

# Autologous transplantation of fresh ovarian tissue in the ICR mice model

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## SUMMARY

During the past 20 years, autologous transplantation of either frozen ovarian tissue for cancer patients or ovarian tissue pretreated with curative medications for premature ovarian insufficiency (POI) patients have been achieved worldwide. POI is characterized by amenorrhea before 40 years of age. As a result, juvenile cancer patients and POI patients have increased chances to give healthy birth based on this achievement. It also indicates that mastering the skills of ovarian auto-transplantation is required for researchers and doctors. In this study, we performed orthotopic auto-transplantation of fresh ovarian tissues by transplanting unilateral half ovarian tissue to the contralateral ovary in the ICR (Institute of Cancer Research) strain of outbred, heterogeneous mice to determine if the transplanted tissue could be functional. The transplantation surgery entailed taking partial ovarian tissue from the unilateral ovary of the adult ICR female mouse and then transplanting into the ovarian sac of the contralateral ovary, in which part of the ovarian tissue had been removed in advance. One week after surgery, we found that the freshly transplanted mouse ovarian tissue survived and functional, as histochemical and immunofluorescence assays have shown that not only both follicles at different developing stages and corpus luteum are available, but the morphology of them are properly maintained within the transplanted tissue. This study approved that for those who are interested in ovarian transplantation study, applying mouse model to practice ovarian tissue auto-transplantation is reliable and easy handled.

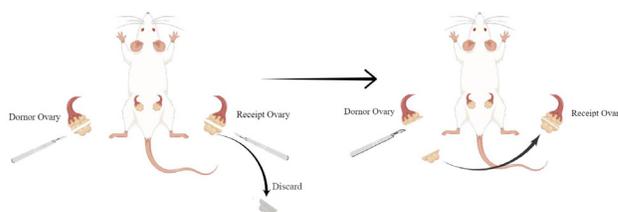
## INTRODUCTION

In order to help female patients who have recovered from malignant tumor treatments to have their own babies, frozen ovarian autologous transplantation surgery was invented in 2004 (1). The goal of the surgery is to take part of the patient's ovarian tissue for cryopreservation before cancer treatment, and then thaw and transplant the tissue back into the autologous body after successful cancer treatment to enable a future pregnancy. In combination with this technique, doctors have used the novel technique of *in vitro* activation (IVA) of ovarian tissue. Using these techniques, doctors have

helped premature ovarian insufficiency (POI) patients who are infertile due to aberrant follicle growth under 40 to have baby in 2013 - 2015 (2-4). By the year 2016, there are 37 babies born worldwide using auto-transplantation technique, and 2 babies born using IVA plus auto-transplantation techniques. Consequently, this significant medical achievement provides an important additional strategy for women to have a healthy birth as an alternative to the preservation strategies of germ cells and early embryos of the patients.

Although ovarian tissue autografting has become the basic technique to assist female reproduction recovery via multiple strategies, it is necessary to improve the technique to meet the needs of clinical practices (5). For instances, to improve the efficacy of IVA plus auto-transplantation in human-assisted reproduction, additional research on the ovarian autografting procedure in mice is needed (6). And, the transplantation of cryopreserved ovarian tissue smaller than half reduced the litter sizes in mice (7), which signals the need for optimizing clinical procedures as well. Therefore, mastering the skills of ovarian auto-transplantation is required for researchers and doctors.

The objective of this study was to replicate fresh ovarian tissue orthotopic auto-transplantation assays in mice, and to investigate whether the transplanted tissue survived and functioned properly, which were verified by histochemistry and immunofluorescence assays. We also wanted to know if seven days after the surgery is enough time to examine the effect of transplantation on ovarian tissue health. We successfully performed contralateral autologous transplantation of fresh ovarian tissues in outbred ICR mice using published protocols (Figure 1) (5, 8).



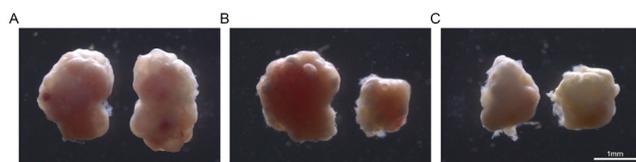
**Figure 1: The surgery procedures of the orthotopic ovarian tissue atograft.** After mice were anesthetized by intraperitoneal injection, the ovarian excision and auto-transplantation under microscope were performed. The sham surgery was performed on the mouse of the control group.

The results showed that the shape of the follicles, the sizes of the individual oocyte within each follicle, the thickness of the granulosa cells outline the follicles were all similar to those found in the control group. Meanwhile, the available of corpus luteum (CL) that were morphologically normal in the transplanted ovarian tissue also indicated that the tissue survived and were functional. In addition, the immunofluorescence assays indicated that the cellular proliferation and the cellular apoptosis both insignificant different between the transplanted and the control group. The results implied that the transplantation surgery was successful, and that seven days after surgery was reliable to evaluate the effect of transplanting surgery.

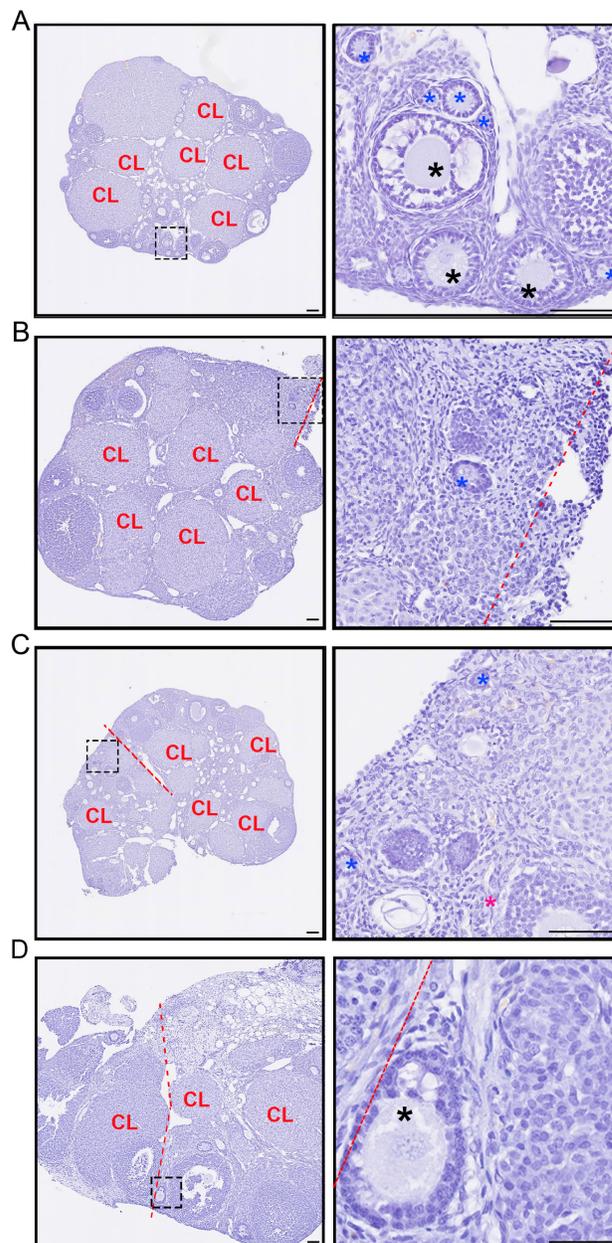
## RESULTS

To verify the effect of autologous transplantation on the ovarian morphology, the size as well as the morphology of the ovarian tissues in the control mouse and the experimental mice seven days after the surgery were examined through histochemical assay. A sham surgery was performed on the mouse. As is shown in the **Figure 2**, the morphology of the control group (**Figure 2A**) was different to that of the auto-transplanted ovaries of 2 mice (**Figures 2B,C**) when we observed the collected ovaries under a microscope. In the control group, the sizes between the two ovaries were uniform and the outline of each ovary was oval shaped (reniform) (**Figure 2A**). However, in the experimental group (**Figures 2B,C**), both the shape and size of individual ovaries were changed. Briefly, the shapes of the donor and the receipt ovaries were more irregular than the control (**Figure 2A**). Additionally, the size of the donor ovary was not only much smaller than the contralateral recipient one (**Figures 2B,C**), but it is also smaller than the ovary in the control group (**Figure 2A**).

To clarify whether the specific ovarian structures of autologous transplanted tissue were properly maintained seven days after transplantation, we dyed ovarian tissue sections with hematoxylin and then examined the structural



**Figure 2: Extracted ovaries and analyzed the shape and the size (n=3).** (A) Control group (sham). Both ovaries were of similar size and oval shaped (reniform), indicating normal ovarian morphology. (B, C) Experimental group (auto-transplantation). (B) The left ovary was the recipient, which showed the similar shape and size to those of the control. The right ovary was the donor ovary, which lacked partial tissues compared to the control. (C) The left ovary was the receipt, which showed smaller size and irregular shape compared with the ovaries in the control group. The right ovary was the donor ovary, which showed even smaller size and irregular shape compared with the ovaries in the control. The pictures were 3 × magnified, The scale bar is 1 mm.



**Figure 3: An overview of the structures of the ovarian slides with hematoxylin staining after surgery.** The figures on the right lane are the enlarged area extracted respectively from the left lane, which are indicated with black square broken lines. (A) The structure of the ovaries in the control group. The developing follicles at different sizes and the corpus luteum (CL) are shown with different colored stars. (B) The structure of the donor ovary in the experimental group. The red broken lines indicate the cutting position during the surgery. (C and D) The structure of the receipt ovary corresponding to the experimental group in Figure 1B and Figure 1C, correspondingly. The red broken line indicates the position of transplantation in each ovary. The healing areas where the donor ovarian tissues and the remaining tissue of the receipt grow together are enclosed in this region. The images are magnified 2 and 20 times on the left and right, respectively. Red star: primordial follicle; blue stars: primary follicles; black stars: secondary follicles; CL: corpus luteum. The scale bars are 100  $\mu$ m.

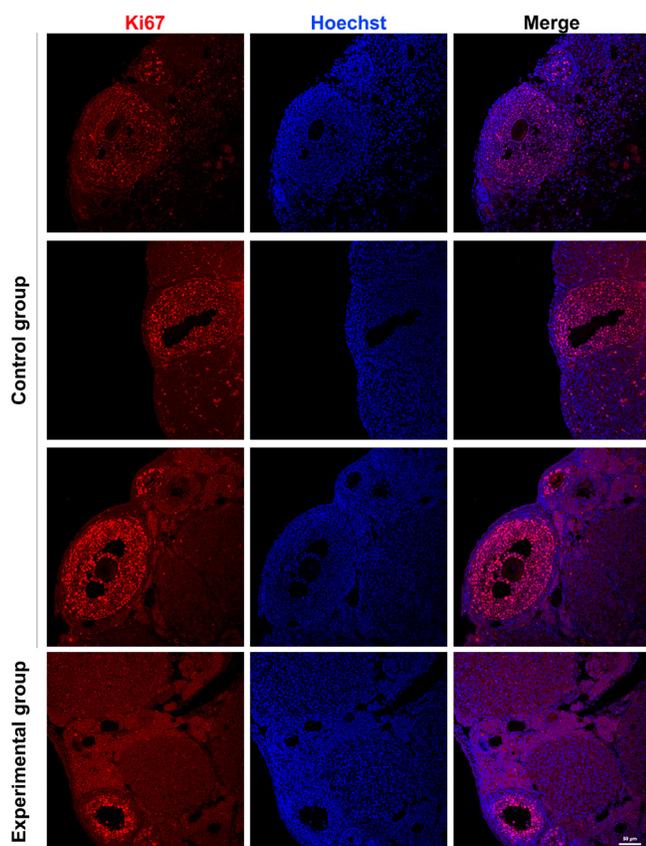
integrity of the tissue under a microscope. The results showed that the shape and the sizes of the ovaries in the experimental group were both different to those found in the control (Figure 3), which was in line with our preliminary examination Figure 2. In the control, the ovaries contained not only early growing follicles with more than one layer of granulosa cells, as was indicated by blue stars, but also secondary follicles with or without antral cavity, as was indicated by black stars. They enclosed CL as well, which is transformed from an ovulated follicle and is responsible for producing progesterone (9) (Figure 3A). In contrast, in the experimental group, the ovarian outline from the donor ovary was incomplete and cracked (Figure 3B, red broken lines). However, the structure of the follicles and the CL in the residue tissues of the donor part were properly maintained (Figure 3B). The donor tissue and the residue of the recipient were physically connected, though there were gaps (red broken lines) in between, indicative of their different origins (Figures 3C,D). Overall, the structure of the primordial follicle, the growing follicles and the CL in the donor ovary (Figures 3C,D) were similar to those in the control (Figure 3A). This implies that the function of the ovarian tissue was unaffected by the surgery, and the

transplantation did not influence the follicle development and ovulation (Figure 3D).

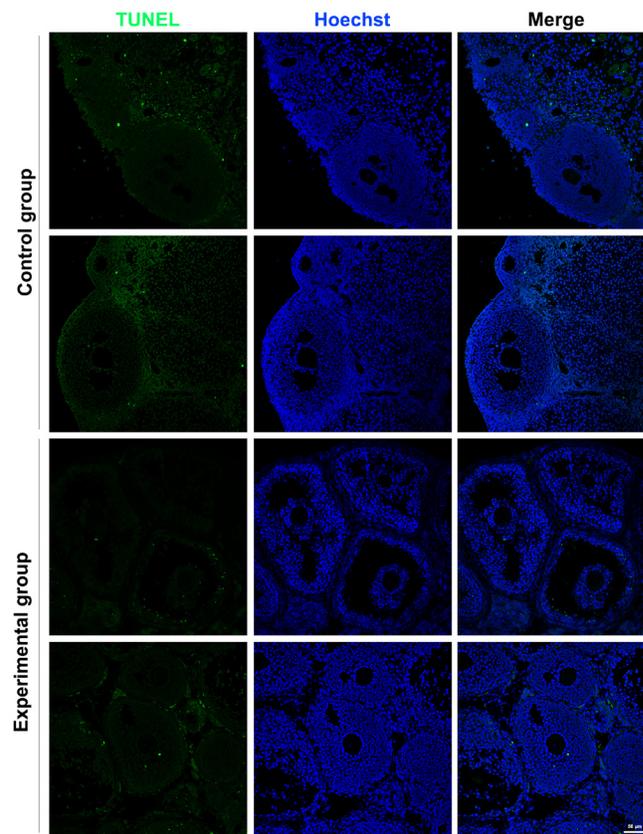
According to our results, the follicles and the CL in the transplanted ovarian tissue are likely functional and not degenerated (Figure 4, 5). In brief, in the immunofluorescent assay, proliferating cells which are experiencing G1 to S stage of a cell cycle are stained by Ki67 protein (red). The results showed that the proliferation of the ovarian somatic cells, especially those granulosa cells within the growing follicles (experimental group), were not different in transplanted ovaries when compared to the control (Figure 4). In addition to that, the transplantation surgery did not induce significantly higher cellular apoptosis in both the follicles and the CL in the autografted ovarian tissue, if compared with the control (Figure 5). In which, cells undergoing apoptosis within either follicles or CL are shown by indicating the DNA double strand break signals in TUNEL assay (green). Therefore, the viability is preserved and the function is maintained in the autografted ovarian tissue seven days after surgery.

## DISCUSSION

In this study, we observed the success of the orthotopic



**Figure 4: The ovarian somatic cell proliferation was examined by checking the Ki67 level.** Proliferating cells that express nucleic protein Ki67 protein (in red) at G1-S stage of cell cycle are shown in the leftmost column of images. The nucleus of each cell was indicated by dyeing with the Hoechst, in blue (center). The merged images are presented on the right. The scale bar is 50  $\mu$ m.



**Figure 5: The ovarian cellular apoptosis was examined by checking the TUNEL signals.** Apoptotic cells with broken double strand DNAs was analyzed using TUNEL staining, indicated by green puncta (left column). The number of apoptotic cells in the auto-transplanted tissue was similar to the control. The nucleus of each cell was indicated by dyeing with the Hoechst, in blue (center). The merged images are presented on the right. The scale bar is 50  $\mu$ m.

autologous transplantation of fresh ovarian tissue in ICR mice model through histochemistry and immunofluorescence assays. These assays applied the principles of either chemical reaction or immunology so as to detect the chemicals or the antigens of tissues, and to locate and quantify them, respectively. Firstly, the morphology as well as the histological structure in the recipient ovary showed that the donor and the recipient ovarian tissue connected to each other and even grew together although there were small gaps between the two parts. Secondly, the recipient ovaries displayed functional units of the ovary, namely follicles and CL, supporting our conclusion that the auto-transplantation process was successful. Follicles are responsible for producing estrogen and mature oocytes, while CL, which is transformed from an ovulated follicle, is responsible for producing progesterone (9). In the orthotopic autograft ovary, not only follicles at different developmental stages were visible, but also some CL structures presented. In general, the estrous cycle of female mice, including ICR mice, is no more than five to six days (10). Thus, the presence of CL in the ovarian tissue seven days after transplanting indicates that the follicles within the tissue survived after transplantation because only growing follicles that experienced a full growth period could respond to the gonadotropin induction to ovulate and form the CL within one estrous cycle in physiological conditions (11). Observing pregnancy in the mice will be a way to validate our results in the future study.

Previous studies have confirmed that orthotopic autologous transplantation of fresh ovarian tissue in mice is useful for scientific research (12,13). For instances, the first live birth after cryopreservation of ovaries was achieved in mice in the 1960s. Since then, scientists have successfully cryopreserved the ovaries from many animals, including humans (14). Also, this technique is helpful to study the effect of different IVA treatments on fresh ovarian tissues, ovarian tissues recovered from frozen samples of cancer patients, or POI patients(13). Our study adds to previous research because the ICR mice are genetically diverse and low cost, while inbred strains like C57BL/6 are genetically identical to each other, making outbred ICR mice potentially better subjects than inbred strains for biomedical studies (11). According to our results, the transplantation was successfully accomplished after verifying through histochemistry and immunofluorescence assays, which was in accordance with our hypothesis that the transplanted tissue would survive after autologous transplantation.

The sample size of this study is a bit small. More robust conclusions can be drawn from future studies that employ a larger sample size and combine more techniques with autografting. For instance, we plan to perform an IVA plus auto-transplantation study one in the future. We want to know if freshly collected ovarian tissues pretreated by TubA, an inhibitor of histone deacetylase 6, could stimulate more primordial follicles to grow in vivo. TubA is believed to activate the primordial follicles in 30 minutes in an IVA study

(13). Further data including mating, ovulation, the levels of sex hormones in the sera, and the litter size could also be collected to validate the technique.

In conclusion, we have demonstrated that auto-transplantation of fresh ovarian tissue in ICR is applicable for answering clinical concerns of this technique and employing immune deficient mice to do IVA/frozen derived ovarian tissue transplantation.

## MATERIALS AND METHODS

The autologous transplantation of fresh ovarian tissues in outbred ICR mice was performed following the published protocols (5, 8). The transplantation surgery entailed taking partial ovarian tissue from the unilateral ovary of the adult ICR female mouse and then transplanting into the ovarian sac of the contralateral ovary, in which part of the ovarian tissue had been removed in advance (**Figure 1**). The results were verified by histological assays accordingly.

### Orthotopic Autologous Transplantation Surgery

In total, three animals were used. We performed the sham surgery on the mouse of the control group, whose ovarian tissues were intact after laparotomy surgery. For the experimental group, we performed orthotopic autologous transplantation surgery for each mouse. Briefly, for each adult ICR female mouse in the experimental group, part of the fresh ovarian tissue from the left ovary (lateral) was cut and surgically transplanted into the ovary sac of the right ovary (contralateral). Before doing this, we had opened the ovarian sac and removed part of the ovarian tissue of the right ovary in advance. For the mouse that was treated as the sham surgery group, we performed the laparotomy surgery on each side of the ovary without touching the ovarian sac and the ovary.

To examine the success of ovarian tissue transplantation, we observed the slides under a microscope after ovarian tissues were embedded with paraffin, sectioned and then either stained with hematoxylin or performed immunofluorescence assays.

### Animals and materials

ICR mice of three months old were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). The mice were housed under controlled lighting (12 hours light/12 hours dark) and temperature (22 – 24°C) conditions with unrestricted access to food and water. All procedures were conducted in accordance with the guidelines of and approved by the Animal Research Committee of the China Agricultural University (No. Aw60502202-3-1).

Surgical instruments were provided by the animal raising facility. Scissors and forceps were used for microsurgery. A fat clip was used to hold the fat near the ovary. Hemostatic filter paper was used to stop bleeding when necessary. Surgical sutures were used to seal the wounds. A stereo microscope (Nikon SMZ745T) was used for stereoscopic observation and surgical operation.

### Surgical procedures

Before surgery, we anesthetized each mouse with 250 mg/kg tribromoethanol intravenously to maintain the anesthesia state for about 10-15 minutes during the surgery.

According to published protocols (5, 8), the abdominal cavity of each mouse was opened to reveal the ovarian tissues, which is generally surrounded by an ovarian sac on each side of the pelvic cavity. Fat tweezers were used to clamp the fat pad surrounding the ovary and the sac to assist fixing the ovary by pulling the pad together with the fallopian tube and part of the uterine tissue out of the abdominal cavity. The mouse was moved under the stereo microscope before making a small cut at the edge of the ovarian sac. We exposed the entire ovary and cut off and removed half of the ovary. The removed tissue was put in a petri dish with cold phosphate buffered saline (PBS) (pH 7.2) for later use. For orthotopic autografting, the donor ovarian tissue from the left ovary was transplanted into the right ovarian sac. We then, pulled back the ovarian sac membrane to cover the donor tissues to make sure the transplanted tissue was placed inside the capsule. Before transplantation, we opened the ovarian sac and removed part of the ovarian tissue of the right ovary. The ovary, the fallopian tube and part of the uterine tissue was replaced back into the abdominal cavity, and the skin was then sutured shut.

After the surgery, the recipient mice were kept warm on a 37°C thermostat pad until they woke up. All animals were housed in their respective cages for one week after surgery for recovery.

### Ovarian tissue sampling, histochemical and immunofluorescence examination

Seven days after the surgery, we sacrificed the animals in each group by cervical dislocation. To verify the transplantation effect, the ovarian tissues were photographed, fixed with formalin, paraffin embedded, sectioned, and dyed with hematoxylin according to Tuo et al. (6). We transferred mouse ovarian tissues into 1.5 mL sterilized centrifuge tubes and added 1 mL of 4% paraformaldehyde solution to fix the tissue at 4°C overnight. The ovaries were dehydrated through a graded series of ethanol, treated with xylene to make them transparent, and embedded in paraffin. Embedded ovaries were cut into 5 µm thick sections. After dewaxing and rehydration of each slide, we performed hematoxylin staining, by which the nucleus was stained blue. Stained sections were sealed with a coverslip and photographed using a Nikon 80i microscope.

To examine if the cellular proliferation and apoptosis of transplanted ovarian tissues were unaffected, we performed immunofluorescence assays (6). In brief, ovarian sections from different groups were deparaffinized and then rehydrated. After the sections were washed in PBS, they were boiled in a microwave for 16 minutes in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen

retrieval. The sections were blocked with 10% donkey serum followed by incubation with primary antibody of Ki67 (CST D385) overnight at 4°C (15). Cells presenting positive Ki67 protein signals indicate the ones who are proliferating. We also applied the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay (Vazyme A112-02) to indicate double strand breaks in DNA of the cells that are experiencing apoptosis (16). Subsequently, the sections were incubated with Alexa Fluor 488-conjugated or Alexa Fluor 555-conjugated secondary antibodies (Thermo Fisher Scientific) at 37°C for 60 minutes in PBS, respectively. Sections were rinsed with PBS and the nuclei of the cells were indicated by staining with Hoechst for one minute. We applied 20 µL Vectashield mounting medium (Applygen, Beijing, China) to each slide, and the sections were sealed with a coverslip. We used Nikon 80i and Nikon A1 cameras (Nikon, Tokyo, Japan) to image the immunofluorescence on the entire section.

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