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RESEARCH ARTICLE

Effects of Melatonin Addition to the Cold Storage Medium on Cumulus Oocyte Complex Apoptosis, Viability and *In Vitro* Maturation Rates of Cat Oocytes

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Abstract: Usage of oocytes obtained from ovaries after long-term cold storage for *in vitro* embryo production is a promising tool for the protection of wildlife and endangered animal species. Mammalian oocytes are susceptible to oxidative stress with regard to the high lipid content of plasma membranes. Melatonin is known as a powerful antioxidant and anti-apoptotic agent due to its ability to eliminate toxic oxygen derivatives and reduce the formation of reactive species. This study was performed to verify the optimal environmental conditions for long-term preservation of cat ovaries *(Felis domesticus)* by adding different concentrations of melatonin (500, 750 and 1000 µM) to the storage medium (0.9% NaCl) as an antioxidant to be preserved at 4°C for 24 h. To determine the effect of melatonin on cat oocytes collected from stored ovaries, the anti and pro-apoptotic gene levels in cumulus oophorus, the *in vitro* maturation rates, the cell membrane and oocytes viability were evaluated. In all melatonin added groups regardless of whether they are stored in the cold; Pro-apoptotic gene levels (BAX) were determined to be upregulated however, anti-apoptotic gene levels (BCL-2) were downregulated in cumulus cells ($P<0.05$). The cell membrane stability and cell viability rates of oocytes began to deteriorate in parallel with the rate of melatonin increase. In parallel with these findings, *in vitro* maturation rates of oocytes were negatively affected as the amount of melatonin increased ($P\leq0.001$). In conclusion the results showed that adding melatonin (500,750 or 1000 µM) to the ovarian transport and storage medium had negative effect on *in vitro* maturation rate, viability and cell membrane structure of cat oocytes.

Keywords: *Cold storage, Melatonin, Cat ovaries, In vitro maturation, Apoptosis, Bax, Bcl-2*

Kedi Ovaryumlarının Soğukta Saklama Solüsyonuna Melatonin Katılmasının, Kümülüs Ooforuslardaki Apoptoz ve Oositlerin Viyabilitesi ve *İn Vitro* Olgunlaşma Oranlarına Etkisi

Öz: *İn vitro* embriyo üretimi amacıyla, ovaryumların soğuk ortamlarda uzun süreli saklanması sonrası elde edilen oositlerin kullanılabilmesi, yaban hayatı ve nesli tükenmekte olan türlerin korunmasına yardımcı olabilir. Memeli gametleri, plazma zarlarındaki yüksek lipid içeriği nedeniyle, oksidatif strese karşı savunmasızdırlar. Melatonin, toksik oksijen türevlerini ortadan kaldırma ve reaktif türlerin oluşumunu azaltma özelliğinden dolayı güçlü bir antioksidan ve anti-apoptotik ajan olarak bilinmektedir. Çalışma, 4°C'de 24 saat süreyle muhafaza edilen ovaryum taşıma sıvısına (%0.9 NaCl) antioksidan olarak farklı dozlarda melatonin (500, 750 ve 1000 µM) eklenerek evcil kedi ovaryumlarının uzun süreli muhafazası için en uygun koşulları belirlemek amacıyla yapılmıştır. Melatoninin soğukta bekletilmiş ovaryumlardan kazanılan kedi oositleri üzerindeki etkisini belirlemek amacıyla; kümülüs ooforuslardaki anti ve proapoptotik gen seviyeleri, oositlerdeki *in vitro* olgunlaşma oranları, hücre membran stabilitesi ve hücre canlılığı gibi parametreler incelendi. Soğukta bekletilsin ya da bekletilmesin, tüm melatonin ilaveli gruplarda; kümülüs hücrelerindeki pro-apoptotik gen seviyelerinin (Bax) yükseldiği, buna karşın anti-apoptotik gen seviyelerinin ise (Bcl-2) düştüğü gözlendi ($P<0.05$). Oositlerin hücre membran stabilitesi ve hücre canlılık oranları da melatonin artışına paralel olarak bozulmaya başladığı görüldü. Bu bulgulara paralel olarak, melatonin miktarı arttıkça oositlerin *in vitro* olgunlaşma oranları da olumsuz yönde etkilendi ($P\leq0.001$). Sonuç olarak, yumurtalık saklama sıvısına 500, 750 ve 1000 µM dozlarında melatonin eklenmesinin, kümülüs hücrelerinde apoptozu teşvik ettiği, kedi oositlerinin *in vitro* olgunlaşma oranları, hücrelerin canlılığı ve hücre membran yapısı üzerinde de olumsuz etkileri olduğu sonucuna varıldı.

Anahtar sözcükler: *Soğukta saklama, Melatonin, Kedi ovaryumları, İn vitro maturasyon, Apoptozis, Bax, Bcl-2*

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Introduction

Most of the wild cat species are facing extinction due to loss of habitat and poaching. Assisted reproductive technologies such as *in vitro* maturation, *in vitro* fertilization and nuclear transfer offers a tremendous potential for conservation of endangered cat species [1]. Domestic cat is not only a convenient model for developing assisted reproduction of endangered felids but also for researching human genetic diseases [2,3]. Many inherited genetic disorders have been identified in cats that resemble humans. In recent years cat model has become widely used for therapeutic studies in human [2-4].

Prolonged preservation of the ovaries under optimal conditions may allow producing new generations from endangered wild felids that undergo ovariectomy or have recently died in their habitat or zoo [5]. However, long-term transport of the ovaries under warm temperatures (35-38°C) has negative effects on oocyte quality in terms of *in vitro* maturation, fertilization and subsequent embryonic development [6]. A number of studies have been conducted to increase oocyte protection by reducing temperatures of transport solution [1,5,7-9]. However, the sensitivity of oocytes to transport conditions was found to vary among animal species [1,5]. Cumulus cells surround the oocyte, keep the oocyte under meiotic arrest, exchange of nutrients and regulate chemical signals related to oocyte development and functions in cytoplasmic maturation of oocytes [10-12]. The viability of the oocyte and cumulus oophorus cells is effective on the cytoplasmic maturation of the oocyte which is crucial for the growth potential of embryos after fertilization [10]. Transport temperature and time may affect the metabolism and thus apoptosis of cumulus cells and *in vitro* maturation rates of cat oocytes [5]. Reactive oxygen species (ROS) have also been shown to cause DNA damage in human embryos and thus induce apoptosis *in vitro* [13]. Apoptosis, also known as programmed cell death, is a physiological process that is defined by the self-destruction of cells and this process is controlled by genes. Reactive oxygen species (ROS) which are known to have mutagenic effects on DNA are produced as metabolites by normal cellular processes. Although all cells own self defense systems containing antioxidant enzymes, their levels are not the same in all cell types [14]. Mammalian gametes are extremely susceptible to oxidative stress due to their high lipid content. Melatonin (N-acetyl-5-methoxtryptamine), which is classified as an indoleamine is a tryptophan derivative synthesized in the pineal gland and regulates seasonal reproductive ability. Melatonin is also known as a direct and indirect antioxidant and anti-apoptotic agent that prevents from oxidative damage due to its ability to scavenge toxic oxygen derivatives and reduce the formation of ROS [15]. Melatonin neutralizes potentially toxic molecules, particularly single oxygen, hydrogen peroxide, nitric oxide and peroxynitrite anion and stimulates several antioxidative enzymes. In the mammalian ovary, as in other organs, both membrane and nuclear receptors for melatonin have been identified [16,17]. Melatonin has been shown to have beneficial effects on stimulating oocyte maturation, embryo production and blastocyst rates *in vitro* in some mammalian species [7-9].

The most effective method for assessing oocyte maturation conditions *in vitro* is the evaluation of the mRNA expression of specific genes related to apoptosis in granulosa cells [18]. BCL-2 is a member of the BCL-2 regulatory family of proteins that regulate cell apoptosis.

BCL-2 is a noteworthy anti-apoptotic gene that inhibits programmed cell death by reducing the formation of ROS, thereby preventing intracellular oxidations necessary for induction of apoptosis. BAX is a member of the BCL-2 protein family and is a pro-apoptotic gene. BAX stimulates apoptosis through interaction with anion channels [19] and the ratio of pro- and anti-apoptotic genes indicates whether a cell undergoes apoptosis [20].

This study proposes the hypothesis that using melatonin in storage media of cat ovaries may have beneficial effects by preventing apoptosis during long-term protection under cold conditions. Therefore, the influence of melatonin addition to the ovary storage medium on COC's apoptosis, oocyte developmental rate and cell membrane viability of domestic cat oocytes were investigated.

Material and Methods

All chemicals were acquired from Sigma Chemical Co. (Saint Louis, MO, USA) except otherwise indicated. Melatonin (M-5250) was dissolved in absolute ethanol before adding to PBS due to its low solubility of in water.

Experimental Design, Collection - Storing of Ovaries and Oocyte Recovery

The ovaries which are obtained from routine cat spay surgeries of 60 cats in total at Istanbul Metropolitan Municipality Animal Sterilization Centre were transported in saline solution (0.9% NaCl) in flask at 4°C and brought to the laboratory within maximum two hours. All of the cats were stray cats, and the cycle period they are in is not taken into account.

The study was performed in two stages. In the first stage of the study, transported ovaries in saline solution added melatonin at different concentrations (500, 750 and 1000 µM) were divided into two parts. Half of ovaries were sliced immediately for the collection of COCs (Fresh Groups) and the others were stored under 4°C for 24 h (Stored Groups). Slicing was done as we did in our previous studies [5]. Briefly, ovarian cortex was gently cut with a scalpel and then washed with warm oocyte washing

medium on watch glass. The COCs with at least 4 rows of cumulus oophorus cell lines, an intact zona pellucida and dark ooplasm were selected. Some of the collected COCs were separated to determine the expression of possible pro-apoptotic (BAX) and anti-apoptotic (BCL-2) genes and some of oocytes were separated and stained for assessment of cell membrane damage and oocyte cell viability and the remaining oocytes were matured *in vitro*.

At the second stage of the study, the stored ovaries at 4°C for 24 h with different melatonin concentrations (0, 500, 750 and 1000 µM) were also sliced and the COCs were collected. Some COCs were separated to determine the expression levels of BAX and BCL-2 genes and several oocytes were separated and stained for assessment of cell membrane damage and oocyte cell viability. Then the others were left for *in vitro* maturation. After IVM period, several COCs were also separated from each group to analyze the expression levels of the studied genes in cumulus cells and the rest oocytes were examined.

In Vitro Maturation

In vitro maturation was performed as described in our group's previous work [5]. Briefly, the maturation medium was modified Synthetic Oviduct Fluid (SOF), supplemented with 10 µg/mL recombinant follicle stimulating hormone (rFSH), 10 µg/mL recombinant luteinizing hormone (rLH), 4% bovine serum albumin (BSA, Fraction V) and antibiotics. The selected COCs were maturated at 38°C for 48 h in four-well petri dishes (NUNC, Denmark) including 500 µL maturation medium covered with mineral oil. A humidified atmosphere at 38°C with 5% CO_2 was used for 48 h for the *in vitro* maturation process *(Table 1, Table 2)*.

Assessment of the Nuclear Maturation

After the 48 h of IVM period, oocytes were denuded by vortexing for 60-90 sec after being transferred into hSOF medium containing 0.2% (w/v) hyaluronidase and pipetting to remove the remaining cumulus cells. The oocytes were evaluated under epifluorescence microscope (IX 70, Olympus, Japan) equipped with a digital camera after 20 min 1 mg/mL Hoechst 33342 staining.

Assessment of Cell Membrane Damage, Oocyte Cell Viability and Nuclear Maturation

The oocytes separated from cumulus cells were stained with the Annexin V-FITC Early Apoptosis Detection Kit (6592, Cell Signal Technology) following the manufacturer's instructions. During the whole process, the reagents were kept at 37°C to avoid false positives. Cells were stained with Annexin-V, a phospholipid binding protein that detects translocation of phosphatidyl-serine to the outer cytoplasmic membrane, which takes place during the early stages of apoptosis. Cells were also stained with propidium iodide (PI), a membrane impermeable stain, to distinguish between live cells and dead cells. PI can only penetrate into the cell if cytoplasmic membrane has lost its integrity. Samples were placed in 35 µL droplets containing Annexin-V buffer, Annexin-V/FITC, PI and 1 mg/mL Hoechst 33342, and incubated for 15 min at 37°C in the dark. After the incubation oocytes were fixed for 10 min using 1% Paraformaldehyde (PFA) on ice. After fixation, the oocytes were washed three times in annexin-binding buffer and then mounted on glass slides which were examined using an (IX 70, Olympus, Japan) epifluorescence microscope equipped with a digital camera.

Denuded oocytes were classified as follows:

I. Live early apoptotic oocytes: Annexin-positive signal on the membrane (more than 20%) (A+/PI–) *(Fig. 1-A)*

II. Necrotic oocytes: PI-positive red nuclei, or cytoplasm (P+) *(Fig. 1-B,D)*

III. Live non-apoptotic oocytes: no annexin staining (A–/PI–) *(Fig. 1-C)*

During the evaluation of the oocytes, the advancement of nuclear maturation was also recorded *(Table 3)*.

Gene Expression Analysis

The differences in the expression of the studied genes related to apoptosis mechanism namely, B-cell lymphoma protein 2 (BCL-2) and Bcl-2-associated X protein (BAX) within the studied groups were analyzed with quantitative real time PCR (qRT-PCR) method using LightCycler 480

Table 1. *The in vitro maturation rates of oocytes recovered from transported ovaries in 0.9%NaCl supplemented with various concentrations of melatonin at 4°C (Fresh Groups)*

Melatonin (µM)	No. of Oocytes Examined (n)	Developmental Stage of Oocytes					
		GV (%)	GVBD (%)	MI (%)	MII (%)	UDNM (%)	Activated (%)
0	307	14.0	16.6^{b}	13.4	48.2^{ab}	6.5^{a}	1.3
500	436	11.5	25.7^{a}	12.6	45.0^{bc}	3.4^{b}	1.8
750	432	15.5	23.8^{a}	16.0	40.3^{c}	3.0^{bc}	1.4
1000	403	11.2	27.5^{a}	15.4	39.5^{c}	4.5^{ab}	2.0

Different superscripts within the same column indicate significant differences (P< 0.05)
GV: *Germinal Vesicle* ***GVBD:*** *Germinal Vesicle Break Down* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

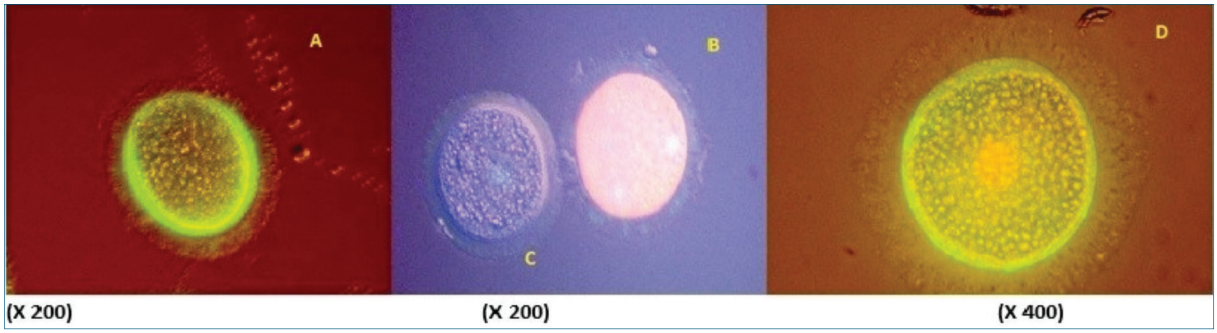

Fig 1. Cat oocytes stained with Annexine V and Propidium Iodide (PI) visualized under an epifluorescence microscope. **A**- An oocyte with membrane defect. Annexine (+) (green fluorescent more than 20%), **B**- A necrotic oocyte. PI (+) (red fluorescent), **C**- A normal oocyte, with healthy membrane and oolplasm, **D**- An oocyte necrotic and with membrane defect (PI+, Annexine+)

Equipment (Roche Applied Science). In this procedure we used a commercial kit (BioRad, SingleShot SYBR Green kit) that enabled the direct use of cell lysates bypassing the RNA isolation step. Briefly, *in vitro* matured cumulus-corona samples were evaluated for the cell count under a light microscope. Dilutions of 10^5 cells were washed in nuclease-free PBS (phosphate buffer solution) and cell lysates prepared in cell lysis buffer (SingleShot Cell Lysis Buffer, BioRad) were snap frozen and stored at -80°C until genetic analyses. For cDNA synthesis, 4 µL of cell lysate was added to reverse transcription mixture (iScript Advanced Reaction Mix, BioRad). Six replicates were done for each group.

Quantitative Real-Time PCR (qRT-PCR) Analysis

The Felis catus mRNA-specific primers for BCL-2 (NM_001009340.1), BAX (NM_001009282.2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_001009307.1) were designed using Primer3 and checked using Oligo-analyzer 3.1 tool *(http://www.idtdna.com)*. The sequence homology was confirmed using the BLAST database *(http://blast.ncbi.nlm.nih.gov/Blast.cgi)*. Primer sequences were 5'-AGTACCTGAACCGGCACCT-3' and 5'-GGACAGC CAGGAGAAATCAA-3' for BCL-2, 5'-TCAAGGCCCT GTGTACCAA-3' and 5'-TGGGTGTCCCAAAGTAGG-3' for BAX, 5'- CAATGACCCCTTCATTGACC-3' and 5'-ATG GGCTTTCCATTGATGAC-3' for GAPDH. qRT-PCR was performed using LightCycler 480 (Roche Applied Science) equipment using SsoAdvanced Universal SYBR Green Super Mix (BioRad). The cumulus cell expression levels of BCL-2 and BAX genes were normalized with GAPDH [21]. The results were expressed as relative quantification (RQ) values and calculated with comparative 2–ΔΔCt method. All samples were studied in triplicates. The specificity of the amplification products was confirmed by melting curve analysis *(Table 4)*.

Statistical Analysis

The statistical analysis was implemented using a purpose-built software (IBM, SPSS Version 14.0). The rates of apoptotic oocytes and nuclear maturation were analyzed by Chi-square test and the significance level was set at $P<0.05$. The comparisons in BCL-2 and BAX expressions (RQ values) in the study groups were evaluated by Mann-Whitney U test. Probabilities of <0.05 were deemed statistically significant.

Results

The outcomes of the study are presented in *Table 1, Table 2, Table 3* and *Table 4*. The meiotic status of oocytes recovered from transported ovaries in 0.9% NaCl supplemented with various concentrations of melatonin at 4°C summarized in *Table 1*. The MII stage oocyte rate was higher in control group than the melatonin trial groups, and this difference increased with accelerating melatonin concentrations ($P<0.001$). The meiotic status of oocytes recovered from ovaries kept at 4°C for 24 h are summarized in *Table 2*. The IVM rates in this groups were parallel to the Fresh groups result.

The apoptosis rate and viability status after IVM of oocytes recovered from ovaries whether kept at 4°C for 24 h or not are summarized in *Table 3*. The rate of apoptosis and dead oocyte rates were increased in parallel with the amount of melatonin added to the medium.

The effects of storing of ovaries for 24 h at 4°C in 0.9% NaCl supplemented with melatonin on BCL-2 and BAX gene expression results were summarized in *Table 4*. The oocytes' cell membrane and viability were affected negatively as the amount of melatonin increased.

After IVM, BCL-2 expression was downregulated in a dose dependent manner whereas BAX expression was not comparable between the groups. The alteration in expression of BCL-2 gene was more prominent with increasing doses of melatonin in both fresh and stored groups. On the other hand, BCL-2/BAX ratio, which is an indicator of apoptosis protection in cells, is significantly decreased with increasing melatonin concentrations ($P<0.05$) and this ratio is mainly influenced by the

Table 2. *The in vitro maturation rates of oocytes recovered from ovaries kept at 4°C for 24 h (Stored Groups)*

Groups	Melatonin (µM)	No. of Oocytes Examined (n)	Developmental Stage of Oocytes				
			GV (%)	GVBD (%)	MI (%)	MII (%)	UDNM (%)
Control Group *(Ovaries not stored)*	0	246	16.7	7.3^{b}	17.9	55.7^{a}	2.8
Trial/Storage Groups *(Ovaries kept at 4°C for 24 h with adding Melatonin)*	0	267	10.5	17.6^{a}	15.0	52.4^{a}	5.6
	500	384	10.9	21.9^{a}	13.8	47.7^{ab}	11.7
	750	376	15.7	21.3^{a}	17.0	41.8^{b}	8.8
	1000	350	12.3	23.4^{a}	15.7	41.4^{b}	13.4

Different superscripts within the same column indicate significant differences (P<0.05)
GV: *Germinal Vesicle,* ***GVBD:*** *Germinal Vesicle Break Down,* ***MI:*** *Metaphase I,* ***MII:*** *Metaphase II,* ***UDNM:*** *Undetermined Nuclear Material*

Table 3. *Apoptosis and viability status of in vitro maturated oocytes recovered from ovaries whether kept at 4°C for 24 h or not*

Groups	Melatonin (µM)	No. of Oocytes Examined (n)	Live Nonapoptotic Oocyte (A-PI-)(%)	Live Apoptotic Oocyte (A+- PI-) (%)	Dead Oocytes (P+) (%)
Fresh Group *(Ovaries not stored)*	0	246	84.1^{a}	2.4^{c}	13.4^{b}
Stored Groups *(Ovaries stored at 4°C for 24 h with adding Melatonin)*	0	267	80.5^{ab}	3.4^{c}	14.6^{b}
	500	384	76.6^{b}	8.3^{b}	14.3^{b}
	750	376	64.6^{c}	11.2^{ab}	23.1^{a}
	1000	350	63.4^{c}	13.1^{a}	23.1^{a}

Different superscripts within the same column indicate significant differences (P<0.05)

Table 4. *Effects of varies storage at 4°C for 24 h or not in melatonin supplemented 0.9% NaCl on BCL-2 and BAX gene expression results*

Groups		Gene Expression Results						BCL-2 /BAX
		BCL-2			BAX			
		Mean Value	Standard Dev.	P Value	Mean Value	Standard Dev.	P Value	
Trial Groups		0.10	0.04	0.86*/0.2**	0.28	0.01	0.38*/0.2**	10
Fresh Groups (Ovaries not stored at 4°C for 24 h with adding Melatonin)								
Amount of Melatonin (µM)	0	0.82	1.00	-	0.96	0.52	-	0.85
	500	0.48	0.90	0.73*	0.58	0.80	0.43*	0.83
	750	1.20	0.69	0.55*	1.31	0.77	0.31*	0.92
	1000	2.86	4.09	0.55*	1.79	1.58	0.69*	1.60
Stored Groups *(Ovaries stored at 4°C for 24 h with adding Melatonin)*								
Amountof Melatonin (µM)	0	0.69	0.51	1.00*	1.17	0.74	0.79*	0.59
	500	0.84	0.81	1.00**	0.92	0.69	0.86**	0.91
	750	0.57	0.53	0.86**	0.90	0.72	0.86**	0.63
	1000	0.34	0.19	0.40**	1.02	0.66	0.86**	0.33

The P value gives the Mann-Whitney U test results between the groups (P<0.05)
** Comparison the values between Fresh and Control Groups; ** Comparison the values between Stored and Control Groups*

expression changes in BCL-2 gene. Photographs of the necrotic, live, apoptotic and non-apoptotic oocytes are given in *Fig. 1*.

Discussion

During the transport of the ovaries to the laboratory, the interruption of blood flow cuts off the energy and puts the ovaries in an ischemic state. Therefore, ROS begin to accumulate in the follicle microenvironment. ROS react with proteins, lipids and DNA of oocyte and cumulus cells, causing cell membrane-lipid peroxidation, DNA damage and ultimately apoptosis and for an effective embryo production program, oocyte oxidative stress caused by ROS should be limited [8,17]. The prolonged time during the transport of the ovaries can cause apoptosis in the cells by disrupting the enzymatic activities that play a role in oocyte maturation. ROS which causes apoptosis in oocytes have also been shown to cause DNA damage in porcine and human oocytes during in vitro culturing [13]. Although it is suggested that storage of cat ovaries at 4°C for 12 h initiates DNA degradation in granulosa cells [22], some researchers stated that cat oocytes have a unique ability regarding to *in vitro* maturation after ovary storage 24 h at 4°C [23]. Our previous study results [1] also showed that storing of domestic cat ovaries in saline up to 24 h at 4°C did not have a negative effect on the ability of *in vitro* maturation of cat oocytes. Parallel to these findings, in this study the *in vitro* maturation rates (MII) in negative control and fresh groups were 55.7% and 52.4% respectively and were statistically similar (P>0.001). These findings are close to our previous IVM results (51%) and support the claim that oocytes can maintain their ability to mature *in vitro* after storage of the ovary for 24 h at 4°C. We have also determined that adding melatonin to storage medium has a negative effect on IVM rates (MII) of cat oocytes and the *in vitro* maturation rates are decreased as melatonin rates increased (P<0.001). Our results contradict the findings that the addition of melatonin in the ovarian storage medium has beneficial effects *in vitro* embryo production rate of sheep oocytes [7]. These different results may be due to the different *in vitro* conditions of the study, the different *in vitro* maturation time of cat oocytes from that of sheep, or other species-specific sensitivities.

Since mammalian gametes have high lipid concentrations, they are highly vulnerable to oxidative stress and melatonin plays an important role as an antioxidant [17]. Supplementation of superoxide dismutase (SOD) of the ovarian storage medium under cold conditions has been reported to decrease cellular apoptosis by increasing BCL-2 expression and reducing BAX expression in sheep [7]. Melatonin has been also reported to downregulate the expression of the BAX and upregulate the expression of BCL-2 by increasing intracellular glutathione and reducing ROS production in some mammals [16,24-26]. Moreover, it was reported that melatonin has a strong antioxidant effect and protect oocytes from free radicals produced during ovulation and improve oocyte maturation in many mammalian species [16]. Some researchers reported that the use of melatonin as an antioxidant in the ovary storage medium had beneficial effects on both the development of sheep oocytes and the quality of the embryos and 700 and 800 (μM) concentrations gave the best results melatonin supplementation in the long-term preservation of the sheep ovaries at even 4 or 20°C temperatures [7]. Contrary of these results, in our study the IVM rates of cat oocytes in both control and fresh group that had no melatonin, were higher than all of the melatonin added groups. Moreover, as the amount of melatonin increased, these rates also decreased (P<0.001). Interestingly, the change in expression of the BCL-2 gene was more pronounced with increasing doses of melatonin in both fresh and stored groups. However, these trends in gene expression levels were not statistically significant. This insignificance may have resulted from the timing of sampling for genetic analysis. It was observed that the similar amount of melatonin used in sheep ovaries does not provide similar benefits in cats, or even harmful to IVM abilities [17].

High amounts of melatonin in follicular fluid have been reported to support the follicle development and protect cumulus oophorus cells and oocyte [16,27,28]. It has been shown that the addition of melatonin to the *in vitro* maturation medium in rats reduces ROS, decreases the expression of pro-apoptotic BAX gene and promotes the expression of an anti-apoptotic BCL-2 gene [24]**.** Although some researchers stated that melatonin supplementation improves oocyte development *in vitro* in some mammalian species [17,25,27-29], there was no statistically significant difference in BCL-2/BAX ratio between melatonin and control groups in both fresh and stored groups (P>0.05). Both our results from our previous study [1] showing that storage of domestic cat ovaries at 4°C for up to 24 h has no adverse effect on the *in vitro* maturation ability of oocytes, as well as our findings here, may suggest that melatonin acts in a species-specific manner and that other cats. It also brings to mind the idea that it may have a different mechanism of action in cat, unlike other species. More studies with different doses and more numbers are needed to find out whether this negative effect of melatonin is due to the concentrations or to a species-specific situation.

In conclusion our present results have shown that unlike most studies in other mammals, adding melatonin (500, 750 and 1000 μM) to the ovarian storage medium had harmful effects on the viability and the structure of oocyte cell membrane in cat.

Availability of Data and Materials

All data sets collected and analyzed during the current study are available from the corresponding author (M. Evecen) on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

M. Evecen planned the present study, conducted this experiment, and wrote this manuscript; K. Ak, S. Pabuccuoğlu, S. Birler, K. Demir, S. Yağcıoğlu, R. Arıcı, A. Eser, G. Bakirer Öztürk, A. Kılıçkap, N. Ersoy and İ. Oruç, conducted and supported this experiment; E. Kömürcü Bayrak and B. Özsait Selçuk performed the genetic analyzes of the study.

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