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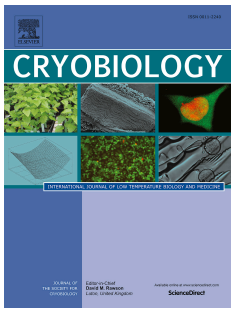

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Evaluation of sheep ovarian tissue cryopreserved by slow freezing or vitrificaton after chick embryo chorioallantoic membrane transplantation

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Abstract

The aim of our investigations was to compare the effectiveness of two methods for cryopreservation of sheep ovarian tissue, slow freezing and vitrification. The quality of cryopreserved tissues was evaluated after 5 days of thawing and chorioallantoic membrane (CAM) transplantation. Follicular structure, stromal integrity and neovascularization were assessed. The areas of fibrosis and necrosis were measured using MICROVISIBLE software, and proliferation was assessed with Ki-67 immunostaning.

After 5 days of culture, the proportion of primordial follicles decreased, whereas the primary and intermediary follicles increased insignificantly ($p>.05$). Only necrosis in the vitrified culture group increased significantly ($p<.05$). It was established also that 5 days CAM culture was not suitable methodology for detection of folliculogenesis. Follicular quality decreased after culture, but was better in fresh and slow frozen tissues than after vitrification ($p<.05$). Cellular proliferative activity fell, but it preserved to some extent in all groups. In conclusion, follicles was preserved better in grafted tissue after slow freezing than vitrification and stroma was more susceptible to ischemia in vitrified rather than conventional freezing in this view. Vitrification may not be a suitable alternative to the slow freezing.

Key words: Ovarian tissue, Slow freezing, Vitrification, Chorio-allantoic membrane, transplantation

Introduction

Survival rates of cancer patients have increased, but treatment regimens are associated with the risk of premature ovarian failure. Nowadays, concern is not only about the quality of life of cancer survivors, but how to preserve their fertility [22, 2]. Before starting treatment, giving appropriate information regarding fertility preservation can reduce levels of psychological distress [25]. There are three main methods for fertility cryopreservation in women: cryopreservation of embryos, oocytes, and ovarian tissues [23, 30, 35]. Cryopreservation of human ovarian tissue is a valuable asset in fertility preservation, because other methods are inappropriate for children, adolescents or single women with no partners or whose clinical course does not permit delay for ovarian hyperstimulation in an ART program [30, 16, 19]. Many studies have investigated two methods of ovarian cryopreservation; vitrification and slow-rate freezing to determine the

effectiveness of the cryotechniques [2, 10], or cryoprotective agents to optimize the cryopreservation [21, 24, 28].

Comparative research on the vitrification and slow freezing of mammalian ovarian tissue is limited, and different conclusions were reported [15]. Slow freezing is relatively time-consuming and costly [1,39], but till 2015, 30 live births in humans had only been achieved after slow-freezing [34]. Isachenko and associates have reported that ovarian slow freezing is a better option [16]. The safety of vitrification is a potential problem in terms of toxicity and osmotic shock due to high concentration of cryoprotectants, and only a few births have been reported after vitrification [9, 12, 21]. Other than autografting back to the patients with the aim of restoring fertility, it is difficult to determine which cryopreservation method is the best, because immediate analysis after warming may not show the cryodamage [37]. There are three experimental methods for evaluating the developmental potential of human ovarian tissue after warming: 1) follicle development after xenotransplantation; 2) in vitro culture; and 3) culture on chick embryo chorio-allantoic membrane (CAM) [17]. Xenografting needs access to the laboratory animals [26] and long term development in vitro culture (IVC) is difficult [36]. Moreover, the IVC is not a suitable model for angiogenic studies [26]. Since the chicken CAM does not have lymphoid system until later in development, it can be used as a natural immunodeficient host capable of showing rapid neovascularization. It can also support the grafted tissues effectively [36, 6]. The CAM structure is similar to the peritoneum and prepares a microenvironment very close to the in -vivo situation [26]. This inexpensive system has been used by embryologists for decades [5], allowing large scale screening without needing the animal testing [27].

Despite the existing controversy regarding methods of freezing, there are many studies that support the effectiveness of the slow freezing [10,34]. So, our hypothesis is that if the ovarian tissues are better preserved after slow freezing, they will be better after transplantation too. To verify this hypothesis, we grafted the ovarian tissue after cryopreservation by slow freezing and vitrification methods, to compare which method yields a better outcome after transplantation.

Materials and Methods

The experimental procedures were reviewed and approved by the Research Council in the Research and Clinical Center for Infertility , Yazd Institute for Reproductive Sciences (approved number; 40668/2017). The use of slaughterhouse derived ovaries and the CAM culture system does not raise ethical or legal concerns, nor does it violate the animal protection laws[19].

Tissue Collection and dissection

Whole ovaries from adult mixed breed sheep (n=10) were obtained from a local slaughterhouse. They were transported to the laboratory in phosphate-buffered saline (PBS) (ATOCEL) supplemented with 5% fetal bovine serum (FBS) and 100 IU penicillin/ml and 100 µg streptomycin sulfate/ml (Life Technologies) on icewater at 4°C. Under the laminar flow hood, the tissues were rinsed three times in PBS for washing off possible blood contamination. From each ovary, the outer 1-mm thick cortex was removed, and then cut into 1×1×5 mm strips. Dissection medium was DPBS supplemented with 10% FBS. We had two main groups of uncultured and cultured and three sub groups of fresh (group I), vitrification (group II) and slow freezinfg (group III). One piece from each sheep ovary was fixed in 7% formalin for future examination, one group was prepared for fresh CAM grafting and two pieces were processed for vitrification and slow freezing for later grafting.

Vitrification and warming

The vitrification protocol was described by Kagawa et al (2009) [19] with some modification at the end immersing into liquid nitrogen. It contained two cryoprotectants ofMe2SO and EG, and the base medium was TCM199 supplemented with 20% serum substitute supplement (SSS). It consisted of two steps of equilibration. Firstly, 7.5% Me2SO+7.5% EG in the base medium for 25 min, and in the second step, 20% Me2SO+20% EG+0.5 mol/L sucrose used for 15 min. All the equilibrations were done at 4°C. At the end of this stage,the tissues were vitrified using the needle immersion method. Thevitrified strips were then transferred into standard 1.8-ml cryovials and put into liquid nitrogen (LN_2) tanks for one week.

For warming, the vials were taken out from the nitrogen tank, and the frozen tissues were extracted and transferred to three warming solutions. The first one was 40ml handling medium (HM, TCM199 supplemented with 20% SSS) solution supplemented with 1.0 mol/l sucrose for 1 min. The second was 15 ml of HM and 0.5 mol/l sucrose, for 5 min, and the third consisted of washing the ovarian strips in HM solution for 10 min twiceat room temperature (RT).

Slow freezing and thawing

The protocol was base on the protocol used by Isachenko et al (2009)[16]with some modifications at the end as described by his group in the other work [13]. Base medium in the slow freezing program was L-15 medium (Leibovitz) with L-glutamine (Sigma) + 10% SSS (Irvine Scientific). The freezing medium contained 1.5 M Me2SO and 0.1 M sucrose which were added to HM. The ovarian tissue pieces (OPs) were incubated in this freezing medium for 30 min,then they were placed in a programmable freezer (Cryologic, CL-8000). We used 5 ml cryovials for this purpose. The freezing program was as follows; starting temperature was 2°C, then at a rate of 2°C/min, temperature decreased from 2 to -6 °C. Manual seeding was done at -6 °C. After crystal formation in the top of the cryovials, the temperature increased to -5.7°C and held at this temperature for 10min. Temperature was cooled from -5.7 °C to -36 °C at a speed of 0.3 °C/min and at the end, the cryovials were plunged into LN_2. The equilibration process was done on the icewater at 4°C.

For thawing, the cryovials were removed from liquid nitrogen and placed for 30sec at RT. Then, they were immersed into a boiling water bath (100°C) until the ice was melted (60sec). The next step was transferring OPs into a 110ml sterile container that contained 10 ml of L-15 medium supplemented with 0.75 M sucrose and 10% SSS. 50 ml of the handling medium was gradually added to the OPs within container. This stage was done under continuous agitation at 200oscillations/min for 15 min at RT[16]. At the end, the OPs were washed three times in DPBS supplemented with 10% SSS at RT. Before transplanting on CAM,tissues were incubated in culture medium for 30 min, in 5% CO_2 and 37 ºC and 98% humidity.

CAM-culture vitrified and slow freezing tissue

After transport to the laboratory, newly laid fertilized eggs of Ross chickens were incubated at 37°C with 60% humidity. They were placed in the incubator on their sharp pole for 6 days. Then, they were taken from the incubator, washed with warm 70% ethanol and placed on the blunt-pole. With a drilling machine, a small hole was made on the blunt-pole of the egg and the hole was covered with a 2x2 cm square of Leukosilk. We created a 1.5-2 cm window in the egg shell with small curved scissors. After sealing the window with Leukosilk, they were incubated again. On day 7, a 0.5 mm thick and 5mm diameter sterile silicone ring was placed on the basal layer of the CAM. CAM has two epithelial layers. The outer layer must be opened gently. We tried to put these rings on the large blood vessels on intact basal layer. With the aid of a microsurgical forceps, the warmed ovarian strips were transferred onto this silicone rings and the window was covered with a tape, and put in the incubator again. On day 5 of culture, the transplanted tissues were retrieved. We evaluated only the pink tissues that were well vascularized.

Morphology of follicles and stroma

Samples were taken from the fresh ovary, after freezing (vitrification or slow freezing), and after 5 days of CAM culture, all were evaluated histologically in a blinded fashion by one person. Cortical tissue was fixed in 7%formalin and embedded in paraffin. 5 mm-thick sections were cut. Since, diameter of an oocyte in primary stage is about 50 µm,we selected and stained only one in every tenth section with Hematoxylin and eosin to avoid double counting.Numbers of each developmental stage of follicles were counted to compare the ovarian follicles in fresh with cultured ovaries. We classified follicles as described by Martins et al (2008) into primordial, intermediary, primary, secondary, pre-antral and antral follicles[27]. Follicles were evaluated and counted at ×400 magnification.

Classifications of follicles were based on follicles and stromal morphology. Three categories of good (A), fair (B) and poor (C) qualities were considered. Follicles were classified as good if their morphology showed regularly shaped granulosa cells, which did not detach from the basement membrane and the oocytes were in contact with their surrounding granulosa cells, with no pyknotic nuclei or signs of shrinkage or

degeneration. Follicles that had pyknotic nuclei and granulosa cells to some extent with detachment from the basement membranes were judged as fair. Follicle were considered poor, when granulosa cells were completely detached from the basement membrane witha degenerated oocyte that lost its contact with surrounding granulosa cells, with pyknotic nuclei and shrinkage of cytoplasm (figure 1).

For analysis of the stroma, three distinguishable zones in the sections were considered as described by Martinez-Madrid (2009) [26].These were healthy, fibrotic and necrotic areas (figure 2). In addition, a clear zone around each grafted section that could be distinguished from the ovarian tissue by the presence of thin and very low density cells (CAM invasion to the grafted tissue) was seen. We measured these zones by using a micros camera and MICROVISIBLE software.

Ovarian tissue viability and transplantation

We considered that all the tissues that grafted successfully to the CAM were viable. Grafts which had only partial adherence to the CAM were excluded and considered as transplant failure. The criteria for a successful transplant were pink color of tissue, neovascularization around tissue with no shrinkage. In this case, avian vessels were easily detectable by nucleated erythrocytes, tight junction of tissue to the CAM, and an important sign of invasion of CAM to the tissue determined by white to gray color tissue that encapsulated the ovarian pieces. This was one of our criteria to determine which tissues grafted successfully (Figure 2).

Proliferation assay

Ki-67, a nuclear antigen was used as a marker of cell proliferation in the ovarian tissue. For detection of this marker, an En Vision monoclonal antibody system was used. If there was at least one Ki-67-positive granulosa cell infollicles, it was considered as proliferating [38, 7]. In addition, we evaluated the stromal cell positive and negative immunoreactivity. All the sections were counted with positive stromal cells and presented as proportion on each group (figure 3).

Statisticalanalysis

Mean and SD were used in presenting continuous data. Independent sample t- test comparisons were done within each group for culture and uncultured groups, alsofor comparisonsof fibrosis and necrosis between two methods of freezing. The variables of fibrosis and necrosis were also compared between three groups of fresh and two freezing groups by using analysis of variance (ANOVA). $p<0.05$ was considered as significant.

Results

Follicular structure

The data showed that the primordial, intermediary and primary follicles were the most abundant in all groups. In analysis, intermediary and primary were classified together in one group. Fifty one percent of the follicles in the uncultured groups, and 47 % in the culture groups were primordial. Although, there was a decrease in primordial and an increase in the growing follicles, the difference wasn't significant. Also no significant differences were found in the percentage of these stages in three cultured groups ($p>.05$; Table 1). In connection with the follicular structure, the follicles had significantly better structure in the control than cryopreserved groups. Between the two cryopreserved groups, there was a better follicular structure after slow freezing rather than after vitrification ($p>0.05$; Figure1; Table 1). After CAM culturing, the quality of follicles decreased ($p<.05$) and it was preserved better in both the fresh and slow cooled groups ($p>.05$), rather than in the vitrified group ($p<.05$).

Stromal morphology

Although, some areas of fibrosis and necrosis were observed after thawing, general stromal appearance was preserved well after cryopreservation. Significantly better stromal cell structure was observed after vitrification as compared to the slow freezing (fibrosis area/mm^2 ($p<.05$)). After CAM culturing, fibrosis areas increased compared to uncultured tissue ($p<.05$), but, there were no significant differences between the groups ($p>.05$) (Figure 2). The necrotic areas increased after culture in all groups ($p<.05$), but was significantly higher only in the vitrification culture group ($p<.05$) (Table 2).

Transplantation status

We transplanted 120 pieces of fresh, vitrified and slow cooled tissues. Fifty one percent of the slow cooled group, 44% in the fresh and 41% in the vitrification group transplanted successfully. Avian vessels were visible more at the border between ovarian tissue and CAM invasion (Figure 2).

Proliferative activity

Ki-67 immunoreactivity was observed in follicles and stromal cells of fresh, and three groups of ovarian CAM cultured tissue. Positive follicles in fresh uncultured group were significantly higher than those in the cryopreserved groups ($p<.05$) and the results were almost the same with slow freezing and vitrification (45.3% and 41.8%, respectively). Although, results in the culture groups were lower than the uncultured groups, there were no significant differences between cultured groups ($p>.05$). The Ki-67 immunoreactivity in the stromal tissue was the same for fresh and vitrified groups, but was lower in slow freezing($p<.05$). After culture, fewer cells were Ki-67 immunoreactive in all groups (Table 3) and there were no significant differences between cultured groups ($p>.05$).

Discussion

Our study showed that CAM culture was unable to activate folliculogenesis in a short period of time. There are similar studies that showed culture onto the CAM hasn't been able to activate folliculogenesis [5, 11, 32]. In this regard, it was reported that in CAM system, the amounts of nutrients and oxygen supply to the grafted tissue are lower than in vivo. In addition, they believed that one specific factor in the chick blood must be responsible for this inhibitory effect [5]. Anti-Mullerian hormone (AMH) that is secreted by chick gonads is one of the inhibitory factors in this system [11]. Despite these negative results pertaining to the folliculogenesis on the CAM, in one study Martinez-Madrid et al. (2009) reported folliculogenesis by CAM. One reason is that the inhibitory or activatory role of AMH is dependent on the species and the age, and different results may be due to different specimens and ages [26]. Data about CAM ability to promoting follicle activation is very low, and more studies must be done to prove follicular activation and maturation in this system.

Our findings showed that slow freezing had better normal follicular structure than vitrification, however differences were not significant. Results about preservation of follicles are controversial, many of them verified the slow freezing method [10, 20, 13], and some others are in favor of vitrification. They reported that there were no differences in the oocyte viability [20] or follicular structure [15] between two methods of freezing. High concentration of cryoprotectant agents (CPA) prohibits formation of ice crystals and results in good morphology, but this dosage is toxic. Growing follicles are more susceptible to the toxic effects. They have larger size and more cells, and CPA doesn't penetrate easily. A lower concentration and longer exposure (what tackes place in slow freezing) may be one solution.

According to our data, after 5 days of culture, follicles in fresh and conventional freezing had better structure than vitrified- warmed culture group. One probable reason may be the fact that follicles preserve better structure after slow freezing, though it was insignificant. To our knowledge, present study was the first that compared follicle steucture between conventional and vitrification methods. Follicle atresia (grade c) also was more obvious in the vitrification cultured tissues. As these criteria weren't significant before culturing, we concluded that slow freezing seems to better tolerate transplantation than vitrified tissues. This result is in accordance with a recent study by Abir et al. (2017) that reported better transplantation of slow freezing tissue after warming compared to vitrified ones in immunodeficient mice [2].

It was shown that stroma preserved well after vitrification [33] and even with better results than slow freezing [20, 14, 10]. In this study, we also observed better stromal cells preservation after vitrification rather than slow freezing. One logical reason for better stromal preservation after vitrification is avoidance of ice crystal formation in this method [20]. If vitrification can preserve stromal cells well, why isn't it true for follicles too? It may be related to the ovarian cortex cellular heterogeneity and different permeability. Other studies also referred to this challenge, and this heterogeneity seems to be the reason why vitrification isn't optimal for the follicles [28, 4]. The findings also showed that necrosis was significantly higher in the vitrified culture group. One reason may be the fact that stroma is more susceptible to hypoxia and oxygen deprivation [3], and vitrified tissue probably transplanted with some delay than slow freezing. This result

is in accordance with other studies that reported more necrotic areas after transplantation of ovarian tissue onto the CAM or grafted to SCID mice[17, 29]. In this regard, there is one theory by Isachenko and colleagues (2012) who reported hypoxia before revascularization can increase ischemic areas [17]. The other one by Nisolle et al. (2000) showed that the interval between neovascularization increased fibrosis zones [29]. This theory also was reported by Martinez-Madrid et al. (2009) who believed fibrosis and necrosis areas appeared before neovascularization, and ischemic damage occurred because of this delay in new vascular supply [26]. We hypothesized that neovascularization doesn't take place at the same time in all grafted tissues and this different delay in timing will be related to higher concentration of CPA that is used in vitrification and this may damage the tissue neovascularization ability. We believe that this episode (ischemic damage) will be unique in each grafted tissue and this different timing in neovascularization onset is an important and evaluable criterion.

After five days culture only pink and well vascularized tissues were fixed in formalin for later evaluations. The low number of successful transplantation was one of our limitations while it was reported at 100% in another study [26]. One reason may be our strict criteria to include a grafted tissue in addition to technical point for transplantation such as the day of egg incubation when a tissue were transferred and equipment as well as non technical factors such as strain of eggs. Our results were in accordance with other studies that showed follicular proliferation can be preserved after vitrification or slow freezing [38, 8] and after CAM ovarian culture [26, 18].

Results showed that stromal cell Ki67 was the same in the fresh and vitrified group, but it was lower in slow freezing and these results after culture were considerably lower in all groups (table 2).

Conclusion

Since culture onto the CAM was unable to promote folliculogenesis, there was not feasible to determine which method of freezing will be better for follicle activation but short term in vivo culture is a valuable method to ensure viability and developmental potential of warmed ovarian tissue. In this situation, follicles were better preserved after slow freezing than vitrification, so it is early to say that vitrification is a viable alternative method to slow freezing. Vitrification and its ability to preserve stroma is applicable only

when the most demanding need (oxygen) fulfilled well and as soon as possible after transplantation. In this study we compared only two distinct protocol for freezing, although changing the times (exposure to the cryoprotectants) and different CPA`s concentration may change the outcomes.

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Table 1: Proportion (%) of follicles in fresh and two freezing method groups and after culturing.

		Primordial (%)	Intermediary & primary (%)	Secondary (%)	Pre antral (%)	Antral (%)
Uncultured	fresh	40.3	30.3	8.8	17.4	3.2
	vit	53.4	29.2	13	3.5	0.9
	Slow	60.3	22.8	10.9	3	3
culture	Fresh/culture	44.1	30.7	9.9	12.9	2.4
	Vit/culture	45.8	35.5	12.7	4.25	1.75
	Slow/culture	50.1	30.1	5.1	14.6	0.1

There were no significant differences in the proportion of these stages before and after culture and between three cultured groups ($p>.05$).

Table 2: fibrosis and necrosis relative area (%) and number of capillaries

		Fibrosis area surface (%)	Necrosis area surface (%)	No. of capillaries/mm^2
uncultured**	fresh*	24.3±5.5	13.6±6.2	-
	Vitrify*	28.8±9	22.2±10.1	-
	Slow freeze*	38.6 ±11.3	24.3±8.7	-
culture**	Fresh ***	48.6±20.5	28.5±13.1	88
	Vitrify***	44.2±18.8	40.3±14.4	63
	Slow freeze***	47.1±11.6	30.5±11.2	71

* The amount of fibrosis was higher after slow freezing rather than fresh and vitrified group ($p<.05$).
**the amount of fibrosis and necrosis increased after culture ($p<.05$)
*** Necrotic area was higher after vitrification rather than fresh and slow freezing culture group ($p<.05$)

Table3. Proportion of follicles and stromal cell positive in cultured and uncultured groups

Groups		Follicle (%)	Stroma(%)
Uncultured **	fresh	55.7*	75.3***
	Vit	41.8*	78.1***
	slow	45.3*	55.4***
Cultured **	Fresh culture	29.9	46.6
	Vit culture	19.2	48.8
	Slow culture	21.6	38.1

* Positive follicles were significantly higher in fresh than freezing groups ($p<.05$)

** Positive follicles and stroma were significantly higher in uncultured than cultured group ($p<.05$)

*** Positive stroma was significantly higher in fresh and vitrification than slow freezing method before culture ($p<.05$)

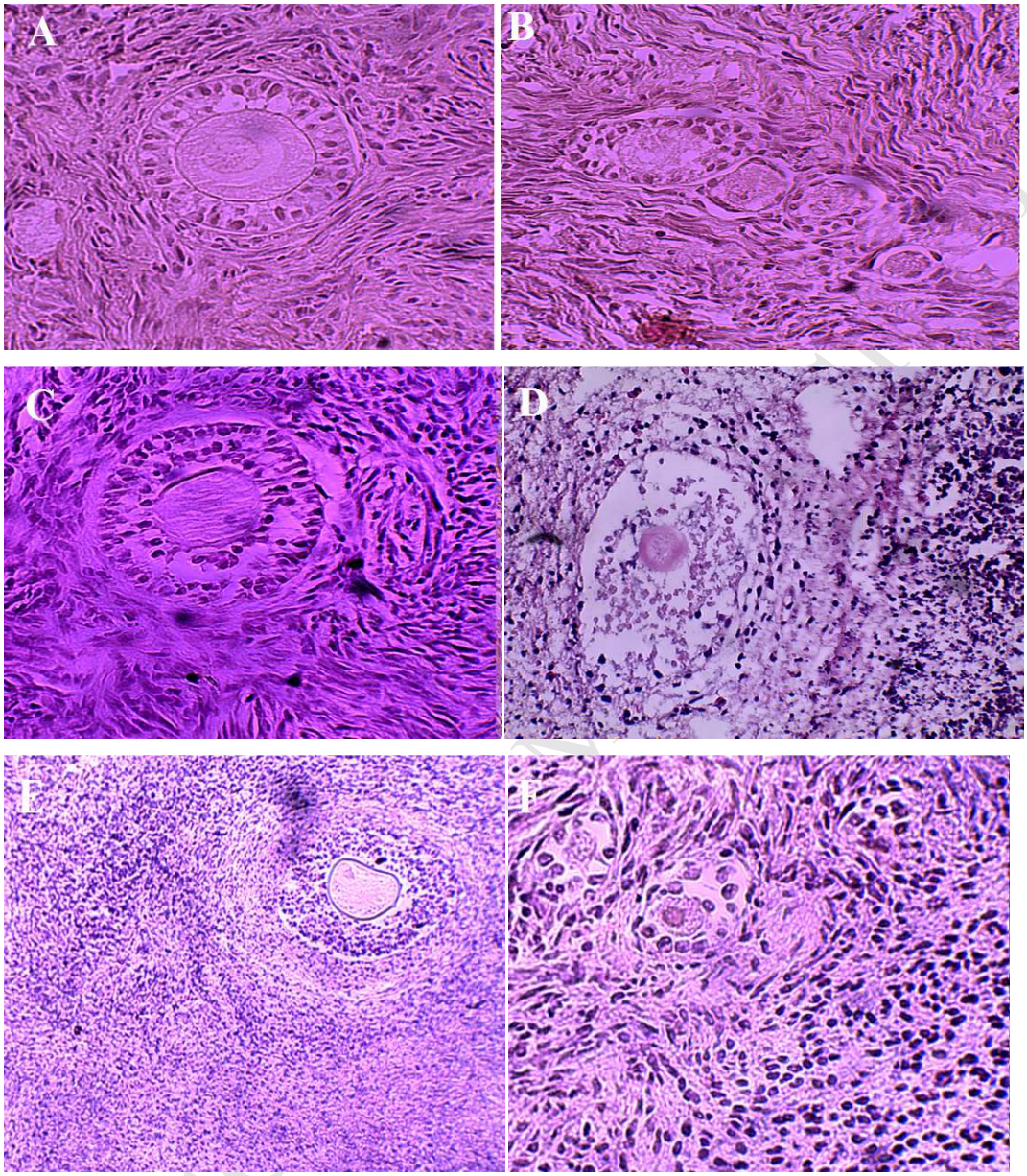

Figure 1. A fresh good quality secondary follicle. B- A fair condition secondary, transitory, primary and primordial follicle after vitrification. C- A fair condition secondary follicle after slow freezing. D- preantral follicle after fresh culture. E- A fair condition secondary follicle after vitrified culture. F- Two fair condition primary follicles after slow culture.

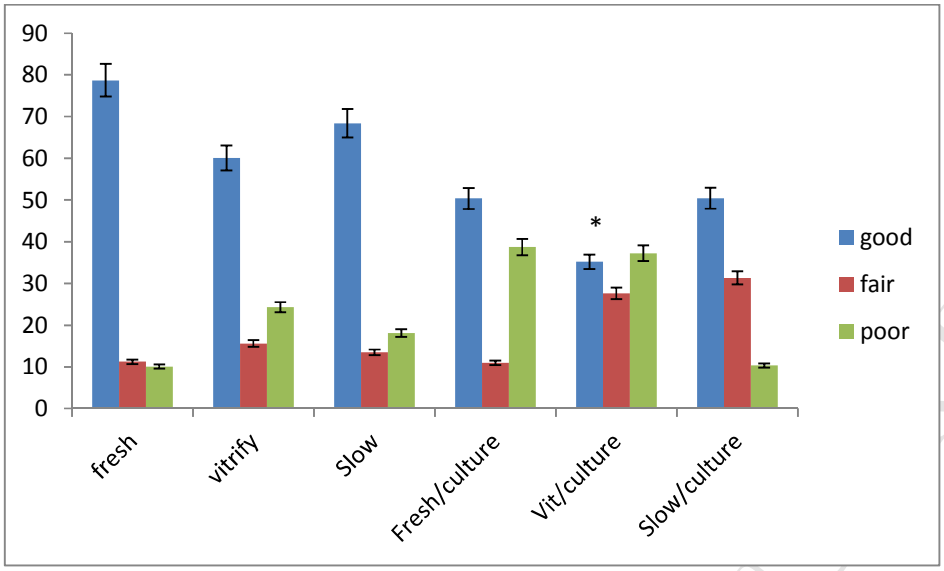

Figure 2. Good, fair and poor quality follicles in each group. In the uncultured groups, the fresh group had the better follicular structure ($p<.05$) There was no significant difference in follicular structure between the two cryopreserved groups ($p>.05$). Good quality follicles decreased significantly after CAM culture and in the vitrified culture group significantly was lower than the other group ($p<0.05$).

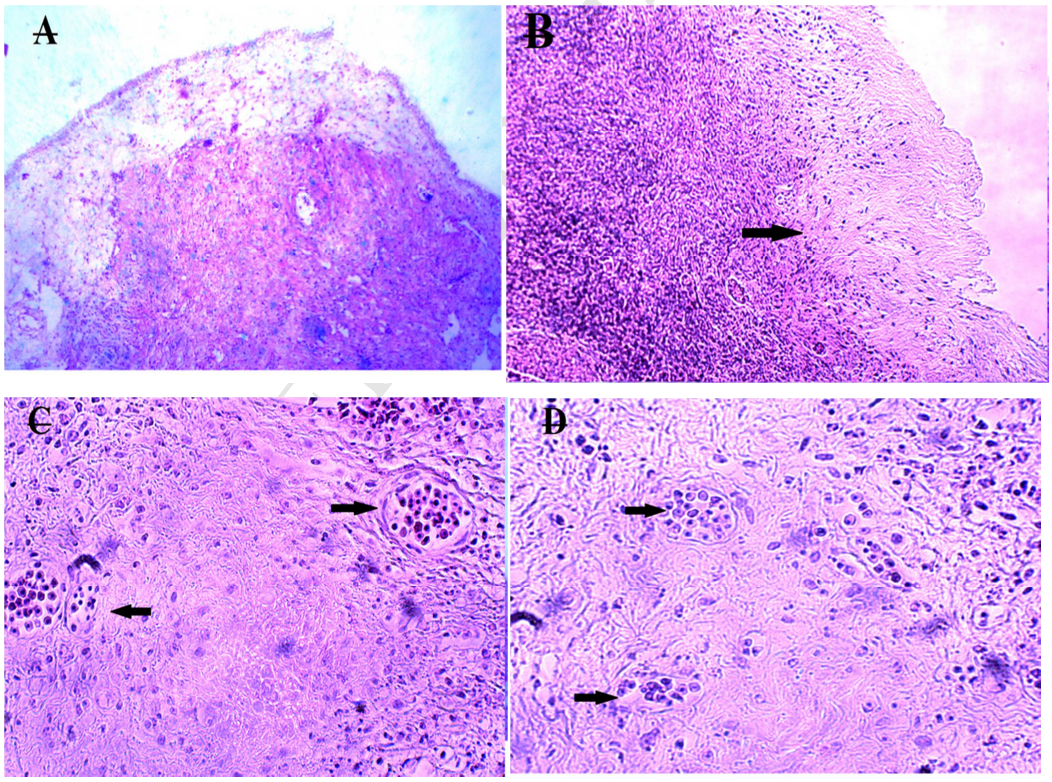

Figure 2: A- Border between CAM and ovarian grafted tissue. B- Invasion of CAM in to ovarian tissue C and D- avian vessels (arrows)

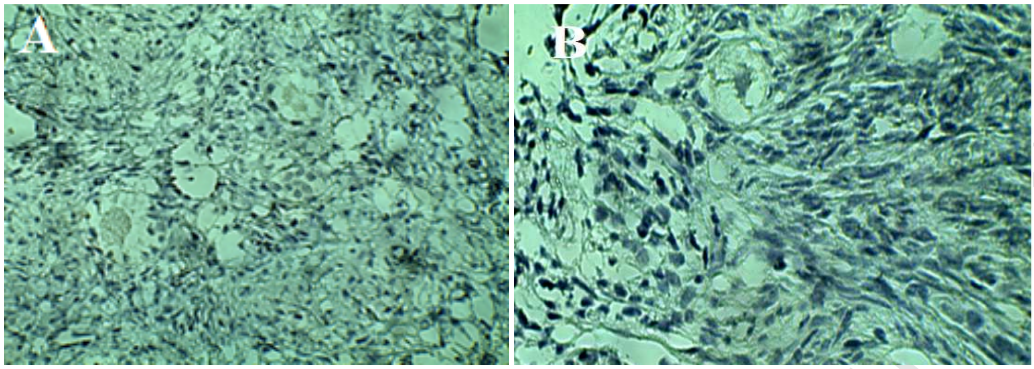

Figure 3: A Ki-67 positive stroma and follicles. B- Ki-67 negative stroma and follicles.