Ovarian tissue transplantation: A new method and site for induction of folliculogenesis in mice as a model for human female

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Abstract:

Background: Ovarian tissue transplantation is a new method of restoring fertility to women whose ovaries are not functioning normally. Young women who undergo chemotherapy or radiotherapy for cancer face serious consequences to their reproductive health and severely affect the ovarian follicular store, especially.

Objective: the aim of this study was to demonstrate induction of the folliculogenesis from ovarian tissue (OT) transplanted under kidney capsule in the presence or absence of gonadotropins support.

Materials and Methods: Forty-eight healthy female mice were anesthetized and abdominal cavity is open. From one side of the body, small piece (~1 X 1 X 1 mm) of OT was transplanted to the subcapsular membrane of kidney at another side, and surgical operation is closed. Then, female mice were classified into three groups according to the time of gonadotropins injection. Group-1: mice injected with sterile normal saline (control group). Group-2: mice injected with gonadotropins directly for four days. Group-3: mice injected with gonadotropins for four days after eight days of surgical operation. Follicular growth, quality of retrieved ova and histological changes for transplanted OT were assessed.

Results: In general, no deletion for transplanted OT pieces and no side effects post-operation on mice of all groups were recorded. Best follicular growth of transplanted OT was achieved for groups 1 and 2. Graafian follicles were obtained from transplanted OT of group-2, and less degree for group-1. However, least degree for follicular growth of transplanted OT was reported for group-3 as compared to other groups. Immature and mature oocytes with corona and cumulus cells collected by squashing of transplanted OT.

Conclusions: The present data demonstrate that the ovarian tissue transplantation is possible to undergo follicular growth subcapsular of the kidney. Also, physiology of the body supports the ovarian follicular growth in another site other than normal position. Further studies are recommended on *in vitro* maturation and fertilization of retrieved ova and embryo transfer.

Keywords: Mice, Ovary, Transplantation, Folliculogenesis, Gonadotropin.

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Introduction:

Therapeutic advances in the treatment of infertility are leading to improved survival and cure. One problem is that exposure of ovaries and uterus to radiotherapy and chemotherapy in childhood and during the reproductive years predisposes to ovarian failure and permanent uterine damage [1,2]. In addition, a significant number of women experience early menopause due to oophorectomy performed for benign indications [3]. Ovarian tissue transplantation (OTT) has been practiced for over a decade, mainly for experimental purposes. It is now being considered as a potential strategy for preserving fertility in young patients. These strategies have been demonstrated

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in animal models and are now undergoing clinical testing [4,5].

It was suggested that the OTT is a new method of restoring fertility to women whose ovaries are not functioning normally. However, after OTT, follicular development and restoration of hormone secretion have been observed in animal and human studies [6]. It was reported "if ovarian transplantation is proven to be safe and effective in humans, fertility preservation might become readily available for young women who need to delay childbearing for medical or social reasons" [2].

A promising field with respect to preservation of gonadal function and fertility is ovarian cortex transplantation. The ability of ovarian allografts to restore fertility has been demonstrated in several species. Furthermore, OTT was achieved within several sites of the body [7]. In recent years, a resurgence in the field of human ovarian transplantation has occurred, with several teams reporting sporadic cases of both fresh and cryopreserved OTT in different clinical scenarios [8]. Therefore, the aim of this study was to demonstrate induction of the follicular development from ovarian tissue transplanted under capsule of the kidney in the presence or absence of gonadotropins support.

Materials and Methods:

1. Animals

Forty-eight healthy adult female mice of Swiss albino strain (age: 8-10 weeks; weight: 25-28 g) were obtained from the animal house at IVF Institute of Embryo Research and Infertility Treatment / Al-Nahrain University and used in this study. Female mice were kept under suitable environmental conditions such as the room temperature was maintained at $24 \pm 2\ ^{o}C$ and exposed to 14-hour day light program. Tap water and food in the form of pellet were accessible freely, *Ad libitum*, to the animals.

2. Ovarian tissue transplantation (OTT) technique

After induction of general anesthesia with intraperitoneal (IP) injection of 100 µL of sodium pentobarbital (Nembutal, Sanofi, France) diluted 1:4 in normal saline. The procedure of OTT used was modified from the method of Gosden *et al*. [9]. Abdominal cavity of female mouse was opened and small pieces of OT cortex (~1 X 1 X 1 mm) were taken from right ovary. The left kidney was exteriorized and small hole torn in the kidney capsule using fine forceps. Only, one piece of OT was transplanted under capsule of left kidney. Finally, abdominal cavity was closed and Aerospray (Chloranfenical, Calier Laboratories, Spain) and Agrsunt (Sulphanilamide, Agropharm Limited Co., Bucks, UK) were used to cover the wound and prevent microbial infection and inflammation. Then female mouse was placed in clean cage for monitoring and special cure until reversed from anesthesia.

3. Experimental design and ovarian stimulation program

Forty-eight female mice were conducted surgical operation for OTT and randomly divided into three groups according to time of gonadotropins injection. Group-1 (G-1; control group) females injected with 0.1 ml normal saline for four days. Group-2 (G-2) females injected with gonadotropins for four days program post-surgical operation directly. Group-3 (G-3) females injected with gonadotropins for four days program after eight days of OTT and surgical operation. Ovarian stimulation program involves IP injections 5 IU/day FSH (Gonal-F, Serono, Italy) for first three days and 5 IU hCG (Profasi, Serono, Italy) at forth day. Pieces of transplanted OT were recovered after 7-8 hours of last injection for either histological processing and examination or ova retrieval and classification.

4. Histological examination and ovarian follicles assessment

Upon the completion of ovarian stimulation program, six mice were killed by cervical dislocation and the pieces of transplanted OT were recovered and

placed in Bouin's fixative for at least 24 hour. Then, dehydrated by series of ethanol alcohol, cleared by xylene, embedded in paraffin wax (melting point 60 ^{o}C), serially sectioned at 5 μm thick and stained with Erlich haematoxylin and eosin [10].

The sections were examined under light microscope for assessment of different types of ovarian follicles and corpora lutea. Ovarian follicles were classified as follows: primordial, primary, pre-antral and antral follicles according to presence of flattened epithelial cells, granulosa cells and antral cavity [7,11]. Different types of ovarian follicles were counted under high power microscope.

5. Ova retrieval and classification

After 7-8 hours of last injection for 10 females, each piece of transplanted OT was recovered from subcapsular kidney with high care and washed with Minimum Essential Medium (MEM; Sigma, UK) twice. Then, piece of transplanted OT was minced in small Petri dish within MEM enriched with 20% bovine serum albumin (BSA) by using special fine forceps. Ova were collected and washed within MEM enriched with 10% BSA. After microscopically examination, ova were classified into immature, mature and atretic according to morphological features and presence of 1st polar body [12].

6. Statistical analysis

Data presented as mean ± standard error of mean (SEM). The crude data were statistically analyzed using statistical computerized package SPSS (Statistical package of Social Science, version-12) to compare among different means of groups by application of Chi-square analysis and ANOVA test. A *p* value of < 0.05 was considered statistically significant.

Results:

The procedure of OTT subcapsular kidney was easy conducted in mice without real difficulties are faced. However, the results of the present study appeared no lose for pieces of transplanted OT subcapsular kidney (Figure 1 A).

Examination the histological changes in transplanted OT (Figure 1 B and C) appeared best results for follicular development in G-2, and this result in regard to number of primordial, primary and antral follicles non significantly (P>0.05) increased as compared to control group (G-1). Meanwhile, significant (P<0.05) differences in the number of pre-antral follicles were noticed between G-2 and G-1 (Table 1). From the same table, the number of all types of ovarian follicles for G-3 revealed significant (P<0.05) reduction as compared to G-2 and G-1.

The results of ova retrieval and classification were presented in the figure (2). The numbers of immature and mature retrieved ova in the G-2 have the best result in the present study (Figure 1 D). Not significant (P>0.05) differences were noticed in the number of immature ova of G-2 when compared to G-1. In contrast, significant (P<0.05) differences in the number of mature ova were reported between G-2 and G-1. Statistically, significant (P<0.05) reduction in the number of immature and mature ova of G-3 as compared to G-2 and G-1. While, no significant (P>0.05) differences in the number of atretic ova among three groups (Figure 2).

Discussion:

Autotransplantation procedures have historically been limited almost exclusively to non-vascular cortex segments grafted to orthotopic or heterotopic locations [13]. It was reported that the human grafts contained large numbers of germ cells about 11000 primordial follicles, an amount that could provide oocytes for a year [14,15]. Here, we described a successful follicular development after ovarian tissue (OT) transplantation (OTT) subcapsular kidney in mice. Figure (1 A) shows the transplanted OT subcapsular kidney in mice. Performance of OTT was easy and has no problems for general health or no side effects on kidney. The same result was mentioned by Bedaiwy *et al.* [5].

Ovarian transplantation appears to be a relatively simple, novel technique to preserve endocrine function in women undergoing sterilizing cancer therapy or surgery [3,16].

The reproductive outcome of the ovarian transplantation depends on the degree of cell damage inflicted over the transplanted tissues upon harvesting, processing and subsequent transplantation [17]. Furthermore, transplantation of ovarian cortical tissue and hemiovaries gave rise to pregnancies/live birth in sheep [9], mouse intact ovaries [18] and rats [19]. The human trials have shown promising results that have some limitations that require some more research [5,15].

In the present work, female mice were killed after 7-8 hours of last injection for ovarian stimulation program to induce follicular development and maturity of oocytes without spontaneous ovulation (Figure 1 C and D). It was reported low dose of hCG support oocyte maturation, while ovulation needs high dose of hCG and required longer period reach 11-12 hour [20,21]. In our study, best follicular growth at all stages of transplanted OT was noticed in the G-2 whose female mice injected gonadotropins for four days program. However, no significant ($P>0.05$) differences in regard to primordial, primary and antral follicles were reported between G-2 and control group (G-1).

Our results following OTT in mice confirm the results of previous work carried out by Weissman and his colleagues [7], they reported that the follicular development after using combined gonadotropins, FSH and LH. Although it is not known what gonadotropin dose is required for ovarian follicles stimulation [8]. The current data demonstrated that the pre-antral and antral follicles were produced in G-1 from transplanted OT in spite of absence of exogenous gonadotropin support. We assume that this reflects presence of normal endogenous gonadotropin milieu and considered sufficient for follicular development. A similar finding was previously reported about importance of hypothalamic-pituitary-gonadal function on transplanted OT [22,23].

It was certified that the follicles do not progress beyond the stage of two granulosa cell layers without gonadotropin support [24]. However, different follicles of development were observed in the grafted tissue whereas, prior to grafting, only primordial and primary follicles were present [22]. Moreover, limited length of ovarian function in some human ovarian transplant cases using non vascularized grafts may be partially due to ischemic injury until revascularization occurs [5,25]. From the results of the present study, highly reduction in the number of primordial follicles after OTT combined with major loss of oocytes. Same result was noticed on dramatic reduction in the number of ovarian follicles [16]. Approximately 50% of the primordial follicle population survives in isologous grafts in mice [26].

From the results of this study, it is clear that the number of antral follicles is too little, which may be as a result of environmental and site effects on transplanted OT. Weissmen *et al.* [7] believed that the kidney capsules are unlikely to be able to support complete human preovulatory follicular development. Although, they mentioned that the OT under the kidney capsule has been shown to develop apparently normal antral follicles. However, it was reported that the follicular development from transplanted OT less than the normal physiologically status [27].

The results of the present study appeared that the number of pre-antral follicles for G-2 was significantly ($P<0.05$) increased as compared to G-1 and G-3. Also, all types of ovarian follicles and immature and mature ova for G-3 were decreased significantly ($P<0.05$) when compared to G-1 and G-2. The reduction in the number of ovarian follicles may be

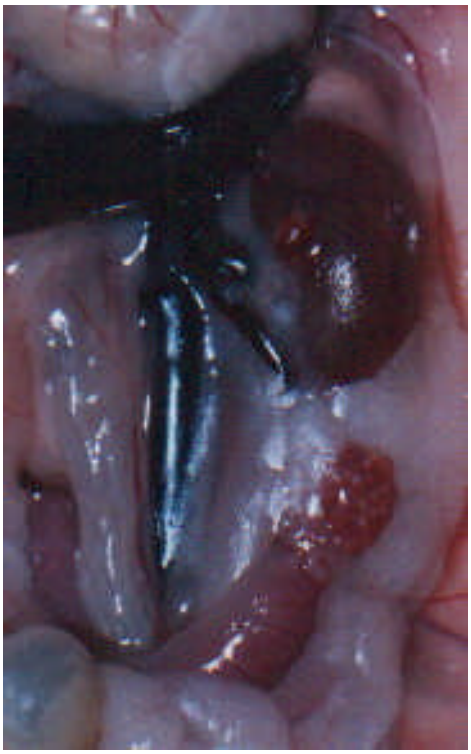
as a result of follicular atresia due to ischemia and apoptosis of primordial and primary follicles, especially [28]. Whether an ischemic insult of the ovarian tissues is associated with comparable alterations in special molecules is still unknown [17,29]. It was known that atretic follicles may reflect the preexisting atretic changes instead of the effect of OTT [5]. Also, it was certified that an important technical limitation of cortical grafting, whether orthotopic or heterotopic, is the potential for follicle atresia during the period of ischemia [2].

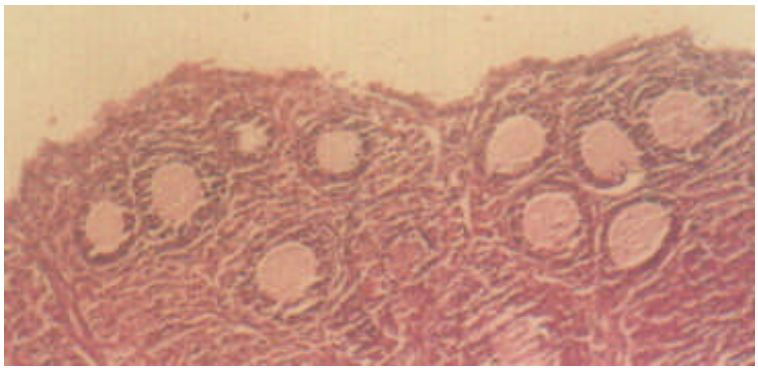
The number of immature ova retrieved from transplanted OT for G-2 was observed non significantly (P>0.05) increased as compared to control group (G-1). Functioning of the graft will be dependent on the initial pool of primordial follicles, the fraction of follicles surviving the grafting procedure, and the local conditions that affect the resting pool of follicles and the fraction of follicles that is recruited from the pool [15]. Knowing that every oocyte reaches full maturity after a fixed timespan and considering the inherent asynchrony in follicle recruitment from the reserve in women, the precise detection of the stage of oocyte maturation becomes an absolute requirement for normal fertilization and embryo development [30].

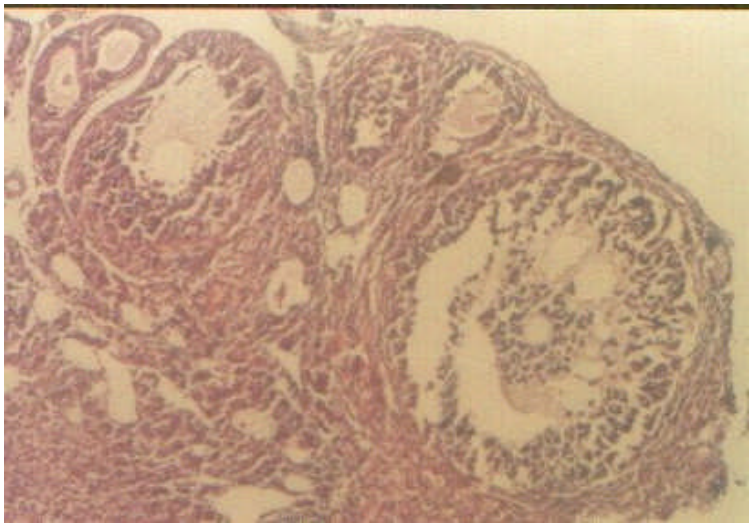
Results of this study appeared that the number of mature ova for G-2 was significantly (P<0.05) increased when compared to G-1 and G-3. Also, significant (P<0.05) reduction was reported in the number of mature ova for G-3 as compared to G-1. The possible explanations for this result are: use of four days ovarian stimulation program causes an increased folliculogenesis and oocytes maturity, but the timing start of this program for G-3 may be reduce outcome and efficiency of transplanted OT as far as from process of OTT. Same result was mentioned by Silber *et al*. [2] who reported that the heterotropic grafts of monkey cortical tissue can generate mature oocytes for IVF. Moreover, several alterations will obviously interfere with the availability of nutrients, hormones and oxygen, which are all essential to obtaining a developmentally competent oocyte [16,27].

Non significant (P>0.05) differences in the number of atretic ova was assessed among three groups of this study. This result may be partially due to technical method for OT recovery and/or retrieval of oocytes. Same result was mentioned by Smitz [15] who reported that the transplant site might it self influence follicle recruitment and atretia. Furthermore, presence of atretic and/or abnormal oocytes, in the present study, may be reflecting wide range of fluctuation of gonadotropins and/or inadequate exposure to gonadotropins which led to abnormal follicular development and subsequently produce abnormal and atretic oocytes. This result is in agreement with results of several investigators [16,18,27]. In contrast, it was mentioned that the elevation level of FSH produces high numbers of abnormal and atretic oocytes [31].

Therefore, the present data demonstrate that the transplantation of ovarian tissue is possible to undergo follicular growth subcapsular of the kidney. Also, physiology of the body supports the follicular development in another site other than normal position. Further studies are recommended on *in vitro* maturation and fertilization of retrieved ova and embryo transfer.







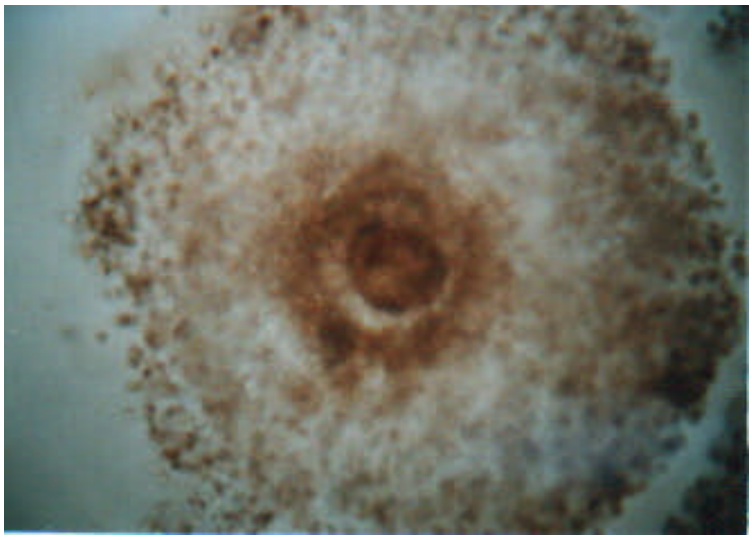


Figure 1: Mice ovarian tissue; (A) transplanted subcapsular kidney, (B+C) processed for histological examination and assessment of follicular development, (D) produced ovum with cumulus cells (Hematoxylin and eosin stain; 100 X).

Table 1: Numbers of different types of ovarian follicles from transplanted ovarian tissues * for three groups of female mice

Types of ovarian follicles	Groups of female mice		
	G – 1; control	G – 2	G – 3
Primordial follicles	7.166 [a] ± 0.771	7.833 [a] ± 0.711	4.50 [b] ± 0.748
Primary follicles	3.33 [a] ± 0.581	4.50 [a] ± 0.339	1.833 [b] ± 0.249
Pre-antral follicles	1.833 [a] ± 0.388	2.666 [b] ± 0.452	0.833 [c] ± 0.249
Antral follicles	1.166 [a] ± 0.249	1.833 [a] ± 0.249	0.50 [b] ± 0.163

*: Number of transplanted OT= 6.
**: Similar letters means non significant (P>0.05) differences.
Different letters means significant (P<0.05) differences.

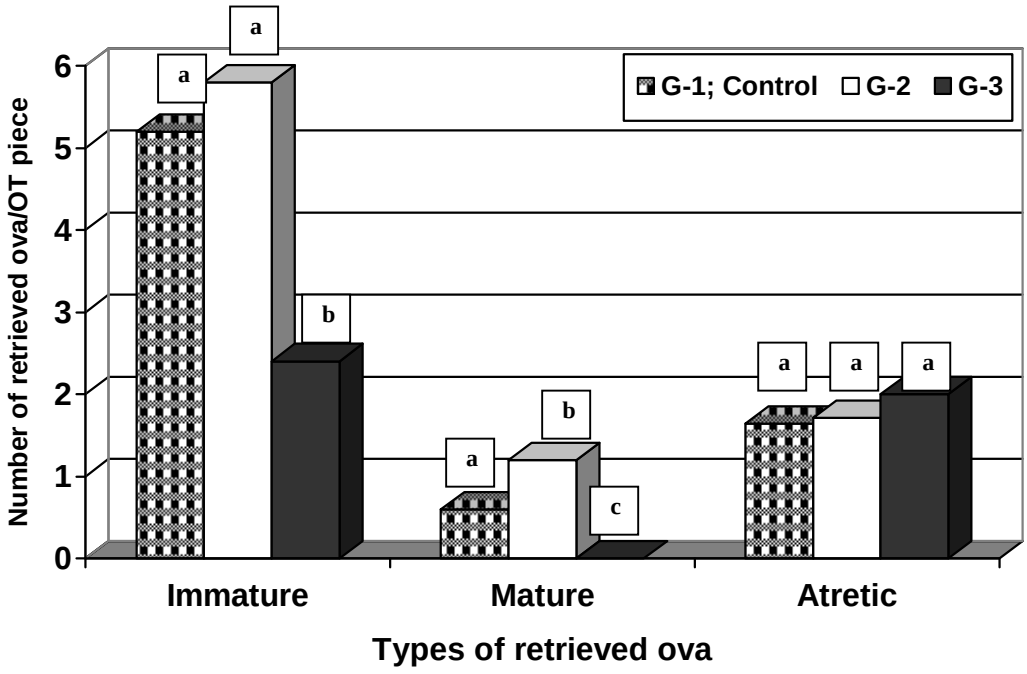


Figure 2: Number of retrieved ova from ovarian follicles of transplanted ovarian tissues * for three groups of female mice

*: Number of transplanted OT= 10.
**: Similar letters means non significant (P>0.05) differences.
Different letters means significant (P<0.05) differences.

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