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Heterotopic Ovary Transplantation Containing Periovarian Adipose Tissue

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Abstract

Introduction: Hypoxia and ischemia occurring after ovarian transplantation lead to significant follicular losses. The aim of this study was to prevent follicular losses during ovarian tissue transplantation through periovarian adipose tissue, without the need for any scaffold, bioengineering applications, or vascular anastomosis.

Methods: In our study, fresh ovaries (maintaining their structural integrity) were transplanted into the dorsal muscles of the rat along with periovarian adipose tissue.

Results: Our findings indicate that paracrine factors secreted by fibroblasts in the tunica albuginea and fibrous sheath, along with adipocytes and mesenchymal stem cells in periovarian adipose tissue, effectively prevent ischemia in ovarian tissue and accelerate angiogenesis.

Discussion and Conclusion: Based on our findings, periovarian adipose tissue effectively prevents ischemia in ovarian tissue and plays a crucial role in preserving the ovarian follicle reserve, without the need for scaffolds, bioengineering applications, or vascular anastomosis

Key words: Heterotopic transplantation; adipose tissue; ovary

Introduction

Ovarian tissue transplantation, a protective experimental medical procedure, has enabled numerous live births in recent years. It is a method used to preserve fertility in women requiring urgent chemotherapy and prepubertal girls (1,2,3). Ovarian tissue transplantation can be **Performed in two ways:**

1. Orthotopic Transplantation-The tissue is transplanted back into its original location (ovarian site).
2. Heterotopic Transplantation-The ovarian tissue is transplanted into different anatomical sites, such as the inner forearm, abdominal wall, fallopian tubes, or under the kidney capsule (4, 5).

Different cryopreservation methods are applied to preserve ovarian tissue. The two main freezing techniques are:

1. Slow Freezing A controlled, gradual cooling process.
2. Vitrification (Rapid Freezing)-A fast cooling process that prevents ice crystal formation.

Currently, no optimal procedure has been established for freezing ovarian tissue. Cells undergoing cryopreservation suffer damage due to intracellular ice crystal formation and dehydration. Cryoprotectants are compounds used to prevent cell dehydration and minimize freezing-related damage. These agents are added to the preservation solution to protect tissues or cells during freezing. Among these techniques, the slow freezing method remains the most commonly applied traditional approach (6,7). In rats, the ovary is surrounded by a fibrous sheath, and adjacent to it lies periovarian adipose tissue, which has a white unilocular structure. This periovarian adipose tissue is absent in humans. Studies have shown that periovarian adipose tissue plays a significant role in follicular development and body lipid metabolism (8). The aim of this study is to prevent follicular loss during ovarian tissue transplantation using periovarian adipose tissue, without the need for vascular anastomosis. With this technique, we aim to significantly reduce ischemia in ovarian tissue and promote faster angiogenesis without the need for scaffolds or bioengineering applications. In humans,

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transplanting ovarian cortex grafts along with visceral adipose tissue after thawing may ccelerate angiogenesis, thereby preventing tissue necrosis. The findings from this study could pave the way for new clinical approaches in ovarian transplantation, leading to the development of novel transplantation techniques in the future.

Materials and Methods

Our study was approved by the Pamukkale University Animal Experiments Ethics Committee with decision number 05.11.2024-E.604534. Two 11-week-old female Wistar Albino rats were obtained from the Pamukkale University Experimental Surgery Application and Research Center. To ensure that the ovarian cycle was synchronized in both rats, vaginal smears were taken before the procedure. When the rats were in the proestrus phase, they were anesthetized with general anesthesia, and a 2-3 cm incision was made to remove the right and left ovaries. The incision was then closed with sutures. The ovaries, along with the periovarian adipose tissue, were placed in 100 mm Petri dishes containing Phosphate Buffered Saline (PBS, Capricorn Scientific, Cat no: PBS-2A, Germany) for preservation. Fresh ovaries containing periovarian adipose tissue were transplanted bilaterally into the dorsal muscles of the rat through a 2-3 cm incision made after anesthesia. The incision was then closed. To avoid damaging the ovarian tissue, a small portion of the fallopian tubes was preserved along with the ovaries, and the ovaries were sutured to the muscle tissue using 6.0 polypropylene sutures (Covidien, Cat no: D8L0624Y, Ireland) (Figure 1).

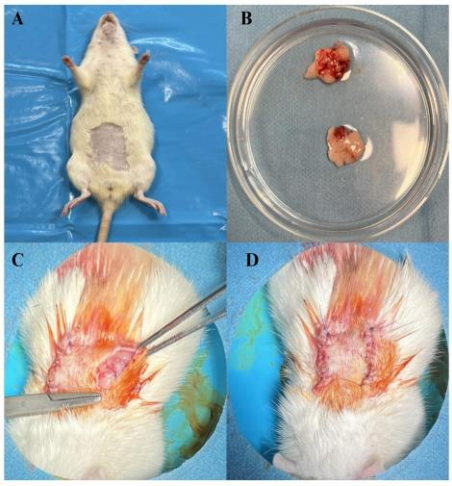

Figure 1: A-B-C-D Ovaries containing periovarian adipose tissue were sterilely removed from rats by ovariectomy. Afterwards, heterotopic transplantation of the ovaries was performed as a whole on the right and left sides in the dorsal region of the rat.

Post-transplantation tissue analysis:
Two weeks after ovarian tissue transplantation, the ovaries were removed from the rat's dorsal muscles and prepared for paraffin embedding.

- Histopathological Evaluation:
- 5 µm-thick serial sections were obtained.
- The sections were stained with Hematoxylin & Eosin (H&E) and Alcian Blue (all from Sigma-Aldrich Chemie GmbH, Germany).
- Samples were examined under a light microscope (BX51 Olympus, Japan).
- Immunohistochemical Analysis:
- Caspase-3 immunoreactivity (Thermofisher, Cat no: 437800, USA) in the ovarian tissues was assessed using immunohistochemistry to evaluate apoptotic activity.

Statistical analysis: Since a statistically significant result could not be obtained due to the limited number of subjects, statistical analysis was not performed.

Results

Fourteen days after transplantation, upon reopening the dorsal skin incision, it was observed that the fascia had regenerated and covered the transplanted ovaries. Periovarian adipose tissue was found to have effectively integrated with both the ovarian and muscle tissues, with angiogenesis occurring in both the ovarian and surrounding tissues. The minor bleeding that occurred during ovarian transplantation had been self-limited into a hematoma, which, after 14 days, was contained and had no adverse effects on the tissues (Figure 2).

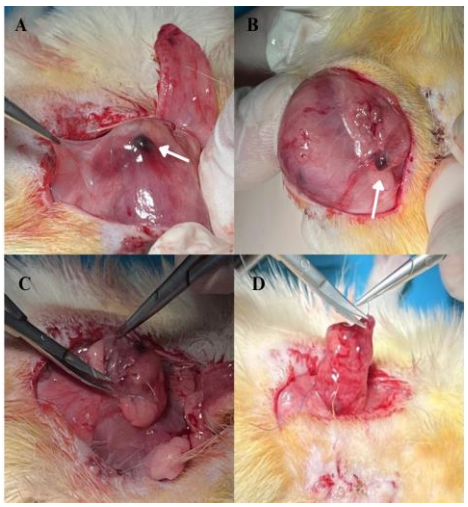

Figure 2: A-B-C-D Ovarian tissues containing periovarian adipose tissue are observed 14 days after transplantation. Adipose tissue was well invaded into the surrounding tissues, new vascularization was observed, both ovaries were intact and covered by fascia. Hematoma is shown with white arrow.

Histological examination revealed that the tunica albuginea, the fibrous capsule surrounding the ovary, the rete ovarii, follicles, stromal cells, and vascular structures in the medulla were preserved, with no fibrosis observed in the parenchyma (Figure 3).

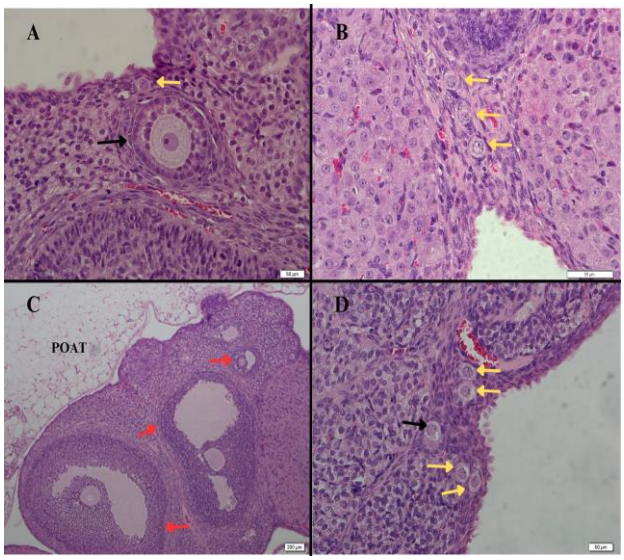

Figure 3: Appearance of primordial and primary follicles in A-B Control rat ovary and C-D Transplanted rat ovary. Primordial follicle is indicated by yellow arrow, primary follicle by black arrow and tertiary follicle by red arrow. (A-B-D Magnification: 400X) (C Magnification: 100X) H&E stain.

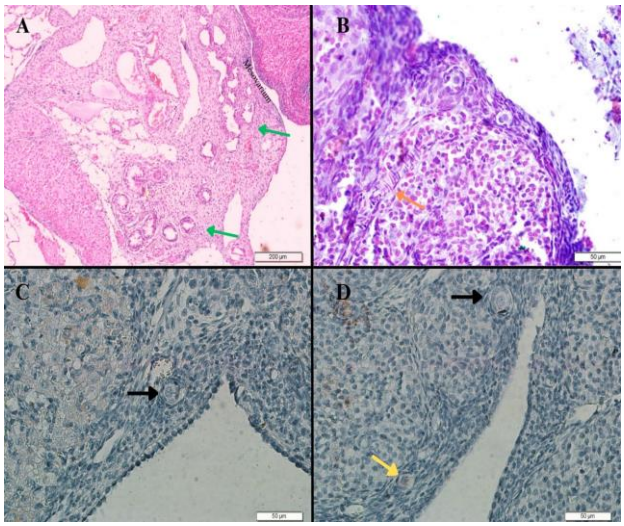

Figure 4: A. Extra rete ovarii structures in the transplanted rat mesovarium and (H&E stain) B. View of the spiral arteries inside the ovary (Alcian Blue stain). Rete ovarii is shown with green arrow and spiral artery is shown with orange arrow. C. Expression of caspase-3 immunoreactivities in follicles and stromal cells in control rat ovary. Primary follicle is indicated by black arrow. D. Expression of caspase-3 immunoreactivities in follicles and stromal cells in transplanted rat ovary. Primordial follicle is indicated by yellow arrow, primary follicle by black arrow. (A. Magnification: 100X) (B. Magnification: 400X) (C. Magnification: 400X) (D. Magnification: 400X)

Rete ovarii structures remained intact within the mesovarium, and spiral arterioles in the cortex and medulla showed no degeneration, maintaining structural integrity. Follicle counting showed no significant difference in follicle numbers between the transplanted ovaries and control rat ovaries. Additionally, caspase-3 immunoreactivity in follicles and stromal cells did not increase compared to the control group, indicating that apoptosis was not significantly induced by the transplantation procedure (Figure 4).

Discussion

Adipose tissue contains adipocytes as well as adipocyte progenitor cells, mesenchymal stem cells, fibroblasts, endothelial cells, pericytes and immune cells (9). It is a dynamic endocrine organ that plays a central role in metabolic regulation. Visceral adipose tissue is found not only around the ovary but also in organs such as the liver, kidneys, intestines, and pancreas (8). Mesenchymal stem cells isolated from adipose tissue are frequently used in clinical research due to their regenerative and tissue repair properties. Adipokine hormones secreted by adipose tissue play a significant role in follicular development in rat ovaries. Additionally, mesenchymal cells within adipose tissue exert a pro-angiogenic effect through the paracrine secretion of bioactive molecules such as VEGF, EGF, bFGF, and HGF (8,10). In order for the tissues transplanted to the patient with autotransplantation to adapt to the environment and to be re-blooded, new vessels must form from both the graft and the surrounding tissues. This process takes more than 10 days and primordial follicles are lost due to ischemia. VEGF is a protein that stimulates the formation of blood vessels and has a critical role in neoangiogenesis in adult tissues (2,11,12). Delayed angiogenesis and hypoxia after ovarian cryopreservation and transplantation may disrupt the expression of genes involved in the transition from primordial to primary follicle in the ovary. When repressor and activator genes are not balanced, it can lead to premature loss of follicles, known as premature ovarian failure. Successful implantation of ovarian grafts depends on the revascularization period, because tissue implants are subjected to ischemic damage until angiogenesis occurs (2,5,12,13). When frozen and thawed ovarian tissue is transplanted, it has been demonstrated that hormonal functions and fertility can be restored (14). Since ice crystal formation is known to cause physical and mechanical damage to cells, optimal vitrification protocols are used for preserving ovarian tissue in

infertility treatments. However, both vitrification and slow freezing protocols lead to follicular cell loss, resulting in a decrease in follicle count (2,3,7). In a study by Wietcovsky et al. the effectiveness of heterotopic transplantation of vitrified ovarian tissue in prepubertal rats was evaluated. Their findings indicated that ovarian tissue vitrification can be used to preserve fertility and restore endocrine functions (6). In another study, Tian et al. investigated the effects of transplanting different volumes of ovarian tissue on reproductive endocrine functions in rats after ovariectomy. Their results showed that larger ovarian tissue transplants experienced lower follicular loss and were able to maintain their functions more effectively than smaller grafts (1). Sheikhveisy et al. reported that when they performed vitrification of the entire ovarian tissue followed by transplantation into the omentum, follicular viability was lower compared to fresh ovarian tissue transplantation. Their study demonstrated that insufficient blood flow after whole ovarian vitrification and omental transplantation led to increased ischemic damage, resulting in a significant reduction in follicle count (4). In another study, Hirayama et al. transplanted ovarian tissue from marmosets under the kidney capsules of immunosuppressed mice. The follicles maintained their viability, and as a result, blastocyst-stage embryos were successfully obtained (15). Similarly, in a study by Taketsuru et al., rat ovarian tissue was transplanted under the kidney capsule of immunosuppressed mice. The follicles remained viable, and embryos obtained via in vitro fertilization (IVF) were subsequently transferred into the uterine tubes of rats, resulting in live births (16). Dong et al. compared the efficacy of ovarian tissue transplantation onto the surface of the biceps femoris muscle versus under the renal capsule in rats. Their findings indicated that, in terms of total follicle count and serum estrogen concentration, transplantation onto the biceps femoris muscle was more successful than renal capsule transplantation (11). In another study, Shiroma et al. applied melatonin hormone during the cryopreservation process to protect ovarian grafts. They observed that melatonin supported follicular activity in ovarian transplantation (2). Terren et al. investigated different transplantation sites to prevent follicular loss after ovarian tissue cryopreservation and transplantation. They transplanted ovarian tissue between the dermal and cartilage layers of the external ear in mice. Due to the high revascularization rate in the ear region, they concluded that this site could be a suitable location for ovarian tissue transplantation (5). Yang et al. investigated the paracrine effects of periovarian adipose tissue (POAT) on folliculogenesis by surgically removing POAT unilaterally in mice. Their results showed delayed antral follicular development and an increase in atretic follicles. These changes were observed only in the ovary lacking POAT, while the contralateral ovary remained unaffected, suggesting that the paracrine interaction between POAT and the ovary is crucial for normal folliculogenesis (8). Day et al. conducted a study in mice with experimentally induced premature ovarian failure via bilateral ovariectomy. To restore endocrine function and prevent graft rejection, the excised ovarian tissue was encapsulated in polyethylene glycol (PEG) hydrogels and transplanted. Their findings demonstrated that ovarian endocrine functions were successfully restored after allotransplantation (3). Kim et al. designed an artificial ovarian tissue using synthetic hydrogel, polyethylene glycol, and vinyl sulfone (PEG-VS) as a supportive matrix containing primordial and primary follicles. After orthotopic transplantation, PEG-hydrogel encapsulated ovarian follicles were shown to function as artificial ovarian tissue, successfully supporting follicular development (14). Man et al. demonstrated that ovarian tissue transplanted along with exogenous endothelial cells prevented follicular functional loss. Exogenous endothelial cells played a crucial role in the formation of functional blood vessels during this process (12). In another study, Kawai et al. subjected mouse ovaries to a brief, low-dose collagenase treatment before exposing them to cryopreservation without hyperosmotic solution exposure. This approach was found to better preserve ovarian function and viability (7). Subcutaneous adipose tissue refers to the adipose layer beneath the skin, while visceral adipose tissue surrounds internal organs. Approximately 80% of body fat consists of subcutaneous adipose tissue, while 20% is visceral fat. Subcutaneous adipose tissue releases its metabolic products into systemic circulation, whereas visceral adipose tissue, found in the mesentery and omentum, drains directly into the liver via the portal circulation. Periovarian adipose tissue (POAT) in rats and mice is intra-abdominal, leading many researchers to classify it as visceral adipose tissue. However, due to its drainage into systemic circulation rather than portal circulation, it should not be considered true visceral adipose tissue (17-19). Adipose tissue has a rich vascular network that allows it to be in close contact with capillary beds. However, the major drawback of autologous

fat transplantation is the unpredictability of graft viability. In clinical applications, a significant issue following fat autotransplantation is its absorption over time. The observed average reduction in the total implanted volume ranges between 25% and 70%. Initially, adipose tissue grafts rely on diffusion for nourishment in the transplanted area. Until neovascularization develops, adipose tissue depends on surrounding tissues for nutrients through diffusion. However, this can lead to atrophy and necrosis of the grafted adipose tissue (20). As demonstrated in our previous studies, the ovarian surface epithelium is covered by a fibrous sheath, and visceral periovarian adipose tissue is adjacent to it (21). In our study, the tunica albuginea, fibrous sheath, and periovarian adipose tissue were transplanted as a single unit along with the ovary. Our findings indicate that paracrine factors secreted by fibroblasts in the tunica albuginea and fibrous sheath, along with adipocytes and mesenchymal stem cells (MSCs) in periovarian adipose tissue, effectively prevent ischemia in ovarian tissue and accelerate angiogenesis. In another study we conducted, we observed that rete ovarii structures remained actively present in the ovaries of rats during both early and late reproductive periods. This finding led us to hypothesize that rete ovarii play a role in maintaining ovarian homeostasis (22). In ovarian transplantation in the clinic, the cortex is generally frozen after being cut into small pieces and transplanted after thawing. In this traditional method, the integrity of the tunica albuginea is mechanically compromised and primordial follicle loss occurs after thawing. The tunica albuginea is still an understudied layer and is thought to play an important role in protecting primordial follicles. Hypoxia-induced ischemia continues to be an important problem in experimental avascular ovarian transplantation in animals. Additionally, we found that the tunica albuginea contributes to the microenvironment of some primordial follicles, playing a significant role in follicle protection and development. Some regions of the tunica albuginea undergo regeneration and proliferation after corpus luteum formation, and this renewal process continues actively throughout reproductive cycles Therefore, we believe that primordial follicles were better preserved in our study, as the tunica albuginea and rete ovarii structures were transplanted intact, maintaining their structural integrity. In experimental animal studies on ovarian transplantation, there has been no ovarian transplantation with periovarian adipose tissue. Therefore, there is no study that we can compare. It was observed macroscopically that the transplanted ovary adapted to the surrounding tissue and new blood vessels were formed both in the ovary and the surrounding tissues. In histologic examinations, vascularization was observed and no areas of vascular congestion were observed. Comparison of the results of our study with the heterotopic method in different transplantation sites and for longer periods may give us new information. Further studies are needed for the clinical application of our study. As known, there is no periovarian adipose tissue in humans. In humans, adipose tissue is found in different localizations of the body. The ability to select the most compatible type for the ovary and to serve as a scaffold for the ovary at the time of transplantation should be investigated in the future in terms of clinical applications.

Study limitations: Our study has some limitations. More samples are requared to establish a suitable technique for future clinical applications. Additionally, longer-term groups should be formed in the study, and the hormone levels of these groups (FSH, AMH and E2) should be measured. The volume and weight of the transplanted periovarian adipose tissue could not be measured before and after the study due to the applied technique.

Conclusion

In conclusion, one crucial aspect to consider in heterotopic transplantation is that the spiral arteries, which normally branch into and nourish the ovary, are absent in the transplanted region. Since the vascular supply type changes, it is essential to investigate how this alteration affects ovarian tissue function. Various scaffolds and controlled-release bioactive molecules have been utilized in transplantation studies to prevent ischemia in ovarian tissue. Research in tissue engineering continues intensively to develop an optimal technique in this field. Based on our findings, periovarian adipose tissue effectively prevents ischemia in ovarian tissue and plays a crucial role in preserving the ovarian follicle reserve, without the need for scaffolds, bioengineering applications, or vascular anastomosis. In this regard, our study introduces a new concept in transplantation and may pave the way for future clinical studies.

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Ethical statement: Our study was approved by the Pamukkale University Animal Experiments Ethics Committee with a decision numbered 05.11.2024-E.604534.

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