Vitrification of Feline Ovarian Tissue on Copper Grid Versus Acupuncture Needle Using Different Rates of Vitrification Solution

Farklı Vitrifikasyon Solüsyonları Kullanılarak Akupunktur İğnesi ve Elektron Mikroskobu Bakır Gridi Üzerinde Kedi Ovaryum Dokusunun Vitrifikasyonu

Ferda Topal Çelikkan[1], Murşide Ayşe Demirel[2], Duygu Baki Acar[3], Sinan Özkavukçu[4], Seçkin Salar[5], Esra Erdemli[1], Ayhan Baştan[5]

[1]Ankara University, Faculty of Medicine, Department of Histology and Embryology, Ankara, Türkiye
[2]Gazi University, Faculty of Pharmacy, Department of Basic Pharmaceutical Sciences, Laboratory Animals Breeding and Experimental Researches Center, Ankara, Türkiye
[3]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Afyonkarahisar, Türkiye
[4]Ankara University, Faculty of Medicine, Assisted Reproduction Center, Ankara, Türkiye
[5]Ankara University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, Ankara, Türkiye

ABSTRACT

Objective: Ovarian tissue vitrification has been utilized in human and veterinary medicine. This study aims to compare two different carrier devices and three different compositions of cryoprotective agent (CPA) solutions in the feline ovarian tissue vitrification using either copper grids (CG) or acupuncture needles.
Methods: The fragments were divided into four groups as control, CG, needle immersed vitrification (NIV)-I and -II. For CG method, the final CPA concentration of the vitrification solution was 20% DMSO, 20% EG and 0.4 M sucrose. The final concentration of vitrification solution in NIV-I group consisted of 15% Dimethyl sulfoxide (DMSO), 15% Ethylene glycol (EG) and 0.5 M sucrose, where in NIV-II group 12% DMSO, 12% EG and 0.5 M sucrose. After equilibration in increasing graded CPAs, tissues were plunged into liquid nitrogen and stored for one week.
Results: The rate of normal follicles in NIV-I group was statistically higher than CG and NIV-II groups. Oocyte and follicle cells' nuclei were intact in normal follicles in control group. Many normal oocytes and follicle cells were seen in vitrified groups, but the number of degenerated oocytes and follicle cells increased. The cell linkages between oocytes and follicle cells were preserved better in the NIV-I group than in the other experimental groups.
Conclusion: It was concluded that NIV-I technique (15% DMSO/EG and 0.5 M sucrose) was more efficient than other two techniques and favourable in preventing follicular damage on cryopreservation of ovarian tissue.

Keywords: Copper grid; cryopreservation; feline ovarian tissue; follicle, needle immersed vitrification; ultrastructure.

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ÖZET

Amaç: Ovaryum vitrifikasyon tekniği insan ve veteriner hekimliği alanında kullanılmaktadır. Bu çalışmada, elektron mikroskobu gridleri (CG) veya akupunktur iğneleri kullanılarak kedi ovaryum vitrifikasyonunda iki farklı taşıyıcıyı ve üç farklı kriyoprotektif ajan (CPA) dilüsyonunun karşılaştırılması amaçlandı.
Yöntem: Ovaryum dokuları kontrol CG, akupunktur iğnesiyle vitrifikasyon (NIV)-I ve NIV-II olarak dört grupta kullanılmak üzere ayrıldı. Vitrifikasyon solüsyonlarının son konsantrasyonları, %20 Dimetil sülfoksit (DMSO)/Etilen glikol(EG); % 15 DMSO/EG; % 12 DMSO/EG (sırasıyla; CG, NIV-I ve NIV-II grupları) olarak oluşturuldu. Ovaryum dokuları dengelendikten sonra sıvı nitrojene daldırıldı ve bir hafta süreyle saklandı.
Bulgular: NIV-I grubunda normal folikül oranı CG ve NIV-II gruplarında olduğundan istatistiksel olarak daha yüksekti. Kontrol grubundaki normal foliküllerde ovosit ve folikül hücrelerinin çekirdekleri sağlamdı. Vitrifikasyon gruplarında birçok normal ovosit ve folikül hücresi görüldü, ancak dejenere ovosit ve folikül hücrelerinin sayısı daha fazlaydı. Ovositler ve folikül hücreleri arasındaki hücre bağlantılarının, NIV-I grubunda diğer deney gruplarına göre daha iyi korunduğu izlendi.
Sonuç: NIV yönteminin (% 15 DMSO/EG ve 0,5 M sükroz) diğer iki yönteme göre daha etkin olduğu ve ovaryum kriyoprezervasyonunda folikül hasarını önlemede daha avantajlı olduğu tespit edildi.

Anahtar Sözcükler: Akupunktur iğnesiyle vitrifikasyon; bakır grid; follikül; ince yapı; kedi ovaryum dokusu; kriyoprezervasyon.

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ORCID IDs. F.T.C.0000-0002-8254-0558, M.A.D.0000-0002-7082-8976,D.B.A.0000-0002-6884-2621, S.O.0000-0003-4525-9027,S.S:0000-0001-9303-6253,E.E.0000-0002-9737-269X,A,B,0000-0001-8291-1147

Address for Correspondence / Yazışma Adresi: Ferda Topal Çelikkan, MD Ankara University, Faculty of Medicine, Department of Histology and Embryology, 06230, Ankara, Türkiye. Email: ftopal@ankara.edu.tr

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INTRODUCTION

Researches on the preservation of fertility have gained importance in recent years (1-5). Many studies indicated that freezing ovarian tissue might be preferable to freezing oocytes as it is possible to store hundreds of oocytes and hormone producing units theoretically at once (1, 4, 6). In women, freezing of the ovarian tissue may be required in some cases such as reduced ovarian reserve and prior to oncological treatments (2, 7-10). Furthermore, the ovarian tissue freezing may be one of the available options for maintenance of fertility in endangered species and economically important animals (4-6, 10-13). The cryopreservation of ovarian tissue can be achieved using slow freezing or vitrification (9, 13-15). During slow freezing, ice crystal formation can cause mechanical damage to cells, and lead to cell death (4, 8). Vitrification is based on an ultra-rapid cryopreservation technique that results with a glass-like solidification in which ice crystal formation does not occur. Besides, this method is convenient and feasible as it does not require a computer-programmed freezer and is done in a short time (2, 10, 13, 15). Therefore, vitrification technique is more advantageous than slow freezing method in many aspects (4, 16). However, because vitrification protocol requires high concentration of the cryoprotective agents (CPA) and ultra-fast cooling rates, the success is operator dependent and CPA toxicity causes cellular damage and osmotic stress (14, 17). Therefore, the type of cell/tissue carriers, their thermal conduction capabilities, cooling rate and CPA equilibration temperature and exposure time in this technique should be optimized (7, 9).

Ovarian tissue freezing using vitrification method has been utilized in the field of human and veterinary medicine (4-6, 9, 12, 13). It is known that the special carriers and CPAs used in vitrification affect the success of the method (18). Several carriers such as cryovials (13), needle (19-21), copper grid (CG) (14, 22), and solid-surface (23) are used for vitrification method. CPAs are divided into two categories as the ones that penetrate into cells such as dimethyl sulfoxide (DMSO), glycerol, propylene glycol (PG), and ethylene glycol (EG) and extracellular CPAs which are trehalose, sucrose, dextrose, and polyvinylpyrrolidone (9, 17, 24). The type and concentration of CPAs, the temperature and duration of exposure to the vitrification solutions are important factors in vitrification procedure.

Still, there is no standard protocol for preserving female cat genetic material by cryopreservation. Moreover, until now, no study has compared vitrification techniques in the cryopreservation of cat ovarian tissue fragments. Therefore, the major aim of this study was to compare the efficiency of different CPA rates and carriers (CG and acupuncture needle) on feline ovarian tissue using brightfield and electron microscope results.

MATERIAL and METHODS

Collection of Tissue Samples

Ovarian tissue samples from 9 healthy, nonpregnant, owned domestic cats (aged 10-24 months) were obtained during routine ovariohysterectomy. All procedures used in this study were reviewed and approved by the local ethics committee of the Afyon Kocatepe University, Faculty of Veterinary Medicine (Decision number: 49533702/86). During cat care, clinical evaluation and performing the study we obeyed the statement of Helsinki Declaration. Both feline ovarian tissues were used in this study. The removed ovaries which were divided into four pieces were designated as the non-frozen control group and vitrification groups using either CG or NIV methods.

Vitrification

***Copper Grid Vitrification*:** The vitrification of ovarian tissue fragments was performed using CG method described by Topal-Celikkan et al. (14). Tissue fragments were held in gradually increasing concentrations of CPA with a final concentration of 20% DMSO, 20% EG and 0.4 M sucrose (Table 1). After equilibration, tissues were loaded onto the copper electron microscope grids with a diameter of 3.05 mm and thickness of 25.40 µm.

***Needle Immersed Vitrification*:** The ovarian fragments were aligned puncturing the tissues by acupuncture needle (0.20 mm diameter and 13 mm length) and incubated in an equilibration solution composed of 7.5% DMSO/EG in Dulbecco phosphate-buffered solution (DPBS) supplemented with 20% Fetal bovine serum (FBS) for 10 min at room temperature. Following the equilibration step, the ovarian fragments on the acupuncture needle were divided into two groups randomly as NIV-I and NIV-II groups (Table 1). The NIV-I method was performed according to Demirel et al. (20). The ovarian tissue fragments in the NIV-I group were plunged into a vitrification solution consisting of 15% DMSO, 15% EG and 0.5 M sucrose for 2 min. The NIV-II method was applied according to Xiao et al. (19). In the NIV-II group, fragments were incubated in the vitrification solution composed of 12% DMSO, 12% EG and 0.5 M sucrose for 2 min.

After incubation, all the tissues were plunged directly into liquid nitrogen before they were inserted into the cryovials under liquid nitrogen and stored for a week.

Thawing: The vitrified ovarian tissues pieces were warmed at 37°C for 30 s. After thawing, the tissues were transferred through a series of media with gradually decreasing concentrations of CPA as summarized in Table 1. Finally, the tissue pieces were transferred to fixative solutions.

Brightfield microscopy

Non-frozen control and vitrified-thawed ovarian tissues were fixed in Bouins' solution for histological examination. The tissues were dehydrated in graded ethanol (75%, 96% and 100%), and then embedded in paraffin blocks, serially sectioned at 5 µm and mounted onto microscope slides. The slides were stained with hematoxylin and eosin (H&E), and observed under brightfield microscope (Carl Zeiss Axioscope microscope, Germany). In brightfield microscopic investigation, the cytoplasm, nucleus and attachments of oocytes, follicle cells and interstitial cells were assessed. As the pool of primordial follicles determines the ovarian reserve, primordial follicles, which were classified as an oocyte surrounded by one layer of flattened or squamous granulosa cells, were counted and evaluated (25).

Transmission electron microscopy

For ultrastructural analysis, non-frozen and vitrified tissue samples were fixed in 2% phosphate-buffered glutaraldehyde and 10% paraformaldehyde solutions at pH 7.2, for 2-4 h at 4°C. The tissues were post-fixed in 1% phosphate-buffered osmium tetroxide. The samples were dehydrated in graded ethanol solutions and embedded in Araldite 6005 (EMS, Fort Washington, USA). Semi-thin (800-1000 nm) sections were stained with Toluidine Blue Azur II and observed under brightfield microscope (Carl Zeiss Axioscope, Germany), and ultra-thin (60-80 nm) sections were stained with uranyl acetate and lead citrate and viewed on a LEO 906-E transmission electron microscope (LEO Elektronen-mikroskopie, Oberkochen, Germany). The ultrastructure, organelles, nucleus of oocytes and follicle cells, integrity of cells and interstitium were evaluated in all groups.

Statistical analysis

The differences in the number of normal and degenerated primordial follicles in the control and vitrification groups were analyzed using Chi square test. The SPSS version 15.0 (SPSS, Chicago, IL, USA) was used for statistical analysis, and $P<0.05$ was considered significant.

Table 1. The vitrification and thawing procedures

	Control group	CG group	NIV I group	NIV II group
Vitrification	No vitrification	*Vitrification* - 5% DMSO + 5% EG + 0.2 M sucrose in L15 (5 minutes) - 10% DMSO + 10% EG + 0.4 M sucrose in L15 (5 minutes) **- 20%** DMSO+ 20% EG + 0.4 M sucrose in L15 (5 minutes)	*Vitrification* 7.5% DMSO+ 7.5% EG in DPBS (10 minutes) **15%** DMSO + 15% EG + 0.5 M sucrose in DPBS (2 minutes)	**12%** DMSO + 12% EG 0.5 M sucrose in DPBS (2 minutes)
		7 days		
Thawing	No thawing	*Thawing* - 20% DMSO + 20% EG + 0.6 M sucrose in L15 (5 minutes) - 10% DMSO + 10% EG + 0.4 M sucrose in L15 (5 minutes) - 5% DMSO+ 5% EG + 0.2 M sucrose in L15 (5 minutes) - L15 (5 minutes)	*Thawing* - 1 M sucrose in DPBS ((5 minutes) - 0.5 M sucrose in DPBS (5 minutes) - 0.25 M sucrose in DPBS (5 minutes) - 20% FBS in DPBS (20 minutes)	
Fixation	**Bouins' fixative and Glutaraldehyde**			

CG: Copper grid; **DMSO:** Dimethyl sulfoxide; **DPBS:** Dulbecco's phosphate-buffered saline; **EG:** Ethylene glycol; **FBS:** Fetal bovine serum; **L15:** Leibovitz's L-15 Medium; **NIV:** Needle immersed vitrification.

RESULTS

Histological evaluation

In brightfield microscopic investigation, a noticeable difference was observed among control and vitrification groups regarding the morphology. The normal follicles were seen containing healthy oocytes and follicle cells. Moreover, follicle cells and oocyte attachments were intact. The degenerated follicles were observed with eosinophilic oocyte cytoplasm and condensed nuclei. Vacuoles in oocyte, which had nuclear condensation (pyknosis) and follicle cells were noticed in degenerated follicles. Furthermore, vacuoles in the interstitial cells were observed. In degenerated follicles, the linkage between oocyte and granulosa cells was distorted. In control group, the number of normal follicles was more than degenerated follicles. In CG, NIV-I and II groups, normal and degenerated follicles were observed whereas the dominant ones were the shrunken follicles (Fig 1).

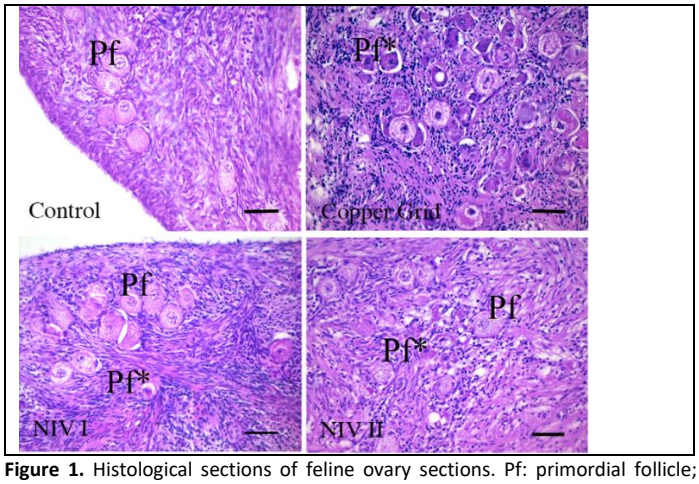

Figure 1. Histological sections of feline ovary sections. Pf: primordial follicle; Asterisks indicate degenerated follicles. Hematoxylin–Eosin staining. Scale bars: 50 µm.

The vitrified (NIV and copper grid) and fresh feline ovarian tissues were compared with regard to the percentage of normal and degenerated primordial follicles in this study (Table 2). A significant difference was found between the control and experimental groups (Table 2; $\chi^2(3)$: 6843.79; P<0.001). There is a significant difference in NIV-I group compared to the copper grid and NIV-II group (P=0.004).

Table 2. The comparison of the number of normal and degenerated follicles between control and experiment (copper grid, NIV-I and NIV-II) groups.

	Groups				
Follicle	**Control**	**Copper grid**	**NIV-I**	**NIV-II**	**Total**
Normal	3127 (90.3%)[a]	139 (6.6%)[c]	201 (9.2%)[b]	184 (7.3%)[c]	3651 (35.5%)
Degenerated	337 (9.7%)	1961 (93.4%)	1985 (90.8%)	2347 (92.7%)	6630 (64.5%)
Total	3464	2100	2186	2531	10281

[abc] The different letters are shown statistically significant differences.
A significant difference in normal follicles assessed between the control and experimental groups P<0.001. A significant difference was noted in NIV-I group compared to the copper grid and NIV-II group (P=0.004).
NIV: Needle immersed vitrification.

In semi-thin sections, correlatively H&E stained sections, degenerated and normal follicles were observed in all groups, but the number of the degenerated follicles was noted more in the CG group when compared to the other experiment groups (**Fig 2**). Noteworthy, the cell interactions in the NIV-I group were preserved better than other experiment groups.

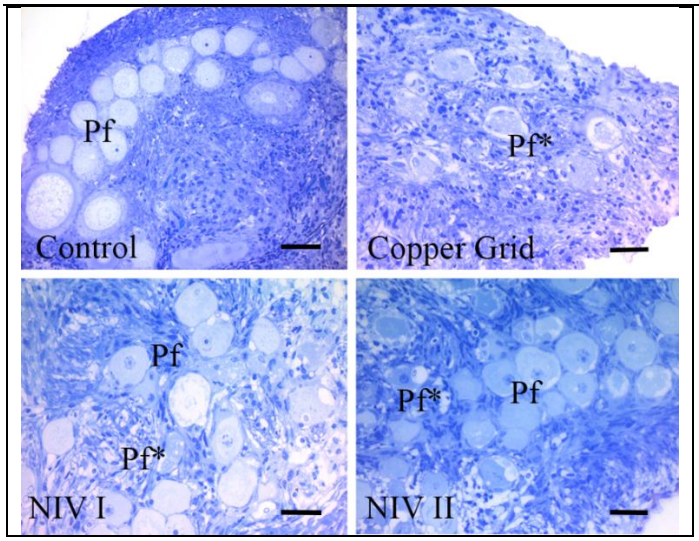

Figure 2. Semi-thin sections of feline ovary sections. Pf: primordial follicle; Asterisks indicate degenerated follicles. Toluidine Blue Azur II staining. Scale bars: 50 µm.

Ultrastructural evaluation

In ultra-thin sections, oocyte and follicle cells' nuclei were intact in normal follicles in the control group. Undegenerated oocytes and follicle cells were seen in vitrified tissues. However, the number of degenerated oocytes and follicle cells increased in vitrified groups (Fig 3). A large number of damaged mitochondria and swollen organelles, chromatin condensation in some follicle cells, irregular spaces between the layer of follicle cells and oocytes were detected in degenerated follicles. Hydrops in extracellular spaces were significant, so dense collagen bundles became apparent (Fig 4). The cell linkages between oocytes and follicle cells were preserved better in the NIV-I group than in other experimental groups. CG and NIV-II groups displayed intracytoplasmic and interstitial vacuoles. Cortical interstitial spaces were irregular and cells were nonadjacent. In the NIV-I group, integrity of cells was well preserved comparable to CG and NIV-II groups.

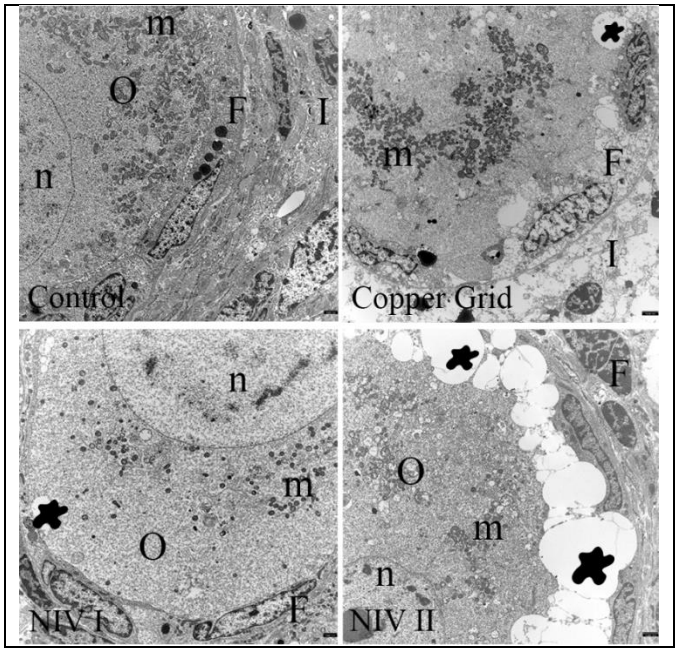

Figure 3. Transmission electron microscopy of feline ovary sections. O; oocyte, n; nucleus, m; mitochondrion, I; interstitium, F; follicle cell, star; vacuole. ×3670 magnification.

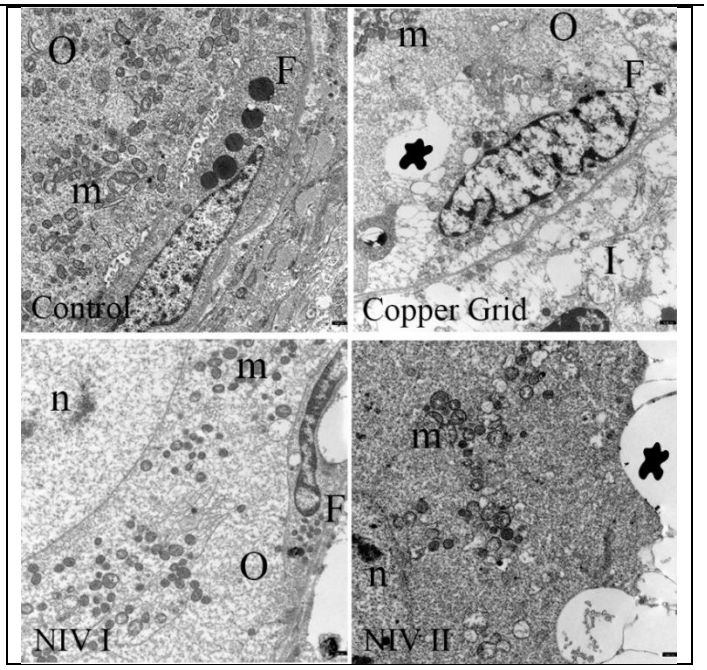

Figure 4. Transmission electron microscopy of feline ovary sections. O; oocyte, n; nucleus, m; mitochondrion, I; interstitium, F; follicle cell, star; vacuole. ×7500 magnification.

DISCUSSION

The present study indicates that NIV method provides better protection for primordial follicles on feline ovarian tissue when compared to CG method. In addition to this, final vitrification solution consisting of 15% DMSO/EG+0.5 M sucrose could be considered as the optimal concentration for NIV technique on feline ovarian tissue.

The vitrification technique that uses high concentrations of combined cryoprotectants can prevent the formation of ice crystals and has been developed as an alternative to slow freezing method (17, 22). Vitrification of ovarian tissue held in a row on acupuncture needle provides to avoid osmotic stress and chemical toxicity of the CPAs (26). Therefore, NIV technique is relatively simple, rapid, cheaper and more convenient to manipulate. NIV is an open vitrification method where ovarian tissues are plunged into the liquid nitrogen directly, such as dropping vitrification, direct cover vitrification, cryotops, cryoloops and CG (27, 28). It was reported that NIV method revealed better results than drop vitrification and slow freezing methods in mouse (27), human (18, 27), Japanese Quail (29) and canine (4) ovarian tissues. Thus, the present study demonstrated for the first time that NIV is superior to the CG method for preserving follicle structures in feline ovarian tissues.

The most convenient combination was DMSO, EG and sucrose to minimize osmotic stress, ice crystals, cryoinjury, CPA toxicity, etc. in cells (19, 30-32). However, optimum CPA concentration is still uncertain. Among various CPA concentrations, lower concentrations demonstrated better preantral follicle morphology and non-degenerated follicle rates in human (19), mice (9), and donkey (30). However, there are limited number of studies on feline ovarian tissue vitrification and there is a lack of optimal CPA volume for this species (33-35). Interestingly, our results revealed that higher concentration of CPA procured less degenerated follicle number and better morphology. The difference between prior studies on different species and our results might have occurred due to feline ovarian tissue cell's natural buffering capacity, membrane permeability or cellular metabolism.

Abir et al. (36), who compared the NIV and slow freezing groups in human ovarian tissue, evaluated the follicles as normal and atretic. Their results indicated that vitrification protocol revealed higher atretic follicle number than slow freezing. Asgari et al. (37) also compared the NIV and slow freezing groups in mouse ovarian tissue. However, no difference was observed between slow-freezing and vitrification protocols. Fathi et al. (7) reported that sheep ovarian tissues were vitrified with the use of the needle.

There was no difference between the percentages of the intact primordial follicles in NIV group and fresh control. Fatehi et al. (1) reported that, NIV method for mouse ovarian tissue was more practical than solid surface vitrification similarly parallel to our fresh ovarian tissue findings. In the present study, both brightfield and TEM results demonstrated that follicle morphology in NIV-I (15%) was preserved better than NIV-II (12%) and CG (20%). It was considered that NIV technique has more advantages than other vitrification methods as mentioned above.

Isachenko et al. (38) investigated standard 0.25 straws or CGs for cryopreservation of human ovarian tissue pieces using cryopreservation medium containing EG, sucrose and egg yolk. There was no statistical difference between straw and copper grid usage in terms of histological changes. In the current study, there was no statistical difference in atretic follicle rate between CG and 12% NIV group. CG carrier may affect the vitrification quality and the follicle structure. In this concern, Hashimoto et al. (39) compared morphologies of follicles vitrified using by on the copper plate or between copper plates. They revealed that the morphologies of follicles vitrified between copper plates were mostly damaged, which is parallel to our research. So, the shape, thickness and application method of the carrier are crucial factors for this issue. As Wei et al. (40) previously published, the different types of CG may be tried and compared in the vitrification of ovarian tissue.

CONCLUSION

In conclusion, 15% DMSO and 15% EG with NIV method could give better results for the vitrification of feline ovarian tissue. Moreover, the variation of vitrification results might differ depending on species. Therefore, future studies need to focus on optimizing CPA volumes for feline ovarian tissue vitrification.

Conflict of interest

No conflict of interest was declared by the authors.

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