenous angiogenic factors that should be administered in a precise time, quantity and selective manner due to the complexity of the process of blood vessel formation, maturation and regression. In this complex process, various angiogenic factors such as VEGF, ANG-1, ANG-2 and bFGF may play a role. An attempt to induce angiogenesis by administering VEGF prior to ovarian transplantation did not improve the ovarian graft activity (Schnorr et al., 2002). The second option

Figure 5. Follicle survival and development 6 days after transplantation. (A, B) Healthy primordial follicles in a graft transplanted into a wound-induced granulation tissue (B, enlargement of the box in A). (C–H) Representative graft transplanted into intact control. A necrotic avascular region with degenerated follicles is marked with an asterisk (E and F higher magnification of C and D). (G, H) Hematoxylin and eosin and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate–biotin nick-end labelling stain of subsequent section, respectively (H is the enlargement of the box in G). (I–L) Representative graft transplanted into wound-induced granulation tissue, note the difference in the size of follicles in K compared with E and G (K and L higher magnification of I and J). Representative sequential sections for each group were stained for smooth muscle cells and pericytes (α-smooth muscle actin; red; C, E, I, K) and functional blood vessels (D, F, J, L) stained with biotin–BSA–GdDTPA (intravenous, 20 min prior to sacrifice; green), shown alone (D, J), or combined with BSA-ROX (intravenous, 1 min prior to sacrifice; red) and nuclear staining (Hoechst; blue; F, L). Bars: 500 μm (C, D, I, J), 100 μm (A, E–H, K–L) and 10 μm (B).
Table III. Developing follicles at 0-time (non-transplanted) and ovarian fragments 1, 2, 3 and 6 days after transplantation

<table>
<thead>
<tr>
<th>Days after transplantation</th>
<th>Wound</th>
<th>n (antral)</th>
<th>Pre-antral follicles</th>
<th>Antral follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number (mean ± SE)</td>
<td>Healthy (%) ± SE</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Healthy</td>
<td>Degenerated</td>
</tr>
<tr>
<td>0</td>
<td>–</td>
<td>3 (3)</td>
<td>38.8 ±10</td>
<td>0.5 ± 0.3</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>5 (1)</td>
<td>10.0 ± 2</td>
<td>23.0 ± 2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5 (0)</td>
<td>8.0 ± 3</td>
<td>33.4 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>7 (2)</td>
<td>4.4 ± 1</td>
<td>23.9 ± 3</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>9 (2)</td>
<td>2.8 ± 1</td>
<td>29.4 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>6 (1)</td>
<td>5.8 ± 1</td>
<td>15.2 ± 2</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>7 (1)</td>
<td>6.0 ± 2</td>
<td>18.3 ± 3</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>5 (4)</td>
<td>10.0 ± 4</td>
<td>1.4 ± 1</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>5 (3)</td>
<td>15.4 ± 4</td>
<td>1.4 ± 1</td>
</tr>
</tbody>
</table>

*In parentheses—number of animals in which antral follicles (either healthy or degenerated) were found.

*Healthy/(healthy + degenerated) × 100. Calculated for individual animals then given as a mean.

*A value of 0% healthy antral follicles was assigned for each individual animal in which no follicles were found.

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Figure 6. Growth of ovarian grafts. Cross-sectional area of the ovarian grafts was determined by magnetic resonance imaging (MRI) on days 8 (n = 4), 16 (n = 3), 28 (n = 12), 29 (n = 8) and 33 (n = 5) after transplantation (mean ± SEM). (A–C) MRI images of a representative graft that was examined on days 16 (A), 28 (B) and 33 (C) after transplantation (graft is indicated by an arrow). (D, E) Histological evaluation of the same graft 33 days after transplantation (D) haematoxylin and eosin staining and (E) smooth muscle cells and pericytes stained (α-smooth muscle actin; red). Bar, 500 μm.

Figure 7. The endocrine effects of rat ovarian grafts on uteri of ovariec-tomized recipient mice. (A) Atrophic uterus of an ovariectomized mouse in the absence of ovarian graft 35 days after ovariectomy. (B) Functional uterus of an ovariectomized mouse 35 days after ovariectomy (28 days after transplantation). (C, D) Histological sections of the ovarian graft affecting the uterus in panel B. (A, C) Hematoxylin and eosin stain. (D) Note smooth muscle cells and pericytes stained red (α-smooth muscle actin). Bars, 500 μm.

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is triggering an endogenous process associated with the formation of new blood vessels such as wound healing.

Formation of new blood vessels in non-pathogenic cases in the adult is limited. Nevertheless, the female reproductive system (the ovaries and the uterus), which undergoes periodic cycles of angiogenesis and vascular regression, is a remarkable exception (Redmer and Reynolds, 1996). Angiogenesis followed by vascular regression is also presented in wound healing, a process that is divided into three phases—inflammatory, proliferative and remodelling phases (Li et al., 2003).

In the inflammatory phase, platelet-derived cytokines recruit white blood cells (leukocytes and monocytes), which produce growth factors that prepare the wound for the proliferative phase in which fibroblasts and endothelial cells are recruited. The integrity of the tissue is restored by deposition of collagen, while angiogenesis helps sustain the new tissue. Many of the signals that promote angiogenesis occur in the inflammatory phase. Capillaries invading a fibrin clot are among the most important cellular components of early granulation tissue, as they deliver nutrients, inflammatory cells and oxygen to the wound site (Lingen, 2001).

As reported here, ovarian grafts transplanted into the granulation tissue were already perfused on the second day, namely at least 24 h prior to the intact control grafts. The quicker connection to the circulation was evident by the shift of the perfused area from the tissue surrounding the graft towards the graft itself. The high vascular permeability and angiogenic activity in the tissue surrounding the graft might be induced in response to the transplantation itself, as well as by proangiogenic factors secreted by the ischaemic graft. It is possible that the decrease in the levels of angiogenic factors and the initial perfusion of the graft lead to the reduction in permeability and angiogenesis in the surrounding muscle.
The wound-healing site provides new emerging blood vessels and possibly contributes also a large number of fibroblasts that penetrate the graft. Fibroblast invasion, a characteristic of the inflammatory phase, was detected inside the ovarian grafts 3 days after transplantation into wound-induced granulation tissue. These fibroblasts might be the cells that promote the creation of the new vasculature in the ovarian grafts. Infiltration of host stroma cells was also shown to be associated with neovascularization in MLS human ovarian tumours (Gilead et al., 2004).

The hypoxic environment during the first days after transplantation results in ischaemia–reperfusion injury by free radicals and lipid peroxidation. Apoptotic death of granulosa cells resulted in positive TUNEL and active caspase-3. In contrast, oocytes and periendothelial cells were stained by TUNEL, but we could not observe positive anti-caspase-3 staining. This could be due to the later stages of apoptosis encountered, which follows the activation by caspase-3. Also in the ovary, other initiator caspases were identified (Yacobi et al., 2004). Finally, it is possible that death of oocytes and vascular cells is due to necrosis. The efficiency of vitamins E and C as antioxidant protective agents was shown previously (Nugent et al., 1998; Kim et al., 2004) and can be potentially combined with the wound site transplantation. In addition, clearance of the degenerated primordial follicles was observed as reported previously (Nugent et al., 1998; Liu et al., 2002).

The improved perfusion achieved by transplantation after wounding resulted in better survival of primordial follicles on each of the days examined and was evident by superior follicular development to pre-ovulatory stages 6 days after transplantation. The wound tissue contributed to earlier restoration of graft vascularization. The improved perfusion during the first days and the subsequent graft protection should lead to improved long-term graft survival and function. Graft transplantation onto the muscle enabled monitoring of the graft in vivo, allowing easy detection and access for oocyte aspiration. Grafts that were followed up to a month after transplantation showed local follicular development that was tracked by MRI, systemic hormonal functionality manifested by the response of the host uteri and the development of healthy oocytes that appear to be suitable for use in IVF.

In summary, the implantation of ovarian grafts into pre-wounded muscle provides improved perfusion of the graft, survival of primordial follicles and restoration of follicle growth. Furthermore, superficial transplantation onto the muscle allows easy monitoring and retrieval of oocytes as shown in this study.

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References


