A study of ovarian autotransplantation without vascular a pedicle in rats

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Abstract *Purpose:* The study aimed to determine effects of FSH applications on follicle survival, development and hormone output and antigenicity of rat ovarian tissue autografts placed at subcutaneous or subperitoneal sites.

Methods: A total of sixteen female rats were used in the study. The animals were divided into three groups. Ovaries were dissected and then transplanted under the peritoneum in the first group animals ($n = 5$) or under the skin in the second group animals ($n = 6$). And the animals in the third group ($n = 5$) were sham operated. Following operations, intramuscular injection of 8 IU of rhFSH were made daily to the animals in first and second groups from the first day of operation through thirty days. Vaginal irrigation samples were prepared daily from the animals for 30 days. The concentrations of serum estradiol and antiovarian antibodies in the blood were determined using ELISA on the last day

of vaginal irrigations. Histopathological examination of the ovaries that were transplanted was made.

Results: Results showed that cyclic variations were noticed in the samples of vaginal irrigation by day 30 in the animals of first and second groups. However, no significant differences were seen between groups. The concentration of blood serum estradiol was higher in the animals of first group. Decrease in numbers of primary follicles were found in the animals of second group and lesser corpus luteum were found in the animals of control group on the histopathological examinations of transplanted ovaries. All rats in the first and second groups were defined as seropositive for antiovarian antibodies. When the OD values were compared between first and second groups, it was identified that the OD values of rats in the first group was higher than it was seen in the second group.

Conclusion: The ovarian transplantation without vascular pedicle in rats is characterized by follicular hyperplasia endocrinologically functional. Being seropositive of all rats in first and second groups in terms of antiovarian antibodies is an indicator to these antibodies does not affect the functions of transplanted ovaries. It is believed that the highness of OD values in the group which is transplanted beneath the peritoneum is based on the highness of estradiol concentrations in these animals.

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Introduction

Ovarian tissue transplantation, started over 100 years ago using both human and animal models. The development of transplantation procedures for ovarian tissue provides one

method of generating or storing genetic material for individuals, including those at risk of becoming infertile. Despite recent advances in this area, optimal transplantation conditions have not yet been established. The success of ovarian tissue transplantation in the long term will depend on the number of follicles that survive and the ability of these follicles to develop and ovulate. Reanastomosis of whole ovaries to blood supply reduces the number of follicles [1, 2].

Oocytes can be obtained from fresh or frozen ovarian tissue by iso-, allo- or xenografting. Transplantation of ovarian tissue back to its normal anatomical position (orthotopic grafting) allows for fertility. Transplantation of ovarian tissue to a position other than its normal anatomical position (heterotopic transplantation) allows the tissue to be placed at a more convenient and easily accessible site for surgery and monitoring of graft function. Some of the common heterotopic sites for grafting ovarian tissue include the kidney capsule, where revascularisation of ovarian grafts is thought to be enhanced due to the large blood supply. However, subcutaneous sites such as the abdominal wall or forearm allows for easy monitoring of the graft [1, 3–11].

Revascularisation of ovarian grafts is estimated to occur within 40–48 h of grafting. In this time, the majority of growing follicles are lost and as much as 50% of the primordial follicle population within an ovarian graft does not survive. This regulation of angiogenic stimulating factors may be mediated by gonadotrophins, and thus may be one of the reasons why ovarian grafts are more successful in bilaterally ovariectomised than intact recipients. Treating the graft recipient (with a combination of FSH and LH) 2 days before and 2 days after grafting increased the number of follicles that survive within the ovarian graft [7, 12, 13].

After the ovarian transplantations, there may appear the stimulation of immunologic response to ovarian tissue, the reentrance of viral infections and malignant cells to the body. There is little information on the relationship of ovarian antibodies and markers of ovarian function. A number of investigators showed an association between infertility and ovarian antibodies. There also appears to be a relationship of ovarian antibodies to poorer outcomes of infertility treatment and with lower pregnancy rates [14–16].

In this study was conducted to determine the effects of FSH applications on the ovarian transplantations performed subcutaneously and subperitoneal without vascular pedicle in rats and the presence of antiovarian antibodies following transplantation.

Materials and methods

Animals

In this study, a total of sixteen Sprague-Dawley female rats weighing between 200 and 250 g were purchased from the

Experimental Investigation Center of Medical Faculty of Firat University (Elazig, Turkey). These animals were maintained in a temperature-controlled environment ($24 \pm 1^\circ\text{C}$), illuminated for 12 h daily and fed with commercial pellets and water ad libitum.

Surgical operations

The animals were anesthetized by rompun (10 mg/kg intramuscular) and ketalar (90 mg/kg intramuscular) anesthesia. The animals were divided into three groups randomly. After bilateral ovariectomy was done to first group ($n = 5$) animals, the ovaries were transplanted completely to the same animals beneath the peritoneum. The ovaries which were taken out bilateral ovariectomy from the animals of second group ($n = 6$) were transplanted subcutaneously to the inguinal plexus of same animals. Third group ($n = 5$) was selected as the control group and sham operation was done.

Hormone administration

After the operations, 8 IU intramuscular rhFSH (Puregon 600 IU, Organon) was injected once daily to the animals in the first and the second groups as from the first day of operation along vaginal irrigations. Placebo was injected to the animals in the third group. Serum estradiol concentrations were measured using ELISA by withdrawing the bloods of rats on the last day of vaginal irrigation.

Vaginal irrigations

Vaginal irrigation samples were prepared daily from the animals for 30 days. And these samples were stained with Giemsa [17]. The intensities of cell types in the prepared samples were assessed as +, ++, +++ [18].

Histological examination

Animals were killed by decapitation on the last day of vaginal irrigations. The ovaries of killed rats were fixed in buffered 10% formaldehyde. The fixed tissues were sectioned (4 micron), and stained with hemotoxylin-eosin. The prepared samples were examined using a light microscope of Olympus B ($\times 200$) follicles with one layer of flattened cells around the oocyte. Primary follicles had one layer of cuboidal follicular cells around the oocyte; and the antral follicles had an antral cavity [7, 19]. The severity of inflammation in ovaries was semiquantitatively assessed as 0; not, 1; mild, 2; moderate, 3; severe [6].

Analysis of antiovarian antibodies, extraction of protein and ELISA

Protein was extracted from homogenized normal ovary with a commercial kit (EZ- RNA total RNA-DNA-Protein isolation kit; Biological Industries Co., Israel). The concentrations of obtained protein were determined according to the instructions of the Bradford Assay (SIGMA, Co., St. Louis, MO, USA). The ELISA was adapted from Risvanli et al. [20]. Briefly, the ovarian homogenate was diluted in pH of 9.6 carbonate buffer to a concentration of 100 mg/ml. 100 μ l of this solution was added to every second well of a 96 well microtitre tray and incubated overnight at +4°C. Plain carbonate buffer (pH 9.6) was added to the remaining wells. All wells were then emptied and washed with 100 μ l/well PBS containing 0.02% tween-20 to block non-specific binding. 100 μ l of 10% horse serum was added to each well and incubated for 2 h. The plate was re-washed and 100 μ l rat serum diluted 1/200 with carbonate buffer was added to all pits. After 2 h at RT the plate was re-washed and peroxidase labeled Goat anti- Rat IgG (SIGMA, Co., St. Louis, MO, USA) diluted 1/5000 and left for 2 h at RT. Following a further 6 washes chromogen solution (0.1 M citrate-phosphate buffer containing 1 mg/mL O-phenyldiamine and 0.003% H₂O₂) was added to each well and left for fifteen minutes in the dark at RT. The reaction was stopped by dropping 100 μ l 1 M H₂SO₄ to each well. The plate was read at 450 nm in an ELISA reader. All serum samples were tested in duplicate.

Statistical analysis

In the study, Kruskal Wallis Variant Analysis was used for the comparison of vaginal irrigation results, histopathological examination results of ovaries and estradiol values among groups by profiting obtained information. The test of Mann Whitney *U* was used for the establishment of difference among groups in the follow-up [21, 22].

Results

In the samples of vaginal irrigation, estrus-like cytological variations were noticed 4.4 times in animals of first group; however those variations were 5.4 times in animals of second group. And estrus signs were seen 5 times in animals of control group. However there was no significant difference among groups (Table 1).

The estradiol concentrations in the animals of first group in which the ovaries were transplanted subperitoneal were higher than the concentrations of other groups (Table 2).

The number and type of follicles and corpus luteum differed between groups Table 3. In the result of histopathological examinations of ovaries, there was not any sign of

Table 1 The vaginal irrigation results according to groups

Data	Group 1 (n = 5)	Group 2 (n = 6)	Group 3 (n = 5)	P
Number of Noticed Estrus	4.4 $\pm$ 0.87	5.8 $\pm$ 0.60	5.0 $\pm$ 0.00	–
Sexual Cycle Span (Day)	7.8 $\pm$ 1.33	5.4 $\pm$ 0.67	6.0 $\pm$ 0.00	–

– Not important ($P > 0.05$).

inflammation in the control group, but inflammation was seen in variable degrees in other two groups, however this achieved result was not found statistically significant ($P > 0.058$) (Figs. 1, 2, 3).

As for the antiovarian antibodies, the OD values higher than the identified cut off values according to the +5x standard deviation of absorbance values that obtained from negative serum examples in ELISA were assessed as seropositive. Although all grafted rats were seropositive for antiovarian antibodies. The OD values in the five rats in the first group were higher than for 4/5 rats in the subcutaneous group.

Discussion

Most follicles in ovarian tissue that survive transplantation are the primordial follicles. On the other hand adult rat ovaries transplanted without vascular anastomosis in rats maintain their hormone secretion and exert an endocrine function in target organs. The ischemia that inevitably occurs during grafting causes the death of a substantial fraction of the primary oocyte pool. The endocrine function of the ovary is suspended temporarily but returns once follicles have been recruited from the surviving primordial follicle store [6, 23, 24]. And in this present study, as parallel findings of other researchers, there were various follicles at different magnitudes although inflammation signs were occurred in the histopathological examination of transplanted ovaries. However, based on the results of statistical analysis, animals of second group displayed lesser primary follicle and lesser corpus luteum was found in control group.

Table 2 The estradiol values according to groups

Group	Estradiol pg/ml
1. group (n = 5)	38.62 $\pm$ 11.43 ^a
2. group (n = 6)	23.96 $\pm$ 8.16 ^b
3. group (n = 5)	16.74 $\pm$ 2.54 ^b
P	*

* $P < 0.001$.

^{a,b}: The difference between different letter carrying averages are important ($P < 0.05$).

Table 3 The results of histopathological examination according to the groups

Group	Primary follicle (piece)	Secondary follicle (piece)	Graffian follicle (piece)	Corpus luteum (piece)
1. group (<i>n</i> = 5)	3.20 ± 1.02 ^b	2.00 ± 0.84	0.20 ± 0.20	15.20 ± 1.46 ^b
2. group (<i>n</i> = 6)	0.50 ± 0.22 ^a	0.33 ± 0.21	0.83 ± 0.31	10.67 ± 2.78 ^b
3. group (<i>n</i> = 5)	3.00 ± 1.00 ^b	0.40 ± 0.24	0.40 ± 0.40	2.40 ± 0.98 ^a
<i>P</i>	**	–	–	**

– Not important ($P > 0.05$).

**The differences between different letter carrying averages in the same column are important ($P < 0.05$).

Callejo et al. [25] showed that vaginal cytology of groups intraperitoneal and subcutaneous ovarian transplantations showed trophic maturation between days 4 and 10 after ovariectomy and insertion of the ovarian tissue implant, whereas the control group remained atrophic. In a study that was done with rats [26], at the first day of vaginal cornification after grafting, plasma estradiol-17 beta (45.8 ± 4.0 pg/ml) was elevated in comparison to controls at metestrus (24.0 ± 2.6 pg/ml), but plasma progesterone (21.5 ± 4.0 ng/ml) was not different (30.6 ± 1.7 ng/ml). Subsequently, at the first metestrus following grafting, plasma estradiol (23.0 ± 3.5 pg/ml) was comparable to control values. In contrast, progesterone was decreased (17.5 ± 1.9 ng/ml). A definite correlation was detected between the vaginal smear and plasma levels of steroid hormones in the castrated female rat with subcutaneous ovarian autographs. Chihal et al. [27] identify that castrates which received ovarian autografts de-

veloped cycling vaginal smears and had increased estradiol (31.9 ± 4.3 pg/ml) and decreased progesterone (18.3 ± 1.9 ng/ml) levels. Also in the present study, there were seen cyclic variations in the vaginal irrigation samples at the group 1 and group 2. The estradiol concentration in the group that was transplanted subperitoneal was higher than that was in the other groups.

In the previous studies, it was proposed that the ovary was an immunologic target or was an organ which contains auto-antigen [15, 16, 28]. Related to this, fertility has affected negatively by antiovarian antibodies in normal females and in the females which had several ovarian diseases. In this sense, the formation of antiovarian antibodies has increased because of the damaging of the ovarian tissue in the course of ovarian transplantations [15]. It was also identified that all rats, both subcutaneously and subperitoneal transplanted, were seropositive in terms of antiovarian antibodies in the offered study.

Fig. 1 Graafian follicle with primary follicle is seen together in the prepared sample of subperitoneally transplanted ovaries HE, X200

Fig. 2 Double arranged primary follicle which oocyte is in the middle is seen in the prepared sample of subcutaneously transplanted ovaries HE, X200

Consequently, although there was not any statistical difference among groups, noticing of cyclic variations in the groups that were transplanted and finding of corpus luteums and follicles at variable number and at different varieties in the histopathological examination of ovaries were signs of the maintenance of the functions of transplanted ovaries. Be-

ing seropositive of all rats in first and second groups in terms of antiovarian antibodies was an indicator to these antibodies did not affect the functions of transplanted ovaries. We believed that the highness of OD values in the group which was transplanted subperitoneal was a result of high estradiol concentrations in these animals.

Fig. 3 Subperitoneal muscle tissue, ovarian tissue and primary follicle is seen in the prepared sample of transplanted ovaries to under the peritoneum HE, X200

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