

Original Article

Successful pregnancy in ovariectomized mice using a combination of heterotopic autotransplantation of ovarian tissues and embryo transfer

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Aim: The present report is the first to show that, after ovariectomy, female mice with autotransplanted ovarian sections can maintain pregnancy after embryo transfer (ET) independent of the transplantation site.

Methods: Three-month-old ICR females were ovariectomized, and sections from their own ovaries were transplanted either under their kidney capsule (KC group) or into a subcutaneous space (SC group) just after ovariectomy. *In vitro* fertilized blastocysts were transferred into uterine horns of the pseudopregnant mice that had received the transplanted ovarian tissues. Cesarean sections were carried out 17 days after ET to deliver any live fetuses that were present, and the numbers of implantation sites and fetuses were noted. Transplanted ovarian sections were removed and fixed for histological analysis.

Results: Of the 10 mice in the KC group that received 107 blastocysts, two females (20%) became pregnant; they showed

12 implantation sites (11.2%) and produced four pups (3.7%). In the SC group, 101 blastocysts were transferred to eight females, and three females (37.5%) became pregnant; there were seven implantation sites (6.9%), and three pups (3.0%) were born. There were no statistically significant differences between the two groups in any of the parameters evaluated. On histological examination, luteinization and vascularization of the ovarian sections that were transplanted in the pregnant SC and KC females were noted.

Conclusion: The pregnancy and full-term fetal development were obtained in ovariectomized mice using a combination of heterotopic ovarian tissue autotransplantation and transfer of embryos produced by *in vitro* fertilization. (Reprod Med Biol 2007; 6: 85–90)

Key words: embryo transfer, heterotopic ovarian transplantation, kidney capsule, mouse, subcutaneous space.

INTRODUCTION

ATROGENIC INFERTILITY CAUSED by a decline in ovarian function after radiotherapy and/or chemotherapy is a serious problem for patients with cancer.^{1–3} Furthermore, ovariectomy in a gynecological cancer patient obviously causes infertility.⁴ In these cases, orthotopic or heterotopic autotransplantation of ovarian tissues can be carried out to recover fertility after cancer therapy or an ovariectomy.^{5–10} Ovarian tissue autotransplantation could preserve fertility in patients who survive cancer.^{11,12} Furthermore, it is possible to recover oocytes from the transplanted ovarian sections.^{13–15}

High levels of follicular stimulating hormone (FSH) and luteinizing hormone (LH) are seen during premature menopause in women who have had ovariectomy or radiotherapy/chemotherapy for cancer.¹⁶ However, the internal hormonal environment can be restored with heterotopic ovarian transplantation in mice,⁸ rats¹⁷ and humans.⁵ Steroid hormones, such as estrogen and progesterone produced by granulosa cells and corpora lutea, are essential for implantation and the maintenance of pregnancy. Although transplanted ovarian tissues can secrete these steroid hormones,¹⁶ little is known about whether these hormones secreted from grafts could control all of the necessary reproductive endocrine functions, especially implantation and pregnancy. Therefore, we investigated whether follicles in heterotopically transplanted ovaries were able to transform into corpora lutea, and whether, after ovariectomy, mice autotransplanted with ovarian tissues could maintain

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pregnancy after embryo transfer. In addition, the site-effect of the transplantation (kidney capsule and subcutaneous space) was compared in terms of the pregnancy and implantation rates of the transferred embryos.

MATERIALS AND METHODS

Animals

FOR OVARY AUTOTRANSPLANTATION, 3-month-old Jcl/ICR, closed colony with white coat color, strain female mice were used. Oocyte donors were BALB/c × C57BL/6 J F_1 female mice, whose offspring are distinguishable by their coat color. ICR male mice were used as sperm donors. All mice were housed at about 22–24°C and under 14-h light/10-h dark photocycle conditions.

Autotransplantation of ovaries

Under anesthesia, ICR female mice were ovariectomized. Each ovary was removed from bilateral bursae that were opened carefully using a scalpel; the ovaries were then dissected into small pieces (about 1 mm³) in CZB-Hepes medium.¹⁸ Just after ovariectomy, the ovary sections were autotransplanted underneath the kidney capsules (KC group) or into subcutaneous spaces (SC group). Ovariectomized mice subjected to a sham operation of tissue transplantation (C-O group) and nonovariectomized mice subjected to a similar sham operation (C-N group) served as control groups. At least 2 weeks later, estrous cycles of some mice in KC and SC groups were checked for 1 week.

In vitro fertilization and embryo transfer

One to two weeks after autotransplantation of the ovaries, the female mice were mated by vasoligature ICR males to induce pseudopregnancy. *In vitro* fertilization was carried out according to a previous report¹⁹ and the zygotes were cultured into the blastocyst stage for 96 h. About 10–20 blastocysts were transferred into each uterus of the 4-day pseudopregnant female mice that had previously received autotransplantation of the ovaries or into the uteri of control group females. Cesarean sections were carried out to deliver live fetuses 17 days after embryo transfer; the numbers of implantation sites and fetuses were noted.

Histological preparations

Transplanted ovarian tissues were removed at the time of cesarean section and prepared for histology. Ovarian

tissues were fixed for 4–7 days in 4% formaldehyde in phosphate buffer saline at pH 7.4, dehydrated in a graduated 70–100% ethanol series, and cleared in xylene. After embedding in paraffin, the tissues were cut into 7- μ m sections and stained with hematoxylin and eosin.

Statistics

Statistical evaluation was carried out using the χ^2 -test to compare the rates of pregnancy, implantation of embryos and production of fetuses.

RESULTS

A TOTAL OF 34 female mice were used for the experiment; 13 underwent autotransplantation of ovarian sections underneath the kidney capsule (KC group), nine underwent autotransplantation of ovarian sections into the subcutaneous space (SC group), eight served as a nonovariectomy (C-N group) control group and four underwent ovariectomy followed by sham tissue transplantation (C-O group) as another control group. Their estrous cycles were checked for 14–21 days after ovarian transplantation. Their normalcy was ascertained by the cytological change in their vaginal smears.

After mating with vasoligature ICR males, females with vaginal plugs were regarded as being pseudopregnant. There were no pseudopregnant mice in the C-O group, whereas ten females (76.9%) in the KC group, eight females (88.9%) in the SC group and seven females (87.5%) in the C-N group were pseudopregnant (Table 1). There was no difference in the rates of pseudopregnancy among the three pseudopregnant groups.

Of the ten females in the KC group that received a total of 107 blastocysts, two females (20.0%) became pregnant; they showed 12 implantation sites (11.2%) and produced four pups (3.7%). In the SC group, eight pseudopregnant females received a total of 101 blastocysts; three (37.5%) became pregnant with seven implantation sites (6.9%) and produced three pups (3.0%). In the C-N group, seven females received a total of 70 blastocysts; four (57.1%) became pregnant and showed 14 implantation sites (20.0%) and produced nine pups (12.9%). The coat colors of fetuses were to be colored and albino, which were expected in backcross progeny. There was no statistically significant difference between the KC and the SC groups in any of the evaluated parameters. However, there was a significant difference in the number of implanted embryos between the SC and the C-N groups ($P < 0.05$), and the number of fetuses was significantly lower in the KC and SC

Table 1 Results of ovarian tissue autotransplantation and embryo transfer

Groups	No. pseudopregnant mice/transplanted mice	No. embryo transferred mice	No. pregnant mice (%)	No. transferred blastocysts	No. implanted (%)	No. fetuses (%)
KC	10/13	10	2 (20.0)	107	12 (11.2)	4 (3.7*)
SC	8/9	8	3 (37.5)	101	7 (6.9*)	3 (3.0*)
C-N	7/8	7	4 (57.1)	70	14 (20.0**)	9 (12.9**)
C-O	0/4	0	–	–	–	–

C-N, subjected to a similar sham operation but nonovariectomized; C-O, ovariectomized and subjected to a sham tissue transplantation operation; KC, tissue transplantation under the kidney capsule; SC, tissue transplantation into the subcutaneous space. ***Values with different superscripts in the same column are significantly different ($P < 0.05$).

groups than in the C-N group ($P < 0.05$). In contrast, no pseudopregnant mice were observed in the C-O group; therefore, embryo transfer could not be carried out in this group. The appearance of estrus on vaginal smears of the KC and SC groups was reflected by the presence of cornified epithelial cells; this suggests that all females who received transplanted ovarian tissues appeared to have an estrus cycle after autotransplantation of the ovaries.

Histological samples of ovarian tissues transplanted underneath the kidney capsule showed growing follicles and corpora lutea, which were thought to be derived from ruptured follicles in the transplanted ovarian sections (Fig. 1). Various stages of folliculogenesis, such as the preantral and antral stages, were found in the histological samples obtained from the KC group (Fig. 2). In the KC group, theca-luteal cells were observed around the corpus luteum, and vascularization into the transplanted ovarian tissues was also observed (Fig. 1). In the histological samples obtained from the SC group, corpora lutea, theca-luteal cells, growing follicles and vascularization were also found (Fig. 3). These histological findings show that ovarian tissues were successfully transplanted, and that the transplanted ovarian tissues might be able to produce estrogen and progesterone needed for implantation of transferred embryos and the maintenance of the pregnancy. Because these samples were collected at the time of cesarian section, the next follicular growth was seen as growing follicles (Fig. 3). However, it remains to be determined whether matured oocytes 'ovulated' within the kidney capsule or subcutaneous space, and/or were retained within the unruptured follicles.

DISCUSSION

IN THE PRESENT study, successful pregnancy and the production of live pups were obtained by a combination

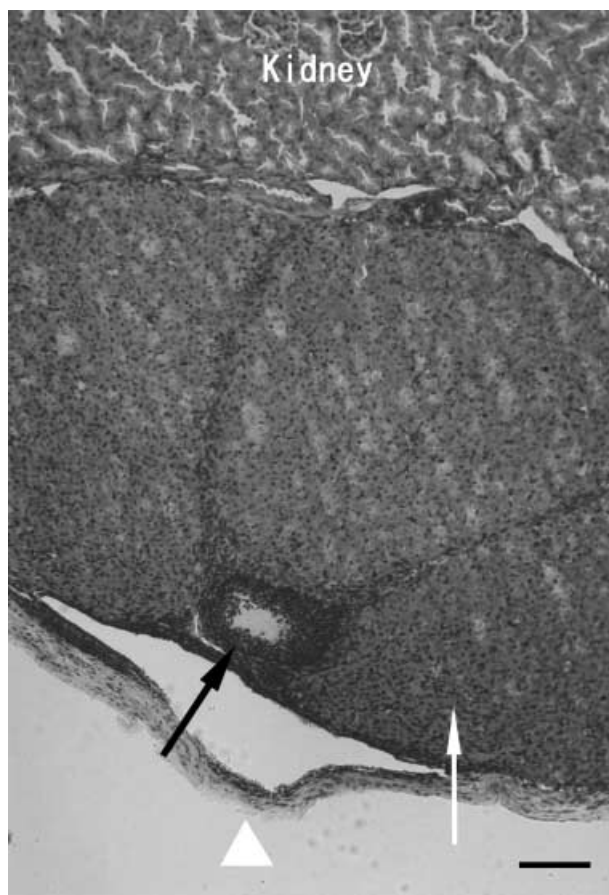


Figure 1 An ovarian tissue specimen autotransplanted underneath the kidney capsule. Corpora lutea (arrow) are seen underneath the kidney capsule (arrow head). Corpora lutea and theca-luteal cells were located adjacent to kidney tissue; these corpora lutea might be derived from follicles that had ovulated. One small follicle (black arrow) is also observed in this ovarian tissue. Bar, 100 μ m.

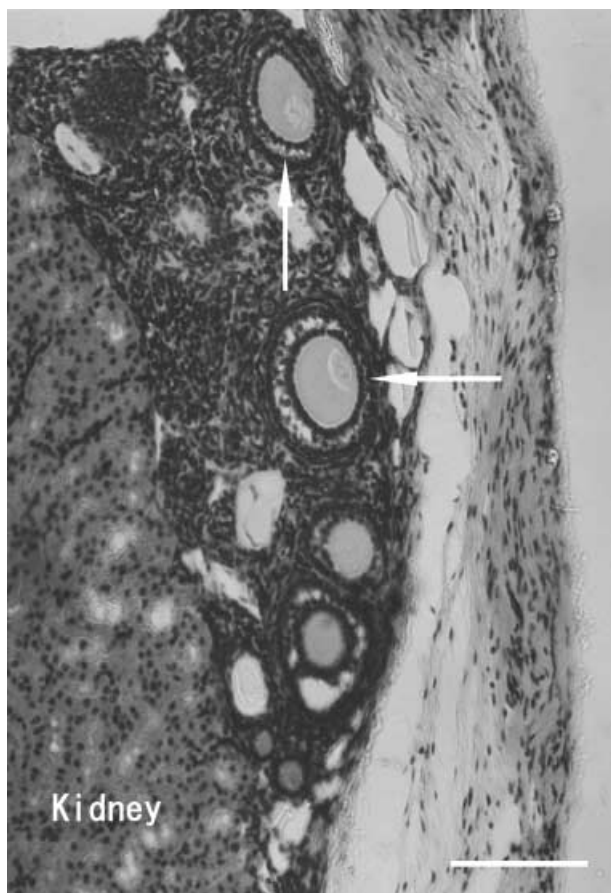


Figure 2 An ovarian tissue specimen autotransplanted underneath the kidney capsule. There are several preantral follicles (arrows) in the transplanted ovarian tissue. The integrity of the follicles was preserved after transplantation and these follicles would be expected to develop to the preovulatory stage. Bar, 100 μ m.

of heterotopic auto-transplantation of ovarian tissues and embryo transfer. The results suggest that the transplanted ovarian sections can secrete estrogen and progesterone, which are essential for the development of uterine endometrium and the maintenance of pregnancy.^{20,21} Indeed, histological examination showed that follicular development, luteinization and vascularization were present in the transplanted ovarian sections of pregnant females in the KC and SC groups.

Heterotopic autotransplantation of ovarian tissues is an effective method for recovering the internal hormonal environment without the need for external hormonal supplementation in the mouse ovariectomy model.¹⁷ A significant decrease of ovarian follicular reserve, and

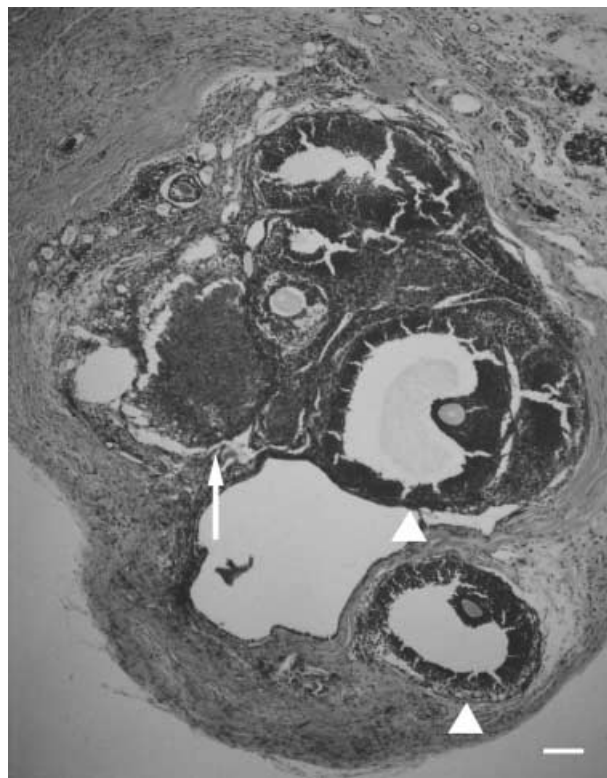


Figure 3 An ovarian tissue specimen autotransplanted into the subcutaneous space. Corpora lutea (arrow) and growing follicles (arrow heads) including oocytes are seen. This ovarian section shows normal ovarian function although it was surrounded by fatty tissue. Bar, 100 μ m.

high serum FSH and LH levels have been reported in women rendered menopausal by radiotherapy and/or chemotherapy for cancer.²² In contrast, a normal estrus cycle with lower FSH and LH levels occurred with heterotopic ovarian transplantation.^{6,23} Most recently, it has been reported that a woman gave birth to a baby after orthotopic and heterotopic autografts of ovarian tissues.²⁴ After transplantation of ovarian cortical strips to the forearm, multiple antral follicles were present in the patients, therefore it was shown that follicles can grow in heterotopically transplanted ovarian tissues under a recovered hormonal environment.²⁵ In the present study, oocytes were certainly ovulated from grown follicles with the effect of LH surge because luteinization is certain evidence of ovulation and sequential copulation in rodents. Thus, ovarian transplantation appears to be a novel technique that preserves endocrine function and also oocyte growth in women treated with certain cancer therapies.¹⁶

Though the values of the parameters studied differed between mice with ovarian sections transplanted underneath the kidney capsule and mice with ovarian sections transplanted into the subcutaneous space, none of the differences was statistically significant. However, the results of both of these groups were significantly lower than those of the C-N group. It is thought that the transplantation site can affect the success rate of autotransplantation of ovarian tissues in terms of vascularization and hormone secretion.^{26,27} Sufficient blood circulation has been found underneath the kidney capsule compared with the subcutaneous space.²⁶ A few days are needed for vascularization to occur into the transplanted ovarian sections¹⁴ and some follicles, including an oocyte, can undergo necrosis during this time.²⁸ Thus, in the present study, delayed and insufficient vascularization might have disrupted the hormonal environment in the KC and SC groups, resulting in low rates of implantation and live fetus production. However, Demeestere *et al.*²⁴ have reported that, in women subjected to heterotopic ovarian transplantation, basal markers (FSH, inhibin B) of ovarian reserve returned to levels that were within normal premenopausal values. Similar results have been reported in rats,²⁹ rabbits³⁰ and sheep.³¹ Furthermore, it has been reported that various stages of folliculogenesis were shown in young murine ovaries transplanted syngeneically into the testes.³² Thus, the site of transplantation of ovarian tissues might be insignificant for the restoration of ovarian function after heterotopic ovarian transplantation.

It has been found that the size of ovarian sections should be reduced, because vascularization can occur earlier in smaller ovarian sections than in bigger ones³³ and gonadotropin administration might be effective in stimulating the follicles that survive within transplanted grafts.³⁴ Furthermore, transplantation of a whole ovary with vascular anastomosis has been investigated as a way to reduce ischemic necrosis and to increase the survival of grafted ovaries.³⁵ The cryopreservation of ovaries will also be useful for clinical ovarian tissue transplantation.³⁶

In conclusion, pregnancy and full-term fetal development were obtained in ovariectomized mice using a combination of heterotopic ovarian tissue autotransplantation and transfer of embryos produced by *in vitro* fertilization. Functional corpora lutea were found in ovarian sections that were transplanted underneath the kidney capsule and in those transplanted into the subcutaneous space. These results suggest that this technique might be able to restore the fertility of women with iatrogenic infertility.

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