Kafkas Univ Vet Fak Derg
19 (Suppl-A): A79-A85, 2013
DOI: 10.9775/kvfd.2012.7986

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Heterotopic Allogenic and Autogenic Ovarian Transplantation in Rabbits: Assessment and Comparison of the Morphological and Endocrine Characteristics [1]

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[1] *This study was supported by the Scientific Research Committee of Kafkas University, (the project number: 2010-TIP-06)*

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Makale Kodu (Article Code): KVFD-2012-7986

Summary

The aim of this study was to evaluate and compare the endocrine function and morphological characteristics of subcutaneous heterotopic allogenic and autogenic ovarian transplantation in immune-suppressed rabbits. Each group included seven rabbits. Group I (allogenic group) underwent freshly subcutaneous allogeneic heterotopic transplantation (n=7) (from group III to group I). Group II (autogenic group) underwent freshly subcutaneous autogenic heterotopic transplantation (n7).Group III was the donor group (n=7). The levels of serum follicle-stimulating hormone (FSH) were significantly lower during the 2nd week (P=0.017) than during the 3rd week in group I, and were significantly higher during the 4th week than at other times (P=0.001) in group II. The levels of serum 17-β estradiol (E_2) were significantly lower during the 3rd week (P=0.008) than during the 1st and 2nd weeks in group I, and were significantly lower during the 4th week than at other times (P=0.001) in group II. The levels of serum progesterone (P_4) were not vary significantly (P=0.441) in group I and were significantly higher during the 1st week than during the 3rd week (P=0.033) in group II according to the ANOVA. Histological examination was performed using light microscopy after staining with hematoxylin-eosin (HE). Our results showed that there is no remarkable difference between autogenic and allogeneic heterotopic freshly transplanted ovarian tissue, especially in terms of the FSH and P_4 levels during a four week period. We found that autogenic and allogeneic freshly transplanted ovarian tissues had similar characteristics in terms of the endocrine and histological characteristics.

Keywords: *Ovarian transplantation, Allogenic, Autogenic, FSH, E_2, P_4*

Tavşanlarda Heterotopic Allojenik ve Otojenik Yumurtalık Nakli: Morfolojik ve Endokrin Özelliklerinin Değerlendirilmesi ve Karşılaştırılması

Özet

Bu çalışmanın amacı, immün sistemi baskılanmış tavşanlara subkutan heterotopik allojenik ve otojenik yumurtalık naklinin endokrin ve morfolojik özelliklerinin değerlendirilmesi ve karşılaştırılmasıdır. Her gruba 7 tavşan dahil edildi. Grup I (allojenik: n=7) taze subkutan heterotopik allojenik transplantasyon (grup III'den, grup I'e), grup II (otojenik: n=7) taze subkutan heterotopik otojenik transplantasyon (kendi ciltaltlarına) ve grup III donor grupları olarak belirlendi. Serum follikül stimülan hormone (FSH) düzeyleri, grup I'de, 2. haftada, 3. haftadaki değerlerden (P=0.017) anlamlı derecede daha düşüktü, ve grup II'de 4. haftadaki değerler, diğer haftalardaki değerlerden anlamlı olarak daha yüksekti (P=0.001). Serum 17-β estradiol (E_2) düzeyleri, grup I'de, 3. haftada, 1. ve 2. haftalardaki değerlerden anlamlı derecede düşüktü (P=0.008) ve grup II'de 4. haftadaki değerler, diğer zamanlardaki değerlerden anlamlı derecede düşüktü (P=0.001). Serum progesterone (P_4) düzeyleri, grup I'de, anlamlı farklılık göstermedi (P=0.441) ve grup II'de, 3. haftadaki değerlerden, 1. haftadaki değerler anlamlı olarak yüksekti, ANOVA testine göre (P=0.033). Histolojik inceleme Hematoksilen-eozin (HE) ile boyanarak ışık mikroskobu ile yapıldı. Bizim sonuçlarımız özellikle dört haftalık dönemde, FSH ve P_4 düzeyleri açısından otojenik ve allojenik heterotopik taze nakledilen yumurtalık dokusu arasında anlamlı fark olmadığını göstermiştir. Sonuç olarak, otojenik ve allojenik taze nakledilen yumurtalık dokularının endokrin ve histolojik yönden benzer özelliklere sahip olduğu belirlenmiştir.

Anahtar sözcükler: *Yumurtalık nakli, Allojenik, Otojenik, FSH, E_2, P_4*

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INTRODUCTION

Currently, ovarian transplantation (OT) is a potential method for the preservation of reproductive function. The first experimental ovarian transplantation was described by Paul Bert in 1863 [1]. The first experimental study of OT using micro-vascular surgery that resulted in pregnancy following the auto-graft transplantation of fallopian tubes and ovaries in a rabbit was published by Wiston and McClure Browne in the Lancet in 1974 [1]. The first successful heterotopic ovarian auto-transplant using microsurgery in a woman was reported by Von Theobald and coworkers in 1987. The heterotopic transplanted ovary in this case had normal endocrine function and follicular development [1].

Although experimental ovarian transplantation was first performed approximately 100 years ago, during last two decades, it has gained great momentum, with many advances in assisted reproductive techniques. Recently, ovarian transplantation has been performed in many different animal models and in humans, but the optimal conditions for ovarian transplantation have not been established. Different transplantation techniques with or without microvascular anastomoses or pedicles, and various placements of the transplanted tissues, including the normal anatomic position and positions outside the normal anatomic positions (subcutaneous, intra-peritoneal, retroperitoneal, inguinal region or inside the kidney capsule) have been described in many different animal models [1-9]. The transplantation of whole ovaries and the grafting of fresh or frozen-thawed ovarian tissues have been successfully reported by many authors in human [10].

In this report, we describe and compare the morphological and endocrine characteristics of transplanted ovaries after autogenic and allogenic heterotopic ovarian transplantation without vascular anastomosis in rabbits. To the best of our knowledge, this is the first published report that compares freshly heterotopic allogenic and heterotopic autogenic ovarian transplantation without vascular anastomosis in rabbits.

MATERIAL and METHODS

This study was approved by the Ethics Committee of Animal Experiments of Kafkas University (the number: 2009-30). In this study, twenty one mature New Zealand white female rabbits were between six and eight months and weighing between 2.8 and 3.9 kg purchased from the Experimental Animal Investigation Center of Ataturk University in Erzurum, Turkey. These animals were maintained in a temperature controlled environment, illuminated for 12 h daily and fed with commercial pellets and water *ad libitum*.

During the follow-up period, before and after surgical procedure, the female rabbits received food and filtered water ad libitum in separate containers and were maintained in individual cages. All procedures were carried out under aseptic conditions in the Laboratory of Experimental Surgery, Department of Veterinary Surgery of Kafkas University. One day before surgery, and for 3 days after surgery, all animals were intramuscularly injected with 1.000 mg of cefazolin sodium (Cefamezin; Eczacıbaşı Drug Co, Turkey.).

All animals undergoing transplantation (in group I and group II) were also received daily an intramuscular injection of 25 mg/kg cyclosporine-A (Sandimmune, Novartis Drug Co., Swiss) throughout the three week period to prevent graft-versus-host rejection, which was primarily a concern for the allogenic group (group I). We also administered cyclosporine-A to the animals in group II (autogenic group) to eliminate any differences between the groups due to the effect of cyclosporine.

Anesthesia was achieved by the intramuscular injection of 25 mg/kg ketamine HCl (Ketasol 10%, Richter Pharma Drug Co., Austria) and 5 mg/kg Xylazine HCl (Rompun 2%, Bayer Drug Co. Animal Health, Germany). Each animal's abdomen was shaved and then disinfected with a Povidone-iodine (PVP-I) solution followed by a 2% alcohol solution of iodine.

The animals were randomly divided into three experimental groups. Bilateral ovariectomies were performed on group I rabbits (n=7). The ovaries retrieved from the donor group (group III) were immediately grafted to lower neck under the skin subcutaneously into group I (allogenic group). On the day before surgery and for four weeks after surgery, blood samples were taken for the analysis FSH, E_2, and P_4. One rabbits died due to immunosuppression on the twenty-ninth day. The other rabbits were euthanized, and subsequently the ovarian grafts were removed for histopathological analysis.

Bilateral ovariectomies were performed on group II rabbits (n=7) (autogenic group) and those ovaries were immediately autologously subcutaneously grafted into the lower neck of the same rabbits. On the day before surgery and for four weeks after surgery, blood samples were taken for the analysis FSH, E_2, and P_4. Blood samples were taken on days 7. 14. 21. 28. after transplantation. Unfortunately, one rabbit in group II died as a result of immunosuppression on the tenth day. The other rabbits were euthanized and subsequently, the ovarian grafts were removed for histopathological analysis.

Blood samples were collected from the marginal artery of the rabbit's ear before surgery and on days 7, 14, 21, 28 after surgery in all animals and centrifuged immediately at 2.500 rpm for 10 min. Serum samples were obtained to measure the FSH, E_2 and P_4 levels. Then, the serum was frozen at-20ºC until the hormone tests were performed using commercially available ELISA kits (Cusabio Biotech Co. Ltd. Hubei Province 430223, P.R. China).

Ovarian tissue samples were immediately fixed in 10% buffered formalin solution and were processed using conventional techniques for light microscopy analysis. Samples were stained with H.E. for histological analysis and observed by light microscopy (Olympus Optical Co., Osaka, Japan).

Statistical analyses (SPSS package version 11.5) were performed using Student's t-test for parametric data and ANOVA variance for multiple groups. Tukey's test was performed to identify the source of significant differences revealed by ANOVA.

RESULTS

We measured serum levels of FSH, E_2 and P_4 on the day before ovariectomy and on days 7. 14. 21. 28. after transplantation in all animals. We started the study with twenty-one rabbits, but one rabbit from group I and one rabbit from group II died due to immunosuppression before the end of the study.

The serum FSH and P_4 levels were not significantly different (P=0.175, P=0.147) between group I (allogenic)

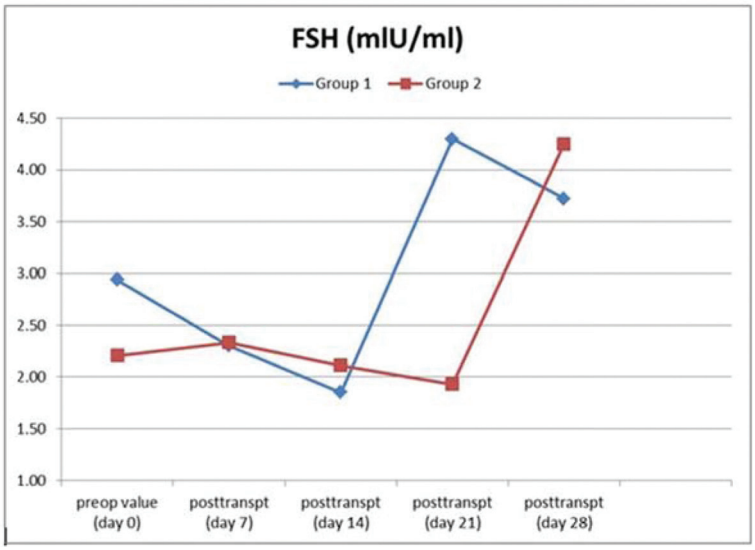

Fig 1. Variation of the serum level of FSH in allogeneic (group I) and autogenic (group II)

Şekil 1. Allojenik (grup I) ve otojenik (grup II) gruplarda serum FSH seviyelerindeki değişim

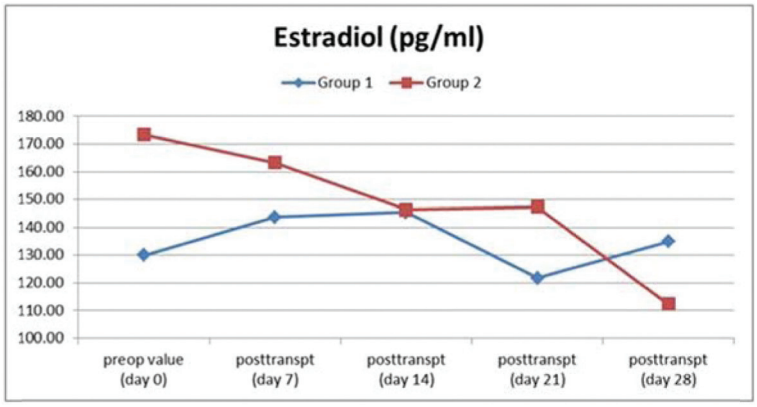

Fig 2. Variation of the serum level of E_2 allogenic (group I) and autogenic (group II)

Şekil 2. Allojenik (grup I) ve otojenik (grup II) gruplarda serum E_2 seviyelerindeki değişim

Table 1. *Serum FSH, E_2, P4 levels of group I (allogenic) and group II (autogenic)*
Tablo 1. *Grup I (alojenik) ve grup II (otojenik) de serum FSH, E_2, P_4 degerleri*

Days	Group I (Allogenic Group)			Group II (Autogenic Group)		
	FSH m IU/ml	E_2 pg/ml	P_4 ng/ml	FSH m IU/ml	E_2 pg/ml	P_4 ng/ml
Day 0 (pre-op.)	2.9±0.3	129.9±6.0	56.6±4.8	2.2±0.2	173.4 ±7.6	69.3±4.5
Day 7 (post-transp.)	2.3±0.3	143.8±2.4	59.0±2.2	2.3±0.2	163.2±11.8	73.2±2.9
Day 14 (post-transp.)	**1.9±0.1****	145.5±4.5	59.5±4.3	2.1±0.1	146.4±4.7	58.7±3.2
Day 21 (post-transp.)	4.3±0.7	**121.8±5.0***	62.9±1.8	1.9±0.1	147.4±7.2	**55.2±5.9****
Day 28 (post-transp.)	3.7±0.8	134.9±4.7	53.6±3.5	**4.3±0.8***	**112.3±6.0***	63.7±3.7

** P<0.01, ** P<0.05*

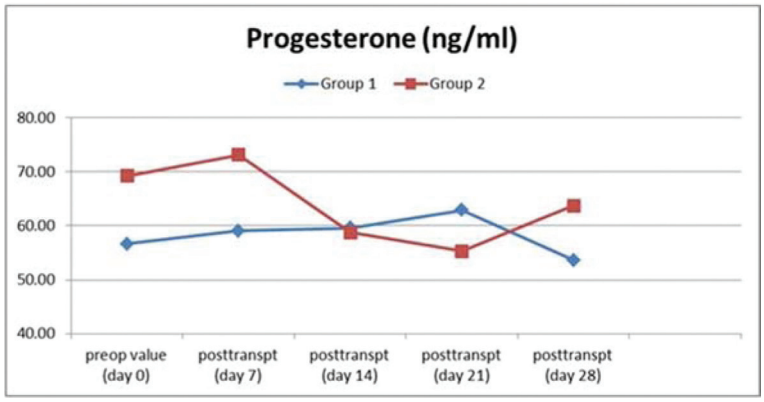

Fig 3. Variation of the serum level of P4 allogeneic (group I) and autogenic (group II)

Şekil 3. Allojenik (grup I) ve otojenik (grup II) gruplarda serum P4 seviyelerindeki değişim

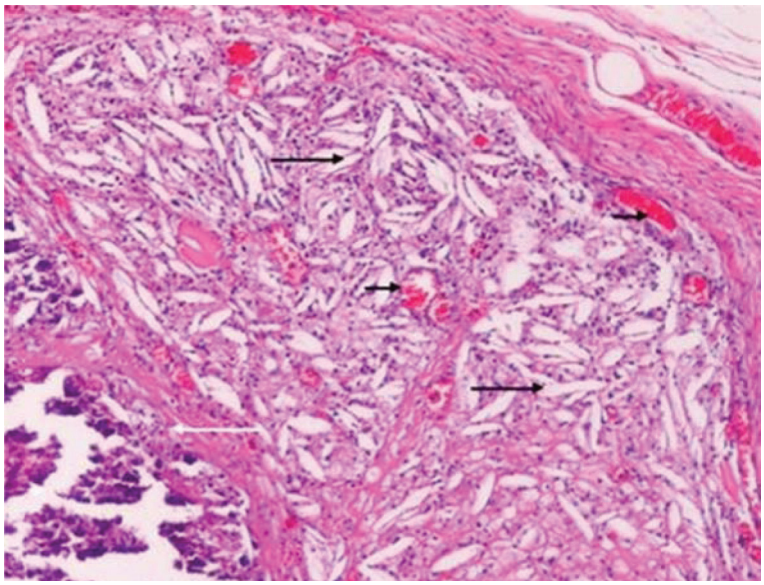

Fig 4. Rabbit ovary from group II (autogenic). No follicles were observed in the cortical regions. Common cholesterol necrosis in the cortical region, cholesterol clefts *(long black arrows)* and vascularization were presented in the necrotic tissue *(short black arrows)*. Severe necrosis of the corpus luteum *(white arrow)* was observed. HEx10

Şekil 4. Grup II (otojenik) tavşan yumurtalığı. Kortekste follikül gözlenmedi. Kortekste yaygın kolesterol nekrozu *(uzun siyah oklar)* ve vaskularizasyon *(kısa siyah oklar)*. Korpus luteumun ciddi nekrozu *(beyaz ok)*

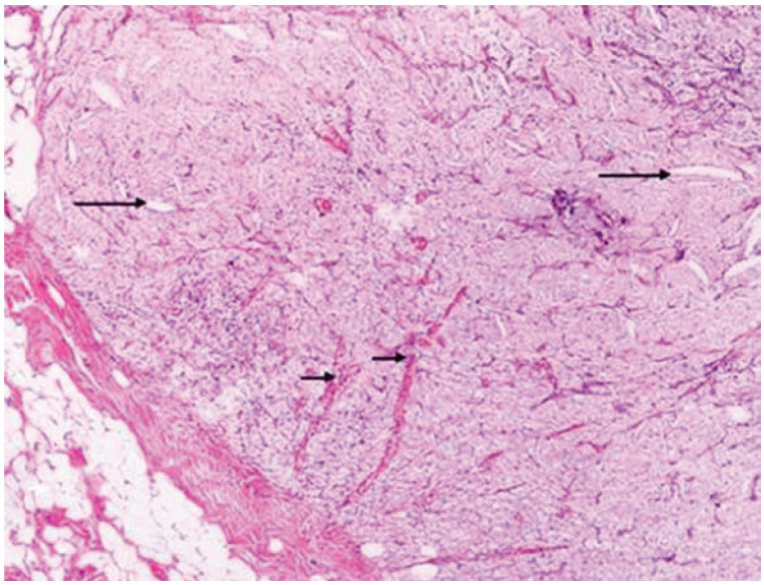

Fig 5. Rabbit ovary from group I (allogeneic). No follicles were observed in the cortical regions. Common cholesterol necrosis in the cortical region, cholesterol clefts *(long black arrows)* and vascularization were present in the necrotic tissue *(short black arrows)*. HE x 4

Şekil 5. Grup I (allojenik) tavşan yumurtalığı. Kortekste follikul gözlenmedi. Kortekste yaygın kolesterol nekrozu *(uzun siyah oklar)* ve vaskularizasyon *(kısa siyah oklar)*

and group II (autogenic), but the serum E_2 levels were significantly different between these two groups (P=0.012) (135.17±2.54 versus 148.53±5.04) *(Table. 1)*.

The difference in the FSH level between the two groups was not significant according to a Student's t-test used (P=0.198). The levels of serum follicle-stimulating hormone (FSH) were significantly lower during the 2nd week (P=0.017) than during the 3rd week in group I, and were significantly higher during the 4th week than at other times (P=0.001)

in group II according to the results of the ANOVA *(Fig. 1)*.

The difference in the E_2 level between the two groups was statistically significant according to a Student's t-test (P=0.021, P=0.023). The levels of serum 17-β estradiol (E2) were significantly lower during the 3rd week (P=0.008) than during the 1st and 2nd weeks in group I, and were significantly lower during the 4th week than at other times (P=0.001) in group II according to the ANOVA *(Fig. 2)*.

The difference in the P_4 levels between two groups was statistically significant according to the Student's t-test (P=0.036 in both groups). The levels of serum progesterone (P_4) were not vary significantly (P=0.441) in group I and were significantly higher during the 1st week than during the 3rd week (P=0.033) in group II according to the ANOVA *(Fig. 3)*.

Histological examinations were performed for the allogeneic and autogenic groups and the features of the ovarian tissues are presented in *Fig. 4* (group II-autogenic) and *Fig. 5* (group I-allogenic).

DISCUSSION

Experimental ovarian transplantation (OT) in animals was first performed in the late 18th century. In the past 20 years, great progress has been made in ovarian transplantation, and these procedures, both in animals and in humans, have become more reliable in recent years. However, there are still few reports on the restoration of ovarian function that show a satisfactory reproductive rate.

The autogenic and allogenic heterotopic ovarian transplantation with micro vascular technique in rabbits were reported by Meraz et al.[1]. The authors, in this study, also in both groups, after hCG administration, values of E2 (1.75 pg/ml versus 148.5 p/ml in autogenic, 0.25 pg/ml versus 135.1 pg/ml in allogenic) and P4 (2.15 ng/ml verus 64.0 ng/ml in autogenic, 0.20 ng/ml versus 58.3 ng/ml in allogenic) were reported remarkably lower than our values [1].

The allogeneic orthotropic transplantation of intact and sliced ovarian tissue without the vascular pedicle were reported by Petroianu et al.[2,3]. These authors analyzed the differences among the groups according to the hormone levels and histological features. The groups of this study were included animals that received intact ovaries (G2A) (n=8), animals that received sliced ovaries (G2B) (n=8), animals that received an intact ovary on one side and a sliced ovary on the other side (G2C) (n=8), and control animals (G1) (n=8). When the hormone levels were compared between the groups and subgroups, the authors found no differences in values, except in subgroup 2C, which showed higher estradiol levels. After observing the rabbits for nine months, the authors showed that intact or sliced orthotropic allogenic OT without vascular anastomosis was viable in rabbits [2,3]. We noted that the hormone values for allogenic group reported by these authors differed from the values that we observed; their values were especially lower than our measured values for E_2 (4.13 pg/ml versus 135.1 pg/ml), FSH (0.13 IU/l versus 3.02 m IU/ml), and P_4 (0.103ng/ml versus 58.3 ng/ml). We couldn't explain any other reason why our all hormone values were higher than those values in both studies but we think that high estradiol values indicate better functional transplanted ovarian grafts.

OT without the vascular pedicle is much better, especially in small animals because this procedure does not require the difficult microsurgery that is necessary to construct the vascular anastomosis. The increase in the operation time due to the need to construct vascular anastomoses may be more risky for small animals [2].

The levels of serum estradiol and morphologies of autologous transplanted ovarian tissue with or without remote ischemic precondition (R-IPC) in rats were studied by Damous et al.[4]. The authors found that R-IPC led to increased levels of serum estradiol (average 65 pg/ml) in most animals. However, there was no significant difference between the groups, although it showed lower values (36 pg/ml). Generally, the grafts were better preserved in the R-IPC group.

The hormone levels and follicular development after heterotopic ovary transplant without the vascular pedicle were studied in syngeneic Lewis rats [5]. The authors observed the recovery of hormone levels to preoperative values within 28 days, and the lowest values were observed at 4 and 7 days after transplantation. In this study, in only autogenic heterotopic group, the authors reported that the values of E_2 on days 7, 14, 21, 28 were 23.4 pg/ml (versus 163.2 pg/ml), 45.2 pg/ml (versus 146.4 pg/ml), 56.6 pg/ml (versus 147.4 pg/ml) and 83.6 pg/ml (versus 112.3 pg/ml), respectively. Also, our E_2 values were higher than those values in this study [5].

The restoration of ovarian function after transplantation with micro-vascular anastomoses of intact frozen-thawed sheep ovaries was studied by Bedaiwy et al.[6]. They reported that 8 of 11 ovaries were nonfunctional due to thrombotic events in the re-anastomosed vascular pedicles.

Recently, a pregnancy and delivery was achieved after the auto-transplantation of whole cryopreserved sheep ovaries with micro-anastomoses, as reported by Imhof et al.[7].

The first study was performed to evaluate the feasibility of transplanting a whole adult mouse ovary, and to compare the live birth rates of mice in the sham-operated, fresh auto-transplanted ovary and cryopreserved auto-transplanted ovary groups [8].

A study of ovarian auto-transplantation without vascular pedicles in sixteen female rats was reported by Risvanli et al.[9]. In this study, animals were divided three groups. All animals except those in the third group (sham operated group,

n=5) underwent bilateral ovariectomy. The transplanted ovaries were placed completely under the peritoneum in first group animals (n=5) and were placed subcutaneous near the inguinal plexus in the second group (n=6). The authors noted that the estradiol concentrations in rats that had sub-peritoneal transplanted ovaries were higher than the concentrations in the other groups, and the results were statistically significant (P<0.001). In addition, there was no sign of inflammation in the sham group, but the other two groups showed varying degrees of inflammation, but the differences were not statistically significant (P>0.058) [9].

Callejo et al.[10] evaluated long-term ovarian function after the heterotopic auto-transplantation of fresh and frozen-thawed human ovarian tissue without vascular anastomoses in four premenopausal patients. These patients were followed for 1 year and did not receive gonadotropins. The women who received either fresh or cryopreserved ovarian tissues without vascular anastomoses at heterotopic sites regained ovarian function. The authors also reported that there was a significant decrease in the serum E_2 level from normal premenopausal values, and menopausal symptoms were observed within 3 weeks after surgery. The hormone functions of heterologous [subcutaneous in the arm in two patients (patients 1 and 2)], auto-transplanted fresh ovarian tissues and heterologous [in the rectus abdominis muscle in one patient (patient 4)], auto-transplanted frozen-thawed ovarian tissues were regained 3-4 months after transplantation [10].

A successful fertilization and pregnancy were achieved using retrieved oocytes from a primate that had undergone fresh ovarian tissue transplantation without any surgical anastomoses to major blood vessels were reported by Lee et al.[11].

The restoration of endocrine function during 14 weeks was reported by Kim et al.[12] after transplantation in a 37-years-old woman who had undergone heterotopic transplantation of cryopreserved ovarian tissue, but the cessation of ovarian function was verified by very high FSH levels 28 weeks after transplantation.

Camboni and Martinez-Madrid [13] evaluated the structural and ultra-structural morphology and viability of grafted tissue, by using transmission electron microscopy after the orthotropic auto-transplantation of frozen thawed human ovarian tissue for 1 year. They reported that primordial and primary follicles were well-protected throughout the 13 months' post-graft period. These authors confirmed that primordial and primary follicles were perhaps more resistant to freeze-thaw procedures and less vulnerable to ischemia than secondary and antral follicles [13].

In another study, Denjean et al.[14] reported the transplantation of rabbit ovaries with vascular pedicles including the ovarian artery and vein using either orthotropic transplantation by end-to-end anastomoses to the ovarian vessels or heterotopic transplantation to the inferior epigastric vessels. They found that the ovulation rate, if the ovary was enclosed in a peritoneal sac, after heterotopic transplantation to the epigastric vessels was high and comparable to that for orthotropic transplantation [14-16].

In a meta-analysis that included 46 women who underwent ovarian transplantation, and the continuation of ovarian function was documented within 60-244 days in 23 women who had have high FSH level (>30) at the time of transplantation was published. Recurrent ovarian failure was observed in four women within 6 months. The authors emphasized that the findings were not sufficient to assess the functions of transplanted ovarian tissue for longer than 12 months [17].The authors explained that the likelihood of the return of ovarian function for fresh transplanted ovarian tissues was higher than for cryopreserved transplanted tissues and that the likelihood of recurrent ovarian failure was much lower for fresh transplanted tissues than for cryopreserved transplanted tissues. They reported that eight of 25 women who attempted to become pregnant pregnancy had become pregnant within 12 months, giving a cumulative pregnancy rate of 37% [17-19].

The primary drawback of ovarian transplantation without vascular anastomoses is the initial ischemia that occurs to varying degrees, depending on the type of transplanted ovarian tissue (whole ovary or sliced cortical ovarian pieces). According to some authors, the reduction in the number of primordial and antral follicles is expected to be 50-65% in some studies, but one study reported a reduction of >90% [20]. In our study, histologic examination showed severe reduction of ovarian cortical follicles, severe necrosis and weakly vascularization in the both transplant groups. Although we estimated that the reduction in the number of primordial and antral follicles was approximately 90% in our study, hormone values showed good functioning of transplanted ovarian grafts in both groups.

It was reported that the ovarian cortex can tolerate ischemia for at least 3 hour at 4ºC in a study that showed a correlation between ischemic damage and the ischemic period for transplanted ovarian tissue [21].

The auto-transplantation of fresh or frozen-thawed ovarian tissue allows the preservation of the fertility of girls or women whose ovaries are damaged due to treatment for diseases such as cancer.

According to the relevant publications, Donnez et al.[22], up to 2010, 11 live births have been achieved after orthotropic re-implantation of cryopreserved ovarian tissue.

The transplantation of whole ovaries or ovarian tissues without a vascular pedicle requires vascularization, which takes 5 days. Oktay and Karlikaya [23] reported one case in which frozen-thawed ovarian tissue was transplanted laparoscopically into a 29 years-old patient who had under-

gone bilateral oophorectomy due to a nonmalignant disease in 2000 [23]. Donnez et al.[24] reported the first case that resulted in a pregnancy and live birth after successfully transplantation of cryopreserved ovarian tissue in 2004.

A live birth after the orthotropic auto-transplantation of cryopreserved ovarian tissue in a patient who suffered from premature ovarian failure after chemotherapy was also published in by Meirow et al.[25].

Two cases in which allogenic orthotropic vascular (case 1) and avascular (case 2) ovarian transplantation were performed on two girls who had been diagnosed with ovarian dysgenesis were reported by Mhatre et al.[26]. Case 1: The patient exhibited spontaneous menstruation and, ovulation, and excellent secondary sexual characters during the 2.5 year follow-up period. Case 2: Serial measurements of the serum E2 level showed a significant increase from 20 pg/ml to 50 pg/ml. Ovarian grafts showed excellent graft vascularization, with the development of small follicles [26].

In conclusion, our results showed that there is no difference between autogenic and allogenic heterotopic transplanted ovarian tissue, especially in terms of FSH and P_4 levels within period of four weeks. We found that autogenic and allogenic transplanted ovarian tissues had similar endocrine and characteristics.

Acknowledgement

The authors thank to Pinar DEMIR for statistics.

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