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An experimental study on autologous transplantation of fresh ovarian tissue in sheep

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ABSTRACT

Objective: To investigate current autologous transplantation methods and sites of ovarian tissues in sheep.
Methods: Sheep ovaries were resected. Ovarian cortices were sliced and transplanted orthotopically into the ovarian mesangial latum and heterotopically into the greater omentum and under groin skin. The grafts were removed two months after transplantation and examined to evaluate the survival of follicles (hematoxylin-eosin staining) and help determining feasible graft sites and transplantation methods.
Results: Graft nodules were found in the transplanted sites. HE staining of the grafts showed that multiple primordial follicles were able to survive in the grafts on both sides of the ovarian mesangial latum, the right side of the greater omentum, and the left inguinal subcutaneous tissue. Secondary or cystic follicles were found in almost all of the grafts.
Conclusion: The ovarian mesangial latum, the greater omentum and the inguinal subcutaneous tissue can be used as autologous transplantation sites, where sheep ovarian tissue can survive and the follicles grow and develop in good condition.

1. Introduction

High-dose radiotherapy and chemotherapy can damage the reproductive function of cancer patients. With the increased incidence of cancer and improved cure rates for young patients, the need to preserve the reproductive ability of these patients became more urgent. There are three main methods of fertility preservation in female cancer patients: embryo freezing, oocyte freezing and ovarian tissue cryopreservation. Ovarian tissue cryopreservation is the only option for preadolescent women with cancer, and is also suitable for patients with hormone-dependent cancer who are unable to perform superovulation induction and without delayed radiotherapy/chemotherapy. At present, more than 130 births have been achieved from ovarian cryopreservation,[1] which has thus become an effective means of fertility preservation and allowed to both secure the fertility and restore the endocrine function of patients.[2,3]

Freezing and ischemic injury are the two main limitations of ovarian tissue cryopreservation and transplantation. However, despite 7%–22% of ovarian follicle damage occurring after cryopreservation due to the use of different cryoprotectants and freezing methods,[4] up to 60%–80% of follicle damage occurs after transplantation because of local ischemia and oxygen deficit. Hence, the damage occurring as a result of transplantation

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is far greater than that resulting from freezing. A critical research point for ovarian tissue cryopreservation and fertility preservation is finding optimal sites and transplantation methods to reduce ischemic injury. Here, we evaluated different transplantation methods and sites of transplantation in sheep by observing the survival and development of ovarian follicles in grafts after transplantation. Our results provide basic research for clinical ovarian tissue transplantation.

2. Materials and methods

2.1. Experimental animals

Two adult female sheep were bought and raised at the Animal Center of Peking University Health Science Center. These individuals were used for autologous transplantation surgery of ovarian tissue.

2.2. Sheep bilateral oophorectomy

After being anesthetized and disinfected, the sheep underwent ventral midline longitudinal incisions, ~6 cm long. The skin, subcutaneous fat, former sheath, muscles and peritoneum were cut in succession. Intraoperative exploration exposed the entire bladder and the uterus behind. The bicorn uterus and the adnexa were exposed after moving the intestines. The uterus had a normal size and range of motion, smooth surface, and no abnormal nodules. The fallopian tubes were circuitous and spiral, approximately 0.2–0.3 cm in diameter. The ovaries were firm and about 1.5 cm in diameter. The right ovary was exposed and the ovarian suspensory ligament and mesovarium were fixed with two curved forceps. The ovary was resected and the remnant was sutured with #4 silk. The left ovary was removed in the exact same fashion (Fig. 1).

2.3. Ovarian tissue slice processing

The ovaries were divided into 5mm × 4 mm × 2 mm sections, according to a previously described method (Fig. 2A).[5]

Transplantation of sheep ovarian cortical tissue into the ovarian mesangial latum, greater omentum and groin subcutaneous tissue.

We exposed the right fallopian tube and the broad ligament near the oviduct fimbria in each sheep. The broad ligament with abundant blood supply in the meso-salpinx junction and the ovarian suspensory ligament were separated, and 3 pieces of ovarian tissue implanted (Fig. 2B) and sutured into place with 2-0 silk sutures. Two pieces of ovarian tissue were placed into the contralateral latum in the exact same fashion. We chose the lower right part of the greater omentum, which contains a rich blood supply. An area of about 1 cm × 1 cm was slit by sharp dissection and three pieces of ovarian tissue were inserted; 1-0 silk was used to suture the breach and mark the transplantation site (Fig. 2C). The peritoneum was continuously sutured with 2-0 silk. The rectus abdominis anterior sheath and some subcutaneous fat were discontinuously sutured in a figure-8 shape, and the skin and subcutaneous fat were stitched using mattress sutures. Slanted longitudinal incisions were made on the left groin. The skin and some subcutaneous fat were excised in succession, and three ovarian tissue sections embedded in the subcutaneous fat (Fig. 2D); the skin and subcutaneous fat were then mattress-sutured with 4 # silk. The incision was disinfected with iodine disinfectant and the procedure was completed.

3. Results

3.1. Observation of the transplanted ovarian tissues during the removal process

Two months after the transplantation, the transplanted ovarian tissues were removed from the ovarian mesangial latum, greater omentum and groin subcutaneous tissue. The longitudinal scar on the midsection of the lower abdomen was eliminated and adhesions along the sheath, subcutaneous fat, muscle and peritoneum were carefully separated. The greater omentum was exposed to exhibit the silk-thread marker left to indicate the anterior ovary grafts. The marker was found on the right lower margin of the greater omentum, and a hard nodule of approximately 0.5-cm (Fig. 3A) was found in the greater omentum and subsequently removed (Fig. 3B). The oviduct fimbria and the mesosalpinx on the left side were exposed, unveiling a 0.6-cm nodule that was then resected (Fig. 3C,D). The graft nodule had a pink surface and a transparent 0.2-cm vesicle, considered as a developmental follicle of the transplanted ovarian tissue. The oviduct fimbria and the mesosalpinx on the right side were similarly exposed and revealed a 0.5-cm nodule that

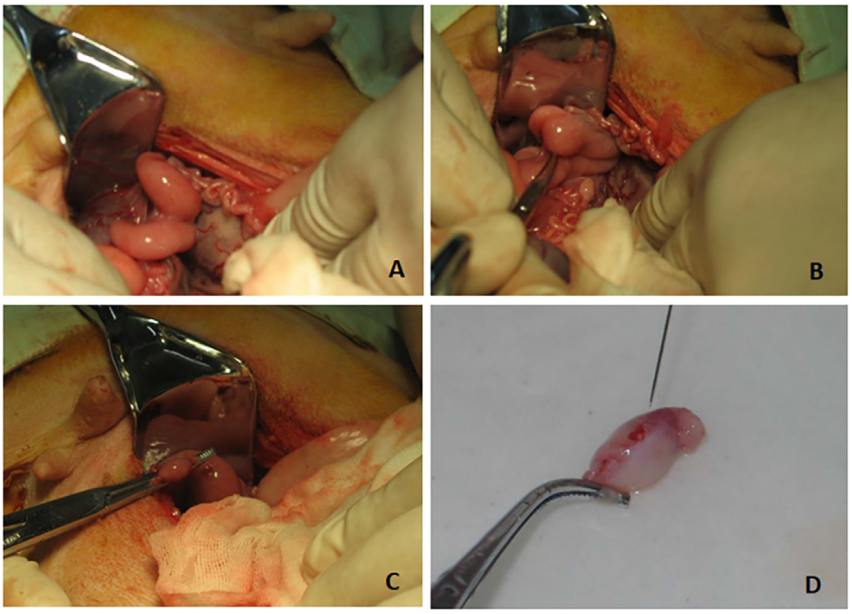

Fig. 1. Sheep bilateral oophorectomy. (A) Bicornuate sheep uterus; (B) Uterus and ovary; (C) Ovariectomy; (D) Removed ovary.

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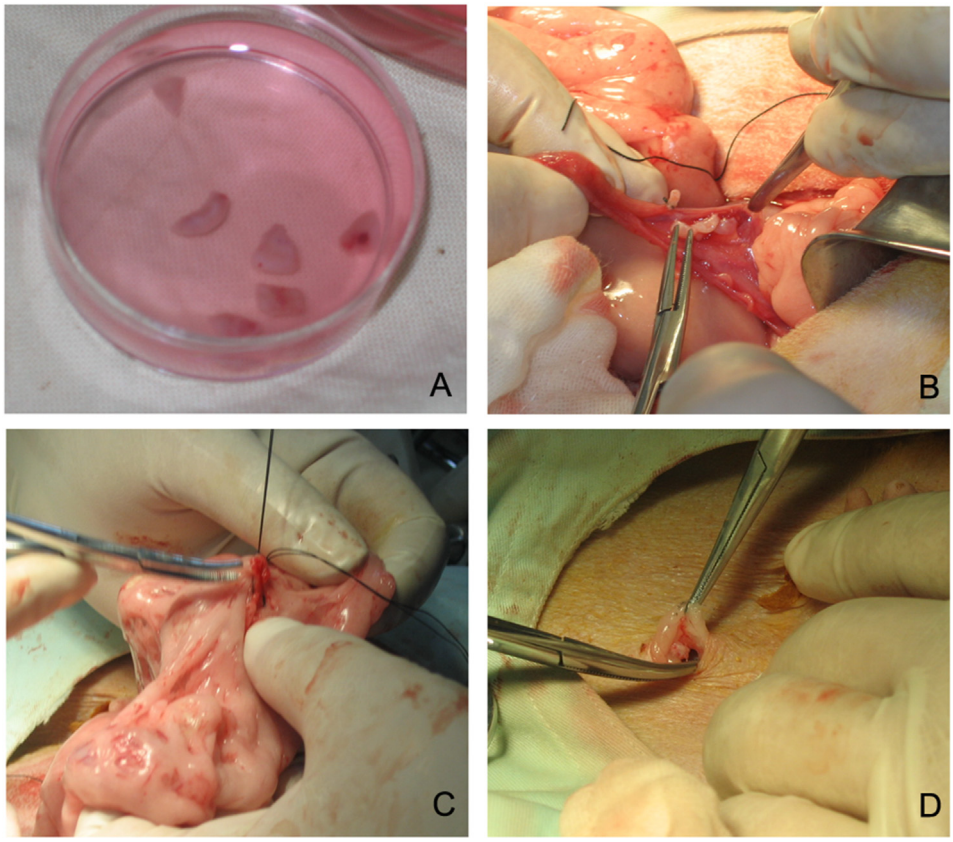

Fig. 2. Transplantation of sheep ovarian cortical tissue into the ovarian mesangial latum, greater omentum and groin subcutaneous tissue. (A) Slices of the ovarian cortex; (B) Placing ovarian tissue into the ovarian mesangial latum; (C) Implanting the ovarian tissue into the greater omentum; (D) Placing the ovarian tissue under the left inguinal subcutaneous tissue.

was excised (Fig. 3E and F). The graft nodule had a pink surface and a ~0.1-cm transparent vesicle; we could not rule out the possibility of a developing follicle. The abdominal cavity was then closed. When we cut the skin, subcutaneous fat and muscle tissue of the left groin, a 1-cm transplanted nodule was found and removed (Fig. 3G and H). The skin and subcutaneous fat were then mattress-sutured with 2-0 silk. The incision was disinfected with iodine disinfectant and the procedure was completed.

3.2. *Graft survival and follicular development in the transplanted sites*

Hematoxylin-eosin staining (HE) was applied and the survival and development of the follicles in the removed grafts were observed. Multiple surviving primitive follicles, and development of primary, secondary or cystic follicles were found in almost all of the grafts in the left and right ovarian mesangial latum, greater omentum and groin subcutaneous tissue. These observations suggest that these areas might be suitable for autologous transplantation (Table 1 and Fig. 4).

4. Discussion

Ovarian tissue transplantation can be handled as autologous transplantation, allogeneic transplantation or xenotransplantation. Autologous ovarian transplantation is the most commonly used clinically because it rarely leads to immune rejection or raises ethical concerns. After ovarian tissue transplantation, the ovarian tissue should undergo ischemia-reperfusion. The ischemia during the formation of new blood vessels can last for 3–7 days. This process will result in the ischemic injury of ovarian tissue and lead to the loss of most of the original follicles and almost all of the growth follicles.[6] Therefore, the rapid generation of neovascularization is particularly important, and it is very important to select graft sites containing a rich blood supply.

The graft sites of autologous ovarian tissue transplantation may be orthotopic or heterotopic. Orthotopic transplantation mainly involves implanting thawed frozen ovarian cortical tissues into ovary remnants or their surrounding tissues through abdominal or laparoscopic surgery with nonvascular anastomosis.[7] After orthotopic transplantation, natural conception may be achieved. Donnez et al. reported that >130 infants were successfully born after freezing and transplantation of ovarian tissue, most of which resulted from orthotopic transplantation. More than 95% of the cases reported after orthotopic transplantation restored the ovarian endocrine function, and more than 40% of the patients regained their fertility.[8] The parts of the body with rich blood supply, such as the abdominal subcutaneous tissue, forearm, breast tissue, the renal capsule, peritoneum and omentum are often chosen in the case of heterotopic transplantations, which are more affected by the local environment. These factors include temperature, pressure, paracrine factor and blood supply factors. Despite the need to develop *in vitro* assisted reproduction technologies in order to achieve pregnancy, the relatively simple operation procedure and graft monitoring make this a popular technique. Successful reproduction resulting from the heterotopic transplantation of ovarian tissue has also been reported. In 2013, Stern et al. reported, for

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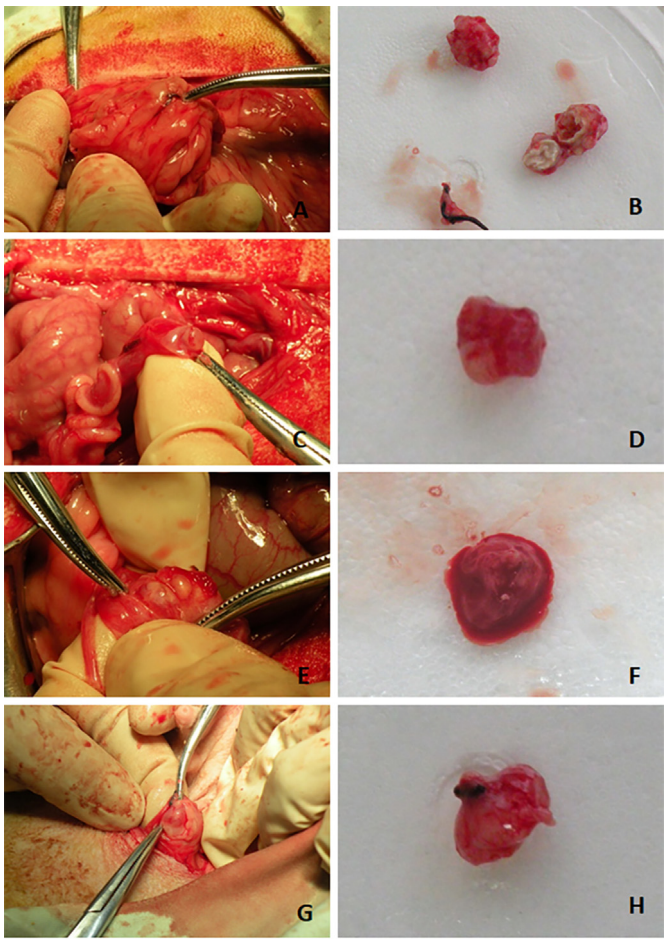

Fig. 3. Removing transplanted ovarian tissues from the ovarian mesangial latum, greater omentum and groin subcutaneous tissue. (A) Removing the graft from the greater omentum; (B) Graft from the greater omentum; (C) Removing the graft from the left ovarian mesangial latum; (D) Graft from the left ovarian mesangial latum; (E) Removing the graft from the right ovarian mesangial latum; (F) Graft from the right ovarian mesangial latum; (G) Removing the graft from the inguinal subcutaneous tissue; (H) Graft from the inguinal subcutaneous tissue.

the first time, a patient with granulosa cell tumor that successfully gave birth to a pair of twins after bilateral oophorectomy by autologous subcutaneous heterotopic transplantation of cryopreserved ovarian tissue in the abdomen.[9]

At present, ovarian tissue freezing and transplantation essentially remain in the experimental stage, and a recognized standardized protocol is currently lacking. Hence, it is necessary to conduct animal experiments before clinical trials to explore and master feasible technical strategies. Dath et al.[10] compared the short-term (3 weeks) effects of human ovarian tissue transplantation in the peritoneum, ovary, subcutaneous tissue and muscle of mice. The authors found that, of these four graft sites, the morphology and structure of follicles in the ovarian tissue implant at the muscle were more complete, with significantly less graft fibrosis. Compared with the commonly used small experimental animals, such as mice and non-human primates (e.g., monkeys), sheep have very similar ovaries to humans, in particular regarding structure and size. These characteristics have made sheep ovaries a commonly used model in ovarian cryopreserved transplantation.[11] In our previous study, we explored different freezing methods of the sheep ovarian tissue, and successfully xenografted it under the skin of immunodeficient mice.[5] In this study, we further explored the site of autologous transplantation and the transplantation method using fresh sheep ovarian tissue. Considering the influence of factors such as blood supply richness, temperature and pressure, as well as the possible locations that are convenient for natural pregnancy and surgical operation and observation, we selected three transplantation sites: the ovarian mesangial latum (orthotopic transplantation), the greater omentum and the groin subcutaneous tissue (heterotopic transplantation). Our results showed that, two months after transplantation, multiple primitive follicles were able to survive. Furthermore, primary, secondary and cystic follicles developed in the grafts placed in the ovarian mesangial latum, the greater omentum, and the subcutaneous groin. These observations indicate that all of these sites are feasible for autologous graft transplantation in sheep, and that ovarian tissue can survive and follicles grow and develop in good

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Table 1
Development of follicles in grafts.

The grafts in different areas (paraffin slices)	Primitive and primary follicles	Secondary follicles	Mature follicles	cystic follicles	Other changes could be seen
Ovarian tissue untransplanted	Multiple	1	0	1	Atresia follicles
Graft in right ovarian mesangial latum	3	0	0	1	None
Graft in left ovarian mesangial latum	Multiple	2	0	1	Atresia follicles
Graft in right greater omentum	Multiple	0	0	1	Foreign body giant cell reaction
Graft in left groin subcutaneous tissue	Multiple	0	0	3	Foreign body giant cell reaction

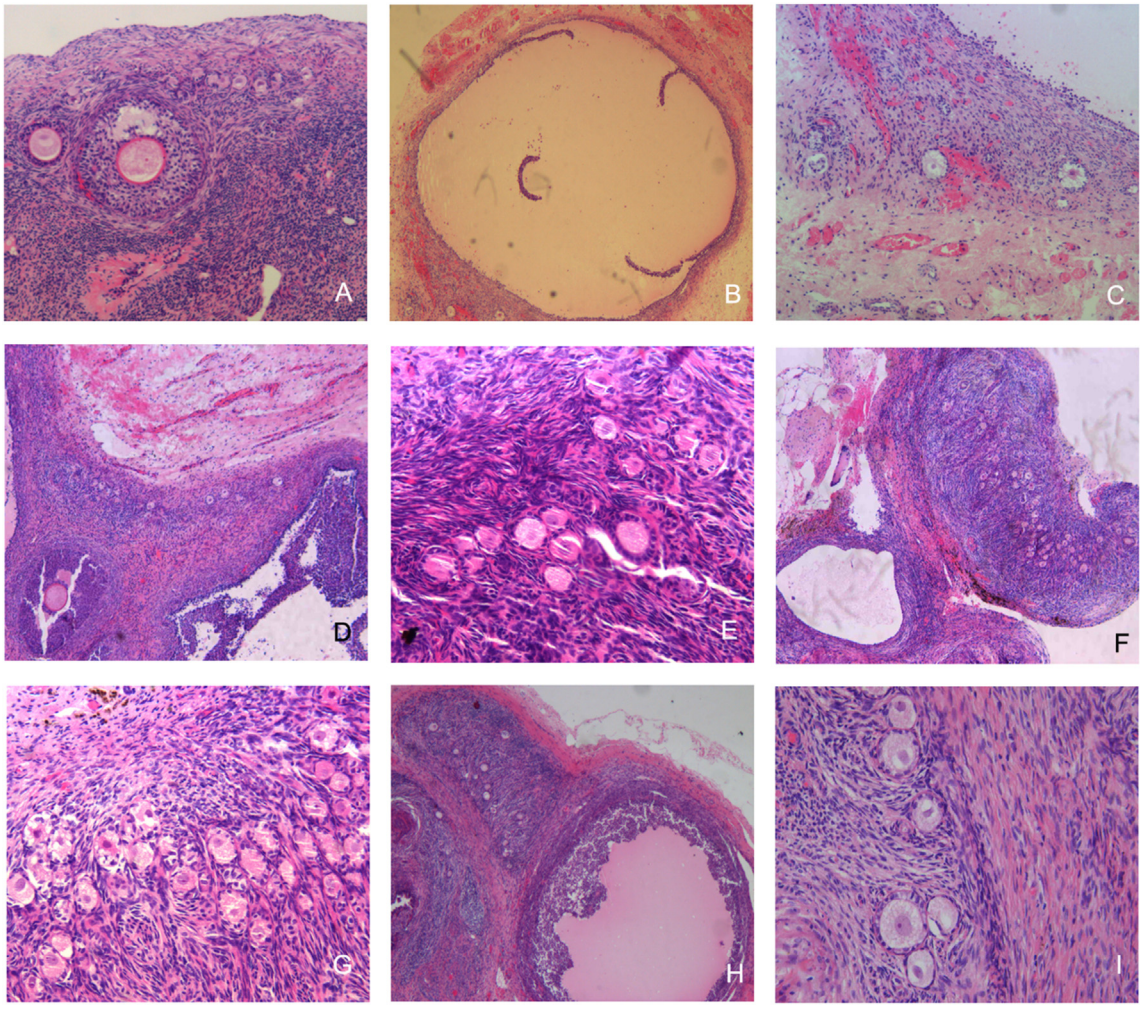

Fig. 4. Observation and evaluation of graft survival and follicular development using hematoxylin-eosin staining. (A) Untransplanted ovarian tissue (100 ×); (B–E) Grafts in the right (B, C) and the left (D, E) ovarian mesangial latum ([B, D] 40 × ; [C] 100 × ; [E] 200 ×); (F, G) Graft in the greater omentum ([F] 40 × ; [G] 200 ×); (H, I) Graft in the inguinal subcutaneous tissue ([H] 40 × ; [I] 200 ×).

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condition.

The ovarian mesangial ligament contains abundant blood supply, which is basically in the ovarian situ, making it an ideal candidate site for orthotopic transplantation. Donnez et al. suggested two schemes for orthotopic ovarian transplantation[12]: in the case of one ovary remaining, the ovarian cortex after cryopreservation was replanted on the ovarian medulla; in the absence of both ovaries, the ovarian cortex was transplanted to the anterior lobe of the broad ligament where the vascular network is visible. In this study, two months after the fresh sheep ovary cortex was transplanted into the broad ligament of the ovary mesangial, there were multiple surviving primitive follicles, developing primary or cystic follicles, and two secondary follicles, indicating that the broad ligament of the ovary mesangial was suitable for transplantation. However, it is also worth noting that follicles were abundant only on the left graft of the ovarian mesangial ligament, which was similar to previous observations in the subcutaneous neck of xenograft mice.[5] Basically, in each transplanted mouse, only one of the two grafts had visible follicles. Hence, it is possible that one graft provides hormonal support for follicular survival and development in the other graft. The specific reasons are unknown, and similar reports could not be retrieved. However, this suggests that in the transplantation of the broad ligament of the ovarian mesangium, multiple tissue grafts should be performed at both sides or multiple points as far as possible.

The greater omentum has sufficient space for tissue transplantation, good flexibility and abundant blood supply, and is recommended as a good heterotopic ovarian tissue transplantation site. In the present study, we found that the graft in the greater omentum seemingly contained more surviving primitive follicles than those in the ovarian mesangial latum and the subcutaneous groin. The differences of survival follicles in grafts can't completely reflect the effect of different transplantation site due to the heterogeneity of the original transplant tissues, which might have different primordial follicle reservation before transplantation. However, it is a good indication that ovarian tissue can survive and that the follicles can grow and develop in the omentum, which may thus constitute an alternative site for transplantation.

Although the majority of successful births result from orthotopic transplantation, heterotopic transplantation outside the pelvic cavity has been favored owing the simple operation procedure and low trauma. Considering the effects of temperature and skin tension on ovarian tissue transplantation, we chose the groin skin, instead of the commonly used rectus or abdominal subcutaneous, for transplantation. Our results showed that there were multiple graft primitive follicles and survival and development of primary or cystic follicles, illustrating that the groin skin can also be used as a suitable transplantation location.

Several studies have previously attempted different ways to promote the formation or regeneration of blood vessels soon after transplantation, in order to reduce the damage of ischemic hypoxia-reperfusion damage to the transplanted ovaries. At present, exogenous intervention factors, including extracellular matrix molecules, vascular epidermal growth factors, antioxidants, and hormone support, are commonly used for ovarian tissue,[13] but are only used for *in vitro* studies. Mechanical stimulation, such as surgical trauma, promotes the formation of new blood vessels. After mechanical injury, the inflammatory phase is accompanied by the deposition of collagen and contributes to the formation of new blood vessels. Therefore, based on this principle, a mechanical injury at the graft site before tissue transplantation can induce the formation of new blood vessels. Donnez et al. suggested that the peritoneal windows should be created in the anterior lobe of the broad ligament, where the vascular network can be seen for ovarian orthotopic transplantation.[12] In this study, we used a sharp device to create mechanical damage at the implant sites, which may promote the formation of new blood vessels and the survival of the transplanted ovarian tissues. Two months after transplantation, we observed multiple surviving primitive follicles and several developed primary or cystic follicles. Furthermore, two secondary follicles were found in the grafts of the broad ligament of the ovary mesangial, suggesting that this transplantation method may be beneficial to the growth and development of transplanted ovarian tissue. We note that these observations are consistent with Donnez's recommendation.

However, some limitations might affect our study. First, more samples are needed for transplantation after cryopreservation to provide a feasible technical solution for future clinical applications. Second, longer studies are needed after transplantation to assess the function of hormone recovery, follicular maturation, and successful pregnancy and reproduction, providing more evidence needed for clinical reference.

Conflicts of interest

The authors wish to declare they have no conflict of interests related to the publication of this article.

Compliance with ethical standards

This study was approved by the Medical Ethics Committee of Peking University People's hospital.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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