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Risks of using Mitoquinone during in vitro maturation and its potential protective effects against lipotoxicity-induced oocyte mitochondrial stress

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Running title: MitoQ during IVM

Abstract

Purpose. Oxidative stress and mitochondrial dysfunction play central roles in reduced oocytes quality and infertility in obese patients. Mitochondria-targeted treatments containing co-enzyme Q10 such as Mitoquinone (MitoQ) can increase mitochondrial anti-oxidative capacity, however their safety and efficiency when supplemented to oocytes under lipotoxic conditions have not been described.

Methods. We tested the effect of different concentrations of MitoQ or its cationic carrier (TPP) (0, 0.1, 0.5, 1.0µM each) during bovine oocyte IVM. Then we tested the protective capacity of MitoQ (0.1 µM) against palmitic acid (PA)-induced lipotoxicity and mitochondrial dysfunction in oocytes.

Results. Exposure to MitoQ, or TPP only, at 1 µM significantly ($P<0.05$) reduced oocyte mitochondrial inner membrane potential (JC-1 staining) and resulted in reduced cleavage and blastocyst rates compared with solvent control. Lower concentrations of MitoQ or TPP had no effects on embryo development under control (PA-free) conditions. As expected, PA increased the levels of MMP and ROS in oocytes (CellRox staining) and reduced cleavage and blastocyst rates compared with the controls ($P<0.05$). These negative effects were ameliorated by 0.1 µM MitoQ. In contrast, 0.1 µM TPP alone had no protective effects. MitoQ also normalized the expression of *HSP10* and *TFAM*, and partially normalized *HSP60* in the produced blastocysts, indicating at least a partial alleviation of PA-induced mitochondrial stress.

Conclusion. Oocyte exposure to MitoQ may disturb mitochondrial bioenergetic functions and developmental capacity due to a TPP-induced cationic overload. A fine-tuned concentration of MitoQ can protect against lipotoxicity-induced mitochondrial stress during IVM, and restore developmental competence and embryo quality.

Keywords: oocyte, CoQ10, MitoQ, IVF, embryo, obesity, oxidative stress

1   Introduction

Mitochondrial (**MT**) dysfunction in oocytes has been shown to play a central role in the pathogenesis of reduced oocyte quality and fertility in humans and animals [1]. Several metabolic diseases are associated with oocyte MT dysfunctions, such as obesity and type-2 diabetes [2, 3]. Exposure of oocytes to elevated concentrations of circulating lipotoxic free fatty acids (**FFAs**) and oxidative stress results in altered MT inner membrane potential (**MMP**), and impacts cellular bioenergetic and metabolic activities [4-7]. This has been demonstrated in high fat diet-induced obese mouse models. Optimal ATP production in oocytes is essential to support several cytoplasmic and molecular changes during early development; including cytoskeletal dynamics, activation of different kinases, and cortical granule activation [8]. Suboptimal MT functions can thus lead to spindle malformation, failure of fertilization, polyspermy, embryo fragmentation, altered embryo kinetics and embryo arrest, as well as reduced embryo quality [9, 10].

MT dysfunction in oocytes is associated with an increased intracellular accumulation of reactive oxygen species (**ROS**) which may have a causative or additive detrimental impact due to oxidative damage [11, 12]. Mitochondria are the main site of ROS production in the cell and they are also the most vulnerable organelles to oxidative stress [13]. Using a well-established and validated bovine *in vitro* embryo production model, it was demonstrated that oocytes matured *in vitro* in the presence of elevated concentrations of FFAs (particularly palmitic acid, **PA** [14], mimicking the intrafollicular conditions seen in obese women [14, 15]) exhibit oxidative stress and increased MT MMP [16-18]. The relative abundance of several MT proteins particularly those involved in unfolded protein responses and redox regulation increase in these oocytes [18]. In this model, alteration in oocyte MT activity was also linked to abnormal epigenetic patterns in the surviving embryos, ultimately leading to deviant blastocyst metabolic and transcriptomic profiles [19, 20]. These alterations persist during post-hatching development even after transfer to a healthy uterine environment (as shown in an *in vitro – in vivo* bovine model [21]). Afterwards, persistent MT dysfunction and epigenetic alterations can increase the risk of miscarriage, neonatal and postnatal diseases [22].

Importantly, we have demonstrated that the endogenous cellular homeostatic mechanisms are not efficient in metabolically compromised oocytes and early embryos cultured *in vitro* [23]. Once cellular stress is elicited, external antioxidant supplementation becomes essential to support development to the blastocyst stage and to safeguard embryo quality [23, 24].

In human IVF clinics, MT dysfunction in oocytes and embryos is increasingly recognized as a main factor that determines the success rate of ART. Early and late pregnancy losses have been linked directly or indirectly to mitochondria-related factors [25-28]. Infertile women who seek ART treatments may already have reduced systemic antioxidant capacity making their oocytes more prone to oxidative stress and MT dysfunction during the ART procedures. A particularly vital example is the deficiency in CoQ10, which is common in humans in their late thirties [29]. CoQ10 is an integral component of the MT electron transport

system, and one of the most effective lipid antioxidants. Its abundance and function limit the generation of free radicals within the MT. The concentration of CoQ10 in the follicular fluid is positively correlated with oocytes maturation, embryo grade and pregnancy rate in women undergoing ART [30, 31]. Obesity as such is strongly linked with systemic oxidative stress which can influence the ovarian microenvironment [32]. In addition, people who suffer from metabolic syndrome and hypercholesterolemia are likely to be treated with Statins which have also been linked with systemic reduction of CoQ10 levels as a side effect [33]. The effects of lipotoxicity-induced MT dysfunction and oxidative stress together with the reduced antioxidant capacity and limited cellular homeostatic machinery can significantly impact oocyte MT functions and put subsequent embryo development at risk.

It might be difficult to modulate MT CoQ10 content and antioxidant capacity in oocytes by dietary supplementation. Studies using radioactive tracing show a high uptake in spleen, liver and white blood cells, while adrenals, ovaries, thymus and heart accumulate lower concentrations [34]. In contrast, a recent paper showed that MitoQ (a mitochondria targeted formulation of CoQ10) could ameliorate ovarian oxidative stress and granulosa cell steroidogenic functions *in vivo* in a DHEA-induced PCOS mouse model [35]. On the other hand, *in vitro* supplementation with MitoQ is increasingly suggested to improve ART success rates. Yet, the safety and efficiency of treating oocytes with MitoQ is under-investigated. In vitro oocyte maturation (IVM) is increasingly used in human ART [36, 37] to minimize the risk of ovarian hyperstimulation syndrome (OHSS). OHSS is a potential risk of IVF, especially in obese women [38]. IVM may thus form a potential window during which oocytes retrieved from obese women can be treated with MitoQ to support their MT functions and minimize cellular stress levels before fertilization. In addition, the ART procedures *per se* involve many factors that can increase oxidative and mitochondrial stress such as handling, exposure to light, ICSI, as well as suboptimal pH, temperature and media composition [39]. Supplementation of antioxidants during IVM may increase the tolerance against sub-optimal physico-chemical conditions, resulting in a better developmental competence.

Unfortunately, from the clinical side, there is a paucity of evidence favoring the protective qualities of antioxidants during IVM [40]. Non-targeted antioxidants (such as Trolox) which exert their action at the cytoplasmic level are not effective in protecting oocyte developmental competence when supplemented during IVM in the presence of lipotoxic concentrations of PA, nor in rescuing embryo development of the exposed oocytes when supplemented during in vitro embryo culture (IVC) [41]. Mitochondria-targeted antioxidants such as MitoQ can be more effective in supporting oocyte developmental competence. MitoQ (Mitoquinone) is composed of CoQ10 conjugated to a lipophilic cation, triphenylphosphonium (TPP$^+$), which has an affinity for the negatively charged mitochondrial membrane and markedly increases the accumulation of CoQ10 within the mitochondria [42]. We have shown that MitoQ can significantly improve blastocyst yields and quality when added during IVC of embryos derived from metabolically compromised oocytes [24]. In contrast, the efficiency of MitoQ supplementation during IVM and its potential protection against lipotoxicity and oxidative stress are underexplored.

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In the present study, our primary hypothesis was that MitoQ can protect the oocytes against lipotoxicity during maturation and alleviate subsequent impact on early embryo development. To test this hypothesis we used our well-established bovine IVM model where oocytes were exposed to pathophysiologically relevant lipotoxic concentration of PA (150µM), in the presence or absence of MitoQ (1µM, as effectively used during IVC in a previous study [24]). In the initial experiments, MitoQ (at 1µM) was found to negatively impact oocyte developmental competence. Therefore, we also hypothesized that exposure of oocytes to the cationic carrier of MitoQ (TPP) can exert cytotoxic effects. Accordingly, a dose-response test was performed for both MitoQ and TPP, separately. Finally, the protective effects of an optimized concentration of MitoQ for IVM (0.1µM) was tested in the presence of PA. The impact on oocyte mitochondrial MMP, ROS levels cleavage and blastocyst development as well as transcriptomic regulation of cellular stress in the blastomeres are reported.

2 Methods

2.1 Experimental design

In the first set of experiments, MitoQ was added during bovine IVM at a concentration of 1µM in the presence or absence of 150 µM of PA. A PA concentration of 150µM is pathophysiologically relevant as measured in the FF of obese women [43] and validated in our previous studies [17, 18, 44]. A MitoQ concentration of 1 µM was effective during bovine embryo culture in our previous study [24]. Here, MitoQ 1µM was found to be toxic for oocyte developmental competence. Therefore, a dose-response test was then performed for MitoQ and for its cationic carrier, TPP, separately at 0.0, 0.1, 0.5, and 1.0 µM each, to see if cytotoxicity is caused by the carrier and to check the effects at lower concentrations. The impact on oocyte mitochondrial MMP, ROS and subsequent embryo development rates were examined. Lower concentrations of MitoQ or TPP only (0.1µM and 0.5µM) did not have a significant negative effect on mitochondrial functions and developmental competence of oocytes in the absence of a lipotoxic insult.

In the second stage of this study, the potential protective effects of MitoQ at a concentration of 0.1µM were tested in the presence or absence of 150µM of PA. TPP was also tested at the same concentration for comparison, to test if the beneficial effects are CoQ10-dependent. The effects on oocyte developmental competence, MMP, and ROS were examined. Finally, to check if MitoQ treatment during IVM can alleviate PA-induced cellular stress that normally persist during early embryo development, the expression of several cell stress-related mRNA markers were examined in the resultant blastocysts.

The number of replicates, and the number of oocytes per treatment per replicate used in each experiment are mentioned in the results. *In vitro* fertilization and embryo culture were performed following standard protocols in a solvent-free media in the absence of PA or MitoQ as described hereafter.

2.2 Collection of bovine cumulus oocyte complexes (COCs) from slaughter house ovaries

Bovine ovaries were collected from a local abattoir immediately after slaughter and transferred within 2-3 hr to the laboratory in warm saline. Ovaries were rinsed in 70% ethanol and then in sterile saline containing Kanamycin (125 µg/mL). Follicular fluid was aspirated from visible antral follicles (2-8 mm in diameter) with 18g needles and 5 mL syringes and transferred to 15mL conical tubes. The cellular precipitate was then mixed with sterile Wash-TALP media (Hepes-buffered Tyrode's albumin lactate pyruvate media) and transferred to a searching dish. Only good quality COCs with more than five layers of compact cumulus cells and dark homogenous ooplasm were selected under a stereomicroscope and further used in the experiments. Temperature throughout the collection and selection procedure was maintained at 37°C either in a water bath or on warm stages.

2.3 *In vitro* maturation and treatments

Good quality COCs were washed and incubated in 4-well dishes containing Maturation media; TCM-199 media supplemented with gentamycin (50µg/mL), sodium pyruvate (0.2mM), cysteamine (0.1mM), L-glutamine (0.4mM), and murine epidermal growth factor (mEGF, 20 ng/ml) [19]. Bovine serum albumin (BSA, fatty acid free, 0.75%w/v) was also added as a carrier for PA. Mitoquinone (MitoQ, Cayman chemical, Sanbio B.V. Research & Diagnostics, Uden, Netherlands), its cationic carrier molecule, triphenylphosphonium (TPP) and PA were first dissolved in absolute ethanol (1000X stock solutions) and then added to the media as described in the experimental design [18]. Stock solutions were aliquoted under nitrogen gas, sealed and stored at -20°C. An equivalent maximum final concentration of solvent was added in the control groups in all experiments (SCONT; 0.2% ethanol). This was previously proven not to influence developmental competence compared to solvent-free controls [17, 45]. COCs were matured in groups of 50±5 in 10 µL media per COC. Plates were equilibrated for at least 2h before transferring the COCs. IVM incubation was for 24h in 5% CO_2 in humidified air at 38.5°C.

2.4 Assessment of mitochondrial activity and intracellular reactive oxygen species in oocytes

Mitochondrial MMP intracellular ROS were determined in oocytes at 24h of IVM (a random sample of 8-10 oocytes per treatment per replicate) using a combined fluorescence staining with JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl-carbocyanine iodide, Invitrogen) and CellROX™ Deep Red (ThermoFisher) as validated by De Biasi, et al. [46] and described by Komatsu, et al. [47] and our previous study [18]. Briefly, oocytes were denuded of their surrounding cumulus cells by repeated pipetting in Wash-TALP. Denuded and partially denuded oocytes were incubated in Wash-TALP containing JC1 (5µg/mL) and CellRox deep red (2.5mM) (from 1000 X stock solutions in DMSO) for 30 min at 37°C. Then they were washed twice and transferred to Wash-TALP droplets under equilibrated mineral oil in pre-warmed 35mm glass bottom dishes. Stained oocytes were immediately examined under a Leica SP8

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confocal microscope equipped with white laser source (Leica WLL) and enclosed in a pre-warmed chamber at 37°C. Images of the oocytes or the surrounding few layers of CCs were acquired at excitation/emission 488/530 nm (green JC-1 monomers), 561/590 nm (yellow JC1-aggregates) and 644/665 nm (red, CellRox as a measure of ROS). The grey scale intensity was quantified using Image-J software. Mitochondrial activity (MMP) was calculated as a ratio of the grey scale intensity at 590 nm : 530 nm. ROS content was presented as the grey scale intensity at 665 nm as such or as a ratio to active mitochondrial (intensity at 665 / (intensity at 590/530)).

2.5   *In vitro* fertilization *and in vitro* embryo culture (IVC)

To determine embryo developmental competence, COCs were washed at 24h of IVM and transferred to Fert-TALP medium containing 1×10^6 sperm/mL in 4-well plates (in pools of 80-100 COCs in 500 µl/well). For that, motile spermatozoa were selected from proven-fertile frozen bull semen by centrifugation through a Percoll gradient (45%-90%) [45]. Straws from the same ejaculate was used in all experiments. COCs and sperms were co-incubated for 20 h in 5% CO_2 in humidified air at 38.5°C. Subsequently at 20h or IVF, presumptive embryos were denuded by vortexing for 3 min and washed in Wash-TALP, then cultured in groups of 25±3 in 96-well plates in 75µL/well of serum-free SOF medium containing 2% w/v BSA [18]. Embryos were incubated in 90% N_2, 5% CO_2, 5% O_2 and 38,5°C until Day 8 post fertilization (p.f.). IVF and IVC media were not supplemented with any treatments or solvent.

Embryo cleavage was assessed at 48 h p.f. by recording the proportions of cleaved embryos, good quality embryos (with ≥4-cells and <15% fragmentation) and fragmented embryos (with >15 % fragmentation of the cellular mass). Blastocyst rates were recorded on day 7 and 8 p.f. and expressed as a proportion of total number of oocytes or per cleaved embryos. Assessment was done under an inverted Olympus CKX41 microscope (Olympus, Aartselaar, Belgium) at 100X magnification.

2.6   Assessment of fertilization rate

In order to check if the effects induced by PA and MitoQ on cleavage and blastocysts rates are due to differences in fertilization rate, extra replicates were performed in which presumptive zygotes were collected at the end of IVF (24h) for pronuclear staining. They were denuded of cumulus cells by vortexing, fixed in paraformaldehyde 4% (PFA 4%) and stained with Hoechst (1µg/mL in PBS-PVP). Stained samples were mounted in DABCO, visualized under Olympus XI71 fluorescent microscope and classified according to their nuclear morphology: the number of pronuclei or an arrest at metaphase I or II (MI, MII) or anaphase I (AI). At this stage, normal fertilized oocytes are expected to have 2 pronuclei.

2.7   Gene expression analysis

Day 8 blastocysts from every treatment were washed and transferred to a 1.5 mL vial in minimal volume of PBS, and immediately snap frozen in liquid nitrogen and stored at -80°C. RNA extraction was carried out using PicoPure™ RNA Isolation Kit (Thermo Fisher Sci.). RNA concentration and integrity were examined using a NanoDrop™ (Thermo) and an Agilent Bioanalyzer (RIN≥7). Total RNA was treated with DNase and reverse transcribed (50ng RNA/sample) using Sensiscript RT kits (QIAGEN) following manufacturer instructions. Quantitative polymerase chain reaction (Real-Time PCR; qPCR) using SYBR Green was used to study the expression of genes related to oxidative stress (*SOD2, GPx1* and *CAT*), mitochondrial UPR (*HSP10* and *HSP60), Endoplasmic reticulum* UPR^{er} (*ATF4* and *ATF6*), as well as mitochondrial biogenesis (*TFAM*). The quantification was normalized using the geometric mean of three housekeeping genes; *18S, YWHAZ* and *H2A* and the relative fold change of each gene was calculated using the 2^(-ΔΔCT) method, as described by Pfaffl [48]. Primers were designed using Primer-Blast based on NCBI Reference Sequences of *Bos taurus* and are all intron flanking (supplementary table 1).

2.8   Statistical analysis

Statistical analyses were performed using IBM Statistics SPSS 26 (for Windows, Chicago, IL, USA) using data derived from at least 3 replicates. Categorical data, such as fertilization, cleavage and blastocyst rates were compared using a binary logistic regression model. Numerical data; cell counts, gene expression data, JC1 and CellRox staining quantification were checked for equal variance and normality of distribution, and means were compared using ANOVA. The interaction between treatment and replicate effects was tested and excluded from the final model if it was not significant (which was the case in all experiments). A Bonferroni post-hoc test was performed to account for multiple familial comparisons. Data were considered significantly different at *P*-values ≤ 0.05, and higher *P* values up to 0.1 were described as tendencies.

3   Results

3.1   Effects of high concentration of MitoQ (1µM) during oocyte maturation in the presence or absence of metabolic stress conditions

Exposure of COCs to 150µM of PA significantly reduced cleavage rate, proportion of good quality embryos at 48h and blastocyst rates at day 8 (P<0.05). Unexpectedly, supplementation of 1µM MitoQ during 24h IVM was found to be toxic for oocyte developmental competence. Cleavage rate was significantly reduced and the percentage of good quality embryos (≥4 cells at 48h p.i.) and blastocysts (at day 8) were

surprisingly very low in compared to the control group ($P < 0.05$). Surprisingly, when added with PA during IVM, MitoQ (1µM) increased the negative impact on development compared to PA only (Fig 1).
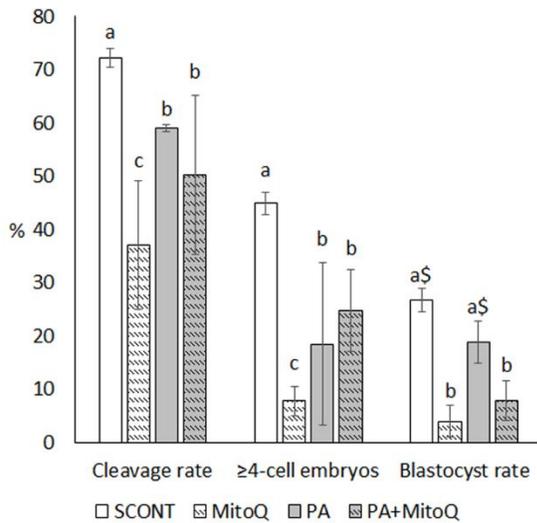

Fig. 1 The effect of PA (150µM) and/or MitoQ (1 µM) supplementation during IVM on early embryo development. Total cleavage and 4-cell-plus embryo rates were recorded at 48h post fertilization, while blastocyst rates were recorded at day 8. Bars with difference alphabets are significantly different at P<0.05. The $ sign indicates a tendency (P<0.1) compared to the corresponding control group. Data are shown as mean percentage ± SEM from three replicates using a total of 735 oocytes (157-198 oocytes per treatment).

3.2   Concentration dependent effects of MitoQ and TPP during IVM on embryo development

We examined if the effects of MitoQ during IVM are concentration dependent and if the detrimental effects of 1µM MitoQ on developmental competence observed above are caused by TPP. Treatment with MitoQ at 0.1 µM or 0.5 µM during IVM did not significantly affect embryo cleavage and development to the blastocyst stage compared to the non-supplemented solvent control (SCONT) (Fig.2A). The negative impact of adding 1.0 µM of MitoQ was confirmed ($P < 0.05$). TPP was found to exert very similar concentration-dependent effects on cleavage and blastocyst rates (Fig 2B). The negative effects at 0.5µM and 1µM of TPP were larger, since relatively less proportions of blastocysts could survive.
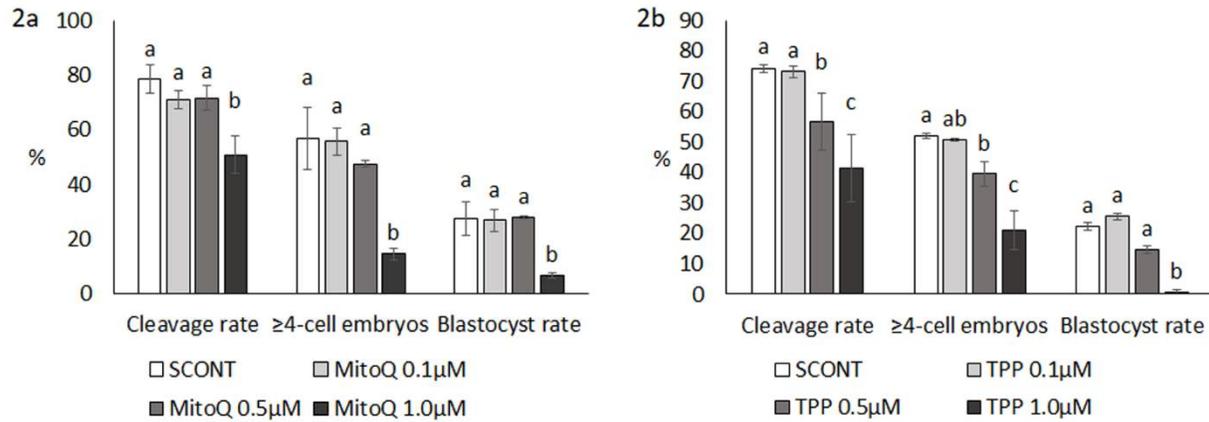
Fig. 2 The effect of different concentrations of MitoQ (**2a**) and TPP (**2b**) (0, 0.1, 0.5, and 1.0 µM) during IVM on early embryo development. Bars with difference alphabets are significantly different at P<0.05. Data are shown as mean percentage ± SEM from three replicates each, using a total of 705 oocytes in the MitoQ experiment and 872 oocytes in the TPP experiment (165-187 oocytes per treatment).

3.3 Concentration dependent effects of MitoQ and TPP during IVM on mitochondrial functions in oocytes

As a next step, we hypothesized that since TPP is a strong cationic compound it may interfere with mitochondrial functions at high concentrations and may also alter ROS production. Therefore, we tested the impact of adding different concentrations of MitoQ and TPP during IVM on mitochondrial MMP and intracellular levels of ROS.

Low concentrations of TPP only and MitoQ (at 0.1 and 0.5 µM) did not have any significant influence on MMP (Fig. 3A) and ROS (Fig. 3B) levels (P>0.1). Whereas the highest concentration of TPP and MitoQ significantly reduced MMP. High concentration of TPP only, but not MitoQ, also significantly reduced ROS concentrations. The impact on MMP was higher than the impact on ROS, hence, an increased ROS per active mitochondria was noticed (P<0.05 for MitoQ 1µM vs SCONT).
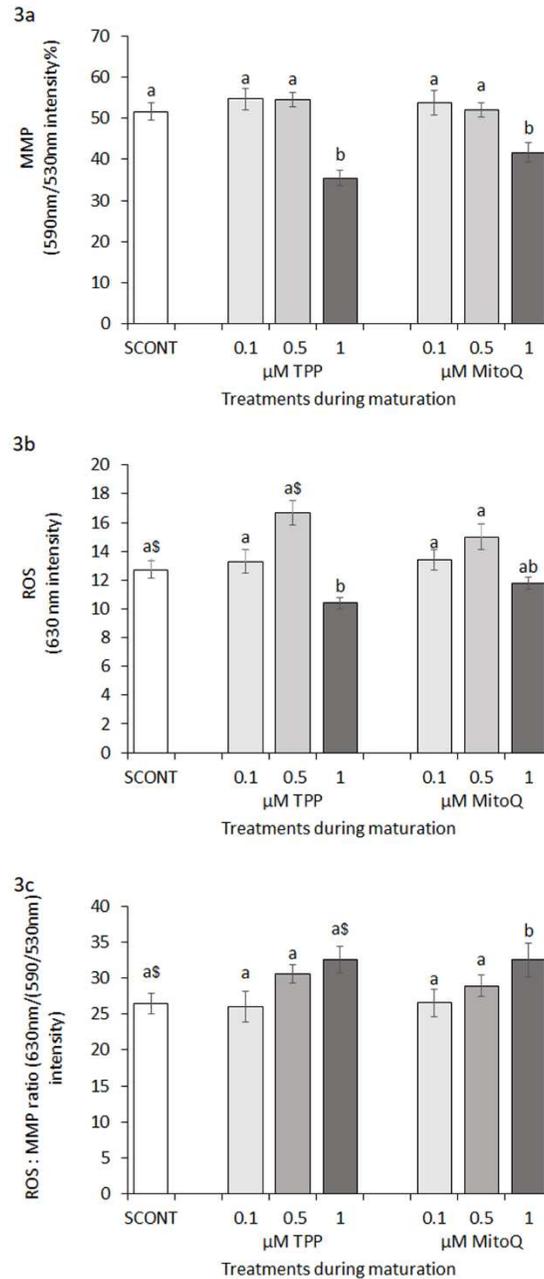
Fig. 3 The effect of different concentrations of MitoQ and TPP (0 (Solvent control, SCONT), 0.1, 0.5, and 1.0 µM) during IVM on **a)** mitochondrial inner membrane potential (MMP) and **b)** intracellular reactive oxygen species (ROS) concentrations in oocytes. This was determined using JC-1 and CellRox Deep Red staining, respectively, and confocal laser scanning microscopy. **c)** The ratio of ROS per active mitochondria is also presented. Bars with difference alphabets are significantly different at P<0.05 within each treatment category (TPP or MitoQ) and compared to SCONT. The $ sign denotes a tendency (0.05< P <0.1) compared to the SCONT. Data are shown as mean percentage ± SEM from three replicates each, using on average 25 oocytes per treatment.

3.4 Protective effects of MitoQ (0.1 µM) during oocyte maturation under metabolic stress conditions on oocyte developmental competence

Based on the previous experiment, MitoQ concentration of 0.1µM was considered optimal. The next experiment aimed to test if MitoQ at 0.1µM can protect the oocyte from the lipotoxic impact of PA. To limit the number of treatment groups, the effect of TPP was not tested further. Exposure of COCs to a high PA concentration (150 µM) during IVM in the absence of MitoQ significantly reduced total cleavage rate and the proportion of 4-cell plus embryos, and increased embryo fragmentation at 48h. PA also decreased total blastocyst rates at day 8 (Table 1). On the other hand, supplementation of MitoQ in the PA+MitoQ group completely protected oocyte developmental capacity as it resulted in restored cleavage and blastocyst rates compared to the control (P>0.1). In contrast, TPP supplementation in the presence of PA (PA+TPP) did not have significant protective effects.

Table 1. Early embryo development after exposure of COCs to high concentration of Palmitic acid (PA; 150µM) during IVM in the presence or absence of MitoQ or TPP (0.1 µM) compared to a solvent control (SCONT).

	SCONT	PA	PA+ TPP 0.1 µM	PA+ MitoQ 0.1 µM
Total COCs (n)	275	272	267	268
Cleaved embryos (%)	190 (69.0%)[a]	154 (56.6%)[b]	149 (55.8%)[b]	186 (69.4%)[a]
4-cell stage or more (%)	137 (49.8%)[a]	93 (34.1%)[b]	97 (36.3%)[b]	136 (50.7%)[a]
Fragmented embryos (%)	11 (4%)[a]	24 (8.8%)[b]	22 (8.2%)[b]	14 (5.2%)[a]
Blastocysts (% from total)	66 (24%)[a]	38 (13.9%)[b]	54 (20.2%)[ab]	65 (24.2%)[a]
Blastocysts (% from cleaved)	66 (34.7%)[a]	38 (24.6%)[a*]	54 (36.2%)[a]	65 (34.9%)[a]

Data is presented as proportions (%). Different superscripts indicate significant differences at P<0.05 within the same row. *Significant from control if Bonferroni correction is not applied.

Extra replicates were performed to check if the increase in early embryo development in the MitoQ supplemented group originates from an increased fertilization rate. Presumptive zygotes were collected at the end of the IVF period (24h), fixed in PFA 4% and stained with Hoechst to assess their nuclear morphology. We found that in the PA-exposed group, the percentage of zygotes with 2 pronuclei was significantly reduced, and the percentage of non-fertilized oocytes (arrested at MII) was doubled

compared to SCONT (P<0.05). Treatment with MitoQ during IVM alleviated these effects and resulted in about 20% increase in normal fertilization rate (Table 2).

Table 2. Fertilization rate after exposure of COCs to high concentration of Palmitic acid (PA; 150µM) during IVM in the presence or absence of MitoQ (0.1 µM) compared to a solvent control (SCONT).

	2PN	1PN	>2PN	MII	MI-AI	Degenerated
SCONT	66/86 (76.7%)[a]	5/86 (5.8%)	6/86 (7%)	8/86 (9.3%) [ab$]	1/86 (1.2%)	0/86 (0%)
PA	52/83 (62.7%)[b]	3/83 (3.6%)	9/83 (10.8%)	16/83 (19.3%)[b$]	2/83 (2.4%)	1/83 (1.2%)
PA+MitoQ 0.1 µM	81/100 (81%)[a]	2/100 (2%)	10/100 (10%)	5/100 (5%)[a]	2/100 (2%)	0/100 (0%)

Data is presented as proportions (%). Different superscripts indicate significant differences at P <0.05 within the same column. PN, pronuclei; MII, metaphase II; MI, metaphase I; AI, Anaphase I. Proportions with $ sign tend to be different (*P*=0.062).

3.5 Protective effects of MitoQ (0.1 µM) during oocyte maturation under lipotoxic conditions on mitochondrial activity and oxidative stress in oocytes

To test if the protective effects of MitoQ on oocyte developmental capacity is mediated through regulation of mitochondrial functions, MMP and ROS levels were measured in the treated oocytes at the end of maturation, after removal of the cumulus cells. As expected, in the absence of MitoQ, PA increased mitochondrial MMP and ROS per active mitochondria (P<0.05 compared to SCONT). These effects were mitigated by MitoQ (P>0.1 compared to SCONT) (Fig. 4).
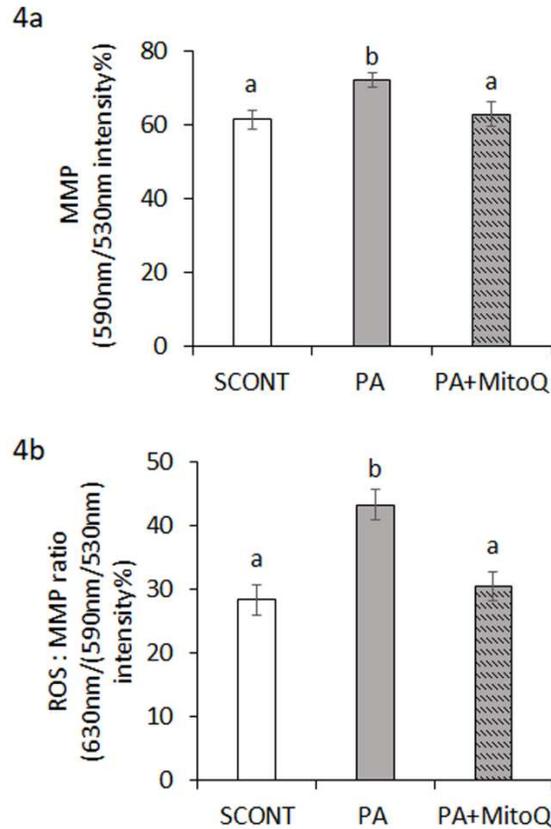
Fig. 4. The effect of PA (150μM) during IVM in the presence or absence of MitoQ (0.1μM) on **a)** mitochondrial MMP and **b)** the ratio of ROS per active mitochondria in oocytes at 24h of IVM. Bars with difference alphabets are significantly different at P<0.05. Data are shown as mean percentage ± SEM from three replicates each, using 20 oocytes per treatment.

3.6 Protective effects of MitoQ (0.1 μM) supplementation on marker genes of cellular stress in blastocysts

Finally, the expression of genes related to cellular stress were examined in day 8 blastocysts produced after PA exposure during IVM in the presence or absence of 0.1 μM MitoQ. Blastocysts produced from PA oocytes exhibited significantly increased expression of mitochondrial HSP60 and a tendency to a higher expression of the mitochondrial HSP10. These effects were partially normalized when MitoQ was also added during IVM (Fig. 5). PA had no effect on blastocyst expression of the ER stress markers ATF4 and ATF6 and only resulted in tendency for an increased SOD2 expression but not GPX1. SOD2 expression was not normalized with MitoQ. PA significantly increased TFAM expression, an effect which was alleviated by MitoQ.
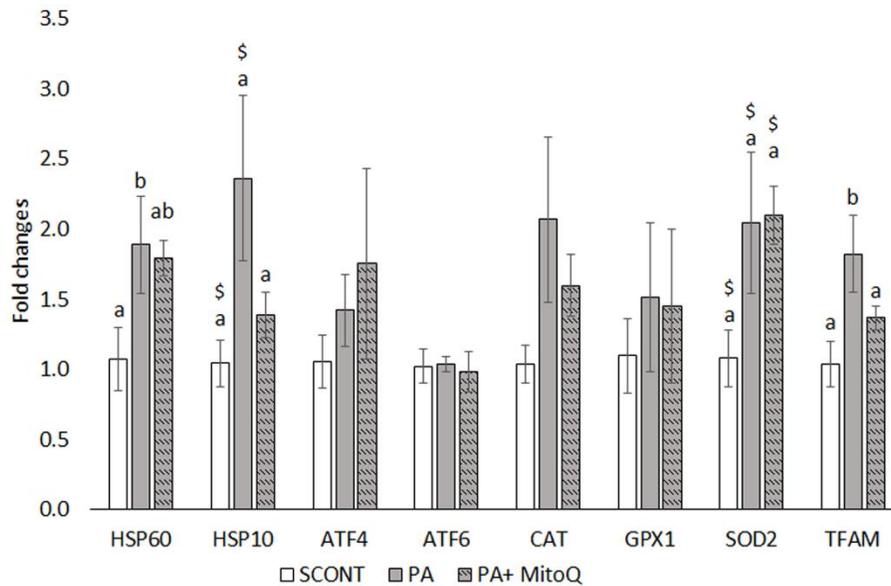
Fig. 5 Effect of PA-exposure during IVM in the presence or absence of MitoQ (0.1μM) on the mRNA expression of cellular stress related genes in blastocysts at Day 8. Data are derived from three replicates using extracts from at least 10 blastocysts per treatment per replicate. Data were normalized based on the expression levels of three housekeeping genes (*18S*, *H2A* and *YWHAZ*) and presented as fold changes relative to the control group. Bars with different alphabets at *P* <0.05 based on the ANOVA results followed by an LSD pairwise comparisons. The $ sign denotes a tendency (0.05< P <0.1) compared to the SCONT.

4   Discussion

The aim of this study was to test the effects of different concentrations of Mitoquinone (MitoQ) and the potential cytotoxic effects of its carrier, TPP, during oocyte IVM. We also aimed to test the capacity of optimal concentration of MitoQ to protect the oocyte against PA-induced mitochondrial stress and to enhance developmental competence.

In the first experiments, using 1 µM of MitoQ during IVM significantly reduced cleavage and blastocyst rates, where only about 5% of the formed zygotes could survive. In contrast, in a previous study [24], using the same MitoQ concentration during IVC did not compromise developmental competence and effectively rescued blastocyst development of PA-exposed bovine oocytes. Here, we found that exposure to only TPP at 1 µM induced the same cytotoxic effects. As mentioned above, MitoQ is composed of the ubiquinone moiety of the endogenous antioxidant CoQ10 [49] loaded on a strong lipophilic cationic carrier;

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triphenylphosphonium (TPP), which enables accumulation of the ubiquinone at the mitochondrial matrix at a 100 to 500 fold higher concentration compared to the cytoplasm [50]. The inner mitochondrial membrane (IMM) possess a distinctive negative potential that arises from the movement of protons required for ATP synthesis. The presence of immobile internal negative charges on the IMM favors the transport of cations. This negative charge is therefore used to enable the delivery of molecules conjugated to phosphonium cations as carriers [51]. However this negative charge is also important to regulate the transportation of other cations across the mitochondria membranes, following the Nernstian behavior of the equilibrium of electrode potential [52]. This involves the primary intracellular cations, namely potassium (K+) and sodium (Na+). The movement of these monovalent cations (osmolytes) is accompanied by the movement of water, contributing to the regulatory mechanism of mitochondrial volume. Furthermore, the negative charge of the IMM also regulates the transport of the divalent cations, calcium (Ca2+) and magnesium (Mg2+) into the mitochondrial matrix [52]. We may thus expect that any cationic overload can disturb mitochondrial functions. This can be demonstrated for example by the fact that calcium overload has been shown to inhibit mitochondrial oxidative phosphorylation when it exceeds a certain threshold [53]. This was associated with detrimental changes in mitochondrial MMP and bioenergetic pathways, leading to lower ATP production rates. In the dose-response experiments performed here, we illustrated that exposure of the oocytes to high MitoQ concentration (1µM) resulted in a significant reduction in the MMP. The exact effect could be replicated by exposure to the same concentration of TPP. This suggests that the high concentration of TPP and MitoQ resulted in a cationic overload on the mitochondria. The reduction in MMP was detrimental for the oocyte developmental capacity as it significantly reduced cleavage and blastocyst rates. As mentioned in the introduction, MMP in oocytes is crucial for several processes during oocyte maturation and subsequent embryo development, including energy provision, cytoskeletal dynamics and intracellular transport, spindle formation and cell division, phosphorylation and enzymatic activation. The reduction in MMP by TPP overload is expected to impact these vital processes, explaining the reduction in subsequent development rates.

In addition, calcium oscillations play a crucial role in oocyte cortical granule reaction, fertilization, and prevention of polyspermia. Upon fertilization, the intracellular concentration of calcium ion significantly increases in the oocyte, which initiates the cortical granule reaction to block polyspermy [54]. Therefore, in addition to MMP and ATP mediated effects, the reduction in cleavage rate after exposure to high MitoQ and TPP concentrations can be mediated by altering the mitochondrial transport of other intracellular cations like calcium in the oocyte. A previous study have demonstrated that TPP itself inhibits the Na /Ca2 exchanger in the mitochondrial membranes and thus increases intra-mitochondrial calcium levels. The degree of inhibition was greater with TPP compared to MitoQ [55]. While this has not been examined in oocytes, we see that the negative impact of TPP on development is more severe compared to MitoQ (Fig.1).

We have previously shown that 1 µM of MitoQ supplementation during IVC (from 22h to day 8 post fertilization) resulted in a significant reduction in ROS levels together with a significant increase in MMP

both in the absence or presence of metabolic stress [24]. The contrast with the effects shown here during IVM (where both MMP and ROS are reduced) suggests that mitochondria are more sensitive to TPP and MitoQ treatments during oocyte maturation compared to embryos during *in vitro* culture. Despite the fact that oocyte mitochondria are immature and posses undeveloped cristae, it has been shown that 65% of the immature (GV) oocyte basal respiration is used for mitochondrial respiration and ATP production via oxidative phosphorylation [56]. This slightly decreases during maturation (to about 50%) [56] and further decreases during early embryo development. Early embryos are known to favor low mitochondrial activity (in accordance with the quite embryo hypothesis) and gradually switch to Warburg metabolism (aerobic glycolysis) [57]. A recent study in our laboratory using a Seahorse Bioanalyzer has shown that over 85% of the ATP production rate by denuded mature oocytes is linked to oxygen consumption (i.e. mitochondrial) whereas only about 15% could be linked to the increase in extracellular acidification rate (glycolytic) [20]. We have also noticed that the intensity of J-aggregate staining in mature oocytes (indicative for a relatively high MMP) is relatively higher that J-aggregate intensity at the 4-cell stage (see supplementary figure 1). Taken together, a higher dependence on mitochondrial bioenergetic functions during oocyte maturation may make the oocytes more sensitive to the TPP cationic overload compared to early embryos. In addition, if disruption of calcium homeostasis plays a role, as suggested above, this can be another reason why developmental competence is more affected if exposure to the cationic overload occur before fertilization.

It is also important to notice that MitoQ did not reduce ROS levels in the oocytes under control conditions (in the absence of lipotoxic metabolic stress). This was evident when using low concentrations of MitoQ (0.1 and 0.5µM). The reduction in ROS observed with 1µM MitoQ appears to be secondary to the reduction in MMP, as it could be replicated using TPP only. It is known that ROS production requires a high membrane potential [58]. The accumulation of positively charged TPP in the mitochondria can decrease the MMP, lowering the ROS production. This mechanism has been illustrated by both MitoQ and TPP in isolated mitochondria [50]. Nevertheless, it is expected that once the MitoQ accumulates within the mitochondria, the ubiquinone moiety should be reduced by accepting two electrons from complex I or II of the ETC, to be then converted to ubiquinol, which is the active antioxidant form [59]. Ubiquinol can scavenge or detoxify ROS, as it is oxidized back to ubiquinone. This process is repeated and has been shown to significantly reduce ROS production, inhibiting downstream lipid peroxidation and oxidative stress after MitoQ administration [59]. Here this was not evident under stress-free conditions. Similarly, many studies have demonstrated that supplementation of other antioxidants, such as vitamin C, vitamin E and Trolox had no beneficial effects on oocyte maturation under standard conditions [60, 61]. High concentrations of antioxidants during IVM may even block spontaneous resumption of meiosis because a minimal concentration of ROS is needed to stimulate vital signaling and activate regulatory molecules required for development [62]. Nevertheless, we noticed that while 0.5µM TPP increased ROS levels (potentially due to disruption of ion transport across the IMM), ROS levels in oocytes treated with 0.5µM MitoQ were not altered. This shows that the ubiquinone moiety may still be able to attenuate ROS production under stress conditions.

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Knowing that low concentrations of MitoQ (especially 0.1 µM) do not impact oocyte developmental competence and may attenuate ROS production under stress conditions, we could proceed with the second set of experiments to examine the potential protective effects of MitoQ against PA-induced lipotoxicity during oocyte maturation. PA is the most predominant FFA in the follicular fluid and its abundance is negatively correlated with positive pregnancy outcomes in women undergoing ART [14, 63]. Several studies from our laboratory and by others have illustrated that in vitro exposure of bovine COCs during IVM to a pathophysiological concentration (150µM) of PA resulted in an increased cumulus cell apoptosis, increased oocyte MMP, ROS and reduced oocyte developmental competence [16-18]. Here, these detrimental effects of PA were confirmed, ultimately leading to about 10% reduction in blastocyst rate.

The toxic effects of PA on oocyte developmental competence can be mediated via several mechanisms. For example, PA has been associated with ceramide accumulation in oocytes, downregulating the AMPK/SIRT3 pathway and consequently inducing mitochondrial protein hyper-acetylation and mitochondrial defects [11]. In addition, a comprehensive proteomic analysis was performed in our laboratory to study the changes that occur in oocytes (after removal of their surrounding cumulus cells) after a 24h exposure to 150µM of PA during IVM. There we could show that 30% of the differentially regulated proteins in the oocytes where mitochondrial proteins, and that the top canonical pathways affected were related to mitochondrial dysfunction, unfolded protein responses and redox regulatory mechanisms [18]. Likewise, here we show that PA significantly increased MMP and almost doubled intracellular ROS levels in the exposed oocytes. The increased MMP is probably a metabolic response to the increased availability of FFAs (PA) which undergo β-oxidation and increase mitochondrial rates of oxidative phosphorylation [64]. Increased MMP and ROS were also reported in oocytes collected from inbred and outbred diet-induced obese mouse models after feeding an obesogenic diet [5, 12, 65]. This illustrates the relevance of our model to search for strategies to support oocyte quality in obese patients.

In addition, MMP and ROS levels are clearly connected, since the ETC in mitochondria is the main source of ROS production [58]. We repeatedly notice that the CellRox Deep Red staining used to quantify ROS is clearly colocalized with the mitochondria stained with JC-1 (see Suppl Fig. 2). This justifies our aim to use a mitochondrial targeted antioxidant to provide protection under these conditions. Low concentration of MitoQ (0.1 µM) could significantly reduce embryo fragmentation rate and completely restore the PA-induced reduction in cleavage and blastocyst rates. This beneficial effect could not be achieved using the same concentration of TPP, which shows that it was driven by the antioxidant capacity of MitoQ. We illustrated that the increased rates of embryo development to the blastocyst stage in the PA+MitoQ (0.1 µM) group originated from an increased fertilization rate. We could also illustrate that this beneficial effect is mediated at least in part through normalizing oocyte MMP and ROS levels, which were both significantly lowered in oocytes treated with PA+MitoQ compared to those exposed to PA only. Increased embryo fragmentation and reduced development are strongly linked to mitochondrial dysfunction and high ROS production in early embryos [66]. A previous study showed that the use of antioxidants that are

not targeted to the mitochondria, such as Trolox, during IVM in the presence of PA, could not reduce MMP and ROS back to normal levels and did not rescue subsequent early embryo development [41]. In contrast, a recent study showed that supplementation with mitochondrial targeted antioxidants such as MitoQ and BGP-15 during IVM normalizes mitochondrial MMP and reverses the spindle and chromosomal abnormalities which occur in oocytes collected from reproductively aged female mice [67]. Deficiency of CoQ10 in oocytes is suggested to be the main reason for the high rates of aneuploidy in aged infertile patients (Bentov et al. 2014). Therefore, specific targeting of the mitochondria with antioxidants appears to be more effective.

Furthermore, we examined the expression of several cell stress related marker genes to check if cellular homeostasis in the blastocysts is completely reestablished, or to identify carry-over effects that might have not been fully restored by the MitoQ treatment during IVM. As expected, exposure to elevated concentration of PA during IVM resulted in an increase or a tendency to increase mitochondrial UPR markers (HSP60 and HSP10), Catalase, SOD2 and TFAM. Markers of ER stress were not affected. This is mostly in line with our previously published data [24]. mtHSP60 and mtHSP10 are known to be upregulated in conditions of mitochondrial stress due to altered MMP, altered OXPHOS or accumulations of misfolded proteins [68]. These chaperones belong to the heat shock protein 70 (HSP70) family and are both necessary for protein import and to support protein folding [69]. Increased TFAM expression may imply that mitochondrial biogenesis might be altered [70]. The increased expression of SOD2 illustrates a persistently high level of oxidative stress during embryo development despite the fact that both fertilization and culture took place in FFA-free (i.e. standard, stress-free) culture condition. It has been previously suggested that early embryos do not have the machinery to establish cellular homeostasis [23], hence the need for exogenous support. Here, the protective effects of MitoQ (0.1 µM) during IVM were associated with a partial or complete normalization of the expression level of most of these markers showing no carryover persistent cell stress. Surprisingly, SOD2 expression was persistently high. Extending the MitoQ support also during IVC may further support embryo redox regulatory mechanisms.

While these results are generated in a bovine IVM model, the data provided here can be useful for human IVF application. Testing the safety and effectiveness of new compounds on human oocyte developmental competence is difficult due to the limited availability of biological material and due to the ethical concerns. Unlike murine models, bovine oocytes share several physiologic characteristics with human oocytes. For example, humans and cows are both mono-ovulatory species, having comparable oocyte characteristics, such as oocyte diameter, the time to reach the 2-cell stage, blastocysts and hatching, the duration of oocyte maturation, lipid content, energy metabolism, and a similar timing of genome activation [71, 72]. Nevertheless, translatability of the data provided here to the human IVF applications should only be done following the necessary validation studies.

In conclusion, the use of MitoQ during oocyte IVM should be done with caution. The cationic carrier of MitoQ appears to disturb oocyte mitochondrial bioenergetic functions and negatively impact early

embryo development. An optimal concentration of MitoQ during IVM can support mitochondrial bioenergetic functions and protect the oocyte against oxidative stress, leading to enhanced developmental competence and lower cellular stress levels in the produced blastocysts. These effects are specific to the CoQ10 content of MitoQ since the carrier molecule TPP had no protective effects. On the other hand, MitoQ has no beneficial effects on oocytes in the absence of lipotoxicity-induced cellular stress.

Competing Interests

Authors have no financial or non-financial conflicts of interests related to this work

Author contribution

WFAM contributed with the main project idea, conducted the experiments, analysed the data and wrote the manuscript. O.M-E performed the confocal microscopy and image analysis. IP helped with the optimization of microscopy and image data analysis. J.L.M.R supervised the project and critically read and edited the manuscript.

Data availability

Raw data files are available at Marei, Waleed (2023), "MitoQ during IVM", Mendeley Data, V1, doi: 10.17632/htrsvbjfk5.1.

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Supplementary figure 1
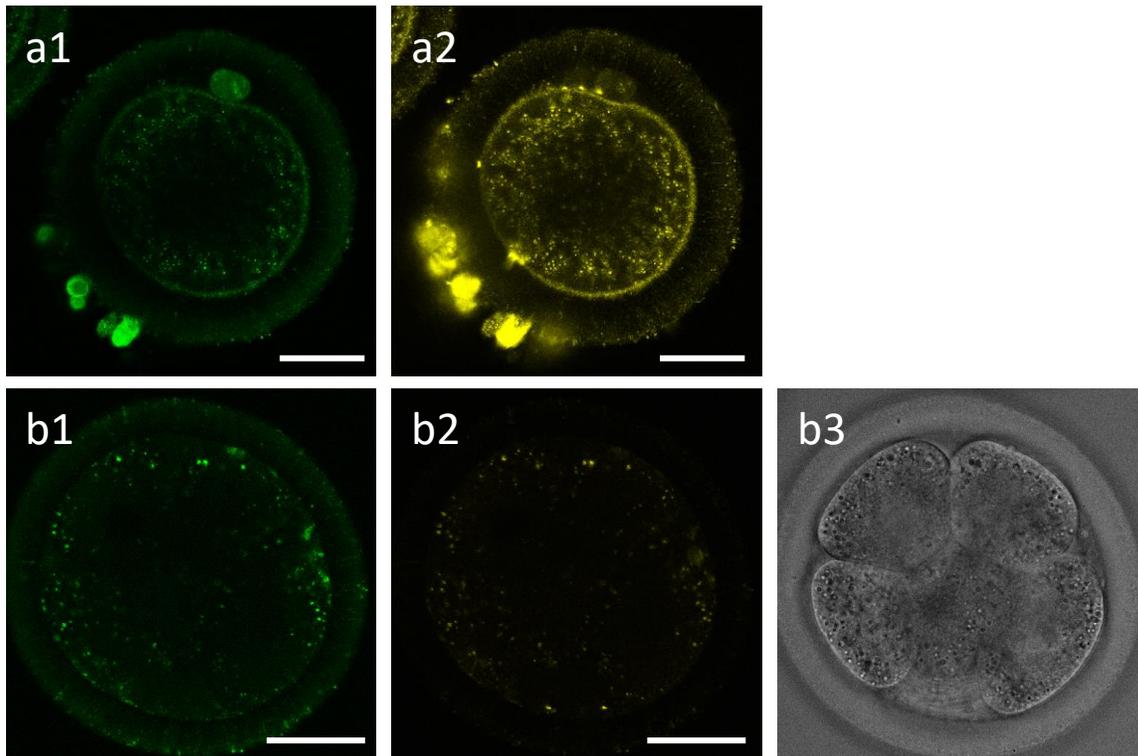
Supplementary figure 1. A comparison between JC-1 staining intensity in mature bovine oocytes at 24h of IVM (a) and in bovine embryos at 48h post insemination (b). J-monomers (a1 and b1) indicate inactive mitochondria, while J-aggregates (a2 and b2) label active mitochondria with high inner membrane potential. b3 is a bright field image of the embryo used in b1 and b2. Scale bar = 50 µm.

Supplementary Table 1. Primer sequences of target and housekeeping genes.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Gene Bank accession number
HSP10	CAACGGTGGTAGCTGTTGGA	TGGTGCCTCCATATTCTGGG	108	NM_174346.2
HSP60	CTACTGTACTGGCACGCTCT	CAATCTCTTCGGGGGTTGTC	159	NM_001166608.1
ATF4	TTTCTGAGCAGCGAGGTGTT	AAGCATCCTCCTTGCTGTTG	214	NM_00134341.2
ATF6	CGAGGATGGGTTCATAGGCA	GACGGCCATGAGCTGAGAAT	126	BC120388.1
TFAM	GCCAAGCTATGGAGGGAACT	AGCTTTACCTGTGATGTGCCA	293	NM_001034016.2
SOD2	TGCAAGGAACAACAGGTCTTATC	CTCAGTGTAAGGCTGACGGTT	181	NM_201527.2
GPX1	AACGTAGCATCGCTCTGAGG	TCTCCTCGTTCTTGGCGTTT	145	NM_174076.3
CAT	CTATCCTGACACTCACCGCC	GAAAGTCCGCACCTGAGTGA	268	NM_001035386.2
18S	AGAAACGGCTACCACATCCA	CACCAGACTTGCCCTCCA	169	AF176811.1
YWHAZ	GCATCCCACAGACTATTTCC	GCAAAGACAATGACAGACCA	120	BM446307.1
H2A	CGGAATTCGAAATGGCTGGC	TCTTTCGATGCATTTCCTGCC	238	NM_174809.2