Effect of different sites for cryopreserved ovarian tissue implantation in rabbit

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BACKGROUND: Autotransplantation of frozen-thawed ovarian tissue has proven to be an effective method to restore endocrine function and fertility. But it remains to be studied which site and which method is most effective and practical. We therefore implanted small pieces of cryopreserved ovarian tissues into different sites in rabbits to find the optimal position. METHODS: Fifteen New Zealand white female rabbits were randomly divided into three groups. In group 1, fresh ovarian tissues were implanted into the mesometrium and ovarian bursa. In group 2, cryopreserved ovarian tissues were implanted into the mesometrium and ovarian bursa. In group 3, cryopreserved ovarian tissues were implanted into the preserved ovary. RESULTS: There were no significant differences among the three groups as to the proportions of normal and morphologically changed follicles in implanted ovarian tissues. The implanted ovarian tissues in the three groups did not show any evident changes in histology and ultrastructure, and all resumed follicle development and revealed maturescent follicles. CONCLUSIONS: Cryopreservation and implantation of small pieces of ovarian tissues are feasible. Generally, the mesometrium, ovarian bursa and ovary are all available sites for implantation and have similar rates of acceptance, despite some differences in the details of implantation.

Key words: cryopreservation/autologous/implantation/ovary

Introduction

Recent advances in cancer therapy have resulted in an increasing number of long-term cancer survivors. Unfortunately, they will face serious or even irreversible ovarian insults as a consequence of treatments such as radiotherapy and chemotherapy. Autotransplantation of frozen-thawed ovarian tissue has proven to be an effective method not only to restore endocrine function (Oktay et al., 2001; Radford et al., 2001; Kim et al., 2004a) but also to restore fertility (Donnez et al., 2004) in humans.

The earliest successful restoration of fertility by autografting of frozen-thawed ovarian tissue was realized in sheep (Gosden et al., 1994; Salle et al., 2002). Almodin et al. (2004a) established the method, which was called ‘sowing’, and succeeded in recovering fertility in rabbits by implanting cortical tissues in a previously irradiated ovary. Subsequently, a live birth after fresh ovarian tissue transplantation in a monkey was reported (Lee et al., 2004).

The current results for ovarian tissue transplantation in humans are very encouraging. To date, there have been several reports about orthotopic (Oktay and Karlikaya, 2000; Kim, 2006) and heterotopic (Callejo et al., 2001; Kiran et al., 2004) autotransplantation of human ovarian tissue worldwide. Two live babies were born after orthotopic transplantation of frozen-thawed ovarian tissue (Donnez et al., 2004; Oktay and Tilly, 2004). The sites of orthotopic transplantation were in the cortex of ovary (Radford et al., 2001) and the peritoneum beneath the ovarian hilum (Donnez et al., 2004; Meirow et al., 2004; Tryde et al., 2004). For heterotopic transplantation, the ovarian tissues were often introduced to the arm, then to the subcutaneous tissue of abdomen, peritoneum, rectus muscle, colic omentum or mesoarium (Oktay et al., 2004).

Despite this progress, human ovarian tissue cryopreservation and transplantation is still in its experimental stage, and it still remains unknown which site is most effective and practical. In this study, we implanted small pieces of cryopreserved ovarian tissues into different sites in rabbits and assessed the histology, ultrastructure and physiological function of the tissues to find the optimal position.

Materials and methods

Animals and programme

The experiments were carried out with New Zealand white female rabbits, aged between 12 and 24 months, with weight varying from 2.0 to 2.5 kg, from Shandong Agriculture Science Academy. Fifteen
rabbits with normal estrous cycles according to vaginal cytology were included in this test. The animals were randomly divided into three groups, with five rabbits in each group. In group 1, small pieces of fresh ovarian tissue were implanted into the mesometrium and ovarian bursa dispersively as soon as the pieces of ovarian tissue were prepared after bilateral oophorectomy. The implanted tissues were resected, and the endometrium was biopsied 20 days later. In group 2, frozen-thawed ovarian tissues were implanted into the mesometrium and ovarian bursa. Ovarian tissues were cryopreserved for 14 days after bilateral oophorectomy and then implanted into the mesometrium and ovarian bursa. Implanted ovarian tissues were removed 20 days after implantation. Small pieces of endometrium were taken for histological analysis during the surgery of implantation and implanted tissue removing. In group 3, cryopreserved ovarian tissues were implanted into the preserved ovary. Ovarian tissues were cryopreserved for 14 days after right oophorectomy. The frozen-thawed ovarian tissues were marked with fluorochrome Hoechst33258 and then implanted into the left ovary dispersively. The left ovary was resected 20 days after implantation. The morphology and ultrastructure of implanted ovarian tissues, follicle survival and development were observed in three groups. Endometrial histology in groups 1 and 2 was examined, and vaginal cytology in all experimental rabbits was observed sequentially.

**Oophorectomy**

Each rabbit was anesthetized by 1 ml/kg pentobarbital sodium i.v. Abdominal fur was sheared, and skin was sterilized with iodine. Medical laparotomy was then performed to isolate and resect the ovary. Grafts in the mesometrium and ovarian bursa were removed as indicated by the silk thread marker.

**Cryopreservation and thawing**

Slow freezing protocol was used according to the published method (Almodin et al., 2004b). The cryoprotectant medium was Dulbecco’s phosphate-buffered saline (PBS) with 1000 mg/l D-glucose, 36 mg/l pyruvate (Gibco), 1.5 mol/l dimethylsulphoxide (DMSO) (Sigma, D5879) and 10% (v/v) fetal calf serum. First, the excised ovary was washed 10 times in PBS solution and cut into small pieces of about 1 mm³. This procedure was completed in 10 min. The pieces were placed in cryoprotectant medium and gently shaken for 30 min at 4°C to promote equilibration. Then, the pieces were drawn up into a 0.5-ml plastic freezing straw (Pacific vet, NO 00-061) with a small volume of cryoprotectant medium. The straws were placed in a programmable cryopreservation system (Freeze Control, Cryologic, Australia) which was pre-cooled to 0°C, cooled at −2°C/min to −9°C, ice-seeded manually, held at −9°C for a further 5 min, cooled to −40°C at −0.3°C/min and finally transferred to liquid nitrogen (LN₂) at −196°C.

For thawing of the ovarian tissue, the straw was taken out from LN₂, held in air at room temperature for 20 s and then immersed in a water bath at 37°C for 10–20 s. The contents of straws were emptied into PBS supplemented with 1000 mg/l D-glucose and 36 mg/l pyruvate (Gibco) at room temperature and washed seven times (−1 min each wash) to remove the cryoprotectant agent. Ovarian tissues were kept in PBS with 10% fetal calf serum in an incubator with 5.0% CO₂ at 37°C until implantation.

**Fluorescence marking and observation**

Before being implanted into the ovary, the frozen-thawed ovarian tissues were cultured in PBS with 1 μg/ml fluorochrome Hoechst33258 (Anaspec Inc. 83219) and 10% fetal calf serum under 5.0% CO₂, 37°C overnight for marking the tissues. In group 3, the left ovary with implanted tissues was resected, and a rapidly frozen section was observed under a fluorescence microscope. Once the ovary was proved to have fluorescence, it was stained with haematoxylin–eosin (HE) and observed with the fluorescence microscope again to confirm the sites of implanted tissue and then analysed under a light microscope.

**Implantation**

The frozen-thawed ovarian tissues were drawn into a self-made tip, which was connected with a 10-ml syringe and injected dispersively into the mesometrium, ovarian bursa or the left ovary (Figure 1A). Much attention was taken to avoid bleeding. The implantation sites in the mesometrium and ovarian bursa were sutured using silk thread to allow subsequent detection of the transplantation sites.

**Vaginal cytology observation**

Vaginal cytology was observed to choose those rabbits with normal estrous cycle before the experiment and to evaluate the recovery of ovarian function after transplantation. From the third day after first surgery, a smear of rabbit’s vaginal mucosa was collected every day and observed under the light microscope after Pap staining for the assessment of general morphological aspects and its correlation to the estrous cycle (Fabbri et al., 2000).

**Histological analysis**

All the samples for histological analysis including fresh ovarian tissue, frozen-thawed ovarian tissue, implanted tissue and endometrium were fixed in formalin (10%), embedded in paraffin, sectioned at 3 μm and stained with HE. The histological sections were observed under a light microscope. Follicles at each developmental stage were counted and classified according the general morphological aspects as follows: (i) Morphologically normal follicles, which have regular oocyte, intact zona pellucida, well-arranged follicle cells and regular corona radiata; (ii) Morphologically changed follicles, which have deformed oocyte, incomplete zona pellucida or disordered follicle cells and (iii) Necrotic follicles, which lose normal morphological structure and appear cytolysis. The morphology of the endometrial gland was also observed under a light microscope.

**Ultrastructure**

Fresh ovarian tissue, frozen-thawed ovarian tissue and implanted tissue in groups 1 and 2 were fixed in glutaraldehyde and then cut into 90 nm sections to be observed under electron microscope.

**Statistics**

The proportions of follicles in each group were compared by means of \( \chi^2 \) test. A value of \( P \leq 0.05 \) was considered significant.

**Results**

**Vaginal cytology**

Daily sequential vaginal cytology started to reveal cornified epithelial cells, which indicated the recovery of ovarian function in groups 1 and 2 from day 14 to 18 after implantation. However, vaginal smears obtained from rabbits in group 2 before implantation showed evidence of absence of ovarian function, with deep cells dominating and several intermediary cells. In group 3, all rabbits appeared to have normal cyclical vaginal cytology before and after implantation because of the normal function of the preserved left ovary.
Figure 1. Implantation, histology and ultrastructure of ovarian tissues. (A) Implanting ovarian tissues into mesometrium. (B) Ovarian tissue implanted in the ovary under fluorescence microscope, showing the boundary of fluorescence. (C) Light microscopy of fresh ovarian tissue and (D) cryopreserved ovarian tissue. (E) Morphologically normal primordial ovarian follicles and (F) secondary ovarian follicle in cryopreserved ovarian tissue implanted in the mesometrium. (G) Morphologically normal mature follicle in fresh ovarian tissue implanted in ovarian bursa. (H) Light microscopy of implanted tissue within fluorescent region in the ovary, showing morphologically normal blood vessels. (I) Secondary ovarian follicle with morphological changes in cryopreserved tissue implanted in the ovary. (J) Atrophic endometrium 14 days after oophorectomy and (K) secretory endometrium 20 days after implantation. Electron microscopy of the follicle and interstitial tissue of cryopreserved ovarian tissue implanted in the mesometrium, (L) part of oocyte in primary follicle, showing lots of mitochondria (MI) in cytoplasm, (M) wall of secondary follicle and interstitial tissue, from inferior part are the outer granulosa cells, a layer of basement membrane, theca cells (TC) and interstitial gland cells with mitochondria and lipid droplet (L) in the cytoplasm. POF, primordial ovarian follicle; SOF, secondary ovarian follicle; AT, albuginea; E, ovarian epithelium; CT, ovarian cortex; PZ, zona pellucida; GC, granulosa cells; BM, basement membrane; O, oocyte; N, nucleus; FC, follicular cells.
Implanted tissues with fluorescence marker in group 3 were identified under the fluorescence microscope. The boundary clearly distinguished implanted tissues from original tissues after implantation (Figure 1B).

**Fluorescence observation**

Implanted tissues with fluorescence marker in group 3 were identified under the fluorescence microscope. The boundary clearly distinguished implanted tissues from original tissues after implantation (Figure 1B).

**Qualitative and developmental evaluation of follicles**

No significant differences were observed in the proportions of normal and morphologically changed follicles between frozen-thawed tissues and fresh ovarian tissues (\( P > 0.05 \)), between cryopreserved tissues and implanted cryopreserved tissues (frozen-thawed group compared with group 2 or group 3, \( P > 0.05 \)) and among implantation groups (groups 1, 2 and 3; \( P > 0.05 \)) (Table I). The percentages of normal follicles in groups 1, 2 and 3 were all fewer than that in fresh tissues (\( P < 0.05 \)). Six and four necrotic follicles were found in the necrotic regions in groups 1 and 2, respectively (Table I).

As shown in Table II, the implanted ovarian tissues in the three groups all resumed follicle development, revealed comparative proportions of follicles in each developmental stage and showed similar percentages of mature follicles.

### Table I. Comparison of follicle morphology in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Morphologically normal follicles (%)</th>
<th>Morphologically changed follicles (%)</th>
<th>Necrotic follicles (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tissue</td>
<td>273 (87.2)</td>
<td>40 (12.8)</td>
<td>0</td>
<td>313</td>
</tr>
<tr>
<td>Frozen-thawed</td>
<td>252 (81.8)</td>
<td>56 (18.2)</td>
<td>0</td>
<td>308</td>
</tr>
<tr>
<td>Group 1</td>
<td>172 (78.2)*</td>
<td>42 (19.1)</td>
<td>6 (2.7)</td>
<td>220</td>
</tr>
<tr>
<td>Group 2</td>
<td>166 (77.6)*</td>
<td>44 (20.5)</td>
<td>4 (1.9)</td>
<td>214</td>
</tr>
<tr>
<td>Group 3</td>
<td>156 (78.8)*</td>
<td>42 (21.2)</td>
<td>0</td>
<td>198</td>
</tr>
</tbody>
</table>

*Compared to fresh tissue, there were significant differences, \( P < 0.05 \).
compared to the fresh tissues ($P > 0.05$). No maturescent follicles were detected in frozen-thawed ovarian tissues. There were significantly more maturescent follicles in groups 2 and 3 compared with frozen-thawed ovarian tissues ($P < 0.05$).

### Histological analysis of ovarian tissue and endometrium

The histology of fresh ovarian tissue is shown in Figure 1C. Under the light microscope, the frozen-thawed ovarian tissue looked healthy and normal, with well-defined presence of germinative epithelium and albuginea. In the cortex of the ovary, there were primordial and primary follicles with primary oocytes and well-arranged follicle cells. The secondary follicles also appeared healthy in that it was possible to identify zona pellucida, granulosa cells and basal membrane. The interstitial cells showed slight tumefaction (Figure 1D).

Small necrotic regions in the implanted tissues were found in groups 1 and 2, but the surviving tissues had no evident structural differences compared with fresh tissues. Follicles in different developmental stages were detected. Most follicles in cryopreserved ovarian tissues implanted in the mesometrium and ovarian bursa were morphologically normal (Figure 1E and F). Most of the secondary follicles survived well with distinct nucleus, intact zona pellucida, multilayer and well-arranged granulosa cells and well-preserved basal membrane (Figure 1F). In Figure 1G, a morphologically normal maturescent follicle can be seen. It developed from fresh ovarian tissues implanted in the ovarian bursa.

All stages of follicular development were detected in the frozen-thawed tissues implanted in the ovary (group 3). The majority were morphologically healthy, and new blood vessels were-established in the interstitial tissue (Figure 1H). But still, 21.2% follicles showed some morphological changes such as irregular oocyte, destroyed zona pellucida, disordered granulosa cells, incomplete basal membrane and/or incomplete follicular theca (Figure 1I). The original ovarian tissues around implantation sites were subject to some structural insults caused by the procedure of implanting, such as disorder and defects of interstitial tissue. The ovarian tissues away from the implantation sites were not affected by implanting.

The endometrium before ovarian tissue implantation (14 days after oophorectomy) in group 2 showed atrophic aspect, with very thin glandular epithelium and rare uterine glands (Figure 1J). Twenty days after implantation, the endometria in groups 1 and 2 had recovered normal reproductive cycle and showed secretory phenomenon, with multiple layers of glandular epithelium cells and large glandular cavities (Figure 1K).

### Ultrastructure of ovarian tissue and follicle

The ultrastructure of ovarian tissue and follicles did not show any evident changes after cryopreservation and implantation. The oocytes had well-distributed chromatin in the nucleus and considerable mitochondria in the cytoplasm. Mitochondria, lipid droplets and other organelles were distributed in the cytoplasm of follicular cells, but tubular cristae were reduced in some mitochondria. The basal membrane was intact, surrounded by well-arranged theca cells. Interstitial tissue was filled with gland cells, which had lots of mitochondria, endoplasmic reticulum and lipid droplets in cytoplasm. In general, the cells and organelles were preserved well (Figure 1L and M).

### Discussion

The optimal sites for transplantation should provide favourable conditions for tissue survival, follicle development and monitoring and oocyte harvesting or spontaneous ovulation. In this study, we assessed the histology, ultrastructure and follicle development of ovarian tissues implanted in the mesometrium, ovarian bursa and ovary. The results indicated that all three sites were suitable for tissue implantation and had similar effects in supporting follicle survival and development. Compared with the ovary, the mesometrium and ovary bursa were easier to implant because of the pultaceous structure and the lack of risk of recipient ovary damage. Furthermore, implantation at the ovarian bursa is expected to result in natural pregnancy because of the site being near to the opening of fallopian tube, but this needs to be proved.

The composition of the ovary is different from those of the mesometrium and ovary bursa, being more complex and compact in structure. It caused some difficulties in implantation and resulted in slight damage to the original ovarian tissue around the implantation sites. Thus, the procedure of implanting should be performed very carefully to avoid any insult to recipient ovary. But transplantation in the ovary yields the possibility of natural conception.

Observations of endometrial histology and vaginal cytology indicated restoration of estrous cycle after ovarian tissue implantation. Before freezing, the ovarian tissues were cut into small pieces. This caused the loss of large follicles. Moreover, the freezing-thawing step also resulted in damage to large developing follicles (Israely et al., 2003). We could not find any large antral follicles in frozen-thawed ovarian tissues. But some small follicles had grown up and reached the maturescent stage from implanted frozen-thawed ovarian tissue in groups 2 and 3, suggesting survival of the implant and developmental
potency of small follicles. The fact that all three implantation
groups showed comparative maturenent follicles compared
with fresh tissues also indicated that the implants survived
well. No distinctive structural changes were found in frozen-
thawed ovarian tissues and implanted tissues. Electron micros-
copy further provided powerful evidence to confirm the
implanted tissues had survived well. These results had sug-
gested the availability of cryopreservation and implantation of
small pieces of ovarian tissue.

One of the most important factors for successful ovarian
graft transplantation is the rapid establishment of a rich blood
supply. This is crucial for survival of the ovarian follicles
(Weissman et al., 1999). Insufficient blood supply will cause
insults to follicles and oocytes and failure to establish a blood
supply can lead to necrosis of the implant (Israely et al., 2003).
In this study, we found that all three groups showed decreased
proportions of morphologically normal follicles than the fresh
tissues. This result suggested that both fresh and cryopreserved
tissue implantation could cause morphological changes to
some follicles mainly because of temporary ischaemia after
transplantation.

But among the morphologically changed follicles, antral folli-
cles and secondary follicles accounted for high proportions,
whereas primordial follicles were rare. Also, according to
some reports, there is a direct relationship between the develop-
ment stages of the follicles and their susceptibility to insuf-
ficient blood supply. Larger antral follicles invariably undergo
damage, whereas smaller ones survive well (Israely et al.,
2003). The main goal of ovarian tissue freezing and transplan-
tation is preservation of the pool of non-growing small folli-
cles, which is responsible for keeping the longevity of grafts
(Israely et al., 2003). On this point, the results of this study are
encouraging. The correlation between follicle morphology and
its development potency has not yet been revealed. In our con-
cluding study, we plan to investigate the possibility of a natural
pregnancy in the recipient and the long-term survival and func-
tion of ovarian tissue implanted in different sites.

Transplantation sites rich in vasculature can provide supe-
rior graft reception compared with those having a poor blood
supply. In this experiment, a small necrotic region was found
in the grafts implanted in the mesometrium and ovarian bursa
in groups 1 and 2, respectively, whereas no necrosis was found
in tissues implanted in the ovary. It seems that the ovarian
environment may meet the condition for sufficient blood sup-
ply better than the mesometrium or ovarian bursa. But we
could not reach a firm conclusion according to only two small
necrotic regions. The necrosis may be overcome by carefully
choosing a rich blood perfusion area in the mesometrium and
ovarian bursa during implantation. Moreover, the surviving tis-
sues in the mesometrium and ovarian bursa had no evidence of
structural differences compared with fresh tissues.

To reduce the ischaemic injury of transplanted tissue, some
measures have been taken. For example, antioxidants and anti-
apoptotic agents such as vascular endothelial growth factors
(VEGF), transforming growth factors (TGF), fibroblast
growth factors (FGF) and vitamin E have been used to relieve
hypoxic tissue damage by promoting neovascularization
(Nugent et al., 1998; Pugh and Ratcliffe, 2003). Kim et al.
(2004b) demonstrated that ascorbic acid, an antioxidant, could
reduce the apoptosis of ovarian cortical stroma under an ischaemi-
environment for 24 h. Recently, it was reported that implantation
of ovarian grafts into angiogenic granulation tissue improved graft
vascularization and follicular survival (Israely et al., 2006).

To assess the effect of implanting small pieces of ovarian
tissue, ovarian tissues as small as 1.0 mm³ were implanted into
different sites in this study, and the results indicated that most
of the implanted pieces survived well despite two small necrotic regions that failed to re-vascularize. Also, one larger
piece of ovarian tissue (as large as one-fifth of the whole
ovary) was transplanted into the mesometrium, but it was
found to be dead 1 week after transplantation. This result gave
more support for the superiority of small pieces for tissue
implantation. In this experiment, we used a self-made injector
to implant the tissues easily and successfully.

Multiple implantations of small pieces of ovarian tissue
show some advantages. (i) It facilitates the cryoprotectant in
penetrating tissue to maintain the viability after thawing. (ii) It
increases the contact of implanted tissue with the surrounding
blood supply, thus promoting re-vascularization and reducing
ischaemia. (iii) It avoids the failure caused by necrosis of the
whole transplant during intact or partial ovary transplantation.

In conclusion, cryopreservation of small pieces of ovarian
tissue is feasible. After autologous transplantation, the cryopre-
served ovarian tissue had no significant morphological and
ultrastructural changes, and the follicles survived and
developed well. The mesometrium, ovarian bursa and ovary
are all available sites for transplantation and have similar rates
of acceptance. Compared with the ovary, the mesometrium and
ovarian bursa are easier to implant because of their pulhtaceous
structure. Transplantation in the ovary offers the advantage of
the possibility of natural pregnancy, but it can cause slight
damage to the original ovarian tissues around the transplanta-
tion sites.

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