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# Lipid modulation during IVM increases the metabolism and improves the cryosurvival of cat oocytes

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ABSTRACT

This study investigated the time course of lipid accumulation during IVM and assessed the role of lipid modulators added during IVM on lipid content, nuclear maturation, oxidative stress, mitochondrial activity, gene expression, and cryosurvival of cat oocytes. First, the lipid content of immature COCs was compared to those subjected to different IVM duration times (24, 28, and 32 h). Then, the lipid content was investigated after the use of different lipid modulators [conjugated linoleic acid (CLA), forskolin (FSK), L-carnitine (LC)]. Subsequently, both the CONTROL group and MIX 18 (CLA+FSK+LC) were compared regarding nuclear maturation, mitochondrial activity, reactive oxygen 19 species (ROS), and glutathione (GSH) levels, to the expression of SDHA, GDF9, BMP15, ZAR-1, 20 PRDX1, SIRT1, and SIRT3 genes (normalized by ACTB and YWHAZ genes); and to vitrification and 21 post-warming viability assessment. When not using any lipid modulator, an increase ($P < 0.05$) in lipid content could be observed after 28 h of IVM. The MIX group showed the greatest ($P < 0.05$) reduction in oocyte lipid content after 28 h of IVM. No difference ($P > 0.05$) was observed in the MII rate in the CONTROL (45%) and MIX (41%) groups and in mitochondrial activity ($(1.00 \pm 0.35$ A U vs $1.19 \pm 0.14$ A U). Although ROS and GSH levels were higher ($P < 0.05$) in MIX than in CONTROL, the redox balance (ROS/GSH) was greater ($P < 0.05$) in the latter (C:$1.00 \pm 0.20^{b}$ vs M:$0.26 \pm 0.06^{a}$ A.U). The *GDF9, HSP70, PRDX1*, and *SIRT1* transcripts were downregulated ($P < 0.05$) in MIX-oocytes, compared to the CONTROL. After vitrification, MIX (74%) presented a higher ($P < 0.05$) viability compared to control (53%). In conclusion, MIX can reduce the total lipid content and improve viability after cryopreservation, however, it seems to affect the oocyte metabolism in a way that still needs to be better understood in the cat biological model.

## 1. Introduction

The domestic cat is an important experimental model for assisted reproductive technologies focused on endangered felids conservation. In this sense, *in vitro* embryo production (IVEP) and cryopreservation represent valuable tools [1,2], however, despite many advances achieved in IVEP, the efficiency of the oocyte *in vitro* maturation (IVM) still limits the application of this biotechnology [3]. Intracellular lipids provide energy for oocyte maturation and the subsequent early embryonic development [4] and exhibit a variety of important cellular functions, including membrane composition, energy storage, and cell signaling [5]. Some species have physiologically a greater amount of lipids in their oocytes, such as pigs, cattle, and cats [6,7] and despite the physiological amount and their use for metabolic functions, there are studies in cattle indicating that lipid content increases abnormally during IVM [8,9].

It is known that the amount and composition of intracellular lipids are important factors in oocyte viability during cryopreservation since one of the main sources of cell damage at chilling temperature is the lipid phase transition that can change and disrupt intracellular processes [10]. Considering that cat oocytes present a high concentration of lipids [6] and the possibility of a lipid accumulation on IVM as reported in bovine species [8], the knowledge of the lipid content behavior on IVM and its modulation became an important aspect to be considered in this

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context.

Depending on the dose and exposure time, lipid modulators may induce a high modification in intracellular lipids and/or reduce lipid content, and the combined use of lipid modulators on IVM already proved to be efficient in promoting changes in lipid content of oocytes in cattle and pigs [11–13]. Different modulators were already reported for such purposes. Forskolin (FSK), a cAMP modulator widely used in IVM of several species, can enhance lipolytic activity and induce the activation of intracellular lipases [3]. L-carnitine (LC) has antioxidant properties and stimulates the metabolism of intracellular lipid stores through the ability to upregulate β-oxidation [5]. Conjugated linoleic acid t10, c12 (CLA) can induce linoleic acid incorporation and triacylglycerols and phospholipids production which improves the lipid cellular lipid profile [14] (Fig. 1).

Even so, studies regarding lipid content and accumulation in cat oocytes are still scarce. Based on that, the aim of this study was to investigate (i) the time course of lipid accumulation during IVM; (ii) the effect of different lipid modulators during IVM on lipid content; and (iii) their role on nuclear maturation, mitochondrial activity, oxidative stress, gene expression, and cryopreservation of cat *in vitro*-matured oocytes.

## 2. Material and methods

Unless otherwise indicated, the chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Ovaries from the domestic cat were collected at local veterinary clinics, as by-products from owner-requested routine ovariosalpingohysterectomies, and for this reason, this study did not require the Ethics Committee of Animal Use approval.

### 2.1. Experimental design

#### 2.1.1. Experiment 1

This experiment was conducted to evaluate the lipid content of cat oocytes.

*1.1. First assay*: To understand the time course of lipid accumulation in this species, and considering the variation found in IVM times in cat studies, the oocytes were submitted to *in vitro* maturation for 24, 28, and 32 h before evaluating the lipid content, and were also compared with an immature group as control.

*1.2 Second assay*: After fixing the IVM time to be used, three lipid modulators (CLA, FSK, and LC) were tested and compared to the control (without lipid modulators) to evaluate the effect on the total lipid content. Concentrations of the modulators used in the present study were

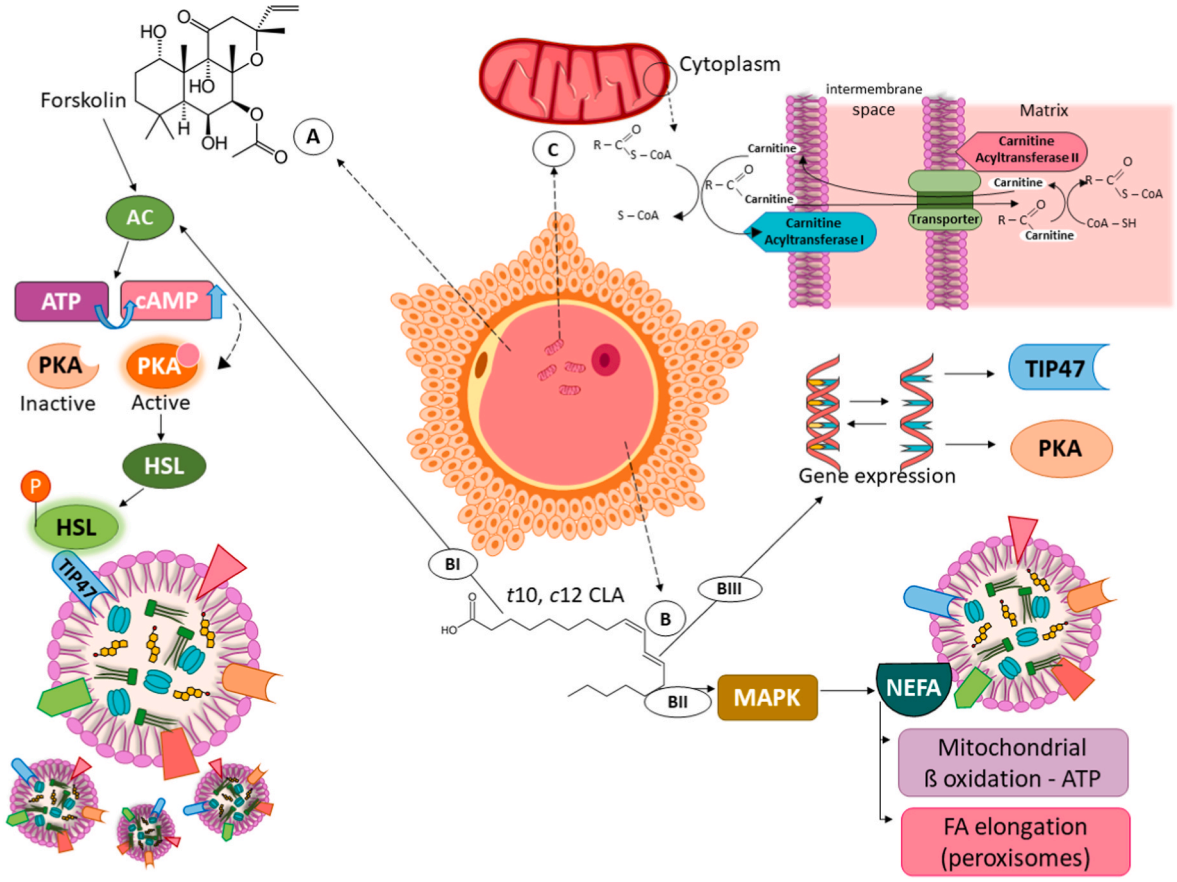


**Fig. 1. Mechanism of action of lipid modulators. (A)**When forskolin [FSK] binds to Adenyl cyclase (AC), cAMP is synthesized from ATP present in the cytoplasm, promoting an increase in cAMP levels that activates protein kinase A (PKA). PKA phosphorylates endogenous lipases such as hormone-sensitive lipase (HSL) and peripelin (TIP47) located on the surface of the lipid droplet (LD). After being phosphorylated, HSL is translocated to the cytoplasm, binding to TIP47 to induce fragmentation of LD into smaller LD, which can be more easily accessed, and the nucleus degraded. **(B)** Conjugated linoleic acid [CLA] is involved in the regulation of lipid metabolism through the stimulation of KA (BI) and mitogen-activated protein kinase (MAPK) (BII) pathways that interfere with LD lipolysis, as well as in the control of oocyte gene expression and protein synthesis, that is, perilipins (BIII). **(C)** L-carnitine [LC] acts as a lipolytic agent promoting the entry of fatty acids (FA) into the mitochondria to be used in ß-oxidation. The FA that are used in mitochondrial oxidation are temporarily linked to the LC forming fatty-acyl-carnitine. These are transferred to the intermembrane space by the action of acyltransferase I and then are displaced to the mitochondrial matrix by facilitated diffusion through the transporter present in the inner membrane of the mitochondria. In the matrix, the transfer of the acyl group to the mitochondrial coenzyme A occurs and the carnitine becomes free to return to the cytoplasm by the same transporter to carry new FA.

based on previous studies on IVM with cats for FSK [15] and LC [16] and with bovine [17] and porcine [12] for CLA.

*1.3. Third assay*: The group with the lipid modulator that showed a better response in the second assay was compared to a new group, the MIX complex group, which was supplemented with the three lipid modulators (CLA + FSK + LC) to observe if a multiple pathway lipid modulator cocktail could be even more effective in reducing lipid content (Fig. 2).

#### 2.1.2. Experiment 2

After validating the IVM time for the occurrence of lipid accumulation and the effect of reducing the lipid content with the tested group (MIX), this experiment aimed to evaluate the effect of this treatment on (2.1) nuclear maturation, (2.2) mitochondrial activity, (2.3) oxidative stress, (2.4) expression of genes related to metabolism and oocyte developmental competence, and (2.5) viability after cryopreservation (Fig. 2).

### 2.2. COCs collection and selection

During the ovariosalpingohysterectomies surgeries, the removed ovaries from each queen were placed in sterile 0.9% (w/v) NaCl solution at 4 °C. The samples were sent to the laboratory within 2–3 h after recovery. In the laboratory, ovaries were washed in phosphate-buffered saline (PBS) to remove the excess blood from surgery. Then, they were sliced and washed into a 60 mm Petri dish with a washing medium (TCM-HEPES supplemented with 3 mg/mL BSA and 0.25 mg/mL pyruvate, 0.15 mg/mL L-glutamine, 0.6 mg/mL sodium lactate, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 0.25 μg/mL amphotericin B) at 37 °C by using a scalpel blade to release COCs. The COCs were selected, and graded, and only grade I and II COCs (surrounded by, at least, two layers of *cumulus* cells with a uniform, dark, and homogeneous cytoplasm) were randomly allocated into the IVM treatments, according to the experiments, for all of them.

### 2.3. IVM and nuclear maturation assessment

For IVM, groups of oocytes were placed in an IVM medium consisting of TCM 199 supplemented with 0.02 IU/mL of FSH/LH, 100 μM of cysteamine, 2.2 g/L of sodium bicarbonate, 3 mg/mL BSA, 0.25 mg/mL sodium pyruvate, 0.15 mg/mL L-glutamine, 0.6 mg/mL sodium lactate, and 0.055 mg/mL gentamicin. COCs were incubated in a four-well dish (Ingamed, Maringá, Brazil) containing 500 μL of IVM medium at 38.5 °C, in an atmosphere with 5% $CO_2$, and in maximum humidity. Depending on the experiment, the IVM duration was different (24, 28, or 32 h). And according to the experiment and treatments, it was added (1) 100 μM CLA, (2) 100 μM FSK, (3) 0.5 mg/mL of LC, or (4) all of them (MIX).

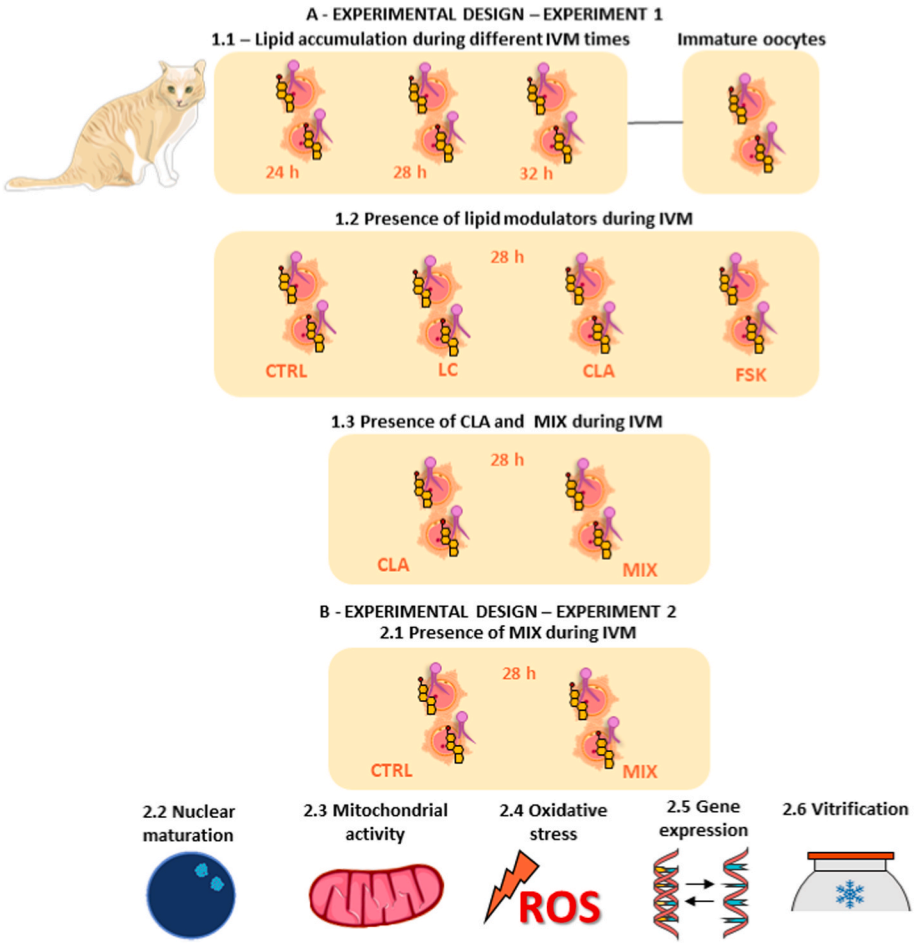


**Fig. 2. Experimental Design. (A)** Schematic illustration of experiment 1: Lipid content was evaluated and compared in immature oocytes and oocytes from different IVM time durations (24, 28, and 32 h); under the effect of three lipid modulators (conjugated linoleic acid [CLA], forskolin [FK], and L-carnitine [LC] and compared to a Control without modulators; and under the effect of conjugated linoleic acid [CLA] and the MIX group [CLA + FSK + LC]. **(B)** Schematic illustration of experiment 2: Oocytes from CONTROL and MIX IVM groups were submitted to nuclear maturation assessment; mitochondrial activity; reactive oxygen species (ROS) and glutathione (GSH) levels analysis, expression of genes related to oocyte quality and metabolism, and cryopreservation. In total, 831 oocytes were used, obtained in 23 replicates.

After IVM, COCs were denuded with hyaluronidase, fixed in 4% paraformaldehyde, stained with Hoechst 33342, and evaluated under fluorescence microscopy (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) at a wavelength between 340 and 380 nm. They were examined to evaluate the meiosis nuclear stage and the presence or absence of the first polar body, and classified as a germinal vesicle (GV) or germinal vesicle breakdown (GVB), Metaphase I (MI), and Metaphase II (MII). The nuclear maturation rate was defined as the number of oocytes at the MII stage per the total number of oocytes subjected to IVM. Oocytes with fragmented or dispersed chromatin were designated as degenerated [18].

### 2.4. Lipid content assessment

After IVM, the COCs were denuded with hyaluronidase for all analyses. The oocytes from each experimental group were fixed in 4% paraformaldehyde solution for 40 min and stored in phosphate-buffered saline at 4 °C. Structures were stained with Oil Red O solution. Oocytes were washed in a 50% ethanol solution (50%ethanol and 50% distilled water) for 2 min. Then, stained for 15 min in 2.45 mg/ml Oil Red O solution 70%/30%destilled water, and washed three times, for 5 min each, in 50% ethanol solution. After, they were kept for 5 min in distilled water before being evaluated. The images were captured with a phase contrast microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) connected to a camera (Pylon viewer, Basler AG, Exton, PA, USA). Analysis was performed using ImageJ software (NIH, USA) for the percentage of stained area from total oocyte cytoplasm on a two-dimensional image [13].

### 2.5. Mitochondrial activity

Denuded oocytes were incubated for 30 min in the dark with 0.5 nM of Mitotracker Green (Invitrogen™, Waltham, MA, USA - M7514). After incubation, they were washed in PBS supplemented with 0.1% BSA, placed on a slide, covered with a coverslip, evaluated under a fluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) and the images were captured (Pylon viewer, Basler AG, Exton, PA, USA). Average fluorescence intensity per oocyte was measured (arbitrary units, A.U.) using Image J software (NIH Image, Bethesda, MD, USA) and normalized to the background average intensity.

### 2.6. Measurement of glutathione (GSH) and reactive oxygen species (ROS) levels

Following the manufacturer's instructions, the measurement of the intracellular ROS and GSH levels was carried out by using 2′,7′-dichlorodihydro-fluorescein diacetate ($H_2$DCHFDA, Invitrogen™, D399), and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin ($CMF_2HC$, Cell Tracker Blue, Invitrogen™, C12881) that detect intracellular ROS and GSH levels as green and blue fluorescence, respectively. Groups of oocytes from each experimental group (CONTROL or MIX) were incubated in the dark for 30 min at 38.5 °C in an atmosphere with 5% $CO_2$ in PBS-BSA containing 10 μM $H_2$DCFDA and 10 μM Cell Tracker Blue. Then, they were washed in PBS-BSA, and the images were evaluated under a fluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) with UV filters (460 nm for ROS and 370 nm for GSH) and captured (Pylon viewer, Basler AG, Exton, PA, USA). Average fluorescence intensity per oocyte was measured using Image J software (NIH Image, Bethesda, MD, USA) and normalized to the background average intensity. Fluorescence analysis was performed in the same was as described above.

### 2.7. Gene expression

Oocytes from each experimental group were frozen dry in identified cryotubes free of RNase and DNase at – 196 °C until molecular analysis. The expression of *SDHA*, *HSP70 GDF9*, *BMP15*, *ZAR-1*, *PRDX1*, *SIRT1*, and *SIRT3* genes was evaluated using quantitative PCR (qPCR) associated with reverse transcription. Total RNA was extracted from three pools of 10 oocytes per treatment (CONTROL and MIX) using the RNeasy Micro Kit (Qiagen Inc., Valencia, USA) obtained in two replicates. RNA extracted from each pool was quantified using a spectrophotometer (Nanodrop Lite, ThermoFisher Scientific, Wilmington, DE, USA).

For reverse transcription, the SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) was used for all samples in the same RNA concentration, and the reverse transcription reaction was performed in a two-step mix. Reactions (20 μL total volume) were prepared using a mixture of 10 μL of GoTaq® qPCR Master Mix (2X) (Promega, Madison, WI, USA), 0.1 μM primers (Table 1), nuclease-free water, and reverse transcribed cDNA (0.5 μL). Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. Template cDNA was denatured at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. For each reaction, primer efficiency was calculated using LinRegPCR software. The primer efficiency average was: 1.88 to *SDHA* (Succinate Dehydrogenase Complex Flavoprotein Subunit A); 1.85 to *HSP70* (70 Kilodalton Heat Shock Protein); 1.85 to *GDF9* (Growth Differentiation Factor 9); 1.87 to *BMP15* (Bone Morphogenetic Protein 15); 1.86 to *ZAR1* (Zygote Arrest 1); 1.88 to *PRDX1* (Peroxiredoxin 1); 1.88 to *SIRT1* (Sirtuin 1); 1.85 to *SIRT3* (Sirtuin 3); 1.91 to *YWHAZ* (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta); and 1.90 to *ACTB* (Beta-actin). Relative quantification was performed in triplicate using qPCR (Applied Biosystems QuantStudio 3, ThermoFisher Scientific, Wilmington, DE, USA). Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software. The expression of each target gene was normalized using the geometric mean of *YWHAZ* and *ACTB* values. The values of the Pearson correlation coefficient observed for the *YWHAZ* ($r2 = 0.945$) and *ACTB* ($r2 = 0.948$) genes demonstrate stability ($p < 0.01$) of these reference genes using the BestKeeper – Excel tool according to the methodology described by Pfaffl et al. (2004) [19].

### 2.8. Cryopreservation/warming and viability assessment

Vitrification was performed according to Colombo and Luvoni (2020) [23] in five replicates after IVM treatment. Groups of five to eight COCs matured *in vitro* from each experimental group were equilibrated (TCM199 + 20% FBS with 7.5% EG and 7.5% DMSO) for 15 min and then, transferred to a vitrification solution (TCM199 + 20% FBS, with 15% EG, 15% DMSO, and 0.5 M of sucrose), in a maximum time of 90 s;

**Table 1**
Details of primers used for the gene expression analysis (sequences and references of the primers used to the PCR in real time of fresh oocytes from MIX and CONTROL groups).

| Gene | Primer sequence | Reference |
|---|---|---|
| *SDHA* | F: AACCTGATGCTTTGTGCTCTGC<br>R: TCGTCAACCCTCTCCTTGAAGT | ENSBTAG00000046019 |
| *GDF9* | F: CAGCCAGATGACAGAGCTTTGAG<br>R: CACTGATGGAAGGGTTCCTGCT | [20] |
| *BMP15* | F: GCCTCGGATCTTAGGGCATC<br>R: TATGTGCCAGGAGCCTCTGA | [20] |
| ZAR-1 | F: CATCCGATGGGAAAGTGCCT<br>R: GCTGTCACAGGATAGGCGTT | XM_023252238.2 |
| *PRDX1* | F: CCCCACGGAGATCATTGCTT<br>R: AATGGTACGCTTGGGGTCTG | XM_003990035.5 |
| *SIRT1* | F: CGCCTTGCAATAGACTTCCC<br>R: GAATTTGTGACAGAGAGATGGTTG | [21] |
| *SIRT3* | F: TGCTTCTGCGGCTCTACAC<br>R: TGTCTCCCAAAGAACACGA | [21] |
| *YWHAZ* | F: GAAGAGTCCTACAAAGACAGCACGC<br>R: AATTTTCCCCTCCTTCTCCTGC | [22] |
| *ACTB* | F: GCCAACCGTGAGAAGATGACT<br>R: CCCAGAGTCCATGACAATACCAG | [21] |

and placed in a Cryotop® device (Kizato, Fuji, Japan) using a minimal volume. The device was immediately immersed in liquid nitrogen and stored until warming.

For warming, the Cryotop® device was removed from liquid nitrogen and immersed in a sequence of three solutions of TCM199 + 20% FBS with decreasing concentrations of sucrose (1 M; 0.5 M; and 0 M) for 1 min, 3 min, and 5 min at 38 °C. Viability assessment was evaluated in two ways: by the COC morphological quality and by Neutral Red (N4638) staining after incubation for 30 min at 15 μg/mL.

Regarding morphological quality, COCs were evaluated under a stereomicroscope (SMZ-800, Nikon®, Tokyo, Japan) and when presenting regular morphology, homogeneous cytoplasm, and no signs of degeneration in the cytoplasm or *cumulus* cells were considered to be of good quality (Grade I or II); when presenting cytoplasm with signs of degeneration, irregular morphology, and rupture of the zona pellucida were considered unviable [24]. Neutral Red staining pattern was read as "viable" when the oocyte was stained (Fig. 3), or non-viable when the oocyte was not stained since it is selectively absorbed by lysosomes [25, 26].

### 2.9. Statistics

The nuclear maturation rate (MII) was evaluated by Fisher's exact test. The viability rates (morphological and metabolic) measured after warming were also evaluated by Fisher's exact test. In the first and the second assays (4 experimental groups) from the first experiment, the results regarding lipid content were submitted to ANOVA, and the Tukey-Kramer test was used for comparison among groups. In the third assay (2 experimental groups), results from lipid content were analyzed by unpaired Student's t-test. Results from mitochondrial activity, GSH and ROS levels, and REDOX balance were also evaluated by unpaired Student's t-test. Regarding cryopreservation, Fisher's exact test was used to evaluate the results from metabolic and morphologic measurements. Analyses were performed in GraphPad INSTAT software Inc. (San Diego, CA, USA), at a significance level of 5%.

## 3. Results

### 3.1. Experiment 1

#### 3.1.1. IVM generates lipid accumulation in cat oocytes after 28 h

To answer whether there is a process of lipid accumulation in the oocyte of domestic cats with advancing IVM time, the lipid content of oocytes was evaluated at 24, 28, and 32 h of IVM and compared with immature (GI) oocytes. The oocytes from GI (46.4%) had a lower ($P < 0.05$) lipid content compared to those of G28 (72.3%) and G32 (74.7%), and similar ($P > 0.05$) to 24 h (67.7%), although the lipid content had increased about 50% in the latter. Considering specifically the three IVM time points (24, 28, and 32 h), no difference ($P > 0.05$) was observed in lipid content (Fig. 4A and B).

#### 3.1.2. CLA can reduce oocyte lipid content during IVM

After establishing the lipid accumulation significantly increased at 28 h of IVM, this assay aimed to use lipid modulators to reduce the lipid accumulation. For that, oocytes were allocated into three experimental groups according to the lipid modulator tested: CONTROL, IVM without any modulator, CLA, FSK, LC, and submitted in IVM for 28 h. Regarding the lipid content, there was no difference ($P > 0.05$) among CONTROL (70.5%), FSK (72.0%), and LC (65.3%) groups. However, the CLA (49.2%) group presented a significant decrease ($P < 0.05$) in the lipid content compared to CONTROL and FSK as can be seen in Fig. 4C.

#### 3.1.3. Lipid modulation for multiple pathways (MIX) was more efficient in reducing oocyte lipid accumulation during IVM

After finding that CLA had a more potent effect in reducing lipid content than the other modulators, we checked whether the combination of modulators would have an even more potent effect in this sense. There was a significant ($P < 0.05$) reduction in oocyte lipid content from the MIX (54.0%) when compared with the CLA (69.1%) group, showing that the combined use of lipid modulators was even more efficient in reducing lipid accumulation (Fig. 4D).

### 3.2. Experiment 2

After establishing a reduction in lipid content by the MIX group, the new experiment aimed to see the effect of this reduction on the maturation rate, oocyte metabolism, gene expression, and viability post-cryopreservation.

#### 3.2.1. The combined use of lipid modulators (MIX) did not impact MII rates

As can be seen in Table 2, there was no difference ($P > 0.05$) in MII or the degenerate rate at 28 h of IVM in the CONTROL and MIX groups.

#### 3.2.2. Mitochondrial activity was not affected by the MIX complex

No difference ($P > 0.05$) was observed between CONTROL (1.00 ± 0.35 A U.) and MIX (1.19 ± 0.14 A U.) in mitochondrial activity as can be seen in Fig. 5A.

#### 3.2.3. GSH and ROS levels were highly impacted by the combined use of lipid modulators (MIX)

Oocytes were evaluated and MIX presented higher ($P < 0.05$) levels of ROS (C:1.00 ± 0.16 vs M:6.84 ± 1.94 A U.) and GSH (C:1.00 ± 0.3 vs M:19.62 ± 4.61 A U.) when compared to CONTROL (Fig. 4). However, the redox balance (ROS/GSH) was greater ($P < 0.05$) in the CONTROL group (C:1.00 ± 0.20 vs M:0.26 ± 0.06 A U.)(Fig. 5B–D).

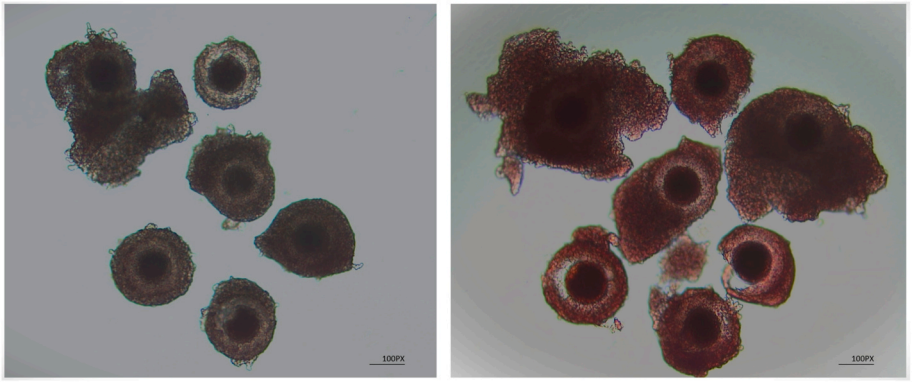


**Fig. 3. Illustration of Neutral Red Staining. (Left)** Morphological viable COCs non submitted to Neutral Red Staining. **(Right)** Morphological viable COCs submitted to Neutral Red Staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

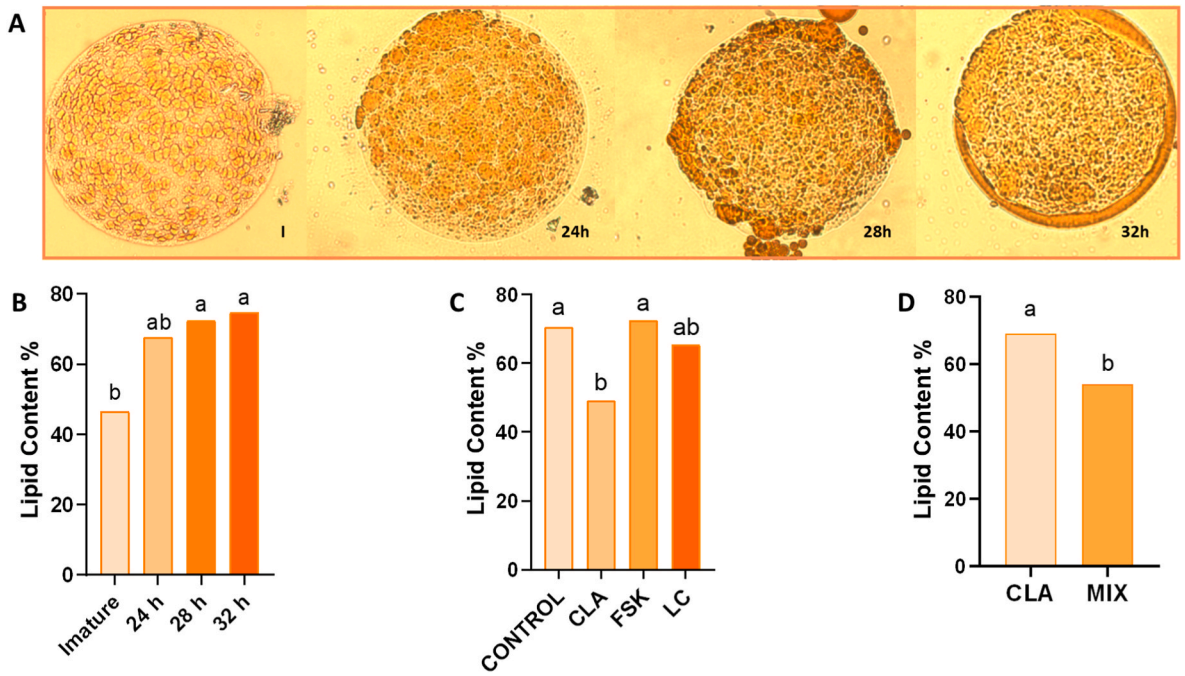


**Fig. 4. Lipid content. (A)** Illustration of oocyte lipid content after Oil red staining in immature oocytes and during 24, 28, or 32h of IVM. **(B-D).** Graphs showing the total oocyte lipid content according to the experiment: (B) among 24, 28, 32 h of IVM and immature oocytes; **(C)** among conjugated linoleic acid [CLA], forskolin [FSK], L-carnitine [LC], and CONTROL groups; and **(D)** between conjugated linoleic acid [CLA] and MIX [CLA + FSK + LC]. Different letters show statistical difference ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**
Effect of *in vitro* maturation (IVM) treatment (MIX or CONTROL) on nuclear maturation of good quality (Grade 1 and 2) oocytes obtained from ovaries after elective surgeries in the domestic cat species.

| IVM GROUP | GV(GVB) | MI | MII | DG | TOTAL |
|---|---|---|---|---|---|
| | %/(n) | %/(n) | %/(n) | %/(n) | |
| CONTROL | 8.4% (8) | 36.8% (35) | 45.2% (43) | 9.4% (9) | 95 |
| MIX | 10.2% (8) | 46.1% (36) | 41.0% (32) | 2.5% (2) | 78 |

n, number of oocytes evaluated; GV, germinal vesicle; GVB, germinal vesicle breakdown; MI, Metaphase I; MII, Metaphase II; DG, degenerated. (Fisher's exact test, at $P > 0.05$).
173 oocytes were obtained in four replicates.

### 3.2.4. *MIX complex downregulated the expression of evaluated genes related to oocyte metabolism and quality*

*HSP70, GDF9, PRDX1,* and *SIRT1* were downregulated ($P < 0.05$) in the MIX group compared to CONTROL; and there were no differences between groups regarding *SDHA*, *BPM15, SIRT3*, and *ZAR1* (Fig. 6).

### 3.2.5. *MIX complex improved oocyte viability after cryopreservation*

After warming, oocytes were morphologically evaluated and assessed for viability by Neutral Red (Table 3). According to the staining, the MIX complex presented a higher ($P < 0.05$) viability rate (73.4% vs 53.6%) after warming than CONTROL; and viability was also higher ($P > 0.05$) in the MIX group through morphological evaluation (72.8% vs 48.3%).

## 4. Discussion

Although some studies reported that lipids can accumulate during IVM in other species, such as cattle [8,9], studies regarding the changes in the lipid content or profile in cats are scarce. Considering that lipid accumulation has been associated with a greater sensitivity to cryopreservation [14,27], lipid modulation could be an important tool in the IVM of domestic cats since this animal model presents relevance in the conservation scenario.

Lipid uptake significantly influences cell metabolism [28] and it is well known that domestic cat oocytes already present a naturally high content of intracellular lipids [6]. So, this study investigated the oocyte lipid accumulation during IVM, and according to the results, it increased at 28 h in our experimental conditions. Even so, there is a lack of knowledge regarding the meaning of this increase in this species. Although the lipid accumulation during IVM seems to be abnormal in species such as cattle, since oocytes matured *in vivo* do not present this accumulation [8], the role of this accumulation in cats remains unknown.

In the bovine model, a family of lipid-binding proteins (FABP) that transport lipids intra and extracellularly during IVM was already reported as a mechanism that explains lipid accumulation in this phase [9]. Considering the possibility of a pathway that also leads to an abnormal accumulation in cats, our next step was to modulate the lipid content through three different agents. As a result, we observed that CLA was the only lipolytic agent used that created a significant reduction in total oocyte lipid content during IVM. CLA can modulate intracellular lipids through transcription mediators such as transcription factors sterol-regulatory element binding protein [14], and it already proved to be effective in reducing the lipid content in a time-dependent manner in pig oocytes [12], that also present a great amount of intracellular lipids [7].

Interestingly, FSK and LC did not reduce the total oocyte lipid content. FSK is widely used in the IVM of several species as a cAMP modulator acting in nuclear maturation, keeping oocytes in meiotic arrest [3,29], and although its role as a lipolytic modulator proved to be efficient in reducing lipid content of pig oocytes during IVM [12,30], it did not affect the lipid content of cat oocytes in the current study. FSK is a stimulator of adenylyl cyclase (AC), an enzyme that synthesizes cAMP. When FK activates AC, keeping the cAMP levels, it also induces the activation of cAMP-dependent protein kinases, which activate intracellular lipases via phosphorylation [3]. However, there is evidence that FSK stimulates the resumption of meiosis in cat oocytes [15], that is, an opposite effect to that reported in other species [29,31], and considering

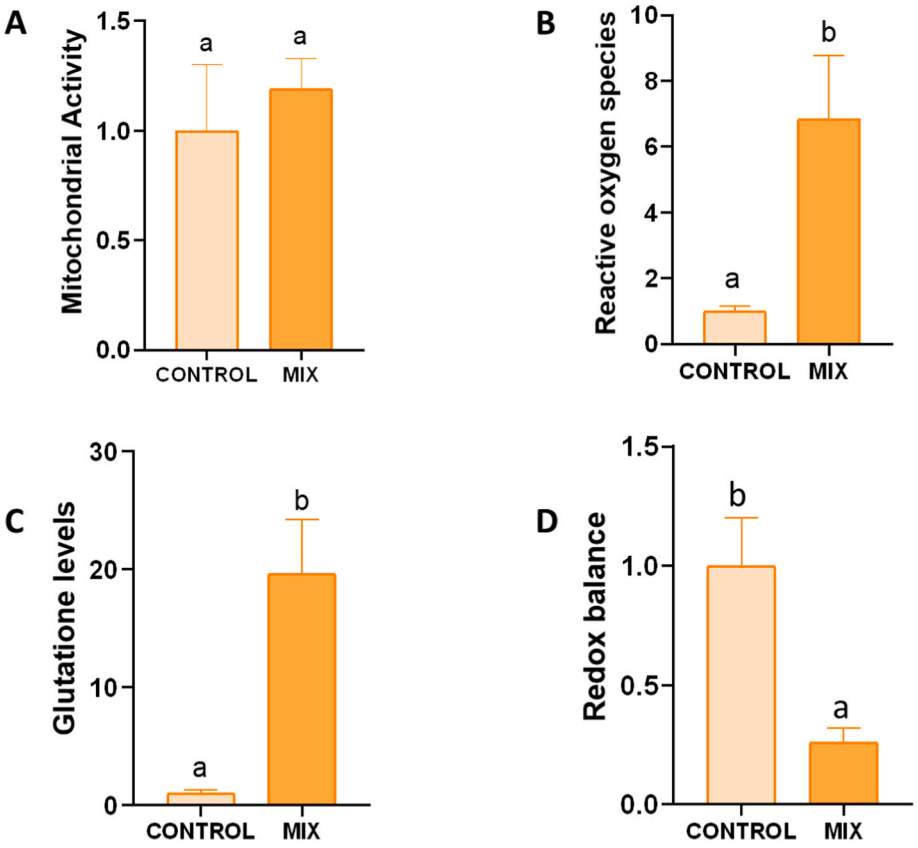


**Fig. 5. Oocyte metabolism**. Graphs showing the relative fluorescence intensity levels (Mean ± SEM of arbitrary units) regarding **(A)** mitochondrial activity, the intracellular levels of **(B)** reactive oxygen species (ROS) and **(C)** glutathione (GSH), and the **(D)** balance redox between oocytes derived from MIX and CONTROL IVM treatments. Different letters show statistical differences ($p < 0.05$).

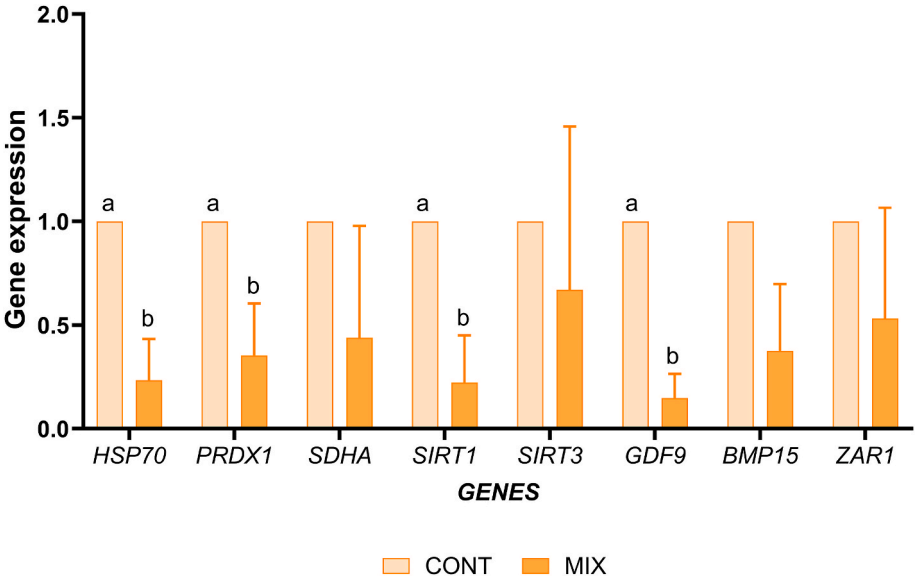


**Fig. 6. Gene Expression.** Gene expression of 70 kilodalton heat shock protein (*HSP70*), peroxiredoxin 1 (*PRDX1*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), sirtuin 1 (*SIRT1*), sirtuin 3 (*SIRT3*), growth/differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), zygote arrest 1 (*ZAR1*) on oocytes derived from MIX and CONTROL IVM treatments. Different letters show statistical differences ($p < 0.05$).

the lipid pathway of FSK, this could be an answer for the result found in this study.

LC was not able to reduce the total lipid content in the cat oocyte, however, it can modify the lipid droplets in pigs [32] and their distribution in bovine oocytes during IVM [33]. Even so, LC is considered an important co-factor in IVM since it can improve oocyte quality via both β-oxidation increasing and reducing oxidative stress [34]. Then, we checked if the MIX complex could be even more effective in reducing lipid content than only the CLA, and according to the results, its use during IVM was more efficient in reducing oocyte lipid accumulation of

**Table 3**
Effect of *in vitro* maturation (IVM) treatment (MIX or CONTROL) on morphological and metabolic viability post cryopreservation of cat oocytes.

| IVM system | Status | n | Metabolic Viability rate (%/n) | n | Morphological viability rate (%/n) |
|---|---|---|---|---|---|
| CONTROL | Fresh | 77 | 87.0% (67) aA | – | – |
| | Vitrified | 123 | 53.6% (66) bA | 124 | 48.3% (60) A |
| MIX | Fresh | 69 | 97.1% (67) aA | – | – |
| | Vitrified | 113 | 73.4% (83) bB | 114 | 72.8% (83) B |

384 oocytes obtained in three replicates; n: Number of oocytes evaluated.
MIX: IVM media with conjugated linoleic acid, forskolin, and L-carnitine.
The "metabolic viability rate" was calculated considering the Neutral Red staining pattern in which the oocyte was considered "viable" when its cytoplasm was stained, or "non-viable" when it was not stained; the "morphological viability rate" was calculated based on morphological quality. Oocytes presenting cytoplasm with signs of degeneration, irregular morphology, and rupture of the zona pellucida were considered "non-viable".
The results from morphological viability after warming (Grade I and II) are indicated only in the vitrified groups.
Within a column, values with different superscripts differ significantly by Fisher's Test ($P < 0.05$).
a,b differ between the status (fresh or vitrified) in the same IVM system and A,B differ between the IVM system considering the status.

cat oocytes. Of note, the association of CLA, FSK, and LC was already tested during IVM in cattle with positive results regarding lipid content reduction, however, it negatively impacted embryonic development [13].

Due to the lack of knowledge of the meaning of the accumulation in the cat oocyte and how the modulation of this accumulation can impact the oocyte, the next steps aimed to answer how the reduction reached in the total lipid content interferes with oocyte development competence, metabolism, and cryosurvival. Oocyte maturation is associated with rapid remodeling of the oocyte lipid content, still, the signaling pathways in which lipid metabolism gives support to oocyte maturation are not completely known [35]. Besides the difference in the lipid content between our two experimental groups (MIX and CONTROL), no difference was observed in the nuclear maturation rates. The mechanism involved in the lipid composition changes and in how they can signal to release the oocyte from the meiotic arrest to reinitiate meiosis still need to be clarified [35], however, the results suggest that lipid content reduction seems to not interfere in the cat oocyte nuclear maturation.

Both growth/differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*) have been associated with oocyte quality because they have an important role in oocyte maturation since they are detectable in oocytes in early follicles stages [36–38]. *GDF9* was downregulated in the MIX group compared to CONTROL, and although there was no difference in our results regarding the expression of *BMP15*, the secretion of these two growth factors by oocytes and the ability of an oocyte to regulate its own environment through these factors, which are also important for cytoplasmic maturation, are essential considering its future development [37]. Based on that, the results suggest that although the combined use of lipid modulators and the reduction in the lipid content did not interfere with nuclear maturation rates, we could not rule out the possibility of an impact on the oocyte development competence.

To understand better the impact of reducing the lipid content through the combined use of modulators, we also checked the effect of it on oocyte metabolism. ROS are naturally formed during cellular metabolism; however, their levels are controlled by enzymatic and non-enzymatic antioxidants. When the production of ROS overcomes the antioxidant capacity, it generates oxidative stress (cellular damage through lipid peroxidation with DNA and protein damage) [34,39]. Our results showed a 5x increase in ROS levels and 18x in GSH levels in the MIX group compared to CONTROL. Both LC and CLA have antioxidant properties [34], and several studies reported the use of the former during IVM as a powerful antioxidant agent able to reduce ROS levels and increase GSH levels in different species [40–43]. In the current study, the modulators present in the MIX group can explain the increase in the GSH levels, still, their use seemed to influence ROS levels as well. It is worth mentioning that besides ROS levels being higher in the MIX group, the redox balance (ROS/GSH) was lower ($P < 0.05$) than CONTROL, which suggested that MIX positively impacted the metabolism, keeping the antioxidant response higher than ROS.

However, peroxiredoxins and HSPs are proteins involved in defense against oxidative stress [44,45] and the expression of both *PRDX1* and *HSP70* was downregulated in the MIX group. There are reports suggesting that the lower abundance of these transcripts after IVM can implicate in oocytes more sensitive to stressful conditions, which may lead to low developmental competence [46]. Even so, it is important to consider that if these genes are involved in the defense against oxidative stress, the lower expression in MIX compared to CONTROL can mean that oocytes are been less challenged regarding the oxidative conditions, and the less oxidative stressful *in vitro* environment can lead to a reduced need for the expression of the antioxidative defense.

In turn, no difference was found regarding mitochondrial activity between groups. The use of oocyte intracellular stores for energy production during IVM is relevant in species with high levels of stored lipids [5,34]. β-oxidation during IVM seems essential for subsequent developmental competence and LC increases beta-oxidation, taking fatty acids from the cytosol into mitochondria [34], however, the mitochondrial activity in the MIX group was not different from CONTROL. Taking together the expression of sirtuins, the MIX group presented a downregulation in *SIRT1* and there was no difference between groups regarding *SIRT3*. Sirtuins are a family of NAD + -dependent deacylases that regulate several physiological processes. This family has seven isoforms in which *SIRT1* is located in the nucleus and can be translocated to the cytoplasm; and *SIRT3* is located in the mitochondria and can be translocated to the nucleus under stress [47]. *SIRT3* is able to control the expression of many mitochondrial proteins and influence major mitochondrial functions [48,49]. In addition, *SIRT1* is involved in the regulation of mitochondrial biogenesis and mitophagy [50]. The results in the expression of *SIRT3* corroborate with mitochondrial activity results in which no difference was found between the two experimental groups. Considering that *SIRT1* can promote mitochondrial biogenesis in conditions of energy deficiency and has a significative role in triggering the dead or replacing the damaged mitochondria [50], its downregulation in the MIX group can suggest that this group was able to maintain a better metabolism balance than CONTROL.

Lastly, based on the association of lipid accumulation with sensitivity to cryopreservation [14,27] we tested the effect of the combined use of lipid modulators on cryopreservation. Although there is no consensus on the real impact of lipid accumulation on the cryotolerance of oocytes and embryos [51,52], the results in this current study showed that the reduction in the lipid content observed in the MIX group generated a higher viability rate ($P < 0.05$) after cryopreservation. On the other hand, this same association of modulators was able to reduce the lipid content of oocytes but negatively impacted embryonic development and embryo cryosurvival rates in cattle [13]. The differences in the cryosurvival due to the lipid content may be species-specific and the knowledge regarding the lipid profile pattern of the domestic cat oocyte and the changes in this profile caused by lipid modulation may be the key factor for understanding the results found in the current study.

Some studies reported that LC during IVM improved buffalo oocyte quality after vitrification by altering the phospholipid composition of vitrified oocyte membranes [53] and there is also evidence that CLA during IVM was able to improve oocyte cryosurvival in cattle [54]. In addition, also in cattle, FSK softened the effect of cryopreservation and developmental abnormalities observed in GV-oocytes during IVM after warming [55]. There is considerable evidence that these modulators are involved with improvements regarding oocyte cryopreservation, however, some of them can be related to the antioxidant defense [34] and

reports in cats remain scarce.

It is worth mentioning that although the viability post cryopreservation seems to be preserved by reducing the through the modulators, there are possible impacts created on the metabolism that can affect oocyte developmental competence, and an IVF would be an important tool to provide more information in this regard.

## 5. Conclusion

It was concluded that IVM can cause lipid accumulation in cat oocytes after 28 h of duration and that the combined use of lipid modulators (CLA + FSK + LC) that act for multiple pathways can reduce their lipid content. In addition, this lipid cocktail seems to improve viability after cryopreservation and to affect the oocyte metabolism, assessed by different fluorescent probes and gene expression. Such changes and their impact on the oocyte development competence still need to be better understood in the cat biological model.

## CRediT authorship contribution statement

**Gabriela Ramos Leal:** Conceptualization, Methodology, Data curation, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Thais de Almeida Oliveira:** Investigation, Data curation, Writing – review & editing. **Mariana Pedrosa de Paula Guimarães:** Investigation, Data curation, Writing – review & editing. **Lucas Francisco Leodido Correia:** Investigation, Formal analysis, Writing – review & editing. **Erlandia Márcia Vasconcelos:** Visualization, Investigation, Writing – review & editing. **Joanna Maria Gonçalves Souza-Fabjan:** Supervision, Funding acquisition, Resources, Writing – review & editing.

## Declaration of competing interest

The authors declare no conflicts of interest.

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