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Research Article

Effects of Ovary Transport and Storage Temperature on *In Vitro* Maturation and Cumulus Cell Apoptosis Rates in Cat Oocytes [1]

Mithat EVECEN [1,a] Kamber DEMİR [1,b] Ramazan ARICI [1,c] Selin YAĞCIOĞLU [1,d] Nur ERSOY [1,e] Nilhan COŞKUN [1,f] Elif ARMUTAK [2,g] Ayça ÜVEZ [2,h] Ebru GÜREL GÜREVİN [3,i] Ahmet ESER [1,j] Hatem ATALLA [4,k] Kemal AK [1,l] Serhat PABUCCUOĞLU [1,m] Sema BİRLER [1,n]

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[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY; [2] Department of Histology and Embryology, Faculty of Veterinary Medicine, Istanbul University, TR-34320 Avcılar, Istanbul - TURKEY; [3] Department of Biology, Faculty of Science, Istanbul University, TR-34134 Vezneciler, Istanbul - TURKEY; [4] Faculty of Veterinary Medicine, An Najah National University, Nablus - PALESTINE

[a] ORCID: 0000-0002-0219-6997; [b] ORCID: 0000-0002-4561-6189; [c] ORCID: 0000-0002-2236-2526; [d] ORCID: 0000-0002-9355-498X; [e] ORCID: 0000-0002-7460-0450; [f] ORCID: 0000-0001-5523-0813; [g] ORCID: 0000-0002-7359-6568; [h] ORCID: 0000-0002-3875-2465; [i] ORCID: 0000-0002-1589-9229; [j] ORCID: 0000-0003-1326-2678; [k] ORCID: 0000-0003-4188-7990; [l] ORCID: 0000-0002-4053-9655; [m] ORCID: 0000-0002-6200-3018; [n] ORCID: 0000-0001-5069-5427

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Abstract

The objective of the present study was to examine the effects of two different transport temperature (37°C vs 4°C) and cold storage of ovaries for 24 h on cumulus cell apoptosis and maturation rates of cat oocytes *in vitro.* Ovaries were collected from 15 ovariohysterectomized domestic cats and maintained and transported to the laboratory in phosphate buffer saline at 37°C and 4°C. In order to determine the effects of storing time, some ovaries transported at 4°C were stored at the same temperature for 24 h. Selected cumulus oocyte complexes (COCs) were matured for 48 h at 38°C in four-well petri dishes containing 500 µL of modified oviduct medium (mSOF) under mineral oil in a 5% CO_2 incubator with nearly 100% humidified. The morphological features of apoptosis were analysed in the cumulus cells at the beginning of *in vitro* maturation in both transporting temperature groups and after 24 h of cold stored group. The degree of apoptosis in cumulus cells were measured by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL). The IVM rates of oocytes were determined using Hoechst (33342) staining. Although the apoptotic morphological features were seen rarely and in similar rates in 37 and 4°C transporting groups (19.40 and 21.55%, P>0.001), it was seen more intensely in the 24 h cold stored group (34.80%, P<0.001). The IVM findings were similar (49.77, 44.55%) at 37°C and 4°C groups (P>0.05), and importantly lower at 4°C transporting and 24 h cold stored groups (18.90%, P<0.05). In conclusion, the results of this study suggest that (I) cumulus cells of cat oocytes are partially exposed to apoptosis during transportation at warm or cold temperature, (II) storing of ovaries for 24 h at 4°C causes apoptosis of the cumulus cells at much higher rates and (III) storing of ovaries for 24 h at 4°C affects negatively IVM rate of oocytes.

Keywords: *Cat, Ovary, Transport temperature, Oocyte, Cumulus, Apoptosis*

Kedi Oositlerinin *In Vitro* Olgunlaştırılması ve Kumulus Hücrelerinin Apoptoz Oranları Üzerine, Ovaryum Taşıma ve Saklama Sıcaklığının Etkisi

Öz

Çalışmanın amacı, ovaryumların iki farklı sıcaklıkta (37°C ve 4°C) taşınma ve soğukta 24 saat bekletilmenin kumulus hücrelerindeki apoptoza ve kedi oositlerinin *in vitro* olgunlaşma oranları (IVM) üzerine etkilerini incelemektir. Kısırlaştırılmış 15 kediden ovaryumlar alındı ve yarısı 37°C, diğer yarısı da 4°C'de olmak üzere, fosfat tampon tuzlu solüsyonunda (PBS) laboratuara taşındı. Soğukta bekletmenin etkilerini belirlemek içinse, 4°C 'de taşınan ovaryumların yarısı, aynı sıcaklıkta olmak üzere 24 saat bekletildi. Seçilen kumulus oosit kompleksleri (COCs), %100'e yakın nemin sağlandığı %5 CO_2'li inkübatörde mineral yağ altındaki 500 µL modifiye Sentetik Ovidukt Medyumu (mSOF) içeren dört gözlü petrilerde olmak üzere 38°C'de 48 saat süreyle olgunlaştırıldı. Apoptozun etkileri, hem iki farklı taşıma grubunda, hem de soğukta 24 saat bekletilen grupta olmak üzere *in vitro* olgunlaşmanın hemen öncesinde kumulus hücrelerinde test edildi. Apoptosis derecesi, deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) test ile ölçüldü. Oositlerin *in vitro* olgunlaşma düzeyleri Hoechst (33342) boyama ile belirlendi. Apoptotik hücre oranları, 37°C ve 4°C taşıma gruplarında sırasıyla; %19.40 ve %21.55 olarak belirlendi (P>0.001). Aynı değer, 24 saatlik soğukta bekletilen grupta ise daha yoğun olarak (%34.80) görüldü (P<0.001). IVM bulguları da sırasıyla 37°C ve 4 °C taşıma gruplarında %49.77, %44.55 (P>0.05) iken, 24 saat soğukta bekletme grubunda ise %18.90 olarak bulundu (P<0.05). Çalışma sonuçları, (I) kedi ovaryumlarının sıcak veya soğukta taşıma sırasında kumulus hücrelerinin, apoptoza kısmen maruz kaldıklarını, (II) ovaryumların 4°C'de 24 saat bekletilmesinin kumulus hücrelerinin apoptozunu önemli derecede artırdığını ve (III) 4°C'de 24 saat bekletmenin, oositlerin IVM oranlarını olumsuz etkilediğini göstermiştir.

Anahtar sözcükler: *Kedi, Ovaryum, Taşıma sıcaklığı, Oosit, Kumulus, Apoptoz*

İletişim (Correspondence)
+90 212 4737070/17262
evecen@istanbul.edu.tr

INTRODUCTION

Domestic cat is a valuable model for researching human genetic diseases and for developing assisted reproduction of endangered felids [1]. Long term storage of ovaries can provide opportunity to rescue oocytes from ovaries of endangered felids recently dead in the field or to rescue ovaries that are ovariohysterectomized for medical reasons [2]. Cumulus cells surround the oocyte and provide nutrients and signals regulating oocyte growth and maturation [3]. Cumulus cells also keep the oocyte under meiotic arrest, transmit the LH signal to the oocyte and participate in meiosis induction and are responsible for cytoplasmic maturation of oocytes [4]. The quality of the oocyte and cumulus cells are not only important for oocyte maturation or fertilization but also affects the cleavage and further embryonic developments. Therefore, the oocytes enclosed within the follicles must remain metabolically active while they are transported to the laboratory in order to preserve their maturation ability *in vitro* [5]. The oocyte cytoplasmic maturation is also crucial for the developmental potential of embryos after fertilization [3]. Transportation of ovaries to the laboratory may result in long transport time which causes some possible post mortem changes in the tissues. Cell metabolism linked to enzymatic activity which depends on the temperature and duration of storage, and the temperature of the ovaries kept could affect the apoptosis and consequently maturation rates of the oocytes *in vitro* [6]. It was shown that oxidative stress during *in vitro* culture leads to changes in maturation and developmental disruption in bovine oocytes [7]. It is also known that reactive oxygen species (ROS) can cause DNA damage and induce apoptosis in human and porcine oocytes during *in vitro* culture period [8].

Programmed cell death (apoptosis) is cell self-destruction under physiological control and is regulated by genes [9]. Apoptosis plays some physiological roles on the ovarian cycle, the growth and the atresia of the follicles, the selection of the dominant follicle and regression of the corpus luteum [10]. The ability to promote apoptosis is always present within the cell nucleus and could be initiated by maintaining the cells under suboptimal conditions [11]. Apoptosis is characterized by a loss of cell volume, nuclear pyknosis and margination of the chromatin and its redistribution against the nuclear envelope. As a bio-chemical perspective, one of the most hallmark features of apoptotic cells is the loss of DNA integrity by endonuclease-mediated fragmentation of the genomic pool [12]. The terminal deoxyribonucleotidyltransferase (TDT)- mediated dUTP-digoxigenin nick end labeling (TUNEL) assay is a widely used approach to label and recognize DNA fragmentation by using the terminal deoxyribonucleotidyl transferase [13].

The aims of the present study were to (I) examine the effect of two ovary transport temperatures (37℃ vs 4℃) and cold storage for 24 h on cumulus cell apoptosis and maturation rate of cat oocytes *in vitro* and based on the results of the study, (II) suggest new practices of transporting cat ovaries.

MATERIAL and METHODS

Ethics Statement

The study was performed in accordance with guidelines for animal research from Istanbul University Ethics Committee on Animal Research (2017/303104).

Collection and Storing of Ovaries and Oocyte Recovery

Ovaries were collected from 15 domestic cats at various stages of the estrus cycle by routine ovariohysterectomy at different local veterinary clinics, and maintained in physiological saline at 37℃ or 4℃ until oocyte recovery for approximately two hours. Some of the ovaries transported at 4℃ were stored at the same temperature for 24 h. Ovaries were sliced and rinsed by washing medium (heparin supplemented HEPES modified TCM 199) at room temperature in order to obtain cumulus oocytes complexes (COCs). The COCs were washed three times with modified synthetic oviduct fluid medium. Large oocytes with darkly pigmented ooplasm and completely surrounded by at least one layer of cumulus cells were selected for *in vitro* maturation (IVM) [14].

In vitro Maturation (IVM)

In vitro maturation was performed as described in our previous study [15]. In brief, the IVM medium was modified Synthetic Oviduct Fluid, supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL luteinizing hormone (LH), 4% bovine serum albumin (BSA, Fraction V) and antibiotics. The COCs were selected and maturated at 38℃ for 48 h in four-well petri dishes (NUNCR, Denmark) including 500 μL maturation medium under mineral oil. *In vitro* maturation was performed at 38℃ in a humidified atmosphere with 5% CO_2 for 48 h.

Assessment of the Nuclear Maturation

At the end of IVM, oocytes were transferred into hSOF medium containing 0.2% (w/v) hyaluronidase and vortexed for 30 secs and then denuded by gentle pipetting. In order to dispersal the chromatins, the oocytes were placed in KCl solution (0.7%, w/v) for 3-5 min and the nuclear maturation rates were examined under a fluorescent microscope after 20-30 min Hoechst (33342) staining.

TUNEL Assay (Tdt-Mediated dUTP Nick-end Labelling)

In order to determine the effects of transporting temperature and storage time on apoptosis rate, 5-10 COCs were randomly selected from each transport temperature and stored groups before *in vitro* maturation procedure. Apoptotic cells in the sections were visualized by TUNEL

(terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) using ApopTag® Plus Peroxidase In Situ Apoptosis Kit (Millipore, S7101, Darmstadt, Germany). Staining was performed according to the manufacturer's instructions. Briefly, the samples (COCs) were fixed in methanol, and then washed in phosphate buffered saline solution (PBS) containing 50% Tween-20 for 15 min. The samples were then treated with 3% H_2O_2 (Merck, 1.08597) in PBS in a dark room to inhibit endogen peroxidase activity. In order to remove H_2O_2 solution, sections were washed three times for two minutes in PBS solution with Tween-20. Then, 50 µL balancing buffer was applied to per each section and waited for 30 min at room temperature. Balancing buffer was wiped off from sections with a napkin, and TdT enzyme was applied to the tissues, and then sections were covered and were allowed to incubate for one hour. To stop TdT enzyme reaction, stop/wash buffer in the kit was applied for 10 min at room temperature, after this sections were washed in PBS with Tween-20 for 15 min (3 times x 5 min). Afterwards, 50 µL of anti-digoxigenin peroxidase enzyme was applied for 30 min at room temperature. After sections were washed in PBS with Tween-20 for 15 min (3 times x 5 min) 3,3'-diaminobenzidine (DAB) substrate was applied as a chromogen. After 20 min of application, the reaction was stopped with distilled water when observed the brown color. Sections were painted with methyl green used as a contrasting paint, then quickly were passed through butanol. Slides were clarified with a total of 15 min (3 times x 5 min) xylol application, closed with entallan and prepared for examination under light microscope.

Calculation of Apoptotic Index

Apoptotic index percentage were determined independently by two observers by counting apoptotic and non-apoptotic cumulus cells in randomly selected five different areas in three different groups at 400x magnification. After cell counting, apoptotic index was calculated using 100x (number of TUNEL-positive cell nuclei/total number of cell nuclei) formula [16]. All sections were photographed by a DM4000 B (Leica) microscope *(Fig. 1)*.

RESULTS

In Vitro Maturation (IVM) Results

The findings of the *in vitro* maturation rates of the study are summarized in *Table 1*. According to the oocytes matured to MII stage, there was no difference between 37℃ and 4°C transport temperature groups (P>0.05) However, the rate of oocytes reached to MII stage in cold stored group was significantly lower than the other two groups (P<0.05).

Findings of TUNEL Method

While apoptotic cells in oocyte sections containing cumulus oophorus were examined by TUNEL method, it was determined that the apoptotic morphological features were seen rarely and in similar rates in 37℃ and 4°C transporting groups (19.40 and 21.55%) *(Fig. 2* and *Fig. 3)* but, it was seen more intensely in the cold stored group (34.80%) (P<0.001). Although apoptotic index percentage of 4°C transportation group was higher than 37°C group, this increase was not statistically significant (P>0.05) *(Fig. 4)*.

Statistical Analysis

The experiments were replicated 5 times. Statistical analysis of IVM rates of oocytes and apoptotic activity rates of cumulus cells were performed using by "Mann Whitney U" test by SPSS for Windows version 13.0.

DISCUSSION

This study is performed to determine the effects of two ovary transport temperature (37℃ vs 4°C) and long term cold storage of ovaries on cumulus cell apoptosis and *in vitro* maturation rates of cat oocytes. The effects of the transporting and storage temperature and time on cat ovaries are controversial. It is known that the modification of the physiological composition of the follicular fluid due to the transport of mammalian ovaries may induce changes in oocyte quality, causing a negative impact on

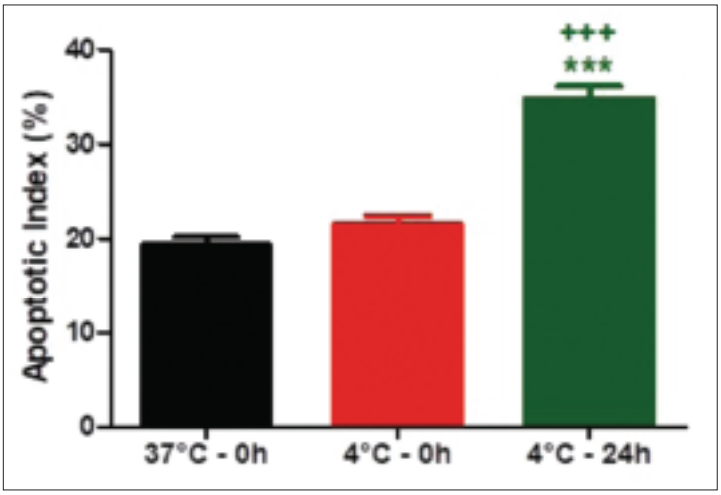

Fig 1. Apoptotic index (%) in all groups (*** P<0.01, compared to 37°C - 0 h group; (+++ P<0.001, compared to 4°C - 0 h group)

Table 1. *In vitro maturation rates of oocytes obtained from ovaries transported in two different temperatures and cold stored for 24 h*

Groups	n	GV n (%)	GVBD n (%)	MI n (%)	MII n (%)	UDNM n (%)
Transported in 37°C	217	3 (1.38)[a]	18 (8.30)[a]	69 (31.80)[a]	108 (49.77)[a]	19 (8.75)[a]
Transported in 4°C	202	10 (4.95)[b]	28 (13.86)[a]	60 (29.70)[a]	90 (44.55)[a]	14 (6.93)[a]
Transported and stored 24h in 4°C	201	32 (15.92)[c]	48 (23.88)[b]	23 (11.44)[b]	38 (18.90)[b]	60 (29.80)[b]

[a,b,c] Values with different superscripts in the same column are significantly different (P<0.05); ***GV:*** *Germinal Vesicle,* ***GVBD:*** *Germinal Vesicle Breakdown,* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

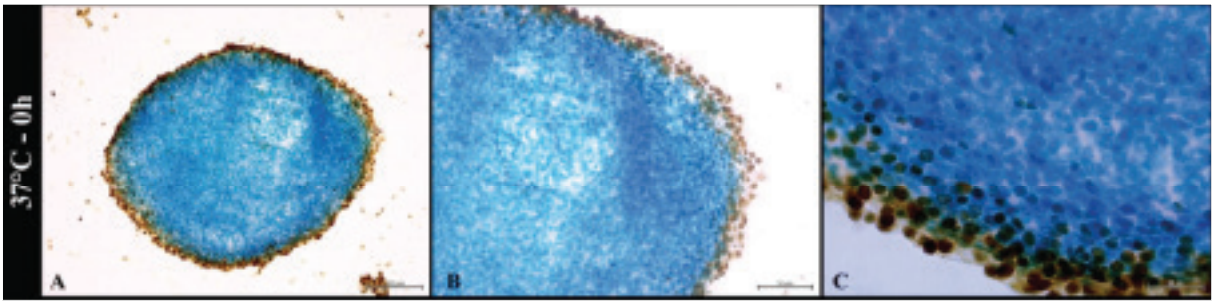

Fig 2. Representative TUNEL assay images of COCs in 37°C transporting group. Scale Bar=100 µm (A), 50 µm (B) and 20 µm (C); **A, B, C:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

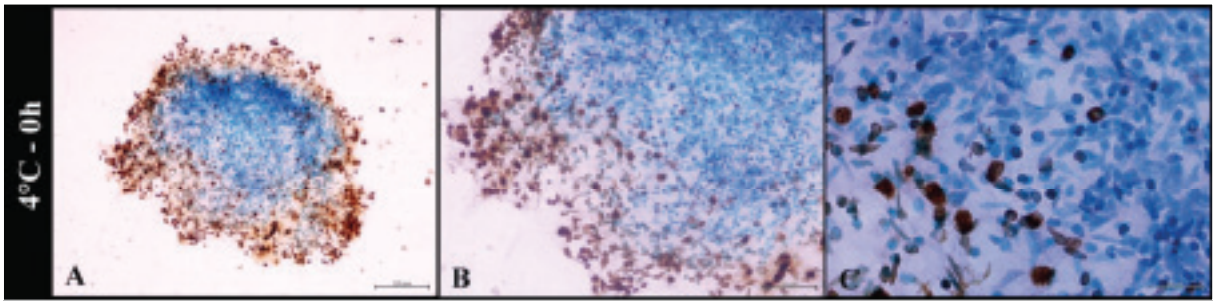

Fig 3. Representative TUNEL assay images of COCs in 4°C transporting group. Scale Bar=100 µm (A), 50 µm (B) and 20 µm (C); **A, B, C:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

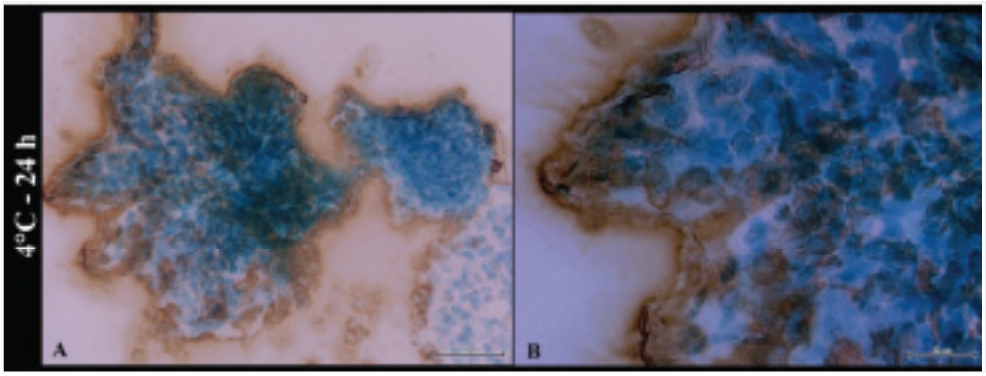

Fig 4. Representative TUNEL assay images of COCs in 4°C 24h storing group. Scale Bar=50 µm (A) and 20 µm (B); **A, B:** Apoptotic cumulus cells show intense brown fluorescence, whereas normal cells appear blue stained with methyl green

maturation [17]. It is indicated that storage of cat ovaries at high temperature (>23°C) for 24 h reduced the meiotic capacity of oocytes and the length of storage at room temperature affected the quality and developmental competence of oocytes [2]. It is also indicated that storage of domestic cat ovaries at room temperature even for a short time can negatively influence the competence of oocytes to undergo nuclear maturation *in vitro* [18]. The

effect of transport temperature on oocytes also vary depending on species. Some researchers [19] stated that warm storage (25°C) of bovine oocytes for up to 11 h resulted in fertilization and development to blastocyst stage, but longer warm storage or storage for any time at 4°C resulted in a few fertilizable oocytes**.** It has been demonstrated that unlike other species cat oocytes have a unique ability to mature *in vitro* after storage of ovaries for 24 h at 4°C [1]. Parallel to this finding, our previous study demonstrated that domestic cat oocytes have the ability to mature successfully *in vitro* after storage of ovaries for 24 h at 4°C [14]. However, it is stated that granulosa cell apoptosis in feline ovaries was increased after 12 h of storage at 4°C and consequently affected IVM results negatively [10]. Although it has been reached to high maturation ratios after the storage of ovaries for 24 h at 4°C, cat oocytes began to lose the fertilization and cleavage ability *in vitro* after the storage [1]. In our study, although the apoptotic index percentage of 4°C transportation group was found higher than 37°C group, this increase was not statistically significant. This result consistent with the researcher's [10] result that transporting ovaries both 37°C and 4°C temperatures have not harmful effect on cumulus cells of cat oocytes. It is stated that to avoid changes in cumulus oophorus morphology the ovaries should be held at 35-37°C and for less than 2 h before processing, and to avoid oocyte chromatin configuration changes ovaries should be stored for less than 6 h [11]. However, it is proposed that storing of bovine ovaries at 10°C for 24 h, did not affect oocyte maturation rates compared with controls [20]. Since the enzymes in animal blood are known to work most efficiently at body temperature, the transport temperature lower than 35-37°C may be expected to delay apoptosis [21]. It is stated that most of the glucose in the follicular fluid is consumed by the granulosa and cumulus cells in the glycolytic pathway and lactate accumulates in the first two hours of the mammalian oocytes in warm temperatures [17]. It is expected that the low temperature decreases the metabolism and slows down the activities of the enzymes. Same researchers showed that transportation ovaries at warm temperatures (25-35°C) for more than two hours decreased follicular pH, increased ROS levels and thus reduced *in vitro* maturation rates [17]. Parallel to this knowledge, it is indicated that storage of porcine ovaries in 35°C for 6 h was efficient to support the developmental competence of oocytes but, these results were lower than those stored at the same temperature for three hours [22]. However, some researchers unexpectedly reported that although the transport temperature of ovaries did not influence the equine oocyte chromatin, it affected cumulus morphology and lower storage temperature resulted in more denuded and expanded COCs [11]. It is possible that due to protein degradation or loss of certain functions in the cumulus cells might cause apoptosis during cold storage. It is stated that long-term storage or transportation of porcine ovaries increased the number of oocytes with DNA fragmented nuclei by inducing acidosis in follicular fluids [22]. In this study, we have found that the rate of oocytes having undetermined nuclear material (UDNM) in cold stored group was significantly higher (29.80%) than the other two groups ($P<0.05$). This finding is inconsistent with our previous results [14] and the researchers claiming that cat oocytes have a unique ability to mature *in vitro* after storage of ovaries for 24 h at 4°C [1]. Some researchers supplemented superoxide dismutase (SOD) to the ovary transport and storage medium to prevent the damage of reactive oxygen species (ROS), and they found beneficial effects of SOD such as lower cellular apoptosis and higher COC survival and *in vitro* embryo production rates. In the same study, researchers obtained blastocysts from *in vitro* fertilized oocytes from cat ovaries stored at 4°C for up to 72 h in transport media supplemented with SOD [23]. These results suggested that different approaches are needed with further studies to identify species-specific factors that would allow feline ovaries to be stored for longer periods.

In conclusion, this study has shown that there is a distinctive relationship between the long storage at cold temperature and COCs quality and IVM rates of the cat ovaries. According to our results, it can be concluded that (I) transporting of the cat ovaries at 37°C or 4°C for a period not longer than two hours provides the appropriate conditions to maintain the quality of COCs and meiotic development *in vitro*, (II) the cold (4°C) storage of cat ovaries for 24 h has deleterious effects on COCs quality and IVM rate of oocytes and (III), cumulus cell apoptosis rate would be a valuable marker for the ability of IVM of cat oocytes.

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