Theriogenology 247 (2025) 117566

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Original Research Article

Antioxidant and antiapoptotic effect of melatonin on cat vitrified oocytes

Martina Colombo *, Alessandra Mascaro, Alessandro Pecile, Jasmine Fusi, Gaia Cecilia Luvoni

Dipartimento di Medicina Veterinaria e Scienze Animali, Università Degli Studi di Milano, Milan, Italy

ARTICLE INFO

Keywords:
Caspase
Cryopreservation
DNA
Feline
Oxidative stress
ROS
TUNEL

ABSTRACT

The development of cryopreservation protocols for feline gametes is a crucial part of conservation efforts. Preservation of oocytes is particularly challenging, since they often degenerate after warming or develop into embryos poorly. Two of the main causes are thought to be oxidative stress and apoptotic cell death. Thus, melatonin was employed in this study for its well-known antioxidant and antiapoptotic properties. After determining suitable concentrations of melatonin (Experiment I), the oxidative status (Experiment II), apoptotic status (Experiment III), and developmental competence (Experiment IV) of cat immature oocytes following vitrification and/or in vitro maturation (IVM) with the supplementation of melatonin were assessed. In Experiment I, 10^{-9} M was the lowest melatonin concentration with favorable maturation of fresh oocytes (58.06 %, p = 0.6 vs. control, 51.48 %). In Experiment II, oocytes vitrified with melatonin showed decreased oxidative stress after IVM, close to negative control (p = 0.849). In Experiment III, the addition of melatonin to vitrification-warming and IVM medium hindered apoptotic signaling by reducing caspase activity (p = 0.9 vs. fresh control oocytes) and enhancing maturation rates (48.39 % vs. 12.12 % in control vitrified oocytes; p = 0.002). In Experiment IV, melatonin brought maturation rates of treated vitrified oocytes close to those of fresh oocytes (46.38 ± 10.68 % and 68.42 ± 10.88 %, respectively, p = 0.145), but there were no differences in embryo development or morphological quality (cleavage rates: melatonin-treated oocytes: 33.29 ± 17.86 %; control vitrified oocytes: 31.59 ± 21.63 %; p = 0.992). In summary, the use of the antioxidant and antiapoptotic melatonin during vitrification-warming and IVM mitigated oxidative and apoptotic stress in vitrified oocytes, though its impact on developmental competence remained limited.

1. Introduction

Oocyte vitrification has potential for fertility and biodiversity preservation [1–3]. It offers the flexibility to exploit gametes that cannot be fertilized at a certain time due to the lack of suitable spermatozoa or to plan the most appropriate combination of male and female gametes for the generation of embryos and offspring later in time, and to be performed rapidly, easily and in field conditions [4]. Significant improvements have been made in human mature oocyte vitrification, but animal gametes present different results [1]. Optimizing protocols is a demanding task, due to gamete biology and species-specific features [1].

In felids, although almost half of the species are threatened with extinction according to the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species [5] and would benefit of the use of assisted reproduction technologies (ARTs) [6], the cryopreservation of female germ cells continues to be a challenge [6]. Studies on domestic cat oocytes, obtained from gonadectomy-derived ovaries and model for rare and valuable wild felids' gametes, are crucial to develop more efficient protocols [6,7].

Although vitrification is more suitable than slow freezing for lipid-rich feline oocytes [8], it still induces several cellular alterations. Immature vitrified oocytes (VOs) are particularly sensitive, and both their ability to resume meiosis during in vitro maturation (IVM) and to develop into embryos after fertilization can be compromised. Although it is difficult to pinpoint the specific causes, as the concurrence of several factors may lead to these outcomes, some cryoinjuries are commonly reported. The list includes cumulus cell loss, zona pellucida hardening and cytoskeleton damages, which may bring about disassembly of the meiotic spindle and DNA alterations [9,10], as well as oxidative stress due to an excessive generation of reactive oxygen species (ROS) which

* Corresponding author.Dipartimento di Medicina Veterinaria e Scienze Animali Università degli Studi di Milano, Via dell'Università, 6, 26900, Lodi, Italy.
E-mail addresses: martina.colombo@unimi.it (M. Colombo), alessandra.mascaro@unimi.it (A. Mascaro), alessandro.pecile@unimi.it (A. Pecile), jasmine.fusi@unimi.it (J. Fusi), cecilia.luvoni@unimi.it (G.C. Luvoni).

https://doi.org/10.1016/j.theriogenology.2025.117566
Received 20 May 2025; Received in revised form 1 July 2025; Accepted 2 July 2025
Available online 3 July 2025

may lead to apoptotic death [11–16].

Previous studies showed that apoptosis is involved in post-warming degeneration of cat VOs [17] and attempted to target the apoptosis pathways to enhance VOs survival with the use of apoptotic pathway inhibitors or regulators, with promising outcomes on maturation and contrasting results in terms of embryo development [17,18]. However, oxidative stress has not been investigated in cat VOs, even though the use of antioxidants is quite common on fresh oocytes [19–22]. Among antioxidants, melatonin has never been tested on cat oocytes IVM, but it proved to be useful in other species thanks to its multiple effects. Melatonin has both antioxidant and antiapoptotic activities and can protect cells from ROS-derived oxidative damage and cell death [23], generally promoting oocyte maturation and/or embryo development in both fresh (e.g., porcine [24–27], bovine [28–30], murine [31], human [32] and others [33,34]) and cryopreserved (e.g., bovine [16], murine [15,35–38], human [39,40] and others [41,42]) oocytes.

The aim of this study was to assess cat VOs cryotolerance, in terms of oxidative status, apoptotic status and developmental competence, following the addition of the antioxidant and antiapoptotic compound melatonin during oocyte vitrification-warming and/or IVM.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were purchased from the Italian branch of Merck KGaA (Darmstadt, Germany) – Merck Life Science S.r.l. (Milan, Italy), unless otherwise stated.

2.2. Experimental design

Four experiments were performed to achieve the aim of the study (Fig. 1).

According to the experiment, immature cumulus-oocyte complexes (COCs) were collected and used as fresh or cryopreserved by vitrification. Moreover, in some cases oocytes were assessed as immature (no IVM), while in others they were assessed after IVM, as better explained below.

First, suitable concentrations of melatonin for cat oocytes were determined in fresh (not cryopreserved) oocytes through a dose-response assessment during IVM (Experiment I). Then, the effects of vitrification, with or without melatonin supplementation, on oxidative stress (oxidation of a fluorescence probe by ROS, Experiment II), on two apoptotic markers (DNA fragmentation and caspase activity, Experiment III), and oocyte developmental competence (in vitro embryo development, Experiment IV) were investigated.

In Experiment I, based on the literature for oocytes of other species, three different concentrations of melatonin were tested (10^{-7} M/10^{-9} M/10^{-11} M) and compared to a standard in vitro matured control (0 M melatonin).

In Experiments II and III, using the concentration of melatonin chosen in Experiment I, oxidative and apoptotic status of cat VOs were assessed right after warming or after 24 h IVM to have a better understanding of the timing of their occurrence. Oocytes (COCs) vitrified with (MVOs) or without (VOs) the addition of melatonin were analyzed after warming using fresh immature COCs (FOs) as negative control and menadione-exposed immature COCs (Menadione, for oxidative stress induction, Experiment II) or hydrogen peroxide-exposed immature COCs (H_2O_2, for apoptosis induction, Experiment III) as positive controls. Some of the MVOs and VOs underwent maturation with (MIVM) or

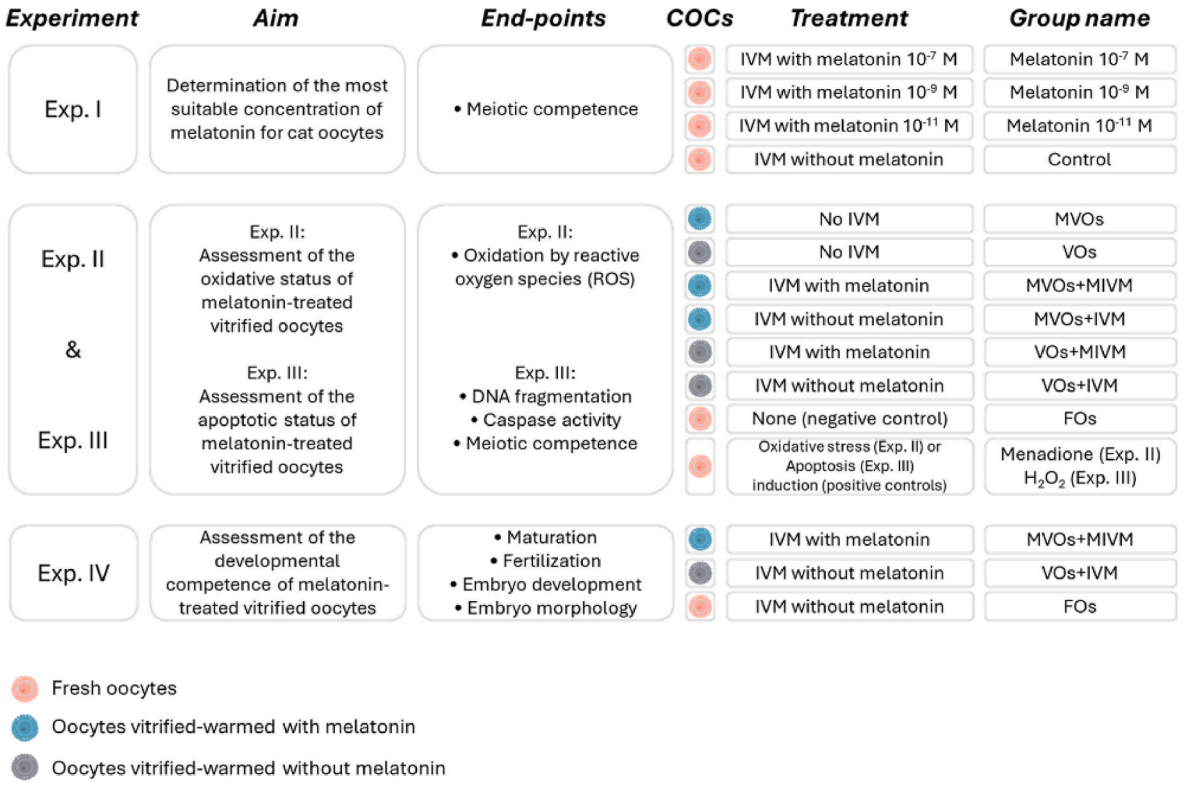

Fig. 1. Graphical depiction of the experimental design of the study. For details, please see Paragraph 2.2. COCs: cumulus-oocyte complexes; IVM: in vitro maturation; MVOs: immature oocytes vitrified and warmed with the addition of melatonin; VOs: immature oocytes vitrified and warmed without the addition of melatonin; MIVM: in vitro maturation in the presence of melatonin; IVM: standard in vitro maturation without melatonin; FOs: fresh immature oocytes (negative control); Menadione: immature oocytes treated with menadione to induce oxidative stress (positive control); H_2O_2: immature oocytes treated with hydrogen peroxide to induce apoptosis (positive control).

without (IVM) melatonin, in all the possible combinations, as follows.

- MVOs + MIVM: COCs vitrified-warmed with the addition of melatonin and in vitro matured in the presence of melatonin;
- MVOs + IVM: COCs vitrified-warmed with the addition of melatonin and in vitro matured without melatonin;
- VOs + MIVM: COCs vitrified-warmed without the addition of melatonin and in vitro matured in the presence of melatonin;
- VOs + IVM: COCs vitrified-warmed without the addition of melatonin and in vitro matured without melatonin.

In Experiment IV, the treatment that gave the most promising results in previous Experiments (MVOs + MIVM) was used for in vitro embryo production to assess maturation and embryo development compared to untreated VOs (VOs + IVM) and fresh (not cryopreserved) gametes (FOs).

2.3. Ovaries and cumulus-oocyte complexes (COCs) retrieval

Ovaries (n = 347) from healthy queens (*Felis catus*) were harvested at random stages of the estrous cycle during routine ovariectomies or ovariohysterectomies. The study did not require ethical approval because cat ovaries were collected as byproducts from routine surgeries.

After surgery, ovaries were immediately placed in phosphate buffered saline (PBS) with a mixture of antibiotics (AB) and antimycotics (100 IU/ml of penicillin G sodium, 0.1 mg/ml of streptomycin sulfate, 0.25 μg/ml of amphotericin B), and transported to the laboratory at room temperature (RT) until processing (within 4 h).

Ovaries were minced in PBS with 0.1 % (w/v) polyvinyl alcohol (PVA) to release the oocytes, and only immature COCs with darkly pigmented ooplasm completely surrounded by 3–4 layers of cumulus cells (Grade I [43]) were selected for the experiments (n = 969), with an average harvest rate of 2.8 COCs/ovary.

2.4. In vitro maturation (experiments I, II and III)

Cumulus-oocyte complexes were in vitro matured for 24 h in groups of 3–14 in a controlled atmosphere (38.5 °C and 5 % CO_2 in air) in 500 μL microdrops of IVM medium [44] [medium 199 supplemented with 3 mg/mL BSA, 10 ng/mL epidermal growth factor (EGF), 0.6 mM cysteine, and 0.5 IU/mL follicle-stimulating hormone+0.5 IU/mL luteinizing hormone (Pluset®, Calier, Spain)] covered by 300 μL mineral oil (Origio Italia s.r.l., Rome, Italy) in Petri dishes.

Melatonin was added at the concentrations of 10^{-7} M/10^{-9} M/10^{-11} M in Experiment I, where a suitable concentration for cat oocytes had to be assessed. Tested concentrations of melatonin were chosen based on the scientific literature on its use for IVM and preservation of oocytes in other species (see Refs. [23,45] for reviews). Then, melatonin was added at the concentration of 10^{-9} M in the following experiments when IVM with melatonin supplementation (i.e., MIVM) was performed (for Experiment II and III, in the groups VOs + MIVM and MVOs + MIVM; for Experiment IV, in the group MVOs + MIVM).

At the end of IVM, in Experiment I, COCs were denuded by a stripper micropipette (The Stripper, BioTipp, Waterford, Ireland) equipped with 155 μm and 125 μm tips (RI-Tip, Gynemed, Lensahn, Germany) and placed on a slide, air-dried, and fixed in 80 % ethanol overnight at 4 °C for the assessment of nuclear status (Paragraph 2.9). In Experiments II and III, at the end of IVM the COCs underwent staining as described below (Paragraphs 2.6 and 2.7).

2.5. Vitrification and warming of immature cumulus-oocyte complexes (COCs)

Cumulus-oocyte complexes were vitrified by the Cryotop method [46,47], as previously described [48]. Briefly, groups of 4–8 COCs were equilibrated at RT in an equilibration solution containing 7.5 % (v/v) ethylene glycol (EG) and 7.5 % dimethylsulfoxide (DMSO) in Medium 199, with 20 % fetal bovine serum (FBS) for 15 min. Then, they were transferred into a vitrification solution (15 % (v/v) EG, 15 % DMSO and 0.5 M sucrose in Medium 199 with 20 % FBS), placed on Cryotop strip, removing the excess of liquid to reduce the volume as much as possible, and directly immersed into liquid nitrogen in less than 90 s.

At warming, the Cryotop strip was immersed for 1 min in a thawing solution at 37 °C containing 1 M sucrose in Medium 199, with 20 % FBS. Vitrified COCs were retrieved and transferred for 3 min in a solution containing 0.5 M sucrose in Medium 199, with 20 % FBS and then for 5 min in a solution without sucrose. Finally, they were washed again in the same solution (Medium 199 with 20 % FBS) and then moved to IVM medium.

In Experiments II, III and IV, for melatonin-treated oocytes, the final concentration of melatonin in all vitrification-warming solutions and IVM media was 10^{-9} M, based on the results of Experiment I.

2.6. Staining for oxidative stress detection (Experiment II)

CellROX® Green Reagent (Thermo Fisher Scientific – Invitrogen, Monza, Italy) was used to stain the oocytes for oxidative stress detection, according to the manufacturers' instructions and the method employed on oocytes by Cavallari et al. [49].

Briefly, COCs were denuded and incubated for 30 min at 38.5 °C and 5 % CO2 with CellROX® Green Reagent (5 μM in Medium 199), washed in PBS/PVA, fixed with 4 % paraformaldehyde and washed again. Fixed oocytes were transferred to microscopy slides, covered with an antifade reagent and coverslipped. Slides were evaluated under an epifluorescence microscope (Eclipse E600, Nikon) equipped with a digital camera (Nikon DS-F12) at 400 × magnification to quantify oxidative status (i.e., green fluorescence) with the fluorescence intensity mean value of ooplasm, measured with the Imaging Software ImageJ, version 1.54f (NIH, USA) [50] – integrated density function. Fluorescence values were corrected for background fluorescence. Images were captured under the same settings. CellROX® Green Reagent maximum excitation/emission wavelengths were 485 nm and 520 nm, respectively.

Eight experimental groups were compared. Fresh immature COCs (FOs, negative control) were denuded, stained and fixed right after collection. In positive control immature COCs (Menadione), oxidative stress was induced with a 1-h incubation in menadione (100 μM in Medium 199) at 38.5 °C and 5 % CO_2 before denuding and incubation with CellROX® Green Reagent. To assess whether the generation of oxidative stress occurs soon after warming or slowly during the following incubation time and how melatonin influences this, vitrified COCs were denuded, stained and fixed right after warming or after 24 h IVM [to have all the combinations of melatonin during vitrification-warming (VOs or MVOs) and/or IVM (MIVM: IVM with melatonin; IVM: standard IVM without melatonin)], as shown in Fig. 1.

2.7. Staining for apoptotic signal detection (Experiment III)

Cell Meter™ TUNEL Apoptosis Assay Kit *Red Fluorescence* (AAT Bioquest, Sunnyvale, CA, USA) and CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific – Invitrogen, Monza, Italy) were used to stain the oocytes for DNA fragmentation and caspase activation, respectively, according to the manufacturers' instructions and our previous work [17]. Briefly, COCs were denuded and fixed with 4 % paraformaldehyde in PBS and incubated for 1 h at 38.5 °C in TUNEL and caspase dyes. Then, they were permeabilized with 0.2 % Triton-X 100 in PBS and counterstained with Hoechst 33342 (0.01 mg/mL) to identify the nucleus. Finally, the slides were covered with an antifade reagent (Fluoromount™ Aqueous Mounting Medium). Dyes maximum excitation/emission wavelengths were as follows.

- TUNEL Assay: 556 nm/579 nm;
- Caspase Detection Reagent: 502 nm/530 nm;

- Hoechst: 352 nm/461 nm.

Slides were evaluated under the same epifluorescence microscope (Eclipse E600, Nikon) and digital camera (Nikon DS-F12) at 400 × magnification to count the number of TUNEL-positive oocytes (showing a bright, strong red fluorescence in the nucleus area) and to quantify caspase activity (i.e., green fluorescence) with the fluorescence intensity mean value of ooplasm, measured with the Imaging Software ImageJ, version 1.54f (NIH, USA) [50] as described above for CellROX® staining for oxidative stress detection.

As in Experiment II, eight experimental groups were compared. Fresh immature COCs (FOs, negative control) were denuded, fixed and stained right after collection. In positive control oocytes (H_2O_2), immature COCs were first denuded, and apoptosis was induced with a 3-h incubation in 100 μM H_2O_2 in Medium 199 [51,52] before fixation and staining. Similarly to Experiment II, to assess whether the activation of apoptotic pathways occurs soon after warming or slowly during the following incubation time and how melatonin influences this, vitrified COCs were denuded, fixed and stained right after warming [vitrified-warmed with (MVOs) or without (VOs) melatonin] or after 24 h IVM (MVOs + MIVM; MVOs + IVM; VOs + MIVM; VOs + IVM), as shown in Fig. 1. Oocytes whose nucleus could not be identified by Hoechst staining were excluded by the analysis, as well as parthenogenetic embryos, identified by the presence of more than one nucleated cell upon staining.

2.8. In vitro embryo production (Experiment IV)

2.8.1. In vitro maturation

Cumulus-oocyte complexes were in vitro matured in groups of 8–31 for 24 h in a controlled atmosphere (38.5 °C and 5 % CO_2 in air) in 500 μL IVM medium (described above), covered by 300 μL mineral oil in 4-well dishes.

2.8.2. Epididymal sperm recovery, freezing-thawing and in vitro fertilization (IVF)

In vitro fertilization was performed with frozen feline epididymal spermatozoa obtained after routine orchiectomy of adult tomcats (n = 2). All the IVF replicates were performed using the same batch of spermatozoa (1 straw/replicate).

Testes were collected after routine surgery. The epididymides were dissected from isolated testicles and placed in a Petri dish in HEPES-buffered Medium 199. Spermatozoa were obtained from vas deferens and cauda epididymis by slicing with a blade at RT. Spermatozoa from the two cats were pooled, and subjective motility and concentration by Bürker chamber were determined.

Spermatozoa were frozen at the final concentration of 25000 motile spermatozoa/μL according to the Uppsala method originally developed for canine semen [53], using egg yolk-Tris-based extenders containing Equex, which was also tested in cat epididymal spermatozoa [54,55]. Briefly, collected spermatozoa were centrifuged (700 g, 5min), the supernatant was removed and spermatozoa were diluted at RT with the first freezing extender. After cooling at 4 °C for 3–4 h, the second freezing extender was added [overall composition of freezing extender: Tris buffer with final concentrations of 5 % glycerol, 1 % Equex STM (Nova Chemical Sales Inc., Scituate, MA, USA) and 20 % egg yolk [53], and spermatozoa suspension was loaded into 0.25-mL straws, which were frozen in liquid nitrogen vapors before plunging into the liquid and storage.

For thawing, straws were exposed to air for 10 s and then immersed in a water bath at 37 °C for 30 s.

For fertilization, after 24 h of IVM, COCs were removed from the IVM dishes, washed twice and transferred into 300 μL microdrops of fresh IVF medium [44] (Medium 199 supplemented with 3 mg/mL BSA, 0.1 mg/mL cysteine, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, 0.055 mg/mL gentamicin and 2.2 IU/mL heparin) covered by 300 μL mineral oil in 4-well dishes. Thawed spermatozoa, previously centrifuged (700 g, 5 min) and resuspended in IVF medium, were added to the microdrops containing the COCs to reach 500 μL volume with a final concentration of 1×10^6 motile spermatozoa/mL. Cumulus-oocyte complexes and spermatozoa were co-incubated in a controlled atmosphere (38.5 °C and 5 % CO_2 in air) for 18 h.

2.8.3. In vitro embryo culture

After IVF, all presumptive zygotes were gently washed in in vitro culture (IVC) medium (Ham's F-10 supplemented with 5 % FBS, 0.11 mg/mL sodium pyruvate, 0.075 mg/mL L-glutamine, 0.6 mg/mL gentamicin) to remove spermatozoa and residual cumulus cells with the help of a stripper micropipette (The Stripper, BioTipp, Waterford, Ireland) equipped with 155 μm and 125 μm tips (RI-Tip, Gynemed, Lensahn, Germany). Presumptive embryos were moved to 500 μL IVC medium covered by 300 μL mineral oil in 4-well dishes, where they were cultured in group for up to 8 days in a controlled atmosphere (38.5 °C, 5 % CO_2). The medium was not changed during embryo culture. During IVC, assessment of embryo development was performed on day 2, 5, 7 and 8 post-fertilization under an inverted microscope at 100 × magnification (Axiovert 100, Zeiss).

Two days after IVF, uncleaved oocytes were deprived of remaining cumulus cells and bound spermatozoa by mechanical displacement with a stripper micropipette equipped with a 125 μm tip, placed on a slide, air-dried, and fixed in 80 % ethanol overnight at 4 °C. Growing embryos were cultured until day 8 or they were removed from culture earlier when they showed signs of degeneration, and then fixed as above.

Embryo quality was subjectively evaluated according to the Manual of the International Embryo Transfer Society [56] based on morphological integrity of embryos (briefly, size, shape, regularity of blastomeres and presence of extruded material; Code 1 - excellent or good; Code 2 - fair; Code 3 - poor; Code 4 - dead or degenerating). All the embryos left in culture on days 2, 5, 7 and 8 post-fertilization were evaluated, regardless of their developmental stage.

2.9. Assessment of maturation, fertilization and embryonic developmental rates

After fixation, chromatin configuration was evaluated by a bis-benzimide (Hoechst 33342) staining to ascertain the nuclear status (Experiments I, II and IV), or the fertilization pattern or embryo developmental stage based on the number of blastomere nuclei (Experiment IV). Briefly, oocytes or embryos on a slide were covered by 10 μl of Hoechst working solution (0.01 mg/mL). After 5 min of incubation in the dark, the Hoechst solution was removed and the slides were covered with antifading solution and a coverslip. Finally, stained oocytes and embryos were observed under a fluorescent microscope (Axiovert 100, Zeiss) at 400 × magnification. Embryo nuclei were counted manually.

Oocyte chromatin configurations were classified as follows [57,58].

- Germinal vesicle (GV): identification of nucleolus and very fine filaments of chromatin;
- Germinal vesicle breakdown–anaphase I (GVBD-AI): identification of different patterns of chromatin condensation (GVBD) or identification of bivalents (AI);
- Telophase I–metaphase II (TI-MII): identification of two groups of chromosomes moving to opposite ends of meiotic spindle (TI) or two sets of chromosomes clearly visible (MII);
- Degenerated: collapsed nucleus or irregular nuclear conformation.

For Experiment IV, maturation stage (metaphase II, MII) in unfertilized oocytes was identified by the presence of a tightly packed group of chromosomes in the form of the first polar body, and another group which were well spread, allowing for the identification of individual chromosomes [58]. The total number of in vitro matured oocytes was calculated as the sum of unfertilized MII oocytes, fertilized oocytes (i.e.

uncleaved but showing pronuclei) and cleaved embryos; accordingly, the number of fertilized oocytes was calculated as the sum of fertilized oocytes and cleaved embryos. For the assessment of embryonic development, cleaved embryos (2–4 cells), morulae, and blastocysts were recorded. An embryo was considered a morula when it had ≥16 cells without a blastocoel or a blastocyst when it had ≥50 cells with a blastocoel [59].

2.10. Statistical analysis

In Experiments I and III, chromatin configuration and maturation rates were analyzed by Fisher's exact test. In Experiment II, oxidative stress levels (i.e. fluorescence intensity) were analyzed by Kruskal-Wallis non-parametric one-way ANOVA followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons (data were not normally distributed according to Shapiro-Wilk test). In Experiment III, TUNEL data were analyzed by Fisher's exact test, whereas caspases activation (i.e. fluorescence intensity) was analyzed by Kruskal-Wallis non-parametric one-way ANOVA followed by Dwass-Steel-Critchlow-Fligner pairwise comparisons (data were not normally distributed according to Shapiro-Wilk test). In Experiment IV, maturation, fertilization and embryo development rates were analyzed by one-way ANOVA (data were normally distributed according to Shapiro-Wilk test and homoscedastic according to Levene's test) followed by Tukey's post-hoc test, and embryo quality by Fisher's exact test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Experiment I

Nuclear status of fresh oocytes after IVM with different concentrations of melatonin is reported in Table 1. No significant differences were found, demonstrating that the addition of 10^{-9} M and 10^{-7} M melatonin did not have any negative effect on cat oocytes and had no influence on degeneration rates ($p = 1$). Among the latter, the lowest concentration was chosen for further experiments on vitrified oocytes.

3.2. Experiment II

The effect of melatonin on the oxidative status of vitrified oocytes is shown in Fig. 2 and Supplementary Table 1. Relative oxidative stress, quantified using the negative control as a reference, showed lower values when melatonin was added during vitrification-warming and/or IVM. Vitrification tended to increase ROS in oocytes, without variations following IVM. In oocytes analyzed after warming, melatonin did not have any effect, although it led towards lower values (MVOs vs. VOs, $p = 0.075$). Among matured oocytes, oocytes that were vitrified-warmed without melatonin (VOs + MIVM and VOs + IVM) had levels of ROS similar to the oocytes analyzed just after warming (VOs; $p = 1$) and higher than negative control (FOs; $p = 0.027$). On the other hand, the oocytes that were cryopreserved with melatonin, regardless of its addition during IVM (MVOs + MIVM and MVOs + IVM), showed lower ROS presence, and were the only groups to not differ significantly from the negative control (FOs; $p = 0.849$). Positive control oocytes expectedly presented the highest fluorescence intensity values (Menadione; $p < 0.001$ vs. the other groups).

3.3. Experiment III

Two oocytes in VOs + IVM, two in VOs + MIVM, two in VOs, one in MVOs and one in FOs were excluded by the analysis because their nuclei could not be identified by Hoechst staining. One parthenogenetic embryo in VOs + MIVM was also excluded.

The effect of melatonin on the apoptotic and nuclear status of vitrified oocytes is shown in Fig. 3 and Supplementary Table 2. For oocytes stained right after warming (MVOs and VOs), DNA fragmentation was comparable to that of negative controls (FOs), although only MVOs showed significantly lower percentages of positive nuclei compared to the positive controls (H_2O_2, $p = 0.0009$). Maturation tended to increase the proportion of oocytes with fragmented DNA, that was significantly higher in all the matured groups compared to negative controls and MVOs ($p = 0.05$). There was no difference in DNA fragmentation among the matured groups.

Caspase activity, quantified using the negative control as a reference, showed an increase following vitrification and beneficial effects of melatonin in the matured groups. Right after warming, fluorescence intensity of MVOs and VOs was significantly higher than in negative controls ($p < 0.001$) and reached the highest levels, similar to those of positive controls ($p = 0.8$). Similar ($p = 0.9$) fluorescent intensity was also reported by VOs + IVM, that showed significantly higher values than the other matured groups ($p = 0.003$). Melatonin, added during vitrification-warming or IVM halved caspases after IVM in comparison to untreated oocytes, but MVOs + MIVM was the only treatment to lower fluorescence levels close to negative controls ($p = 0.9$).

Maturation rates followed a similar trend. Melatonin addition seemed beneficial for meiosis resumption of VOs and MVOs, and there was a significant improvement in full maturation rates of MVOs + MIVM ($p = 0.002$ vs. VOs + IVM, which was the standard protocol). Similar but opposite results were obtained for degeneration rates. While degeneration generally increased with IVM, the addition of melatonin both during vitrification-warming and IVM was able to hamper degenerative processes ($p = 0.04$ vs. VOs + IVM, $p = 1$ vs. non-matured VOs).

3.4. Experiment IV

Maturation, fertilization and embryonic developmental rates of VOs + IVM and MVOs + MIVM, as well as those of fresh control oocytes, are shown in Table 2. Maturation rates of the vitrified groups did not differ ($p = 0.639$), although the value of MVOs + MIVM was slightly higher and was the only one that did not differ from fresh control oocytes ($p = 0.145$). Fertilization and cleavage rates of vitrified oocytes with (MVOs + MIVM) or without (VOs + IVM) melatonin were not different from FOs ($p = 0.913$ and $p = 0.992$, respectively). However, vitrified oocytes, regardless of the presence of melatonin, gave significantly lower morula and blastocyst rates than FOs ($p = 0.038$). Melatonin addition during vitrification-warming and IVM slightly decreased the number of degenerating oocytes and embryos, with a rate that was closer to fresh

Table 1
Nuclear status of fresh domestic cat oocytes following in vitro maturation (IVM) with different concentrations of melatonin (6 replicates). No significant differences among groups ($p > 0.05$).

Oocyte treatment	n.	Immaturity n. (%)	Partial meiosis resumption n. (%)	Full maturation n. (%)	Deg/N.A. n. (%)
Melatonin 10^{-7} M	31	1 (3.23)	5 (16.13)	18 (58.06)	7 (22.58)
Melatonin 10^{-9} M	31	0 (0)	6 (19.35)	18 (58.06)	7 (22.58)
Melatonin 10^{-11} M	31	0 (0)	9 (29.03)	12 (38.71)	10 (32.26)
Control (Melatonin 0 M)	35	1 (2.86)	8 (22.86)	18 (51.43)	8 (22.86)

Immaturity: germinal vesicle (GV) oocytes; Partial meiosis resumption: oocytes in germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI); Full maturation: oocytes in telophase I (TI), metaphase II (MII); Deg/N.A.: degenerated oocytes/not assessable.

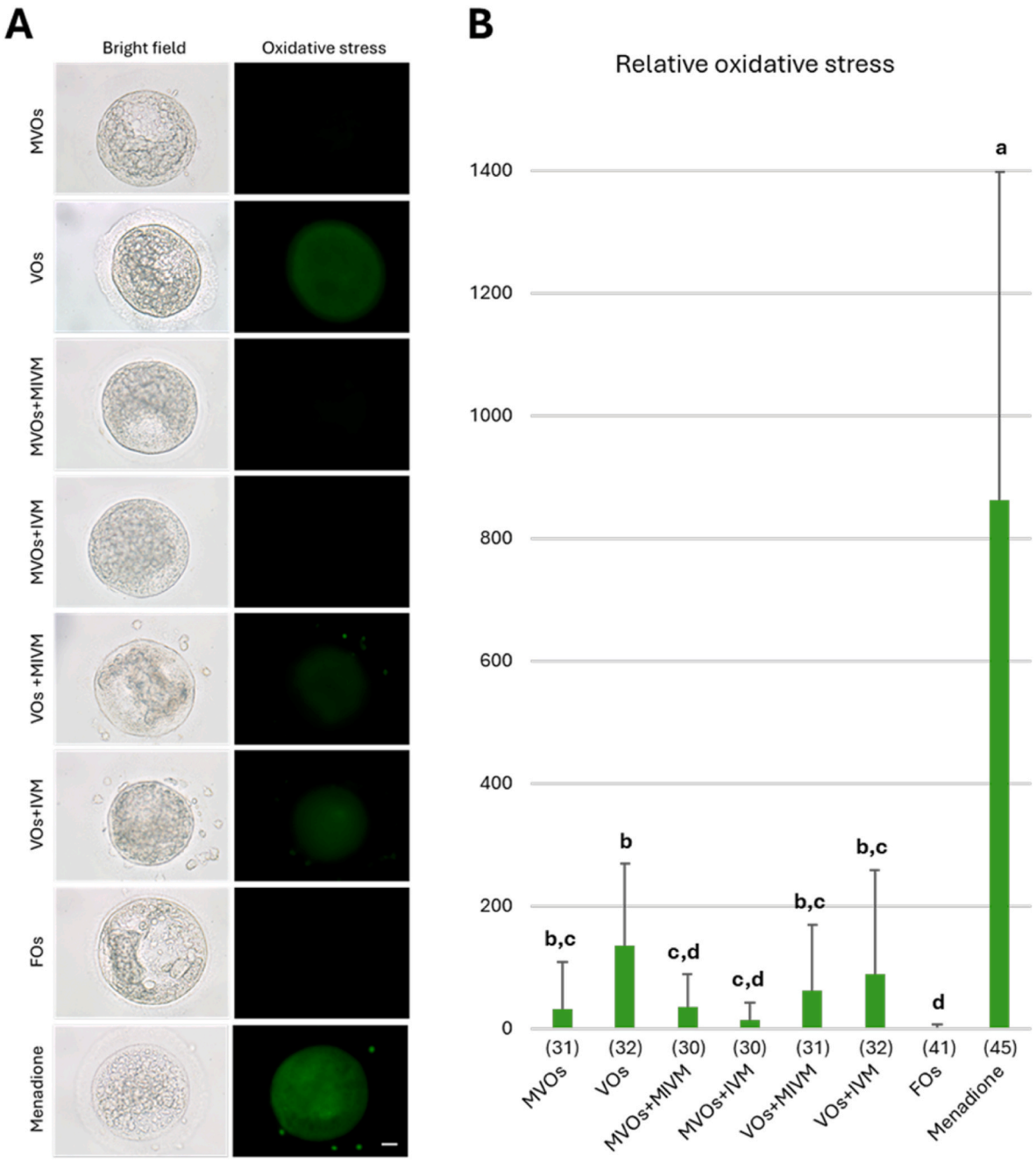

Fig. 2. Oxidative stress in vitrified and fresh domestic cat oocytes stained with CellROX® Green Reagent A) Representative fluorescence micrographs. Bright green fluorescence in the ooplasm (column "Oxidative stress") estimates, according to its intensity, the presence of reactive oxygen species. Scale bar: 20 μm. B) Relative oxidative stress fluorescence intensity (mean value ± SD; 4 replicates). The number of oocytes in each group is depicted in parenthesis under each bar. Different superscripts (a,b,c,d) above bars indicate significant differences among groups ($p < 0.05$). MVOs: immature oocytes vitrified and warmed with the addition of melatonin; VOs: immature oocytes vitrified and warmed without the addition of melatonin; MIVM: in vitro maturation in the presence of melatonin; IVM: standard in vitro maturation without melatonin; FOs: fresh immature oocytes (negative control); Menadione: immature oocytes treated with menadione to induce oxidative stress (positive control). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

controls ($p = 0.116$), although not different from the standard vitrified group (VOs + IVM; $p = 0.521$).

The evaluation of embryo morphological quality (Table 3) gave better results for fresh controls, as expected. These showed higher proportions of code 1 (excellent or good) embryos than both the vitrified groups, regardless of melatonin addition ($p < 0.00001$ on days 2 and 5; no statistical significance on days 7 and 8, likely due to the small number of embryos). No differences were observed between MVOs + MIVM and VOs + IVM at any time-point.

4. Discussion

The use of melatonin in reproduction is gaining even more importance in the latest years [60], including applications in oocyte maturation and gamete cryopreservation [45,61]. In this study, we assessed its effects on vitrified immature cat oocytes oxidative status (ROS generation), apoptotic status (DNA fragmentation and caspase activity) and developmental competence.

Feline oocytes could particularly benefit of an improvement in IVM and cryopreservation outcomes, since, compared to other species,

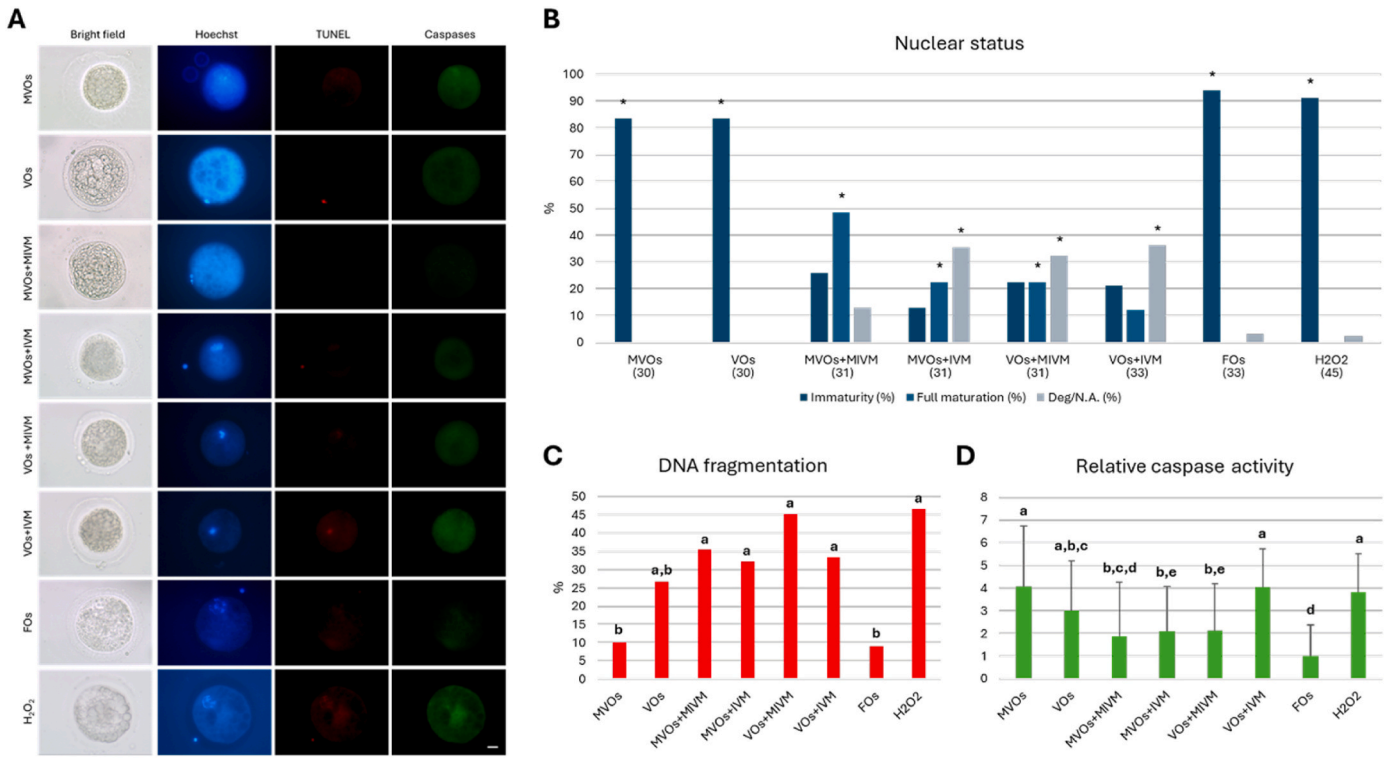

Fig. 3. Nuclear status and activation of apoptotic pathways, assessed as DNA fragmentation (TUNEL assay) and caspase activity, in vitrified and fresh domestic cat oocytes. A) Representative fluorescence micrographs. Bright blue fluorescence (column "Hoechst") indicates the nuclear material. Bright red fluorescence (column "TUNEL") in the nuclear area indicates DNA fragmentation by TUNEL assay. Green fluorescence in the ooplasm (column "Caspases") indicates, according to its intensity, the extent of caspase activity. Scale bar: 20 μm. B) Nuclear status (%), C) DNA fragmentation (%), D) Relative caspase activity fluorescence intensity (mean value ± SD). The number of oocytes in each group is depicted in parenthesis under each bar only in B), and it is the same for C) and D). Presence of asterisks (*) or different superscripts (a,b,c,d,e) above bars indicate significant differences among groups ($p \leq 0.05$). MVOs: immature oocytes vitrified and warmed with the addition of melatonin; VOs: immature oocytes vitrified and warmed without the addition of melatonin; MIVM: in vitro maturation in the presence of melatonin; IVM: standard in vitro maturation without melatonin; FOs: fresh immature oocytes (negative control); H_2O_2: immature oocytes treated with hydrogen peroxide to induce apoptosis (positive control). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Maturation, fertilization, embryonic development and degeneration rates of vitrified and fresh domestic cat oocytes (4 replicates). Different superscripts (a, b) within the same column indicate significant differences among groups ($p < 0.05$).

Oocyte treatment	n.	Maturation rate[a]	Fertilization rate[a]	Embryo developmental stage			Deg./N.A.
				Cleavage (2–4 cells)	Morula	Blastocyst	
		% (mean ± SD)	% (mean ± SD)	% (mean ± SD)	% (mean ± SD)	% (mean ± SD)	% (mean ± SD)
VOs + IVM	88	36.68 ± 20.68^{a}	31.59 ± 21.63^{a}	31.59 ± 21.63^{a}	10.86 ± 9.09^{a}	0 ± 0^{a}	60.31 ± 19.19^{a}
MVOs + MIVM	85	46.38 ± 10.68^{a,b}	37.43 ± 18.83^{a}	33.29 ± 17.86^{a}	6.26 ± 5.29^{a}	0 ± 0^{a}	47.80 ± 16.49^{a,b}
FOs	87	68.42 ± 10.88^{b}	52.99 ± 19.93^{a}	52.13 ± 19.27^{a}	47.22 ± 18.70^{b}	14.84 ± 12.25^{b}	22.90 ± 9.82^{b}

VOs + IVM: oocytes vitrified and warmed without the addition of melatonin + standard in vitro maturation without melatonin; MVOs + MIVM: oocytes vitrified and warmed with the addition of melatonin + in vitro maturation in the presence of melatonin; FOs: fresh oocytes (control); Deg/N.A.: degenerated oocytes/not assessable.
[a] Maturation rate was calculated including unfertilized metaphase II oocytes, fertilized oocytes (i.e. uncleaved but showing pronuclei) and cleaved embryos; accordingly, the fertilization rate was calculated as the sum of fertilized oocytes and cleaved embryos.

oocyte maturation and embryo development rates are rather low (50–60 % maturation and 20–30 % blastocyst rate for fresh oocytes [19] and around 30 % maturation rate with sporadic blastocyst formation [62] for cryopreserved oocytes). Despite the challenges, efficient protocols for the cryopreservation of immature COCs need to be developed to salvage cells that in some cases, such as sudden deaths or spaying of valuable individuals, might be the only source of genetic material or germ cells to be used in basic ARTs (i.e., without the use of advanced techniques such as somatic cell nuclear transfer or stem cell technologies).

Taking into consideration the effects of cryopreservation on cellular oxidative and apoptotic status, the addition of antioxidant or anti-apoptotic compounds could be a valuable option to improve the developmental competence of preserved gametes. Several non-enzymatic antioxidants, including melatonin, were applied during vitrification, warming, and/or culture to reduce or control the oxidative damage, with positive effects on oocyte maturation and development in other species [63]. Melatonin, naturally produced mostly by the pineal gland, is exogenously supplemented in vitro for its actions, that are both receptor-dependent and independent [64]. Melatonin receptors have been identified on oocytes [65–68], although not on feline ones, and allow melatonin to activate phospholipase C and a cascade that finally leads to an increase in the expression of antioxidant enzymes [64]. Instead, receptor-independent mechanisms (melatonin can diffuse freely through the cell membrane) include scavenger activities carried out by both melatonin itself and its metabolites [69]. Its beneficial effects

Table 3
Morphological quality classification of embryos obtained in vitro from vitrified and fresh domestic cat oocytes (4 replicates). Codes for embryo grading were given following the Manual of the International Embryo Transfer Society (IETS). Different superscripts (a,b) within the same column indicate significant differences among groups ($p < 0.05$).

Day of embryo culture	Oocyte treatment	n.	Code 1 (Excellent or good)	Code 2 (Fair)	Code 3 (Poor)	Code 4 (Uncleaved, dead or degenerating)
			n. (%)	n. (%)	n. (%)	n. (%)
Day 2	VOs + IVM	88	7 (7.95)[a]	10 (11.36)[a]	11 (12.50)[a]	60 (68.18)[a]
	MVOs + MIVM	85	4 (4.71)[a]	11 (12.94)[a]	9 (10.59)[a,b]	61 (71.76)[a]
	FOs	87	32 (36.78)[b]	12 (13.79)[a]	3 (3.45)[b]	40 (45.98)[b]
Day 5	VOs + IVM	27	6 (22.22)[a]	10 (37.04)[a]	2 (7.41)[a]	9 (33.33)[a]
	MVOs + MIVM	26	8 (30.77)[a,b]	8 (30.77)[a]	2 (7.69)[a]	8 (30.77)[a]
	FOs	46	24 (52.17)[b]	12 (26.09)[a]	3 (6.52)[a]	7 (15.22)[a]
Day 7	VOs + IVM	13	2 (15.38)[a]	6 (46.15)[a]	3 (23.08)[a]	2 (15.38)[a,b]
	MVOs + MIVM	17	2 (11.76)[a]	9 (52.94)[a]	2 (11.76)[a]	4 (23.53)[a]
	FOs	41	16 (39.02)[a]	18 (43.90)[a]	7 (17.07)[a]	0 (0)[b]
Day 8	VOs + IVM	10	1 (10.00)[a]	3 (30.00)[a]	4 (40.00)[a]	2 (20.00)[a]
	MVOs + MIVM	11	1 (9.09)[a]	7 (63.64)[a]	1 (9.09)[a,b]	2 (18.18)[a]
	FOs	41	11 (26.83)[a]	24 (58.54)[a]	4 (9.76)[b]	2 (4.88)[a]

VOs + IVM: oocytes vitrified and warmed without the addition of melatonin + standard in vitro maturation without melatonin; MVOs + MIVM: oocytes vitrified and warmed with the addition of melatonin + in vitro maturation in the presence of melatonin; FOs: fresh oocytes (control).

during cryopreservation led to consider it as a cryoprotectant [61].

In the present study, melatonin supplementation during COCs vitrification and IVM was tested for the first time in domestic cats. Suitable concentrations for fresh cat oocytes proved to be 10^{-7} M and 10^{-9} M (Experiment I), similarly to other studies (for a review: [45]). To prevent possible damages, the lowest concentration was chosen for further experiments on vitrified oocytes, since higher concentrations were reported to possibly have no or negative effect on oocyte developmental competence [45].

Bearing in mind that ROS are physiologically present during many biological processes, including embryo development, where they play pivotal roles [70], their excess could be harmful, and a balance is needed [71]. In the present study, vitrification induced an increase of ROS in cat immature oocytes, and its extent was first estimated hereby (Experiment II). The addition of melatonin during vitrification-warming could bring ROS level closer to those of fresh oocytes (negative control), suggesting that it can have a role in the reduction of ROS generated because of the cryopreservation procedure, without a marked effect on post-warming culture, as we already observed for ROS in vitrified cat ovarian cortex [72]. In other species, results were consistent with ours and showed a general decrease in oxidative stress in oocytes treated with melatonin during vitrification-warming and/or IVM compared to controls [15,16,35,39,41,73,74].

As expected and previously reported [17,75], vitrification induced apoptosis in cat immature oocytes, causing a marked increase in caspase activation (Experiment III). Only the addition of melatonin in both vitrification-warming and IVM was useful to lower caspase activity to levels that were similar to those of fresh oocytes and increase full maturation rates compared to standard vitrified-warmed and matured oocytes (without melatonin). Similar results on apoptosis and maturation were reported in other species [16,39], even if melatonin was only added in vitrification-warming solutions [16,39].

The difference between the present study and those performed in other species, instead, mainly lies in the effects of melatonin on developmental competence. Although mostly reported to be beneficial for the developmental competence of both fresh and cryopreserved oocytes ([45,63] for recent reviews), in the present study melatonin did not have an effect on embryo development and morphology (Experiment IV). The increase in full maturation rates due to the presence of melatonin in Experiment III was however confirmed, since the maturation rate, calculated during in vitro embryo production as the sum of unfertilized mature oocytes, fertilized uncleaved oocytes and cleaved embryos, was similar between melatonin-treated vitrified oocytes and fresh controls. Yet, embryo development until late in vitro stages was not affected, and melatonin was not helpful in ameliorating the well-known scarcity of blastocysts produced by immature cat vitrified oocytes.

It is known that oocyte morphological abnormalities, including those caused by exogenous factors, might affect human embryo development and quality [76]. After vitrification, some morphological alterations might occur, thus it seems plausible that cryoinjuries affecting preserved mammalian oocytes might translate into suboptimal quality in deriving embryos. While it has been reported that melatonin addition during IVM can benefit embryo quality [68,77], the morphological quality of embryos derived from vitrified oocytes in the present study remained poor, regardless of melatonin addition.

Altogether, these results lead us to think that the poor developmental competence of vitrified immature cat oocytes is not strictly linked to oxidative stress and apoptotic cell death, but other cellular injuries are likely to be involved in post-warming degeneration. In addition, there are other reports of lack of beneficial effects of melatonin on oocyte developmental competence. For instance, addition of melatonin during IVM had no effects in one study on pig fresh [78], and vitrified oocytes [79], and even if accompanied by modifications in ROS levels or gene expression, melatonin happened to have no effect on development in mice mature cryopreserved oocytes [74].

In the domestic cat, the use of antioxidants has been tested a few times on fresh (not cryopreserved) oocytes. Results generally showed beneficial effects when antioxidants such as superoxide dismutase, catalase and taurine were added to the IVM medium [20,21] and some are commonly included during maturation, such as cysteine [19], which was also included in all the IVM media in the present study. Instead, less work has been done to study the effect of antioxidants on cryopreserved oocytes, but a recent study reported a beneficial effect of resveratrol on embryo development of feline oocytes vitrified at the MII stage [80]. The choice of the meiotic stage for oocyte preservation is surely a critical factor, since mature oocytes tend to develop at higher rates than immature after cryopreservation [81,82], partly because competent oocytes are already pre-selected before the preservation procedure [81], and thus they might be more responsive to antioxidant or other ameliorative treatments. A recent abstract is the only report of the use of an antioxidant (i.e., resveratrol) on cat immature vitrified COCs, and it reported a reduction in the mitochondrial activity [83]. In addition, differences in the outcomes after antioxidant treatment might be explained because of the different action mechanisms of these compounds, compared to melatonin. Enzymatic antioxidants such as superoxide dismutase and catalase can transform the superoxide radical anion to hydrogen peroxide and hydrogen peroxide into water, respectively, while both resveratrol and melatonin can increase the expression of antioxidant and directly scavenge free radicals [64,84,85].

Although melatonin was hypothesized to support cat oocyte cryosurvival, considering its multiple, receptor-dependent and independent actions and its ability to cross the cellular membranes, more work is

needed to better address the remarkable cryosensitivity of cat oocytes, which is unfortunately well known [86]. Considering the generally promising results of antioxidants, a suitable option could be the use of alternative forms of these compounds. For instance, nanoparticles can effectively deliver the antioxidant into the cells and prolong its release compared to the standard molecule, which has a shorter half-life, and a recent study showed that melatonin-loaded nanoparticles could increase the cryotolerance of murine oocytes [87].

5. Conclusions

Vitrification induced an increase in ROS, DNA fragmentation and caspase activity in cat immature oocytes. The use of the antioxidant and antiapoptotic melatonin during vitrification-warming and IVM partially rescued vitrified oocytes from these damages, with beneficial effects on oxidative (ROS levels) and apoptotic (caspase activity) status compared to standard vitrification-warming. However, effects on oocyte developmental competence were limited, with some effects on maturation rates but no influence on embryo development or morphological quality. In order to efficiently preserve feline female germplasm, specific mechanisms of action of melatonin on cat vitrified immature oocytes remain to be investigated, as well as alternative approaches to control cryoinjuries and enhance embryo development.

CRediT authorship contribution statement

Martina Colombo: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Alessandra Mascaro:** Writing – review & editing, Methodology, Data curation. **Alessandro Pecile:** Writing – review & editing, Resources. **Jasmine Fusi:** Writing – review & editing, Resources. **Gaia Cecilia Luvoni:** Writing – review & editing, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements/Funding

This work was supported by “Piano di Sostegno alla Ricerca 2023 (Linea 2 Azione A)”, Università degli Studi di Milano. We also acknowledge financial support under the National Recovery and Resilience Plan (“PNRR”), Mission 4, Component 2, Investment 1.1, Call for tender No. 1409 published on September 14, 2022 by the Italian Ministry of University and Research (MUR), funded by the European Union – NextGenerationEU – PRIN 2022 PNRR n. P2022PRFM7 – Project Title “Bioengineered 3D ovarian follicle for biodiversity preservation - Bio3-versity” – CUP G53D23007780001 – Grant Assignment Decree No. 1377 adopted on September 01, 2023 by the Italian Ministry of Ministry of University and Research (MUR). This manuscript reflects only the authors’ views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.117566.

References

[1] Picton HM. Preservation of female fertility in humans and animal species. Anim Reprod 2018;15:301. https://doi.org/10.21451/1984-3143-AR2018-0089.
[2] Bolton RL, Mooney A, Pettit MT, Bolton AE, Morgan L, Drake GJ, et al. Resurrecting biodiversity: advanced assisted reproductive technologies and biobanking. Reprod Fertil 2022;3:R121–46. https://doi.org/10.1530/RAF-22-0005.
[3] Luvoni GC, Colombo M. Cold case: small animal gametes cryobanking. Theriogenology 2020;150:445–51. https://doi.org/10.1016/j.theriogenology.2020.02.047.
[4] Zahmel J, Jänsch S, Jewgenow K, Sandgreen D-M, Simonsen KS, Colombo M. Maturation and fertilization of African lion (panthera leo) oocytes after vitrification. Cryobiology 2021;98:146–51. https://doi.org/10.1016/j.cryobiol.2020.11.011.
[5] Cat conservation compendium | IUCN CatSG n.d. https://www.catsg.org/compendium (accessed January 2, 2025).
[6] Jewgenow K, Zahmel J. Preservation of female genetic resources in feline species. Theriogenology 2020;156:124–9. https://doi.org/10.1016/j.theriogenology.2020.06.040.
[7] Pope C. Embryo technology in conservation efforts for endangered felids. Theriogenology 2000;53:163–74.
[8] Amstislavsky S, Mokrousova V, Brusentsev E, Okotrub K, Comizzoli P. Influence of cellular lipids on cryopreservation of mammalian oocytes and preimplantation embryos: a review. Biopreserv Biobanking 2019;17:76–83. https://doi.org/10.1089/bio.2018.0039.
[9] Brambillasca F, Guglielmo MC, Coticchio G, Mignini Renzini M, Dal Canto M, Fadini R. The current challenges to efficient immature oocyte cryopreservation. J Assist Reprod Genet 2013;30:1531–9. https://doi.org/10.1007/s10815-013-0112-0.
[10] Moussa M, Shu J, Zhang X, Zeng F. Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. Sci China Life Sci 2014;57:903–14. https://doi.org/10.1007/s11427-014-4689-z.
[11] Savitskaya MA, Onishchenko GE. Apoptosis in cryopreserved eukaryotic cells. Biochemistry 2016;81:445–52. https://doi.org/10.1134/S0006297916050023.
[12] Men H, Monson RL, Parrish JJ, Rutledge JJ. Degeneration of cryopreserved bovine oocytes via apoptosis during subsequent culture. Cryobiology 2003;47:73–81.
[13] Vallorani C, Spinaci M, Bucci D, Porcu E, Tamanini C, Galeati G. Pig oocyte vitrification by cryotop method and the activation of the apoptotic cascade. Anim Reprod Sci 2012;135:68–74. https://doi.org/10.1016/j.anireprosci.2012.08.020.
[14] Trapphoff T, Heiligentag M, Simon J, Staubach N, Seidel T, Otte K, et al. Improved cryotolerance and developmental potential of in vitro and in vivo matured mouse oocytes by supplementing with a glutathione donor prior to vitrification. Mol Hum Reprod 2016;22:867–81.
[15] Pan B, Yang H, Wu Z, Qazi IH, Liu G, Han H, et al. Melatonin improves parthenogenetic development of vitrified–warmed mouse oocytes potentially by promoting G1/S cell cycle progression. Int J Mol Sci 2018;19. https://doi.org/10.3390/ijms19124029.
[16] Zhao XM, Hao HS, Du WH, Zhao SJ, Wang HY, Wang N, et al. Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. J Pineal Res 2016;60:132–41. https://doi.org/10.1111/jpi.12290.
[17] Colombo M, Zahmel J, Jänsch S, Jewgenow K, Luvoni GC. Inhibition of apoptotic pathways improves DNA integrity but not developmental competence of domestic cat immature vitrified oocytes. Front Vet Sci 2020;7:588334. https://doi.org/10.3389/fvets.2020.588334.
[18] Arayatham S, Tiptanavattana N, Tharasanit T. Effects of vitrification and a rho-associated coiled-coil containing protein kinase 1 inhibitor on the meiotic and developmental competence of feline oocytes. J Reprod Dev 2017;63:511–7. https://doi.org/10.1262/jrd.2017-004.
[19] Luvoni GC, Colombo M, Morselli MG. The never-ending search of an ideal culture system for domestic cat oocytes and embryos. Reprod Domest Anim 2018;53:110–6. https://doi.org/10.1111/rda.13331.
[20] Cocchia N, Tafuri S, Del Prete C, Palumbo V, Esposito L, Avallone L, et al. Antioxidant supplementation to medium for in vitro embryo production in Felis catus. Pol J Vet Sci 2019;22:573–9. https://doi.org/10.24425/pjvs.2019.129966.
[21] Ochota M, Pasieka A, Nizański W. Superoxide dismutase and taurine supplementation improves in vitro blastocyst yield from poor-quality feline oocytes. Theriogenology 2016;85:922–7. https://doi.org/10.1016/J.THERIOGENOLOGY.2015.10.042.
[22] Piras AR, Ariu F, Falchi L, Zedda MT, Pau S, Schianchi E, et al. Resveratrol treatment during maturation enhances developmental competence of oocytes after prolonged ovary storage at 4°C in the domestic cat model. Theriogenology 2020;144:152–7. https://doi.org/10.1016/j.theriogenology.2020.01.009.
[23] Cruz MHC, Leal CLV, Cruz JF da, Tan DX, Reiter RJ. Role of melatonin on production and preservation of gametes and embryos: a brief review. Anim Reprod Sci 2014;145:150–60. https://doi.org/10.1016/j.anireprosci.2014.01.011.
[24] Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK, et al. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. J Pineal Res 2009;46:22–8. https://doi.org/10.1111/J.1600-079X.2008.00602.X.
[25] He B, Yin C, Gong Y, Liu J, Guo H, Zhao R. Melatonin-induced increase of lipid droplets accumulation and in vitro maturation in porcine oocytes is mediated by mitochondrial quiescence. J Cell Physiol 2018;233:302–12. https://doi.org/10.1002/jcp.25876.
[26] Shi JM, Tian XZ, Zhou G Bin, Wang L, Gao C, Zhu SE, et al. Melatonin exists in porcine follicular fluid and improves in vitro maturation and parthenogenetic development of porcine oocytes. J Pineal Res 2009;47:318–23. https://doi.org/10.1111/J.1600-079X.2009.00717.X.
[27] Do LTK, Shibata Y, Taniguchi M, Nii M, Nguyen TV, Tanihara F, et al. Melatonin supplementation during in vitro maturation and development supports the

development of porcine embryos. Reprod Domest Anim 2015;50:1054–8. https://doi.org/10.1111/RDA.12607.
[28] El-Raey M, Geshi M, Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE, et al. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. Mol Reprod Dev 2011;78:250–62. https://doi.org/10.1002/mrd.21295.
[29] Zhao XM, Wang N, Hao HS, Li CY, Zhao YH, Yan CL, et al. Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. J Pineal Res 2018;64. https://doi.org/10.1111/jpi.12445.
[30] Pang Y, Zhao S, Sun Y, Jiang X, Hao H, Du W, et al. Protective effects of melatonin on the in vitro developmental competence of bovine oocytes. Anim Sci J 2018;89:648–60. https://doi.org/10.1111/asj.12970.
[31] Keshavarzi S, Salehi M, Farifteh-Nobijari F, Hosseini T, Hosseini S, Ghazifard A, et al. Melatonin modifies histone acetylation during in vitro maturation of mouse oocytes. Cell J 2018;20:244–9. https://doi.org/10.22074/CELLJ.2018.4860.
[32] Wei D, Zhang C, Xie J, Song X, Yin B, Liu Q, et al. Supplementation with low concentrations of melatonin improves nuclear maturation of human oocytes in vitro. J Assist Reprod Genet 2013;30:933–8. https://doi.org/10.1007/S10815-013-0021-2.
[33] Saeedabadi S, Abazari-Kia AH, Rajabi H, Parivar K, Salehi M. Melatonin improves the developmental competence of goat oocytes. Int J Fertil Steril 2018;12:157–63. https://doi.org/10.22074/IJFS.2018.5204.
[34] Fathi M, Salama A, El-Shahat KH, EL-Sherbiny HR, Abdelnaby EA. Effect of melatonin supplementation during IVM of dromedary camel oocytes (Camelus dromedarius) on their maturation, fertilization, and developmental rates in vitro. Theriogenology 2021;172:187–92. https://doi.org/10.1016/J.THERIOGENOLOGY.2021.05.021.
[35] Zhang Y, Li W, Ma Y, Wang D, Zhao X, Zeng C, et al. Improved development by melatonin treatment after vitrification of mouse metaphase II oocytes. Cryobiology 2016;73:335–42. https://doi.org/10.1016/j.cryobiol.2016.09.171.
[36] Wu Z, Pan B, Qazi IH, Yang H, Guo S, Yang J, et al. Melatonin improves in vitro development of vitrified-warmed mouse germinal vesicle oocytes potentially via modulation of spindle sssembly checkpoint-related genes. Cells 2019;8. https://doi.org/10.3390/CELLS8091009.
[37] Yang J, Guo S, Pan B, Qazi IH, Qin J, Zang S, et al. Melatonin promotes in vitro maturation of vitrified-warmed mouse GV oocytes potentially by modulating MAD2 protein expression of SAC component through MTRs. Cryobiology 2021;102:82–91. https://doi.org/10.1016/J.CRYOBIOL.2021.07.008.
[38] Guo S, Yang J, Qin J, Qazi IH, Pan B, Zang S, et al. Melatonin promotes in vitro maturation of vitrified-warmed mouse germinal vesicle oocytes, potentially by reducing oxidative stress through the Nrf2 pathway. Animals 2021;11. https://doi.org/10.3390/ANI11082324.
[39] Zhang Z, Mu Y, Ding D, Zou W, Li X, Chen B, et al. Melatonin improves the effect of cryopreservation on human oocytes by suppressing oxidative stress and maintaining the permeability of the oolemma. J Pineal Res 2021;70. https://doi.org/10.1111/JPI.12707.
[40] Zhang C, Yang D, Ding D, Fan Y, Yang H, Wang J, et al. Melatonin application during cryopreservation improves the development and clinical outcomes of human vitrified-warmed oocytes. Cryobiology 2024;115. https://doi.org/10.1016/J.CRYOBIOL.2024.104902.
[41] Clérico G, Taminelli G, Veronesi JC, Polola J, Pagura N, Pinto C, et al. Mitochondrial function, blastocyst development and live foals born after ICSI of immature vitrified/warmed equine oocytes matured with or without melatonin. Theriogenology 2021;160:40–9. https://doi.org/10.1016/J.THERIOGENOLOGY.2020.10.036.
[42] Kandil OM, Rahman SMA El, Ali RS, Ismail EA, Ibrahim NM. Effect of melatonin on developmental competence, mitochondrial distribution, and intensity of fresh and vitrified/thawed in vitro matured buffalo oocytes. Reprod Biol Endocrinol 2024;22. https://doi.org/10.1186/S12958-024-01209-7.
[43] Wood TC, Wildt DE. Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. J Reprod Fertil 1997;110:355–60.
[44] Jewgenow K, Fernandez-Gonzalez L, Jänsch S, Viertel D, Zahmel J. Brilliant cresyl blue staining allows the selection for developmentally competent immature feline oocytes. Theriogenology 2019;126:320–5. https://doi.org/10.1016/j.theriogenology.2018.12.021.
[45] Nadri P, Zahmatkesh A, Bakhtari A. The potential effect of melatonin on in vitro oocyte maturation and embryo development in animals. Biol Reprod 2024;111:529–42. https://doi.org/10.1093/biolre/ioae077.
[46] Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online 2005;11:300–8. https://doi.org/10.1016/S1472-6483(10)60837-1.
[47] Cobo A, Domingo J, Pérez S, Crespo J, Remohí J, Pellicer A. Vitrification: an effective new approach to oocyte banking and preserving fertility in cancer patients. Clin Transl Oncol 2008;10:268–73. https://doi.org/10.1007/s12094-008-0196-7.
[48] Colombo M, Luvoni GC. Minimum volume vitrification of immature feline oocytes. JoVE J 2020;160:e61523. https://doi.org/10.3791/61523.
[49] Cavallari FDC, Leal CLV, Zvi R, Hansen PJ. Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during in vitro maturation. Zygote 2019;27:180–6. https://doi.org/10.1017/S0967199419000236.
[50] Schneider CA, Rasband WS, Eliceiri KW. NIH image to ImageJ: 25 years of image analysis. Nat Methods 2012;9:671–5. https://doi.org/10.1038/nmeth.2089.
[51] Chaube SK, Prasad PV, Thakur SC, Shrivastav TG. Hydrogen peroxide modulates meiotic cell cycle and induces morphological features characteristic of apoptosis in rat oocytes cultured in vitro. Apoptosis 2005;10:863–74. https://doi.org/10.1007/s10495-005-0367-8.
[52] Zhou Z, Jia R-X, Zhang G, Wan Y, Zhang Y, Fan Y, et al. Using cysteine/cystine to overcome oxidative stress in goat oocytes and embryos cultured in vitro. Mol Med Rep 2016;14:1219–26. https://doi.org/10.3892/mmr.2016.5395.
[53] Linde-Forsberg C. Regulations and recommendations for international shipment of chilled and frozen canine semen. Recent Adv Small Anim Prod 2001;21:1–5.
[54] Buranaamnuay K. Determination of appropriate cryopreservation protocols for epididymal cat spermatozoa. Reprod Domest Anim 2015;50:378–85. https://doi.org/10.1111/rda.12496.
[55] Colombo M, Morselli MG, Zahmel J, Luvoni GC. Ultra-rapid freezing preserves morphofunctional integrity and fertilizing ability of epididymal cat spermatozoa. Front Vet Sci 2022;9:866953. https://doi.org/10.3389/fvets.2022.866953.
[56] Stringfellow DA, Givens MD, editors. Manual of the international embryo transfer Society. fourth ed. Champaign, Illinois 61822 USA: International Embryo Transfer Society; 2010.
[57] Bolamba D, Borden-Russ KD, Durrant BS. In vitro maturation of domestic dog oocytes cultured in advanced preantral and early antral follicles. Theriogenology 1998;49:933–42.
[58] Hewitt DA, England GCW. Synthetic oviductal fluid and oviductal cell coculture for canine oocyte maturation in vitro. Anim Reprod Sci 1999;55:63–75. https://doi.org/10.1016/S0378-4320(98)00162-6.
[59] Sananmuang T, Tharasanit T, Nguyen C, Phutikanit N, Techakumphu M. Culture medium and embryo density influence on developmental competence and gene expression of cat embryos. Theriogenology 2011;75:1708–19. https://doi.org/10.1016/j.theriogenology.2011.01.008.
[60] Yong W, Ma H, Na M, Gao T, Zhang Y, Hao L, et al. Roles of melatonin in the field of reproductive medicine. Biomed Pharmacother 2021;144. https://doi.org/10.1016/j.biopha.2021.112001.
[61] Marcantonini G, Bartolini D, Zatini L, Costa S, Passerini M, Rende M, et al. Natural cryoprotective and cytoprotective agents in cryopreservation: a focus on melatonin. Molecules 2022;27. https://doi.org/10.3390/molecules27103254.
[62] Colombo M, Alkali IM, Luvoni GC. Microenvironment factors promoting the quality of vitrified cat oocytes. Theriogenology 2022;196:275–83. https://doi.org/10.1016/J.THERIOGENOLOGY.2022.11.027.
[63] Cao B, Qin J, Pan B, Qazi IH, Ye J, Fang Y, et al. Oxidative stress and oocyte cryopreservation: recent advances in mitigation strategies involving antioxidants. Cells 2022;11. https://doi.org/10.3390/cells11223573.
[64] Silva BR, Silva JRV. Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during in vitro follicle growth, oocyte maturation, and embryo development. Anim Reprod Sci 2023;249. https://doi.org/10.1016/j.anireprosci.2022.107186.
[65] Sampaio RV, Conceição DSB, Miranda MS, Sampaio L, de FS, Ohashi OM. MT3 melatonin binding site, MT1 and MT2 melatonin receptors are present in oocyte, but only MT1 is present in bovine blastocyst produced in vitro. Reprod Biol Endocrinol 2012;10:1–7. https://doi.org/10.1186/1477-7827-10-103/TABLES/1.
[66] Lee S, Jin JX, Taweechaipaisankul A, Kim GA, Lee BC. Stimulatory effects of melatonin on porcine in vitro maturation are mediated by MT2 receptor. Int J Mol Sci 2018;19. https://doi.org/10.3390/IJMS19061581.
[67] Tian X, Wang F, Zhang L, He C, Ji P, Wang J, et al. Beneficial effects of melatonin on the in vitro maturation of sheep oocytes and its relation to melatonin receptors. Int J Mol Sci 2017;18. https://doi.org/10.3390/IJMS18040834.
[68] Soto-Heras S, Catalá MG, Roura M, Menéndez-Blanco I, Piras AR, Izquierdo D, et al. Effects of melatonin on oocyte developmental competence and the role of melatonin receptor 1 in juvenile goats. Reprod Domest Anim 2019;54:381–90. https://doi.org/10.1111/RDA.13378.
[69] Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M, Qin L. Melatonin as an antioxidant: under promises but over delivers. J Pineal Res 2016;61:253–78. https://doi.org/10.1111/JPI.12360.
[70] Deluao JC, Winstanley Y, Robker RL, Pacella-Ince L, Gonzalez MB, McPherson NO. Oxidative stress and reproductive function: reactive oxygen species in the mammalian pre-implantation embryo. Reproduction 2022;164:F95–108. https://doi.org/10.1530/REP-22-0121.
[71] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 2005;3:28. https://doi.org/10.1186/1477-7827-3-28.
[72] Alkali IM, Colombo M, Luvoni GC. Melatonin reduces oxidative stress and improves follicular morphology in feline (Felis catus) vitrified ovarian tissue. Theriogenology 2024;224:58–67. https://doi.org/10.1016/j.theriogenology.2024.05.011.
[73] Wu Z, Pan B, Qazi IH, Yang H, Guo S, Yang J, et al. Melatonin improves in vitro development of vitrified-warmed mouse germinal vesicle oocytes potentially via modulation of spindle assembly checkpoint-related genes. Cells 2019;8:1009. https://doi.org/10.3390/cells8091009.
[74] Li W, Cheng K, Zhang Y, Meng Q, Zhu S, Zhou G. No effect of exogenous melatonin on development of cryopreserved metaphase II oocytes in mouse. J Anim Sci Biotechnol 2015;6. https://doi.org/10.1186/s40104-015-0041-0.
[75] Turathum B, Roytrakul S, Changsangfa C, Sroyraya M, Tanasawet S, Kitiyanant Y, et al. Missing and overexpressing proteins in domestic cat oocytes following vitrification and in vitro maturation as revealed by proteomic analysis. Biol Res 2018;51:27. https://doi.org/10.1186/s40659-018-0176-5.
[76] Balaban B, Urman B. Effect of oocyte morphology on embryo development and implantation. Reprod Biomed Online 2006;12:608–15. https://doi.org/10.1016/S1472-6483(10)61187-X.

[77] Marques TC, da Silva Santos EC, Diesel TO, Leme LO, Martins CF, Dode MAN, et al. Melatonin reduces apoptotic cells, SOD2 and HSPB1 and improves the in vitro production and quality of bovine blastocysts. Reprod Domest Anim 2018;53: 226–36. https://doi.org/10.1111/RDA.13097.
[78] Martinez CA, Cuello C, Parrilla I, Maside C, Ramis G, Cambra JM, et al. Exogenous melatonin in the culture medium does not affect the development of in vivo-derived pig embryos but substantially improves the quality of in vitro-produced embryos. Antioxidants 2022;11. https://doi.org/10.3390/ANTIOX11061177.
[79] Gonzalez-Plaza A, Brullo C, Cambra JM, Garcia M, Iacono E, Parrilla I, et al. Equilibration time with cryoprotectants, but not melatonin supplementation during in vitro maturation, affects viability and metaphase plate morphology of vitrified porcine mature oocytes. Reprod Domest Anim 2022;57(Suppl 5):58–63. https://doi.org/10.1111/RDA.14158.
[80] Nowak A, Kochan J, Gabryś J, Kij-Mitka B, Witarski W, Prochowska S, et al. The effect of resveratrol on the developmental competence of feline oocytes vitrified at the metaphase II stage. Theriogenology 2024;230:101–6. https://doi.org/10.1016/J.THERIOGENOLOGY.2024.08.028.
[81] Luvoni GC, Pellizzari P. Embryo development in vitro of cat oocytes cryopreserved at different maturation stages. Theriogenology 2000;53:1529–40.
[82] Sowińska N, Zahmel J, Niżański W, Hribal R, Fernandez-Gonzalez L, Jewgenow K. Meiotic status does not affect the vitrification effectiveness of domestic cat oocytes. Animals 2020;10:E1371. https://doi.org/10.3390/ani10081371.
[83] De Almeida Oliveira T, Leal GR, Pedrosa de Paula Guimarães M, Francisco Leodido Correia L, Lucia Rosa e Silva Maia A, Gonçalves Souza-Fabjan JM. The effect of resveratrol during immature oocyte vitrification on the mitochondrial activity in feline. Anim Reprod 2022;19:22163.
[84] Reiter RJ. Melatonin: lowering the high price of free radicals. News Physiol Sci 2000;15:246–50. https://doi.org/10.1152/PHYSIOLOGYONLINE.2000.15.5.246.
[85] Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: a review. Eur J Med Chem 2015;97:55–74. https://doi.org/10.1016/J.EJMECH.2015.04.040.
[86] Tharasanit T, Thuwanut P. Oocyte cryopreservation in domestic animals and humans: principles, techniques and updated outcomes. Animals 2021;11. https://doi.org/10.3390/ani11102949.
[87] Lee S, Kim HJ, Cho HB, Kim HR, Lee S, Park JI, et al. Melatonin loaded PLGA nanoparticles effectively ameliorate the in vitro maturation of deteriorated oocytes and the cryoprotective abilities during vitrification process. Biomater Sci 2023;11: 2912–23. https://doi.org/10.1039/D2BM02054H.