Original Research Article

Melatonin reduces oxidative stress and improves follicular morphology in feline (*Felis catus*) vitrified ovarian tissue

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A B S T R A C T

Ovarian tissue vitrification is associated with multiple events that promote accumulation of ROS (reactive oxygen species) which culminate in follicular apoptosis. Thus, this study was aimed at evaluating the role of melatonin in vitrification and culture of feline (*Felis catus*) ovarian tissue. In phase 1, domestic cat ovaries were fragmented into equal circular pieces of 1.5 mm diameter by 1 mm thickness and divided into four groups (fresh control and 3 treatments). The treatments were exposed to vitrification solutions supplemented with melatonin at 0 M, 10^{-9} M, and 10^{-7} M, then vitrified-warmed, histologically evaluated and assayed for ROS. Consequently, phase 2 experiment was designed wherein ovarian fragments were divided into two groups. One group was exposed to vitrification solution without melatonin and the other with 10^{-7} M melatonin supplementation, then vitrified-warmed and cultured for ten days with fresh ovarian fragments as control prior to assessment for histology, immunohistochemistry (Ki-67, MCM-7 and caspase-3) and ROS. Concentration of ROS was lower (p = 0.0009) in 10^{-7} M supplemented group in addition to higher proportion of grade 1 follicles. After culture, proportions of intact and activated follicles were higher (p < 0.05) in melatonin supplemented group evidenced by higher expression of Ki-67 and MCM-7. Follicular apoptosis was lower in melatonin supplemented group. In conclusion, melatonin at 10^{-7} M concentration preserved follicular morphological integrity while reducing ROS concentration in vitrified-warmed feline ovarian tissue. It has also promoted the follicular viability and activation with reduced apoptosis during in vitro culture of vitrified-warmed feline ovarian tissue.

1. Introduction

During the mid-20th century, ovarian tissue cryopreservation was introduced following some successes recorded in gamete freezing [1]. It became the clinical standard cryopreservation technique of ovarian tissue in human clinics known as slow freezing (for additional review check [2]). Later, ice free cryopreservation or vitrification of ovarian tissue came to limelight in the early 21st century [3,4]. It is a technique devoid of both intracellular and extracellular ice crystals, technically simple and field feasible thus preferred over slow freezing [5–7]. These positive traits of vitrification make it suitable for genetic salvage of endangered wildlife such as the Felidae, in which case the domestic cat may serve as a model.

Regardless of the technique, ovarian tissue cryopreservation is associated with real and yet unresolved challenges including but not limited to osmotic stress which results from the required tissue fluid replacement with cryoprotectants (CPAs) during equilibration [8]. It is

at this point that CPA toxicity occurs, involving a plethora of cellular damaging events such as oxidative injury and protein damage [9]. Furthermore, mitochondrial alterations, vacuolations and extracellular matrix swelling have also been reported [10]. Another challenge is cryoinjury which is generally mechanical and bidirectional, that is to say that ice crystallization can occur during cryopreservation or due to recrystallization during warming [11]. In addition, ovarian tissue handling during ovarian fragmentation may be another source of cellular injury and oxidative stress [12]. Above all, ischemic injury may be a compounding factor in transported ovaries especially over long periods in an unideal condition [13].

Most of these cryopreservation related tissue damages can be somehow mitigated. For instance, incorporating non permeable CPAs in cryopreservation media reduces osmotic stress by enabling a more controlled osmotic balance [14]. Similarly, melatonin supplementation mitigates the detrimental effects of osmotic stress on isolated bovine ovarian stromal cells [8]. Adopting vitrification with a precise high

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cooling rate and warming rate preferably automated [7] will eliminate cryodamage. However, the major causes of cryopreservation related ovarian damage is oxidative stress [15].

Oxidative stress, characterised by supraphysiological levels of reactive oxygen species (ROS) can be a byproduct of all the other cryopreservation related tissue insults [16]. Oxidative stress is said to occur whenever there is an imbalance between ROS production and antioxidant system in a given cell. This is because ROS are involved in modulation of a number of cellular pathways such that certain level of ROS is essential for proper follicular development [17]. However, it is one of the triggers of apoptosis and participates in nucleic acids degradation and membranous damage through lipid peroxidation and eventual ferroptosis [9,18]. Additional ameliorating measures include the use of antioxidants [2] such as melatonin [9], resveratrol [19], vitamin E [20], anethole [15,21], and antiapoptotic agents such as Sphingosine-1-phosphate [22], and melatonin [23].

Melatonin (N-acetyl-5-methoxy-tryptamine), primarily an indoleamine neurohormone, has now been described as one of the biological compounds with multiple physiological functions [9]. Its activities are both receptor and non-receptor mediated and can be realized both in vivo and in vitro [24,25]. Most notable is the circadian rhythmic control in vivo; others include antioxidant and antiapoptotic effect [23]. Direct scavenging of multiple ROS species through neutralisation with electron transfer is the prominent antioxidant role [26]. Transcriptionally, it up regulates antioxidant genes and activates antioxidant enzymes via the Nrf2 signaling pathway [9,23,27]. These antioxidant roles are robust such that its metabolites also act in the same manner [24]. On apoptosis, melatonin activates anti-apoptotic genes (Bcl-2) while simultaneously supressing apoptotic genes (Bax) and the caspase cascade [9]. Apart from being antioxidant and antiapoptotic, melatonin also renders cryoprotective effect on ovarian tissue during freezing through induction of heat shock proteins [28,29]. This is the reason why some authors consider melatonin as a CPA [9]. Additionally, the versatility of melatonin is evident in its ability to cross lipid membranes thanks to its composition [30].

Thus, the general aim of this study is to evaluate the role of melatonin in vitrification and culture of feline ovarian tissue while specific objectives include: to evaluate the effect of melatonin supplementation of vitrification solution on superoxide anion concentration in vitrified-warmed feline ovarian tissue; to determine a suitable concentration of melatonin for feline ovarian tissue banking and to evaluate the effect of melatonin supplementation on the in vitro culture of feline ovarian tissue.

2. Materials and methods

2.1. Reagents and chemicals

Chemicals and/or reagents presented in this study were procured from Sigma–Aldrich (Taufkirchen, Germany) or as specifed in the text.

2.2. Ovary transport and preparation of ovarian fragments

A total of 50 ovaries from 25 cats were obtained from animal shelter and/or veterinary clinics and transported to the laboratory at room temperature (~22 °C) in a transport solution composed of phosphate buffered saline (PBS) and mixture of antibiotics and antimycotics (100 IU/ml penicillin G sodium, 0.1 mg/ml streptomycin sulphate, and 0.25 μg/ml amphotericin B). Ovaries were fragmented into equal circular pieces using 1.5 mm diameter biopsy punch (Kai medical, Oyana, Japan). The fragments were punched from an approximately 1 mm thick feline ovarian cortex. Since ovaries were surgical byproducts, no approval was sought from ethics committee of use of animals for experiments.

2.3. Experimental design

The experiment was divided into two phases (Fig. 1). Phase 1 involved dividing ovarian fragments into four groups. One group (FR) was immediately fixed in form-acetic [31,32] as the fresh control while the remaining three groups were placed on 30G needles (4 fragments per needle) and exposed to vitrification solutions [33] supplemented with 0 M (VI), 10^{-9} M (VII) and 10^{-7} M (VIII) melatonin respectively then vitrified and stored in cryotubes for at least 1 week before warming. After warming, fragments were processed for histology and assayed for ROS (superoxide anion).

In phase 2, ovarian fragments were placed on 30G needles (4 fragments per needle) and divided into two groups. One group was exposed to vitrification solution without melatonin and the other with 10^{-7} M melatonin supplementation (the most suitable concentration from phase 1). They were vitrified, placed in cryotubes and stored in liquid nitrogen for minimum of 2 weeks prewarming. Subsequently, fragments were warmed and placed in culture for ten days with fresh ovarian fragments as control. Fragment were eventually processed for histology, immunohistochemistry and assay of ROS.

2.3.1. Vitrification

The vitrification technique was based on our previous submission [33] as follows: The composition of vitrification solution (VS) included 10 % DMSO, 26 % EG, 2.5 % polyvinylpyrrolidone (PVP, MW 10 000), 1 M sucrose and 20 mg/ml bovine serum albumin (BSA) in minimum essential medium (MEM) (Gibco, Bleiswijk, Netherlands). Three equilibration steps were employed using different concentrations of VS (VS1 [25 % VS in MEM + 20 mg/ml BSA]; VS2 [50 % VS in MEM + 20 mg/ml BSA] and VS3 [100 % VS]) at room temperature (~22 °C). The first equilibration in VS1 lasted for 3.5 min then the samples were moved to VS2 for 2 min and finally transferred to VS3 for 1.5 min. Needles with the fragments were then removed and dabbed with sterile gauze to remove excess VS before plunging the needles into liquid nitrogen for vitrification.

2.3.2. Warming

Warming was carried out at 37 °C in four warming solutions (1–4) containing 20 mg/ml BSA in MEM. Warming solution (WS) 1, 2 and 3 were supplemented with 1 M, 0.5 M and 0.25 M sucrose respectively. Each needle was warmed in WS1 for 15 s then moved through WS2 to WS4 for 5 min each. For the melatonin supplemented groups, each WS (1–4) was supplemented with melatonin concentrations identical to the corresponding VS (precisely, the warming solutions were also supplemented with either 10^{-9} M or 10^{-7} M melatonin just like the vitrification solutions).

2.3.3. Culture

The culture medium was MEM supplemented with 50 μM ascorbic acid, 0.3 % (w/v) Polyvinyl alcohol, 10 μg/ml follicle stimulating hormone, 1 % (v/v) insulin, transferrin, and selenium mixture (ITS+1) and 1 % (v/v) penicillin streptomycin solution (Gibco, Bleiswijk, Netherlands). Additional supplementation with 10^{-7} M melatonin was included for the melatonin supplemented group. The culture system involved the use of agarose gel inserts prepared as follows: agarose (1.5 % [w/v]) in distilled water was heated until fully dissolved and made into gel by allowing it to cool in a 100 mm dish under a laminar flow cabinet. Thereafter, gel inserts of (~10 × 10 × 5 mm) were aseptically dissected and then culture media was added and allowed to soak for 24 h. Twenty-four well culture plate was prepared by placing the agarose inserts in the wells followed by addition of culture media (400 μl) such that the agarose inserts were not fully submerged. Unused wells were filled with 500 μl distilled water to augment local humidity. Four ovarian fragments were cultured on each agarose gel insert for 10 days and 200 μl of medium was replaced every other day. Fragments were fixed at day 2, 6 and 10 of culture.
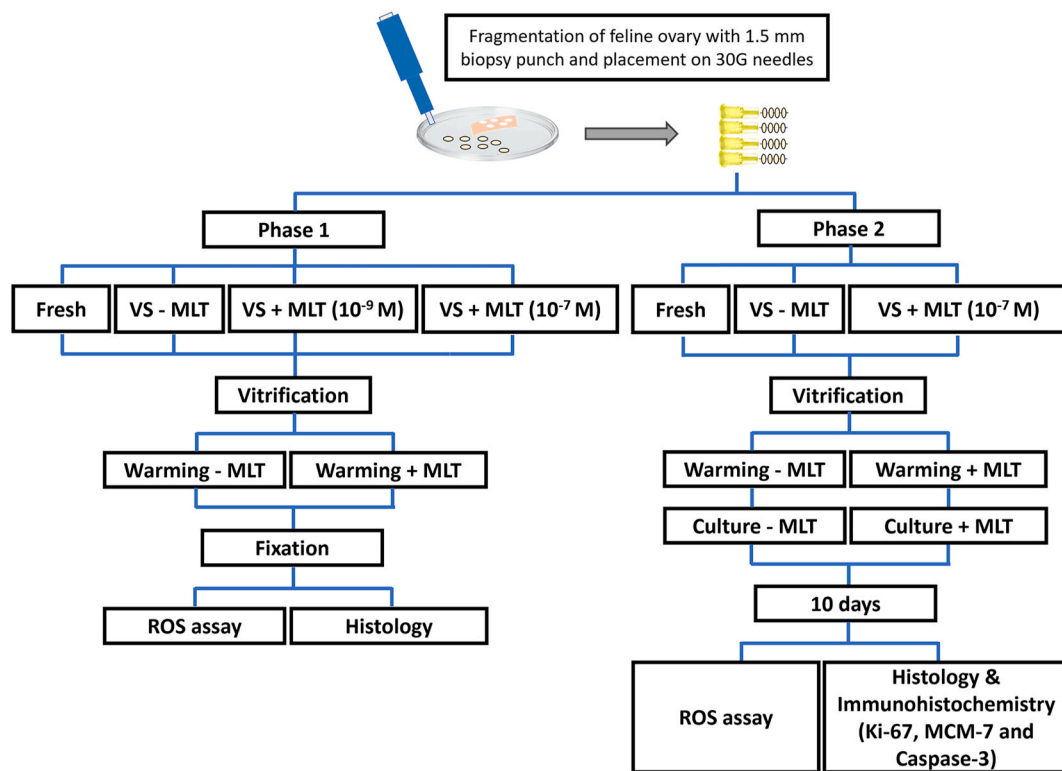
Fig. 1. Flow chart of the experimental design showing the two segments of the study. VS: Vitrification solution; MLT: Melatonin; ROS: Reactive oxygen species; MCM-7: Minichromosome maintenance protein complex component-7.

2.3.4. Histology

Routine histology was executed according to our previous description [31] as follows: fragments were dehydrated in serial concentrations of ethanol and treated with xylene prior to paraffin impregnation and embedding. Manual sectioning of paraffin embedded blocks to 5 μm sections was carried out using a semi-automatic microtome (Microm HM335E, Walldorf, Germany). Sections were placed on glass slides (while skipping 5 consecutive sections between each selected section) and allowed to dry overnight at 37 °C. Hematoxylin and Eosin staining was performed manually wherein slides were submerged in xylene to remove the paraffin followed by rehydration in reducing concentrations of ethanol and then submerged in distilled water. At this point the slides were stained with Hematoxylin and counterstained with Eosin and dehydrated before cover slipping. The slides were allowed to dry under a chemical hood and digital scanning ensued using NanoZoomer S60 (Hamamatsu, Shizuoka, Japan). Follicles were graded into 4 morphological grades and classified into 4 developmental stages as follows: Follicles with spherical shape and a spherical oocyte characterised by intact nucleus and homogenous cytoplasm enclosed by evenly distributed follicular cells in an intact surrounding stroma were considered grade 1. The second grade (grade 2), exhibited all of the grade one features except that oocyte nucleus was misshapen and/or without homogenous cytoplasm. Grade 3 was a spherically disposed follicle that was detached from the stroma while grade 4 was when the follicular cells were detached from the stroma and/or the oocyte was misshapen, vacuolated, with pyknotic nucleus and/or disorganized follicular cells. The follicles were further classified into primordial (oocyte surrounded by squamous follicular cells), transitional (at least one follicular cell cuboidal), primary (oocyte surrounded by cuboidal follicular cells), and secondary follicles (multilayered cuboidal follicular cells surrounding the oocyte with antral spaces) [31].

2.3.5. Immunohistochemistry

Immunostaining was performed based on our previous report [34]

but manually executed. Briefly, the expression of 3 protein targets were assessed which included 2 markers (Ki-67 and MCM-7 [minichromosome maintenance protein complex component-7]) of proliferation [35], and an apoptotic marker (activated caspase-3). Tissue sections with the thickness of 5 μm were made from the paraffin embedded blocks and attached to immunohistochemical slides (Dako, California, USA), one for each marker, and a section of feline tonsil was placed on each slide as a positive control. The slides were deparaffinized in xylene followed by rehydration using decreasing concentration of ethanol in distilled water. Antigen retrieval was performed by heating the slides to 97 °C in antigen retrieval solution (citrate buffer pH 6.0 for Ki-67 and pH 9.0 (Dako EnVision Flex, Glostrup, Denmark) for MCM-7 and activated caspase-3). Slides were then cooled, washed with wash buffer (Dako EnVision Flex, Glostrup, Denmark) and treated with endogenous peroxidase blocking reagent (Dako EnVision Flex, Glostrup, Denmark) before incubation with primary antibodies as follows: Ki-67 (clone MiB-1; Dako EnVision Flex), MCM-7 (1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and activated caspase-3 (1:400; Cell signaling, Massachusetts USA). Thereafter, slides were treated with HRP (horseradish peroxidase) cocktail (Dako EnVision Flex, Glostrup, Denmark) before visualisation with a DAB (diaminobenzidine) chromogen (Dako EnVision Flex, Glostrup, Denmark). Slides were counterstained with hematoxylin (Dako EnVision Flex, Glostrup, Denmark), dehydrated, cover-slipped and digitally scanned. Using a viewer software NDP.view.2.9.29 (Hamamatsu, Shizuoka, Japan), the slides were evaluated by counting the follicles and classifying positive follicles. Follicles were considered immunopositive where at least one follicular cell was immunolabelled. In addition, follicles were also considered positive when the oocyte was immunostained in the case of caspase-3. Data were presented as percentages of positive follicles from the total number of follicles counted.

2.3.6. ROS assay

Superoxide anion (O2•(−)) kit (Bioworld Minnesota, USA) was used

adhering to manufacturer's instructions to measure the concentration of $O_2\bullet(-)$ in the ovarian tissue homogenate. Briefly, ovarian tissue fragments were weighted and placed in biomasher tubes (Funakoshi, Tokyo, Japan), snap frozen in liquid nitrogen and stored at $-80\,°C$ until assay. Fragments were removed from the freezer and homogenised within the tubes using biomasher pestle on ice. Tubes were centrifuged (8000 g) for 10 min at $4\,°C$. The supernatant was immediately collected and kept on ice until used for the assay. Flat bottom 96 well plate was prepared with the standards, and test wells were prepared by mixing the supernatant with two reagents provided in the test kit. The plate was then incubated at $37\,°C$ for 40 min before absorbance measurement with a microplate reader (Agilent, California, USA) at 530 nm wavelength. Superoxide anion concentration was interpolated from the formular provided by the manufacturer based on tissue weight.

2.4. Statistical analysis

Discrete data collected from counting follicles were presented as percentages of the total follicle counts and analysed using chi-square while continuous data curated from ROS assay were analysed using one-way analysis of variance (ANOVA) with Tukey's post hoc tests. Confidence interval of 95 % was considered for all analyses.

3. Results

3.1. Phase 1

This phase involved ten experiments carried out to test the effect of two different concentrations of melatonin on the vitrification and warming of feline ovarian tissue.

3.1.1. ROS

Superoxide anion concentration was significantly lower in VIII (VS + melatonin 10^{-7} M) than VI (not supplemented), details in Fig. 2A. Additionally, VIII was not statistically different from FR (fresh control).

3.1.2. Histology

Morphologically, the proportion of grade 1 follicles was higher in VIII than all the other treatments with a corresponding lower percentage of grade 4 follicles (Fig. 2B) and as expected since thin ovarian cortex was used for the study, most follicles were primordial with little variation between treatments (Fig. 2C). Details can be found in Supplementary Table 1 and representative photomicrographs of hematoxylin and eosin stained sections of FR and the treatments are presented in Fig. 3.

3.2. Phase 2

Here, a total of six experiments were conducted to test the effects of melatonin supplementation on both vitrification and culture of feline ovarian tissue.

3.2.1. Histology

Fig. 4 presents the distribution of follicles based on different grades. Proportions of morphologically intact (grade 1) follicles were consistently higher ($p < 0.05$) in melatonin supplemented group than the other throughout the 10-day culture period. However, both treatments were not statistically different with regard to grade 4 (poorest morphology) follicles. Data on follicular activation and development can be found in Fig. 5 wherein reduction in the proportion of primordial follicles and corresponding increase in transitional follicles was evident in the melatonin supplemented group through day 6 of culture. However, more than 60 % of follicles were at their primordial stage even after a 10-day culture. Detailed data has been presented in Supplementary Tables 2 and 3 while hematoxylin and eosin stained sections are presented in Fig. 6.
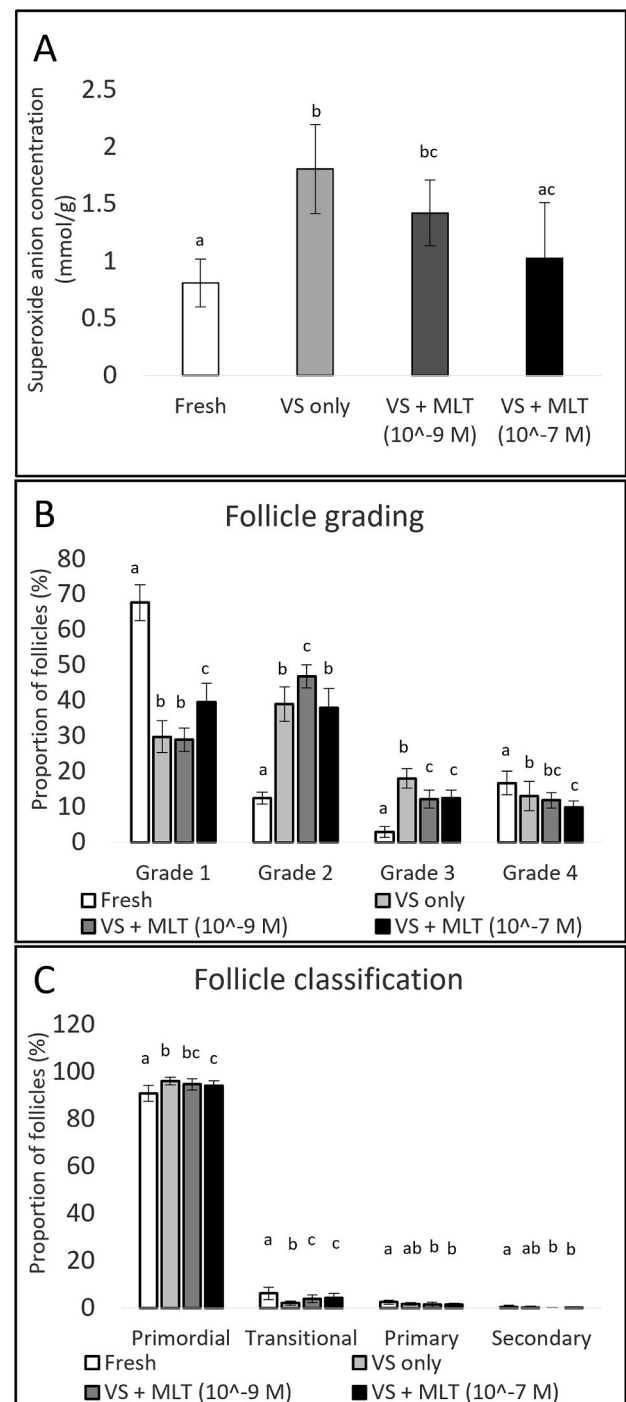

Fig. 2. Graphical presentation of data obtained from vitrified-warmed feline ovarian tissue, A: Chart of concentrations (mean ± standard deviation [error bars]) of superoxide anion in the different groups, B: Proportions of follicles based on morphological grades in the different groups, C: Classification of follicles based on developmental stages, VS: Vitrification solution; MLT: Bars accompanied with a common superscript (a, b, or c) in the same column are not statistically different ($P > 0.05$) and error bars on chart B and C represent the standard error of the mean.

3.2.2. Immunohistochemistry

Similar to the morphological classification, the expressions of proliferative markers (Ki-67 and MCM-7) were higher ($p < 0.05$) in melatonin supplemented group at day six of culture but indifferent at the day 10 between the treatments (Fig. 5B and D). The data also corresponds to the general morphological quiescence of the follicles as the expression of
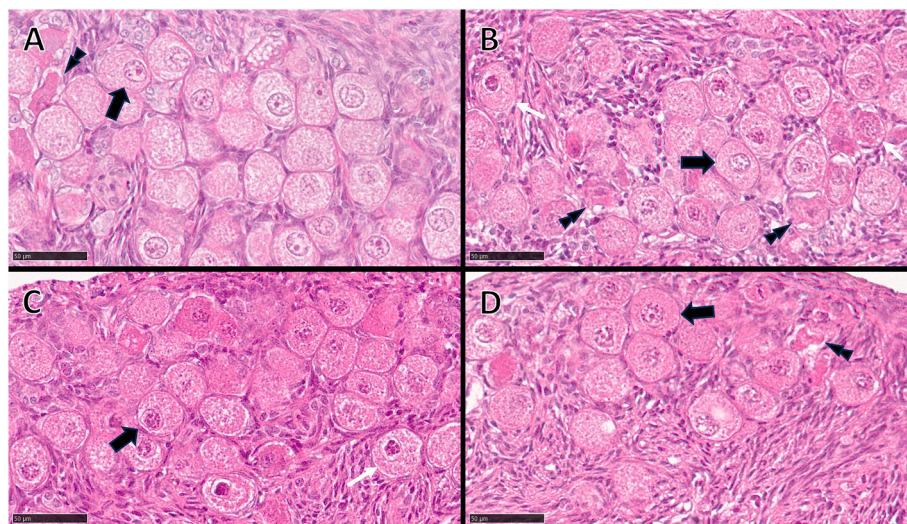
Fig. 3. Micrographs from hematoxylin and eosin stained sections of fresh (A), vitrified-warmed feline ovarian tissue (B), vitrified-warmed with melatonin (10^{-9} M) supplementation (C) and vitrified-warmed with melatonin (10^{-7} M) supplementation (D) showing grade 1 follicles (Black block arrow), grade 2 (white line arrow) and grade 4 (double arrowheads). Scale bar = 50 μm.

the markers was in below 30 % of the total follicles. Caspase-3 activity has been kept at lower expression in melatonin supplemented group throughout the culture period although only significant up to day 6 of culture. This can be found in detail in Supplementary Table 4. Immunostained sections showing positive follicles can be found in Fig. 7.

3.2.3. ROS

Chart E of Fig. 4 shows the distribution of superoxide anion concentrations in the different groups across the 10-day culture period. However, no significant difference (p > 0.05) was detected within and among the treatments.

4. Discussion

The main aim of this study was to evaluate the role of melatonin in the vitrification and culture of feline ovarian tissue, and we have demonstrated in our phase 1 experiment that supplementation of vitrification and warming solutions with melatonin had considerable cryoprotective effects. These effects included significant reduction in tissue ROS generation which is obviously one of the main causes of cryotoxicity and apoptosis at supraphysiological concentration [17,18]. This exceptional role of melatonin in mitigating cryoinjuries lead to its recognition as a cryoprotectant by some authors [9]. Secondly, morphological integrity was significantly higher in melatonin supplemented groups evident with high proportion of grade 1 follicles and a concomitant low proportion of grade 4 follicles. These desirable effects were profound in the group treated with higher (10^{-7} M) melatonin concentration (VIII). The two melatonin concentrations evaluated in this study were chosen based on previous reports in in vitro studies on ovarian tissues and embryos [36–40]. Our findings are in congruence with most of these studies, for instance, Shiroma et al. [38], reported that melatonin supplementation at 10^{-7} M improved the ovarian tissue graft viability evident with early return to cyclicity and ample number of maturing follicles in addition to low apoptosis.

In vitro studies have shown that melatonin permeate membranes and exerts its effect through direct scavenging of ROS [41] and indirect modulation of pathways, enzyme activity and induction of heat shock proteins [23,27,28,42]. Melatonin also suppresses apoptosis through upregulation of antiapoptotic genes such as Bcl-2 [43]. Some of these indirect effects are exerted through membrane receptors MT1 and MT2 which have been earlier demonstrated in follicles and oocytes [43,44] and regulate several signal transduction pathways [45]. Moreover, apart

from the many transcriptomic pathways regulated by melatonin, there is evidence that it is involved in ribosomal pathways which suggest its contribution in translational processes [42]. In some cases, role of melatonin has been associated with epigenetic modulation [46].

The second phase of this study focused on functional evaluation of ovarian tissue vitrification involving the in vitro culture of vitrified-warmed feline ovarian tissue for 10 days. Here, the role of melatonin on follicular viability and development was also ascertained. We found higher preservation of follicular morphological integrity in the melatonin supplemented group throughout the culture period or even similar to the control at day six of culture. This indicates the positive effect of melatonin in maintaining structural vigour of follicles in vitro through the prevention of oxidative stress-induced membrane damage [14]. Our findings agree with previous report on bovine ovarian tissue culture wherein the proportion of morphologically intact follicles were not different from the control [36]. Similar report from Najafi et al. [2], compared the effects of supplementing melatonin and a calcium chelator (BAPTA-AM) on both vitrification and slow freezing media and the author concluded that both supplements improved the morphological integrity of follicles.

In addition to structural integrity, melatonin supplementation in the present study has maintained a steady lower expression of caspase-3 activity. Although only significantly different from the unsupplemented group at day 6 of culture, it has demonstrated its antiapoptotic role in the in vitro culture of feline vitrified-warmed ovarian tissue. Sun et al. [23], have reported that antiapoptotic function of melatonin in vitrified-warmed rat ovarian tissue is achieved through downregulation of pro-apoptotic protein (Bax) and concomitant upregulation of anti-apoptotic protein (Bcl-2). However, this is not the only pathway, other authors have shown the anti-apoptotic role of melatonin especially through oxidative stress induced apoptosis in various cell types [8,9,18, 38].

In this study, we have seen a significant turnover of primordial follicles to transitional follicles through day six of culture with higher percentage in the melatonin supplemented group. This was confirmed by the corresponding significant increase in the expression of proliferative markers (Ki-67 and MCM-7) until day six of culture. This indicates the positive effect of melatonin in follicular activation and development which is a remarkable finding especially in the feline where several studies have reported the global lack of primordial follicle activation in both in vitro culture and ovarian tissue grafts [33,47–49]. On the contrary, most human studies reported overactivation of follicles and
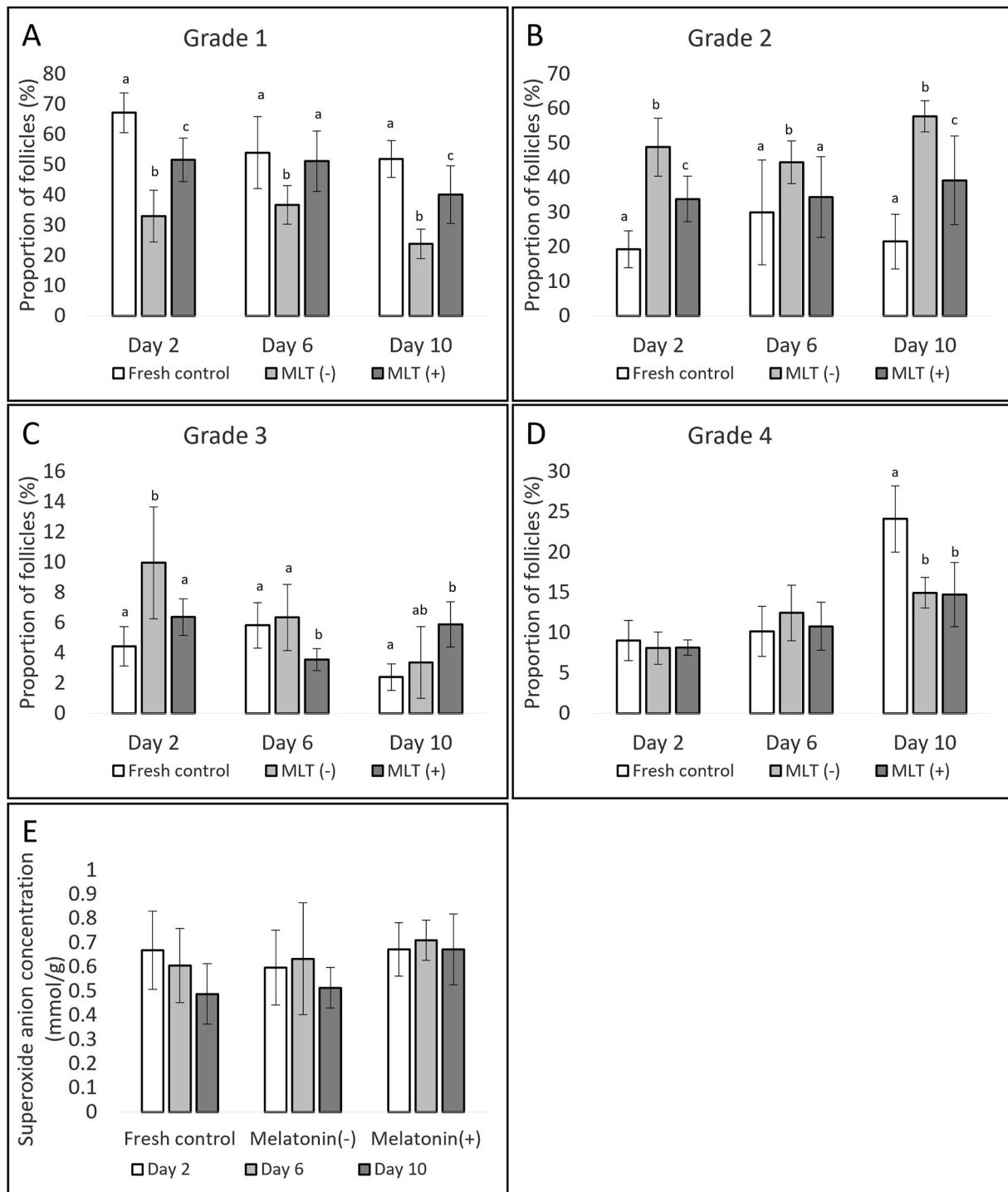
Fig. 4. Follicle grading and superoxide concentration in fresh and vitrified-warmed and cultured feline ovarian tissue with (+) or without (−) the addition of melatonin (MLT). A: Proportion of morphologically intact follicles, B: Proportion of grade 2 follicles, C: proportion of grade 3 follicles, D: Proportion of grade 4 follicles, E: Concentrations (mean ± standard deviation [error bars]) of superoxide anion in the different groups across the 10-day culture period. MLT: Melatonin. Bars accompanied with a common superscript (a, b, or c) in the same column are not statistically different (P > 0.05) and error bars on chart A, B, C and D represent the standard error of the mean while those on chart E represent the standard deviation.

consequent follicular depletion after cryopreservation procedures [50, 51]. This, therefore delineated the necessity of more studies to understand the feline folliculogenesis. The results in this study are in line with studies reported on in vitro bovine ovarian tissue culture [36] as well as transplanted rat ovarian tissue [38]. In vivo study on rat ovary also indicated the enhancing role of melatonin on follicular development [52]. More recently, Silva et al. [53], reported that melatonin promotes

primordial follicle activation in cultured bovine ovarian tissue through mTORC1 pathway.

Being a potent ROS neutraliser, melatonin has been the prime mover of antioxidants in recent years due to its dual antioxidant role, that is both direct [26] and indirect through modulating pathways and enzyme systems [27,41]. However, the second phase of our study failed to record any significant variation in ROS concentration between the different
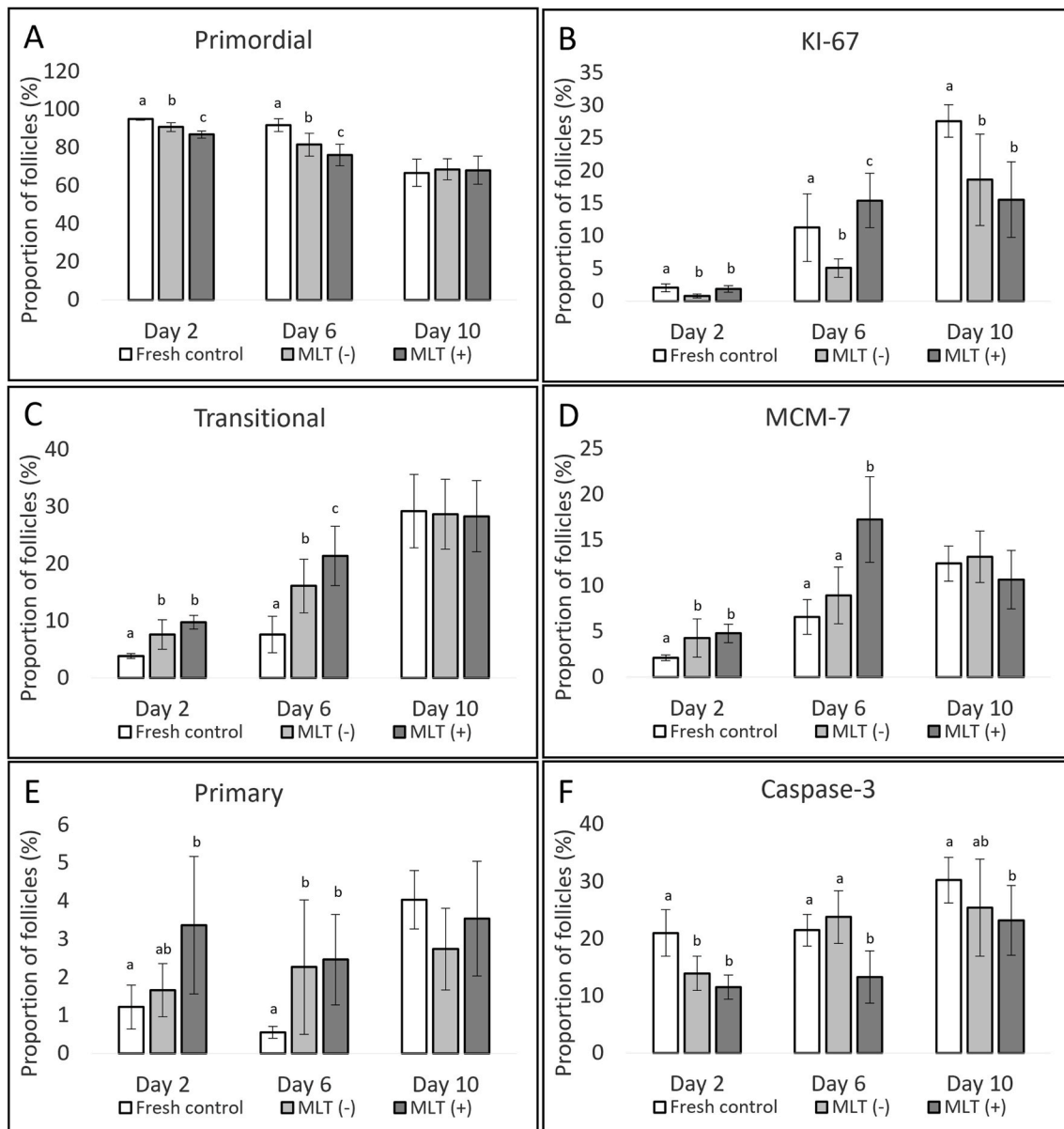
Fig. 5. Follicle development and expression of immunohistochemical markers in fresh and vitrified-warmed and cultured feline ovarian tissue with (+) or without (−) the addition of melatonin (MLT). Charts A, C, and E represent follicle classification based on their developmental stages along 10 days of culture viz: primordial, transitional, and primary respectively. Charts B, D, and F represent immunohistochemistry data obtained along 10 days of culture as follows: B, expression of Ki-67 marker in follicles; D, expression of minichromosome maintenance protein complex component-7 in follicles; F, expression of caspase-3 activity in follicles. Bars accompanied with a common superscript (a, b, or c) in the same column are not statistically different ($P > 0.05$) and error bars represent the standard error of the mean.

groups across the 10-day culture period, perhaps because only one parameter (superoxide anion) was evaluated. It is worthy of note that, ROS in the form of superoxide is often associated with cryopreservation and is considered the precursor of other ROS [54,55]. Albeit superoxide anion is not as damaging as hydroxyl radical (•OH), the later can be generated from superoxide through the Fenton and Haber–Weiss (HW) reaction [54]. Recently, Najafi et al. [56], demonstrated that ROS concentration alone may not be a reliable predictor of follicular viability, instead a ratio between total antioxidant capacity and ROS may provide a better understanding of follicular viability. Nevertheless, to the best of our knowledge this is the first report of melatonin supplementation on feline ovarian tissue vitrification and culture. However in future studies, this ratio between ROS and total antioxidant capacity should be considered in conjunction with more studies for full elucidation of the in vitro role of melatonin on feline ovary.

In conclusion, melatonin at 10^{-7} M concentration preserved follicular morphological integrity while reducing ROS concentration in vitrified-warmed feline ovarian tissue. It has also promoted the follicular viability and activation with reduced apoptosis during in vitro culture of vitrified-warmed feline ovarian tissue.
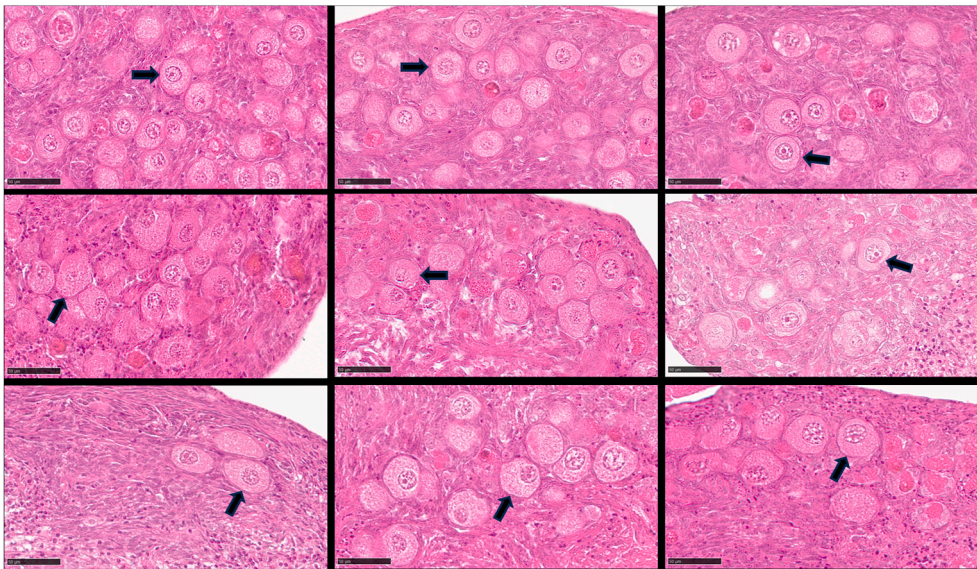
Fig. 6. Micrographs from sections stained with hematoxylin and eosin representing fresh and vitrified feline ovarian cortex cryopreserved and cultured with (+) or without (−) the addition of melatonin across the 10-day culture period, showing grade 1 follicles (Black block arrow) in all the groups. Scale bar = 50 μm.
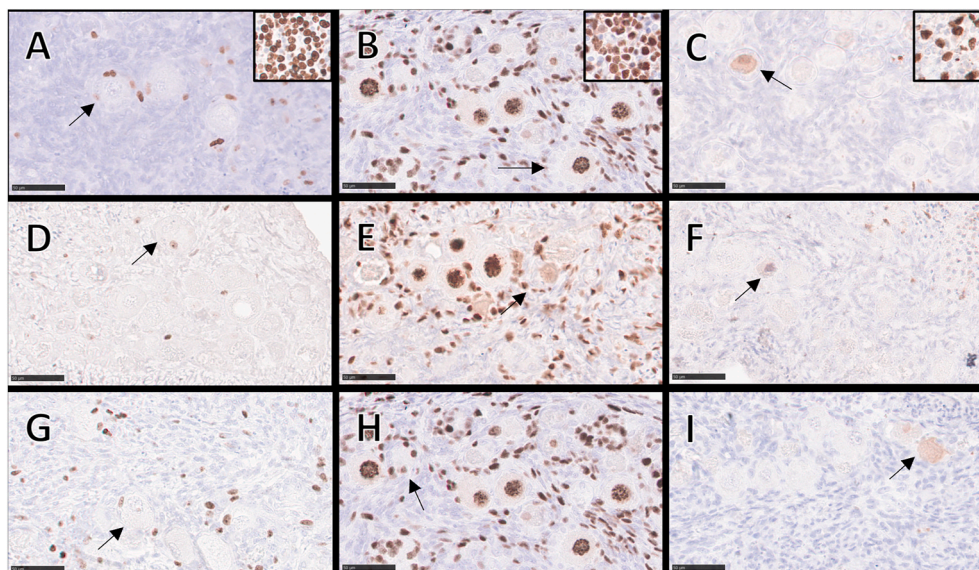

Fig. 7. Immunohistochemistry representative micrographs from the sections of fresh and vitrified feline ovarian cortex cryopreserved and cultured with (+) or without (−) the addition of melatonin showing positive follicles (black line arrows). The thumbnails at the top right of each column represent the positive control. Scale bar = 50 μm.

CRediT authorship contribution statement

Isa Mohammed Alkali: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martina Colombo:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Gaia Cecilia Luvoni:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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