

# Ultrastructural Analysis of Vitrified Rat Ovarian Tissue Follicles after Heterotopic Autotransplantation

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**Abstract:** ***Objective:** To evaluate and compare the efficiency of vitrification technique and heterotopic autotransplantation with different duration on morphology and ultrastructure of rat ovarian tissue follicles. **Methods:** Twenty 5-6-week-old female SPF SD rats were randomly divided into two groups, with ten rats in each group. Freshly isolated ovaries saved as a control (group 1; fresh ovaries) in formalin-fixed or vitrified immediately after dissection (group 2; vitrified ovaries). Ovaries in vitrified groups were processed into thin slices then vitrified in cryotubes using vitrification medium and kept in liquid nitrogen for 21 days, rapidly thawed and transplanted into back muscles for 2 and 4 weeks. All rats in vitrified experimental group were sacrificed and tissue grafts were collected and fixed in 4% formaldehyde solution. Then non-vitrified and vitrified heterotopic autotransplanted ovarian tissue was compared by light and transmission electron microscopic morphology of the follicles within the tissues. **Results:** There was no significant difference between the percentages of normal primordial (28% vs 41%), primary (41% vs 33%), secondary (23% vs 29%) and antral (30% vs 28%) follicles in both fresh control and vitrified transplanted groups ( $p>0.05$ ). No clear differences in the structures of the follicles, the stromal cells and bundles of collagen fibers were well preserved. By mean of TEM, ovarian tissue follicles showed a well-preserved ultrastructure however the number of mitochondria-SER aggregates and Golgi apparatus were highest in 2 weeks vitrified transplanted group than the 4 weeks vitrified transplanted group. The intact contact between granulosa cells was highly visible in 4 weeks vitrified transplanted tissue whereas irregular shaped mitochondria with no cristae and widened gap junction with slight edema between granulosa cells were only observed in 2 weeks vitrified transplanted group. **Conclusions:** Vitrification technique with heterotopic autotransplantation of ovarian tissue is efficient to maintain their morphological and ultrastructural features at different durations (2 and 4 weeks) similar with fresh tissue, while mild ultrastructural changes in preantral follicles in 4 weeks transplanted tissue was noticed. Although these data are encouraging, further studies are necessary to optimize vitrification protocol and to confirm the accuracy of these observations.*

**Keywords:** Ovarian tissue, Vitrification, Heterotopic, Ultrastructure, Transplantation

## 1. Introduction

According to the World Health Organization (WHO), infertility is considered as a public health issue which affects the human population worldwide with significant psychological consequences [1], [2]. In most of the developed countries approximately 9-14% of infertile couples confronted with infertility problem [2]. Females with cancer or other benign pathologies require chemotherapy, radiation and/or bone marrow transplantation which exert irreversible gonadotoxic effects which cause premature ovarian failure and infertility [1], [3], [4], [5]. According to the recommendations by the American Society of Clinical Oncology (ASCO), cryopreservation of embryos, oocytes or ovarian tissue and oophoropexy are currently proven effective fertility preservation alternatives in patients who wish to preserve their reproductive potential prior to chemotherapy or radiotherapy [6],[7]. Other numerous attempts such as autotransplantation, xenografting and follicular cultures also have been considered as experimental strategies for restoring ovarian functions[3]. Cryopreservation of ovarian tissue can be performed by two main methods: slow-rate freezing and vitrification technique. Several groups have reported that slow cooling is more successful than vitrification [8], [9], however, based on

animal and human studies vitrification technique seems to have greater efficiency in ovarian tissue preservation over slow freezing protocol[1],[4],[10],[11].With regard to the vitrification technique, different studies have reported that it is an attractive procedure as it preserves large, heterogeneous samples of complex biologic tissue without ice crystal formation and apoptosis in mouse and human ovarian tissue after warming [4], [10], [12], [13], [14]. According to Eimani et al [15] cryopreservation and transplantation of ovarian tissue and in vitro maturation of follicles or oocytes are two emerging methods which allow more appropriate choices to preserve fertility with patient's specific situation. However, it also has been proven that autotransplantation of cryopreserved thawed ovarian tissue is an effective method which restore both endocrine function and fertility in humans as well [16],[17]. To date, several studies have been carried out to try to evaluate the best place for ovarian autotransplantation and the competence of cryopreserved ovarian graft but the best site for transplantation still remains unknown [1],[15].After the first successful live birth in adult patient following orthotopic transplantation of cryopreserved ovarian tissues in 2004, until now only about 86 live births (only 2 births from vitrified ovarian tissue) with orthotopic sites (cortex of ovary and peritoneum beneath the ovarian hilum ) or with heterotopic sites (arm muscle, subcutaneous tissue of

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abdomen, peritoneum, rectus muscle, colic omentum and mesoarium) have been recorded [1],[12],[15],[18]. However, heterotopic autotransplantation of frozen-thawed ovarian tissue has become a debatable topic with many conflicting opinions as no clinical pregnancy was recorded in humans [19]. Despite the good results have been obtained by ovarian cryopreservation and transplantation, the main drawback of this method to successfully restore fertility from frozen-thawed ovarian cortex are graft adhesions, massive ischemic damage to follicles until neovascularization develops, inadequate penetration of cryoprotectant through stroma and granulosa cells to the oocytes or possible cryoprotectant toxicity [4],[5],[20]. Merdassi et al [21] in his study concluded that it is crucial to validate the freezing protocol in order to evaluate the success of xeno-or auto-graft transplantation of frozen-thawed ovarian fragments though, surgical technique improvements will also be necessary to reduce the delay in recovery of endocrine function. Moreover, several best known techniques can be used to evaluate the competence, viability and cryodamage of cryopreserved ovarian tissue graft including DNA fragmentation, morphological analysis by hematoxylin-eosin staining, ultrastructural analysis by transmission electron microscopy, cytological analysis of vaginal smears in animals, serum levels of pituitary and gonadal hormones, and follicular in vitro culture [1],[21]. Kim et al [19] reported that there are insufficient experiences and knowledge on heterotopic ovarian tissue transplantation in humans so need more investigation to verify the validity and efficacy of this technique. Our earlier report evaluated the survival and morphology analysis by HE stains in cryopreserved-thawed ovarian tissue grafts after heterotopic autotransplantation at different duration 2 and 4 weeks respectively, and demonstrated good survival of follicles, abundant blood vessels, stroma and a normal histological structure in all ovarian components after thawing [3]. Despite these positive results, it is essential to conduct a thorough examination of the effects of vitrification method and heterotopic autotransplantation at different duration on cryopreserved-thawed ovarian tissue grafts. Therefore, the aim of this present study was to further investigate and compare the efficiency of vitrification technique and heterotopic autotransplantation at 2 and 4 weeks on morphology and ultrastructural features of rat ovarian tissue follicles and compared with fresh control tissues.

## 2. Materials and Methods

### 2.1 Animal care and feeding

All the experimental procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (Wuhan, China) and were in accordance with the guide for Care of Laboratory Animals published by the National Research Council [22]. The 5-6-week-old female SPF SD rats (n=20; weighing 200-250gm) purchased from the Hunan SJA Laboratory Animal Co., Ltd, China were randomly housed five female rats/cage, given free access to water, and fed a standard laboratory animal feed

(Beijing Bio-Technology Co., Ltd). Animals were housed in cages with a controlled temperature (22-25°C) and humidity (50±5°C) and 12/12 h light/dark cycle.

### 2.2 Experimental design

The animals were randomly divided into two groups, with five rats in each group. In fresh control group (fresh ovaries=10), fresh control fragments were fixed in formalin. In experimental group (vitrified ovaries=10), freshly isolated ovaries were vitrified immediately after dissection and transplanted into back muscles of rats for 2 and 4 weeks respectively.

### 2.3 Surgical procedure

The rats were anesthetized by 10% Chloral Hydrate (0.3ml/100gm) i.p injection. All the following surgical procedures were performed aseptically. A single median longitudinal skin incision was made on the lumbar portion to expose the subcutaneous tissue over the ovary. A small incision was made on the fascia and muscles immediately above the ovary, thereby exteriorizing the reproductive tract, to isolate and resect the ovary. In the end of surgery rats were injected with 16000IU of penicillin in 1ml 0.9% NaCl by intraperitoneal.

### 2.4 Cryopreservation of ovarian tissue

At the laboratory, the recovered ovarian pairs (n=10) were stripped of adhering tissue and fat and transported immediately in cell culture dish (Corning) containing 2ml [HTF medium (M&C Gene Technology)] and then ovarian cortex was processed at room temperature (25 degree Celsius) and cut with a scalpel into approximately 1mm x 1mm x 1mm (1mm<sup>3</sup>) fragments. Ovarian cortical sections were transferred into cell culture dishes in stepwise manners, containing vitrification solution 1 [15% EG +15% DMSO (Sigma-Aldrich) + 0.5M sucrose (Sigma-Aldrich) with 10ml HTF-HEPES (In Vitro Care, Frederick, USA)] remained for 30~45 seconds, then transferred in solution 2 (7.5% EG + 7.5% DMSO) remained for 3 minutes and finally transferred in solution 3 (12% HTF-HEPES) remained for 3 minutes at room temperature. The ovarian tissues were then transferred in cryogenic vials and plunged into liquid nitrogen (LN<sub>2</sub>) for 21 days storage.

### 2.5 Thawing procedure

After the storage time for 21 days in liquid nitrogen (LN<sub>2</sub>), the cryopreserved ovarian tissue were thawed rapidly (~100°C/min) by agitating in a warm water bath (35°C) for 2 to 3 minutes and washed in a stepwise manner to minimize osmotic damages (1.0M sucrose + 10ml HTF-HEPES for one minute, 0.5M sucrose + 10ml HTF-HEPES for 3 minute and 12% HTF-HEPES for 3 minutes) at room temperature prior to transplantation.

## 2.6 Transplantation procedure

All rats in experimental group were anesthetized by 10% chloral hydrate (0.3mL/100g) i.p injection. All the following surgical procedures were performed aseptically. The skin and muscle of back incised and frozen-thawed ovarian tissues were immediately inserted inside the muscle gap (made with ophthalmologic tweezers), then the incision was closed with stitches of nylon sutures. In the end of surgery, rats were injected with 16000 IU of penicillin in 1ml 0.9% NaCl by intraperitoneal. From the first day after transplantation, the vaginal cytology was observed every morning to monitor the resumption of cyclic ovarian activity after transplantation.

## 2.7 Rats killing and tissue sampling

In control group, the freshly isolated ovaries were fixed in formalin. In experimental group, the freshly isolated ovaries were vitrified immediately and cut into thin slices. After stored in liquid nitrogen (LN<sub>2</sub>) for 21 days, the tissues of experimental group were rapidly thawed and transplanted into back muscles of rats for 2 and 4 weeks, respectively. After 2 and 4 weeks, all rats in experimental group were killed by cervical dislocation and the ovarian tissues removed from the transplantation site and fixed in 4% formaldehyde solution.

## 2.8 Light microscopy

The morphology of the follicles, granulosa cells and ovarian stroma of fresh control and vitrified transplanted fragments was analyzed by LM. The procedure for ultrastructure evaluation by LM was conducted according to Sheikhi et al [4]. Vitrified transplanted and non-vitrified control tissues were fixed in freshly prepared 4% formaldehyde solution. The fixed tissues were embedded in paraffin wax. Serial sections of 4 mm thickness were prepared and every 11<sup>th</sup> section of each tissue piece was mounted on glass slides and stained with HE. To prevent double counting, each follicle was followed through neighboring sections and counted only once. The development stages of the follicle were classified by the number of layers and shape of granulosa cells surrounding the oocyte, as defined by Jafarey et al [14]. Briefly, primordial follicles were classified as those containing a single layer of flattened granulosa cells, primary follicles were those with a complete single layer of cuboidal granulosa cells, secondary follicles had two complete layers of cuboidal granulosa cells with or without irregular spaces among granulosa cells and tertiary follicle with a single antrum and cumulus oophorus.

## 2.9 Ultrastructure evaluation

The procedures for ultrastructure evaluation by TEM were conducted according to Lunardi et al [7]. Briefly, ultrastructural studies were carried out using fresh control fragments and from transplanted fragments that underwent vitrification treatments. For these, tissue fragments with a maximum dimension of 1mm<sup>3</sup> were fixed in 2%

paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 hours. After fixation and five washes, specimens were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1M sodium cacodylate buffer for 1 hour at room temperature. Subsequently, the samples were dehydrated through a gradient of acetone solutions (31-100%) and the tissues were embedded in Spurr. Semi-thin sections (3µm) were stained with Toluidine blue. The ultra-thin sections (60-70nm) were contrasted with uranyl acetate and lead citrate, and examined under a Jeol JEM 1011 transmission electron microscope (Jeol, Tokyo, Japan). The following elements were evaluated for qualitative assessment of ultrastructural preservation: nuclear content, membrane integrity, density of the cytoplasm and intramitochondrial matrix, cytoplasmic organelles (quality, type and microtopography), and intercellular contacts (between oocytes and granulosa cell).

## 2.11 Statistical Analysis

IBM SPSS Statistics software (version 19) was used for data evaluation. Chi-square test was used to compare the two groups as appropriate. A value of  $P > 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Light microscopy

The morphology and different developing stages of follicles (n=132) in both non-vitrified control and vitrified transplanted tissues were evaluated using an inverted microscope. From the follicles found, 69 were at the primordial stage and 74 follicles at the primary stage. The remaining follicles were 52 secondary and 58 antral stage are presented in Table 1.

There was no significant difference between the percentages of normal primordial (28% vs 41%), primary (41% vs 33%), secondary (23% vs 29%) and antral (30% vs 28%) follicles in fresh control and vitrified transplanted groups ( $p > 0.05$ ). No clear differences could be found in the structures of the follicles in either group when analyzed at the light microscopic level from hematoxylin/eosin stained sections. The stromal cells and bundles of collagen fibers were as well preserved in the vitrified transplanted tissue as it was in the non-vitrified control tissue (Fig.1).

### 3.2 Transmission electron microscopy

The total number of follicles analyzed from each group was as following: 30 non-vitrified fresh control group and 30 vitrified transplanted group. The ultrastructure of the oocytes in both 2 and 4 weeks vitrified transplanted groups were evaluated and compared with control group. The ultrastructure of the oocytes was well preserved in both non-vitrified control tissues and vitrified transplanted tissues with similar morphology.

### 3.2.1 Ultrastructure of 2 weeks vitrified transplanted follicles

The ultrastructure of the oocytes in 2 weeks vitrified transplanted tissues Fig.2(B) are very alike to non-vitrified controls Fig.2(A) except few differences. The presenting oocytes in 2 weeks vitrified transplanted tissues with a large central nucleus well-defined by a nuclear envelope containing distinct nuclear pores (not shown).

Well-defined structures were uniformly distributed throughout the homogenous cytoplasm with lower number of vesicles, empty interstitial area and vacuoles than control group with numerous vesicles and vacuoles Fig.2(A and B). The round or ovoid-shaped mitochondria and endoplasmic reticulum were the most abundant organelles in the oocytes. Moreover, it had highest aggregates of mitochondria-smooth endoplasmic reticulum (M-SER aggregates) same as control group. The endoplasmic reticulum was well defined with slight increase in density. The mitochondrial density was lower than control group while few swollen mitochondrial cristae was found same as control group Fig.2(A and B). The Golgi complexes were also well defined. The oocyte was surrounded by flattened follicular cells on a continuous basal membrane and the contact between the oocyte and granulosa cells in both groups was sharp containing gap junctions and microvilli Fig.2(A and B). In both groups the

granulosa cells showed uniform contact and have a normal morphology and cytoplasm organelle distribution. The granulosa cells contained a voluminous, indented nuclei have euchromatin in the inner part and a small peripheral part of heterochromatin Fig.3(A and B). In 2 weeks transplant group there was an apparent shrinkage of the cell membrane and the follicular cytoplasm exhibit a few variable vesicles, Golgi complex, endoplasmic reticulum, a large number of mitochondrial vacuolization with no cristae Fig.3(B) than control group. Gap junctions connecting neighboring cumulus granulosa cells was widened, edematous and not clearly visible in 2 weeks vitrified transplanted group Fig.3(B). Compared to control group, well-preserved follicular stromal tissues contained abundant collagen fibers observed in 2 weeks vitrified transplanted group. The stromal cells had hetero and euchromatic containing nuclei distributed homogenously in the nuclear envelope while the distribution of collagen fibers was uniform as compared to control group Fig.3(A and B).

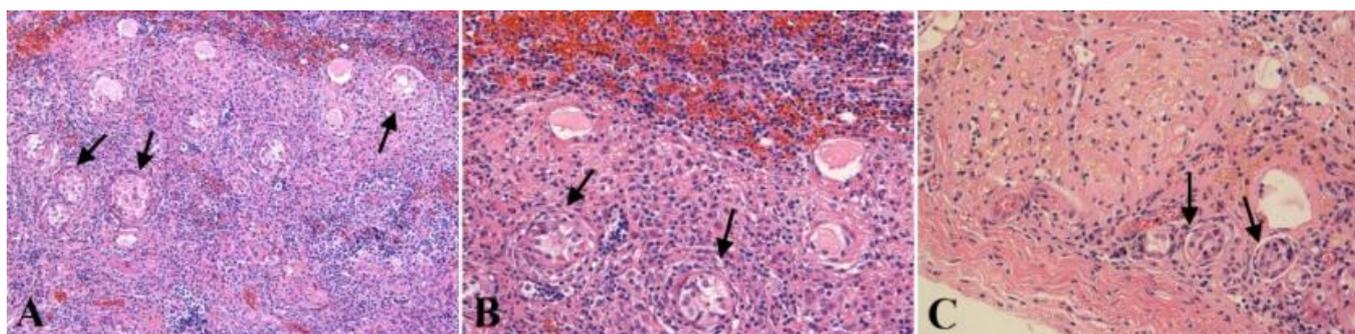
### 3.2.2 Ultrastructure of 4 weeks vitrified transplanted follicles

The ultrastructure of the oocytes in 4 weeks vitrified transplanted tissues Fig.2(C) was same as non-vitrified controls Fig.2(A).

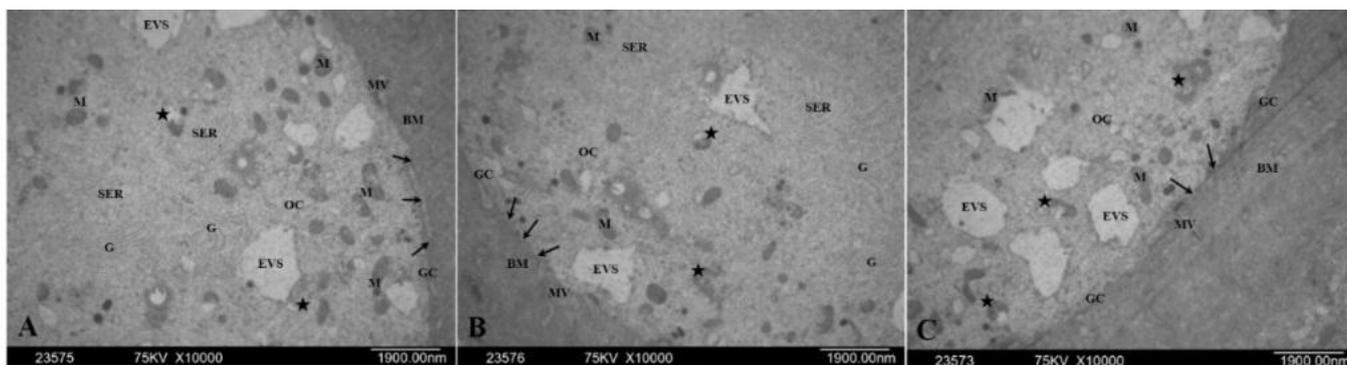
**Table 1:** Number of follicles analyzed in the rat's ovarian tissues assessed by LM

Groups	Total no. of follicles	PmF (%)	PrF (%)	SF (%)	AF (%)
Non-vitrified control	64	28 (44)	41 (64)	23 (36)	30 (47)
Vitrified transplanted	68	41 (60)	33 (49)	29 (43)	28 (41)

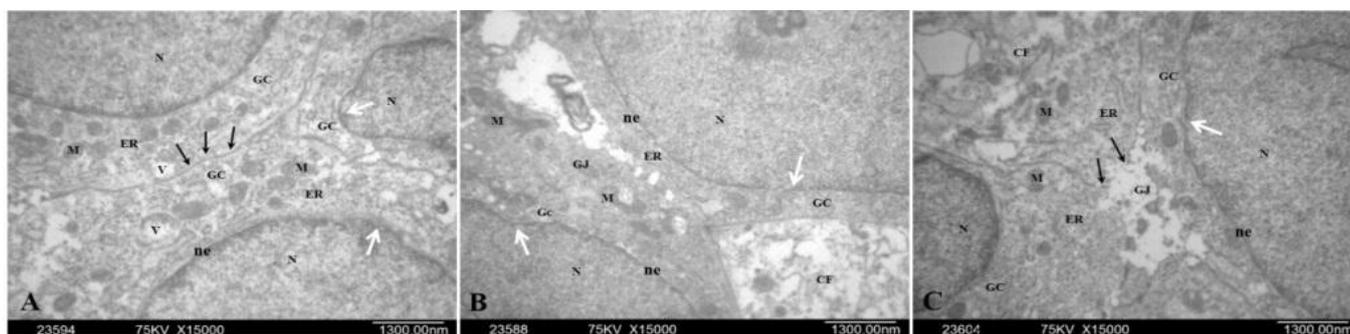
Note: PmF, Primordial follicles; PrF, Primary follicles; SF, Secondary follicles; AF, Antral follicles.  
No significant difference between the two groups ( $P > 0.05$ ).



**Figure 1.** Morphological images of rat ovarian cortical histological sections showing follicles at different developmental stages in non-vitrified control (A) and vitrified 2 and 4 weeks transplanted (B and C) groups. Well-preserved morphology of pre-antral follicles with one to two layers of cuboidal granulosa cells (black arrows) within vitrified transplanted tissue (B and C) was seen, like non-vitrified tissue follicles.



**Figure 2.** Transmission electron microscopy images of primordial follicles within rat ovarian tissues in three groups of non-vitrified control (A) and vitrified 2- and 4-weeks transplanted (B and C) tissues showing the oocyte (OC) encircled by single layer of flattened granulosa cells (GC), well defined gap junctions (arrows) as well as microvilli (MV) and contact with basement membrane (BM) is uniform. The homogenous cytoplasm of the oocyte contains uniform mitochondria (M) with some swollen and disrupted cristae (asterisk), extravascular spaces (EVS) in (A, B and C). Note well defined smooth endoplasmic reticulum (SER) and Golgi apparatus (G) seen in (A and B) but not visible in (C). TEM: (A, B and C) =  $\times 1900\text{nm}$ .



**Figure 3.** Transmission electron microscopy images of granulosa cells of secondary follicles. (A) non-vitrified control, (B and C) vitrified 2- and 4-weeks transplanted tissue showing homogenous appearance, intact nucleus (N) contain hetero- and euchromatin in their nuclear, slight shrinkage (white arrows) but intact nuclear envelope (ne) and cytoplasmic organelles, mitochondria (M) and endoplasmic reticulum (ER) are well-defined. The intact contact between granulosa cells (GC) is seen (black arrows) in (A and C) but not cleared in (B). Note vesicles (V) in (A), irregularly shaped mitochondria (M) with little or no distinguishable cristae, widened gap junction (GJ) between granulosa cells with slight edema and stroma containing abundant collagen fibers (CF) in (B and C). TEM: (A, B and C) =  $\times 1300\text{nm}$ .

The presenting oocytes in both groups exhibited a large central nucleus well-defined by a nuclear envelope containing distinct nuclear pores (not shown) and uniform distribution of cytoplasmic organelles with most vesicles, empty interstitial space and vacuole, round and ovoid shaped highly organized mitochondria, well defined endoplasmic reticulum and Golgi complexes. Although the mitochondria-smooth endoplasmic reticulum (M-SER aggregates) and Golgi complexes were not well defined in 4 weeks transplanted tissues Fig.2(C). The mitochondrial density was a little lower than control group Fig.2(A and C) but a little bit higher than 2 weeks transplanted tissues Fig.2(B). The presence of few swollen mitochondrial cristae was also found similar to control group. The oocyte was surrounded by flattened follicular cells on a continuous basal membrane and the contact between the oocyte and granulosa cells in both groups was sharp containing gap junctions and microvilli Fig.2(A and C). In both groups the granulosa cells showed uniform contact and have a normal morphology and cytoplasm organelle distribution. The granulosa cells contained a voluminous, indented nuclei have euchromatin in the inner part and a small peripheral part of heterochromatin with no sign of cell death observed, while the intact follicular cells cytoplasm exhibit multivesicular bodies, Golgi complex, many mitochondria with cristae and endoplasmic reticulum Fig.3(A and C). Gap junctions connecting neighboring cumulus granulosa cells was widened, edematous but still clearly visible in 4 weeks

vitrified transplanted group Fig.3(C) than control tissues with tight and visible junction Fig.3(A). Compared to control group, well-preserved follicular stromal tissues contained distributed collagen fibers observed in 4 week vitrified transplanted group Fig.3(C). The stromal cells had hetero and euchromatic containing nuclei distributed homogeneously in the nuclear envelope while the distribution of collagen fibers was uniform than control tissues Fig.3(A and C).

#### 4. Discussion

Despite significant progress in human ovarian tissue cryopreservation and transplantation over the last few years, more improvement is obligatory to attain ideal results, therefore several animal models have been established to enhance cryopreservation and transplantation methods [23]. It is considered that ovarian tissue vitrification and autotransplantation is a promising approach for fertility preservation however still is an experimental and pilot technique [23], [24]. According to Varghese et al [25] the advantages of cryopreservation and transplantation of ovarian tissue are: immediate harvesting (treatment can start without delay), no male partner/gamete donor is needed, allows complete gonadotoxic or ionizing radiation treatment without exposure, allows endocrine function to be resumed, allows resumption and preservation of reproductive function, suitable for prepubertal girls and avoid immunosuppressant treatment while the disadvantages include: requires surgical

procedures for tissue harvesting and transfer, possibility of reintroducing malignant cells, and low success rate. To date, heterotopic autotransplantation of frozen-thawed ovarian tissue has become a debatable topic with many conflicting opinions as no clinical pregnancy was recorded in humans [19]. However, it was reported that the vitrification protocol significantly affects the survival of follicles due to its minimal changes in morphology and ultrastructure of ovarian tissue [11],[23]. In this research we intended to evaluate the application of vitrification technique with heterotopic (back muscles) autotransplantation on the follicular morphology and ultrastructure of rat ovarian tissue. Our results of H & E staining showed that the well-preserved morphology of the preantral follicles of ovarian tissue appeared similar in both fresh and vitrified transplanted groups however, fresh control primordial follicle count was lower than 2 and 4 weeks vitrified transplanted group but not statistically significant. These results indicated that vitrified follicles can maintain their natural appearance after transplantation may be due to the cryoprotectants (such as EG and DMSO) used for vitrification-warming have no obvious detrimental effect on transplanted ovarian tissue [3] or a combination of cryoprotectant (ethylene glycol, propylene glycol, and sucrose) at different concentrations have achieved sufficient dehydration to successfully vitrify all components of ovarian follicles [11] or that the transplantation time was enough to allow the angiogenic factors to work or to induce neovascularization [23]. According to Aubard and Hernandez-Fonseca et al [26],[27] any growing follicles present in the graft tissue within days after transplantation are supposed to be derived from primordial follicles present at the time of transplantation. In our study an increased proportion of primordial follicles in vitrified transplanted group may indicate the fact that undeveloped primordial follicles are more resistant to cryo-injury compared with mature and immature oocytes, due to their relatively small size, low metabolic rate, absence of a zona pellucida, peripheral cortical granules in their oocytes and the cell cycle stage is arrested at the prophase of meiosis I [28],[29]. Moreover, an increased number of viable follicles may indicate better survival rate of follicles during vitrification [11]. The improved Follicular morphology reported in the present study confirms our previous observations [3],[14] and is in contrasts to the poor morphology of primordial follicles in human ovarian tissue previously reported using slow freezing technique [30]. In fact, at nuclear level the most likely consequence of vitrification would be the necrosis process, which can be better demonstrated using careful morphological and ultrastructural analysis [11],[31],[32]. Furthermore, ultrastructural evaluation by Transmission electron microscopy is the best-known method to demonstrate the cryodamage [4],[5] so we applied TEM to complete our ovarian tissue ultrastructural evaluation in both groups. Our ultrastructural studies by TEM showed that after transplantation of vitrified ovarian tissues at 2 and 4 weeks, there was no noticeable change in the organelles of oocytes and follicular cells when compared to fresh controls and the integrity of cell organelles was well preserved. The mitochondrial organization, cytoskeletal arrangement and

vacuolization are crucial factors for cryopreservation assessment[33],[34]. Several studies have demonstrated that the mitochondria are very important to the energy (ATP) production, metabolic activation and regulating cell survival [23],[33],[34], however its structural and metabolic defects cause failures of oocyte maturation and developmental arrest of embryo[23]. Yan et al [35] mentioned that mitochondrial organization is dependent on microtubule assembly though vitrification seriously disturbs mitochondrial distribution and behavior in mouse oocytes. Previous studies showed that vitrification solutions containing DMSO can induce an abnormality in microtubule assembly, which might not affect mitochondrial behavior in vitrified oocytes but when the oocytes developed to early-stage embryos, the abnormality became obvious and led to abnormal mitochondrial localization [36],[37]. Moreover, several studies mentioned that only primordial or small preantral follicles are more resilient and survive to cryo-damage due to a direct consequence of detrimental effect by cryoprotectant or low temperatures [38]. In vitrified ovarian tissue transplanted at 2 and 4 weeks, our results showed that some mitochondria appeared elongated, swollen with disrupted cristae consistently present in all the primordial follicular stage. These results suggest that perhaps mitochondria were going through an apoptosis process [33] maybe caused by oocyte microtubule damage induced by toxicity of cryoprotectants [33],[38], an alteration of the osmotic equilibrium process[39], low temperatures, or due to the lack of post-graft vascularization of primordial follicle[38]. M-SER aggregates are considered as a precursor complex of mitochondria and endoplasmic reticulum vesicles, have essential role in the regulation of free calcium concentrations and ATP production[2] while any ultrastructural changes of M-SER aggregates in vitrified oocytes may lead to impaired calcium homeostasis and reduction in fertilization rate [40]. In this study, reducing of M-SER was observed in 4 weeks vitrified transplanted tissues may be related to use of ethylene glycol in vitrification solution to damages from the cooling, the long-time cooling of ovarian tissue [2], unequal size of ovarian graft, certain environmental factors, cryoprotectants used in high concentration or may not have developed blood anastomosis after transplantation[3], can confirm the potential reduction of oocyte competence for fertilization because of calcium homeostasis disruption [40]. Despite of such mild ultrastructural alteration in secondary follicles of 4 weeks vitrified graft, it showed different stages of antral folliculogenesis with intact morphology may indicating the fact that vitrification with heterotopic transplantation don't have any detrimental effect or less toxic effects of EG and DMSO used in vitrification solution may have resolved quite rapidly and preserved ovarian tissue follicle morphology and functions as well[40]. Gap junctions are aggregations of intracellular channels composed of protein connexins that allow movements of ions, metabolites, and signaling molecules from cell to cell is an important facet for fertilization and embryogenesis[41]. However, the regulation of antral follicular development and oocyte maturation dependent on gap junctions to exchange a significant amount of metabolites in the granulosa-oocyte

interface[28]. This study demonstrates intact contact between oocyte and granulosa cells containing wide gap junctions with mild edema in both 2 and 4 weeks vitrified transplanted ovarian tissue when compared with fresh control. This indicates that DMSO appeared a more effective vitrification protocol in to maintain the granulosa-oocyte interface in vitrified ovarian tissue [38]. Previously it was reported that granulosa cells are more vulnerable to ischemia and hypoxia before the grafted fragment is revascularized[41] while our results didn't demonstrate a loss in integrity of granulosa cell-oocyte interface instead noticed antral stage follicles after transplantation of vitrified ovarian tissue proving this hypothesis that folliculogenesis is known to be gap-junction dependent, may be due to certain factors such as transplantation site induced angiogenesis, nontoxic effect of DMSO cryoprotectant, complete vitrification process (prevent ice crystal formation) or long duration of tissue transplantation, prevent granulosa cells ischemia then begin folliculogenesis. The gap junction protein connexin establish important communication between oocyte and cumulus cells for oocyte development and fertility and permit the exchange of water, sugars, amino acids, lipid precursors, nucleotides and cryoprotectants through plasma membrane diffusion process[42]. Though in vitrification solution a high concentration of permeating cryoprotectants is a major concern for cryotoxicity [42,43] but our TEM ultrastructural results showed granulosa cells with wide gap junctions and slight edema without any cytotoxic damage in both vitrified transplantation group which may indicate this fact that the permeability to water or high permeating cryoprotectants ethylene glycol (EG) and DMSO was low in rat oocytes by simple or facilitated diffusion [43], so cells undergo slight swelling with no sign of apoptosis. Stromal cells certainly play an important role in proliferation and differentiation of granulosa cells while co-operation between granulosa cells, stromal cells and the oocytes is necessary for ovarian function and preservation of these component integrity[4]. In our study, the ultrastructure of stromal cells within both 2 and 4 week vitrified transplanted tissue showed a good quality stroma, composed of collagen fibers and stromal cells and there were not any sign of fragmentation of nucleus within the stromal cells which indicate that ovarian stroma was significantly better preserved after vitrification and transplantation at both 2 and 4 week duration.

Our results agree with those reported by other authors, Sheikhi et al [4] compared the ultrastructural analysis of non-vitrified and warmed-vitrified human ovarian tissue follicles by light and electron microscopy. The results showed that the ultrastructure of pre-antral follicles within the vitrified human ovarian tissue was well preserved by using cryotube device, concluding that vitrification is the method of choice for preservation of follicular morphology of human ovarian tissue. Xiao et al [30] compared a novel vitrification technique (needle immersed vitrification method) and slow freezing in term to preserve primordial follicles in human thawed ovarian tissue, reported that percentage of morphologically abnormal primordial follicles was significantly lower with vitrification than with slow

freezing. Nasrabadi et al [33] showed that vitrification of mouse ovarian tissue with optimal cryoprotectant solutions (EG + DMSO) is the most effective for preserving the morphological integrity and ultrastructure of follicles and ovarian tissue don't undergo osmotic shock after vitrification. Luyckx et al [44] reported the first-time transplantation of human cryopreserved pre-pubertal ovarian tissue to mice and demonstrated that frozen-thawed preantral follicles from prepubertal patients can successfully survive and develop after transplantation.

## 5. Conclusions

In conclusion, the present study showed well preservation of follicle morphology, organelles of granulosa cells and oocytes and stromal cell integrity, as also reported by other authors in the literature. This finding indicates that vitrification technique with heterotopic autotransplantation of ovarian tissue is efficient to maintain their morphology and ultrastructure at different durations (2 and 4 weeks) compared with fresh control, because detrimental effect on the follicles at the different developmental stages are avoided. To maintain the ultrastructural level of vitrified transplanted ovarian tissue, the cryoprotectants (EG and DMSO), concentrations and volume of cryoprotectants, angiogenesis, low temperature and certain environmental factors are considered the main factors. Despite of mild ultrastructural alteration in primordial and secondary follicles in 4 weeks transplanted tissue, presence of different stages of antral folliculogenesis in vitrified ovarian graft suggests that vitrification with heterotopic autotransplantation don't have any detrimental effect and can preserve their morphology and ultrastructure as well. Therefore, additional studies are needed and essential to optimize vitrification protocol and to confirm the accuracy of these observations by long-term grafting and heterotopic autotransplantation of human ovarian tissue. Nevertheless, immunohistochemistry, TUNEL, and tissue culture might be excellent alternative methods to demonstrate viability of follicle within the tissue.

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