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Mitochondrial function, blastocyst development and live foals born after ICSI of immature vitrified/warmed equine oocytes matured with or without melatonin

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Abstract

Oocyte vitrification is considered experimental in the horse with only three live foals reported. The oxidative conditions induced by vitrification could in part explain the poor results and melatonin, a powerful antioxidant, could stimulate ROS metabolization and restore mitochondrial function in these oocytes. Our objective was to determine the oxidative status of vitrified equine oocytes and to analyze the effect of melatonin on mitochondrial-specific ROS (mROS), oocyte maturation, ICSI embryo development and viability. Immature, abattoir-derived oocytes were held for 15 h and vitrified in a final concentration of 20% EG, 20% DMSO and 0.65 M trehalose. In Experiment 1, overall ROS was determined by DCHF-DA; vitrification increased ROS production compared to non-vitrified controls (1.29±0.22 vs 0.74±0.25 a.u.; P= 0.0156). In Experiment 2, mROS was analyzed by MitoSOX™ in vitrified/warmed oocytes matured with (+) or without (-) supplementation of 10^{-9} M melatonin; mROS decreased in vitrified and non-vitrified oocytes matured in presence of melatonin (P< 0.05). In Experiment 3, we assessed the effect of melatonin supplementation on oocyte maturation, embryo development after ICSI, and viability by pregnancy establishment. Melatonin did not improve oocyte maturation, cleavage or blastocyst rate of non-vitrified oocytes. However, vitrified melatonin (+) oocytes reached similar cleavage (61, 75 and 77%, respectively) and blastocyst rate (15, 29 and 26%, respectively) than non-vitrified, melatonin (+) and (-) oocytes. Vitrified, melatonin (-) oocytes had lower cleavage (46%) and blastocyst rate (9%) compared to non-vitrified groups (P < 0.05), but no significant differences were observed when compared to vitrified melatonin (+). Although the lack of available recipients precluded the transfer of every blastocyst produced in our study, transferred embryos from non-vitrified oocytes resulted in 50 and 83% pregnancy rates while embryos from vitrified oocytes resulted in 17 and 33% pregnancy rates, from melatonin (+) and (-) treatments respectively. Two healthy foals, one colt from melatonin (+) and one filly from melatonin (-) treatment, were born from vitrified/warmed oocytes. Gestation lengths (considering day 0 = day of ICSI) were 338 days for the colt and 329 days for the filly, respectively. Our work showed for the first time that in the horse, as in other species, intracellular reactive oxygen species are increased by the process of vitrification. Melatonin was useful in reducing mitochondrial-related ROS and improving ICSI embryo development, although the lower pregnancy rate in presence of melatonin should be further analyzed in future studies. To our knowledge this is the first report of melatonin supplementation to an *in vitro* embryo culture system and its use to improve embryo developmental competence of vitrified oocytes following ICSI.

Keywords: equine, oocyte, vitrification, melatonin, ICSI, pregnancies

1. Introduction

Equine assisted reproductive technologies such as ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) have become widespread among practitioners in recent years. In addition, the advancement of equine cloning and the large numbers of oocytes required in this procedure, has resulted in a growing interest in oocyte cryobanking. Unfortunately, the mammalian oocyte is one of the hardest cells to cryopreserve by traditional, slow freezing [1,2]. Vitrification, a method that results in glass-like structure without the formation of ice crystals, was first reported by Luyet in 1937 [3]. For vitrification to occur, the oocytes must be exposed to relatively high concentrations of permeating and non-permeating cryoprotectants, which act by lowering the freezing point of the cytoplasm and causing oocyte dehydration. Once equilibrated, the sample must be cooled at ultra-rapid rates by direct plunging into liquid nitrogen. In humans, the first live birth from a vitrified/warmed oocyte was reported by Kuleshova et al. in 1999 [4]. Since then, the procedure has gone from experimental to clinical application and is now an integral part of human reproductive medicine.

In the horse, oocyte vitrification is still considered an experimental procedure due to its low success, with only three live foals reported [5,6]. In 2002, Maclellan et al. [5] collected oocytes from live donors; these *in vivo* matured oocytes were vitrified/warmed, surgically transferred to the oviduct of inseminated recipients and ultimately resulted in the birth a foal ("Vitreous") and a filly ("Ethyl"). Sixteen years later, Ortíz-Escribano et al. [6] reported the birth of one foal ("VICSI") from vitrified/warmed, *in vitro* matured oocytes fertilized by ICSI. Although these live births represent a significant accomplishment, the efficiency of the procedure remains extremely low. Several contributing factors have been associated to the lack of progress in the equine species, including the length of exposure to cryoprotectant combinations [6,7,8,9], nuclear maturation status [8,10,11,12], presence or absence of cumulus investments [13], cytoskeletal damage and microtubule depolymerization [6,7], meiotic spindle and chromosomal aberrations [14]. Other factors affecting equine oocyte vitrification were recently reviewed by De Coster et al. [15]. Clearly, a better understanding of the vitrification/warming process and its effects on the oocyte is needed to improve the use of this technology in the horse.

The oocyte requires a considerable stock of functional mitochondria to support early embryonic division and mitochondrial health has been associated with energy reserves during meiosis, calcium modulation, lipid oxidation and successful embryo development [16,17,18].

Mitochondria plays an important role in the reactive oxygen species (ROS) balance during the process of oxidative phosphorylation and their dysfunction results in harmful accumulations of superoxide anions within the cell. Interestingly, vitrification/warming has been linked both to increased ROS levels and mitochondrial dysfunction in several species, including pigs [19], cattle [20], mice [21,22] and humans [23]. Although there is currently no information regarding the oxidative status of the vitrified/warmed equine oocyte, our group recently demonstrated impaired mitochondrial function and lower membrane potential after vitrification/warming, particularly with long exposure to cryoprotectants [9]. Mitochondrial function and increased ROS levels in vitrified/warmed oocytes may contribute to the reduced developmental competence observed in the horse.

Melatonin (N-acetyl-5-methoxy tryptamine), an indole compound found in vertebrates to modulate circadian rhythm, has been reported to stimulate ROS metabolization, improve cell membrane fluidity and restore mitochondrial function [24,25]. The importance of melatonin as antioxidant has been associated with several unique characteristics such as its lipophilic and hydrophilic nature, its ability to by-pass membranes, and its accumulation in all cell compartments including mitochondria [25, 26]. As antioxidant, it has been shown to improve the development of porcine [27], bovine [28,29] and murine [30] oocytes after *in vitro* maturation. Several authors have suggested mitochondria are the main targets of melatonin's antioxidant action, by improving electron transport chain and reducing mitochondrial DNA damage [24,31].

To date, there are no reports about the oxidative status or mitochondrial-related ROS of the vitrified/warmed equine oocyte. We hypothesize ROS may increase after vitrification and melatonin's antioxidant capacity could act as a free radical scavenger thus improving embryo yield after vitrification. Therefore, our objective was to determine the overall oxidative status of vitrified equine oocytes and to evaluate the effect of melatonin supplementation on mitochondrial-specific ROS (mROS), oocyte maturation, ICSI embryo development and viability by pregnancy establishment following transfer of *in vitro* produced equine blastocysts.

2. Materials and Methods

2.1. Media and chemicals

Except otherwise indicated, all chemicals were obtained from Sigma-Aldrich®.

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157 *2.2.General Procedures*

158 *2.2.1. Oocyte harvest and holding*

159 Equine ovaries were obtained from local abattoir (Frigorífico Lamar, Buenos Aires,
160 Argentina), transported at room temperature to the laboratory in an insulated container (2-h transit
161 time) and processed by follicular slicing followed by scraping using bone curettes of various sizes.
162 All visible follicles up to 35 mm in diameter were processed; this was done because larger-size
163 follicles tend to be hyaluronan-rich and will typically render a tight matrix that makes the recovery
164 of oocytes problematic. In addition, follicles with evidence of intrafollicular blood were also
165 avoided. The contents of curettes were rinsed in a centrifuge tube containing 25 mL of Hepes-
166 TALP medium [32], allowed to sediment to the bottom and transferred to a search dish. The
167 cumulus-oocyte complexes (COCs) were isolated in a stereomicroscope at 50X magnification.
168 No separation of expanded versus compact cumulus was made; all COCs with no evidence
169 of degeneration were included. Previous reports regarding vitrification of immature equine oocytes
170 suggested that leaving only a few layers of cumulus cells surrounding the oocyte would not affect
171 subsequent IVM and may be beneficial in the process [6]. Therefore, the excess cumulus cells of
172 all the COCs were removed in Hepes-TALP medium using an oocyte stripper (Stripper®, Cooper
173 Surgical, USA) fitted with a 150 µm pipette tip immediately after isolation.
174 Oocytes were held overnight (~15 h) in 2 mL Eppendorf tubes filled to the top with holding
175 medium, sealed with parafilm and placed in a thermostatic water bath set at 22^{o}C. Holding medium
176 consisted of 40% Medium 199 Hanks's salts, 40% Medium 199 Earle's salts and 20% fetal bovine
177 serum (FBS) supplemented with 25 µg/mL gentamicin.
178

179 *2.2.2. Oocyte vitrification and warming*

180

181 Immature oocytes were vitrified and warmed using a three-step short protocol adapted from
182 Lane et al. (1999) [33,34]. The description of vitrification media composition, exposure times and
183 working temperatures is shown in Table 1. Concave microwells of Universal GPS® petri dishes
184 (LifeGlobal, Cooper Surgical, USA) were used to move oocytes trough sequential
185 cryopreservation solutions without mineral oil overlay. A pulled, borosilicate Pasteur pipette

connected to a 0.22 µm filter and mouth tubing was used to move oocytes through solutions with minimal volume. The Cryotop® was selected as the vitrification device, in which oocytes were loaded in groups of 5 with minimal volume of vitrification solution number 3 (VS3). Excess medium was quickly removed, thus creating a thin film layer and causing the oocytes to flatten onto the surface of the device. The Cryotop® was then plunged into liquid nitrogen; the action of plunging was conducted in one single, rapid movement while maintaining horizontal agitation in order to minimize the Leidenfrost effect. Vitrified oocytes remained stored for at least one week at -196ºC prior to removal and warming. Warming was conducted in 4-well plates (USCryotec, USA); oocytes were moved sequentially through warming solutions. The description of warming media composition, volumes, exposure times and working temperatures is shown in Table 1.

Both vitrified/warmed and non-vitrified oocytes were allowed a 90 min cytoskeletal stabilization period prior to fluorescent staining or exposure to IVM medium with gonadotropins. This stabilization was conducted in pre-equilibrated IVC medium (composition described in 2.2.7.) at 38.2ºC in a humidified atmosphere of 6% CO_2 in air.

2.2.3. Determination of total reactive oxygen species (ROS)

The level of intracellular ROS of equine non-vitrified and vitrified immature oocytes was measured using 2″, 7′-dichlorodihydrofluorescein diacetate (DCHF-DA, Molecular Probes, Eugene, OR, USA) as previously described for other species [35,36].

Due to lack of published information about ROS measurement in equine oocytes, incubation temperature was adjusted to fit the equine requirements. Oocytes were gently and completely denuded from cumulus cells using an oocyte stripper fitted with a 130-µm diameter micropipette in TALP medium without hyaluronidase supplementation. Then, oocytes were washed and incubated in 10 µM DCHF-DA in Hepes-TALP for 30 min at 38.2˚C, in total darkness and humidified air. Fluorescence emission was measured in an epifluorescence microscope with bandpass green filter block (465-495 nm excitation/515-555 nm emission) immediately after mounting.

2.2.4. Determination of mitochondrial-specific reactive oxygen species (mROS)

MitoSOX™ Red (Molecular Probes, Eugene, OR, USA) stock solution (5 mM) was prepared according to manufacturer instructions by diluting the content of 1vial (50 µg) into 13 µL of sterile DMSO and stored in 1 µL aliquots in a vacuum packed and sealed container. Oocytes were denuded as described in 2.2.3. and incubated in a final concentration of 5µM MitoSOX™ in Hepes-TALP (without phenol red) for 30 min at 38.2°C in the dark in a humidified chamber. After 30 minutes, the oocytes were washed three times in 100 µL TALP droplets and placed for visualization in groups of 5 oocytes/concave slide. Fluorescence emission was evaluated in an epifluorescence microscope with bandpass red filter block (528 nm excitation/575 nm emission) immediately after mounting.

2.2.5. Images analysis

The acquired images of ROS and mROS determination were processed using ImageJ software (version 1.51, National Institutes of Health, USA). Micrographs were converted into greyscale images and the integrated density of the signal was quantified individually. Background fluorescence was subtracted in every repetition; Mean fluorescence intensity (arbitrary units, a.u.) was plotted graphically with standard error of mean.

2.2.6. In vitro maturation with and without melatonin supplementation

The melatonin working concentration was selected based on previous reports for pigs [27,37] because the low number of available oocytes precluded a preliminary dose-response curve. Melatonin (M5250) working stock was prepared weekly in Medium 199 while protecting from light and used immediately; it was added to oocyte IVM medium to a final concentration of 10^{-9} M.

In vitro maturation was conducted in Medium 199 with 10% FBS, 1 µL/mL insulin-transferrin-selenium, 1 mM sodium pyruvate, 100 mM cysteamine, 0.1 mg/mL FSH (Folltropin-V; Vetoquinol, USA) and 25 µg/mL gentamicin. Maturation was conducted in groups of up to 10 COCs per 100 µL droplets under mineral oil. Cumulus-oocyte complexes were cultured for 28 hours at 38.2°C in a humified atmosphere of 6% CO_2 in air.

2.2.7. Intracytoplasmic sperm injection (ICSI) and embryo in vitro culture (IVC)

Only oocytes with a visible first polar body were considered mature and subjected to conventional ICSI without Piezo drill. Frozen semen from a single stallion of previously proven fertility for ICSI was used. A 3-5 mm portion of the semen straw was cut under liquid nitrogen and transferred to a centrifuge tube containing 3 mL of Hepes-TALP. Sperm was allowed to swim up for 25 min at room temperature, protected from light. A 1 µL aliquot of the upper layer was added to a 3µL droplet of 7% PVP solution (Sage, Cooper Surgical, USA). Micromanipulation was conducted in a Nikon TiU inverted microscope fitted with Narishige micromanipulators and oil microinjectors. Mature oocytes were placed in a 100 µL Hepes-TALP droplet; a single sperm immobilized and injected using a 7 µm (o.d.) micropipette (Origio, Cooper Surgical, USA).

Presumptive zygotes were cultured in DMEM-F12/Global Total LP® (54:40) with 6% FBS (GE Healthcare HyClone™ Fetal Bovine Serum, U.S., Standard, SH30088.03), 0.1 mM sodium pyruvate and 10 µg/mL gentamicin and cultured at 38.2ºC in 5% CO_2, 5%O_2 and 90% N_2 in a modular incubator chamber MIC-101 (Billups-Rothenberg Inc., CA, USA) placed inside a traditional water-jacketed CO_2 incubator.

2.2.8. Evaluation and transfer of ICSI embryos

Cleavage assessment was conducted on day 3 (day 0= ICSI). On day 5 of IVC, medium was completely renewed, unfertilized oocytes were removed and those with 16 or more visible blastomeres were recorded as well as embryos with evidence of cytoplasmic fragments. From days 7 to 10 of IVC, blastocyst development was monitored daily.

Embryo grading system was based on our own laboratory observations and also on grading described by Tremoleda et al. (2003) for equine embryos produced by piezo-assisted ICSI [38]. Embryos were graded from 1 to 4 (1= Excellent; 2= Good; 3= Poor; 4= Degenerate); grade 1 blastocysts were symmetrical and evenly colored, with a noticeable blastocele cavity, refringent trophectoderm layer, absence of cytoplasmic fragmentation and frequently thinned zona pellucida; grade 2 may include irregular shape, minor cytoplasmic fragmentation and less evident trophectoderm layer; grade 3 embryos were characterized by substantial cytoplasmic granules, large perivitelline space, and absence of distinguishable trophectoderm; grade 4 was characterized by overall cytoplasmic fragmentation, absence of trophectoderm layer or noticeable blastocoel cavity, and may include broken zona pellucida. Only embryos grades 1 and 2 were transferred.

Embryos were transferred to recipients from a commercial polo embryo transfer herd during the reproductive season for the Southern Hemisphere. Available recipients with ovarian follicles ≥ 35 mm diameter were synchronized by hCG administration on the same day of the ICSI procedure and subsequently monitored for ovulation. Embryos were rinsed, loaded into sterile 0.5 mL straws in Syngro® holding medium (Vetoquinol, USA), transported to the farm (2 h-transit time) and non-surgically transferred to the uterus of a recipient on day 5 post-ovulation. Pregnancy was checked by rectal ultrasonography on day 14 and confirmed for the presence of an embryo with cardiac activity on day 26; fetal sexing was conducted between days 50 and 60 after transfer by an experienced technician.

2.3. Experiments and treatments

2.3.1. Experiment 1: Determination of oxidative status

The overall oxidative status of fresh and vitrified/warmed equine oocytes was studied. For this, the total ROS of immature oocytes randomly allocated to Non-vitrified or Vitrified treatment groups, was measured by DCHF-DA staining in 2 replicates (n=25 oocytes per group).

2.3.2. Experiment 2: Determination of mitochondrial-specific reactive oxygen species

Mitochondrial function was analyzed by measurement of mROS using MitoSOX™ Red fluorescent assay for live cells. Immature oocytes were randomly allocated to Non-vitrified, melatonin (-), Non-vitrified, melatonin (+), Vitrified, melatonin (-) or Vitrified, melatonin (+) groups. After IVM, only the oocytes with a visible first polar body were used for fluorescent staining. The mROS level was measured in 3 replicates (n=45 *in vitro*-matured oocytes per group).

2.3.3. Experiment 3: In vitro embryo development after ICSI and transfer to recipients

The developmental potential of non-vitrified and vitrified oocytes, with and without melatonin supplementation was analyzed by ICSI embryo production. The viability of the resulting embryos was evaluated by transfer to synchronized recipients; this experiment was conducted in 4 replicates during the reproductive season for the Southern Hemisphere. A total of 452 immature oocytes were randomly allocated to Non-vitrified, melatonin (-) (n=137), Non-vitrified, melatonin (+) (n=100), Vitrified, melatonin (-) (n=106) or Vitrified, melatonin (+) (n=110) groups. Only day-

7, grades 1 or 2 blastocysts were selected for transfers to the available synchronized recipient mares.

2.4. Statistical analysis

Continuous variables in Experiment 1 and 2 were analyzed using the non-parametric Kruskal-Wallis and Dunn's test for multiple comparisons with GraphPad Prism software (version 6.0c for Mac, GraphPad Software, La Jolla California, USA); values were expressed as Mean fluorescence intensity ± SEM. The effect of melatonin supplementation on *in vitro* oocyte maturation rate, embryo development and viability (Experiment 3) was analyzed using a probit link function with binomial error distribution; results were expressed as mean (%) within the text, and dispersion values (SEM) were included in Table 2. InfoStat software (Versión 2020, Centro de Transferencia InfoStat, FCA, Universidad Nacional de Córdoba, Argentina) was used to perform the generalized linear model. Statistical significance level was set at $P<0.05$.

3. Results

3.1. Experiment 1: Determination of oxidative status

Non-vitrified and vitrified/warmed immature equine oocytes showed differences in their intracellular ROS levels. Intracellular ROS was higher in vitrified/warmed oocytes; Mean fluorescence intensity increased significantly after a vitrification/warming cycle (1.503±0.32 vs 2.394±1.01 a.u., $P<0.05$) (Figure 1).

3.2. Experiment 2: Determination of mitochondrial-specific reactive oxygen species

There was no difference in mROS levels of non-vitrified and vitrified oocytes without melatonin supplementation. However, supplementation with 10^{-9} M melatonin during oocyte IVM resulted in a decrease in mitochondrial-specific ROS, both for non-vitrified (1.184±0.08 vs 0.787±0.04 a.u., $P<0.05$) and vitrified oocytes (1.048±1.16 vs 0.560±0.02 a.u., $P<0.05$) (Figure 2).

3.3. Experiment 3: In vitro embryo development after ICSI and transfer to recipients

All the vitrified oocytes (n=216) survived (morphologically) the vitrification/warming cycle. Non-vitrified and vitrified/warmed equine immature oocytes were matured *in vitro*, with or without supplementation of 10^{-9} M melatonin for a period of 26-28 h. The addition of melatonin

to IVM did not have an effect on the proportion of oocytes reaching the metaphase-II stage, although a slight increase in maturation rate was observed for oocytes in the non-vitrified, melatonin (+) group (P= 0.19).

Melatonin supplementation had no effect on embryo development after ICSI of non-vitrified oocytes in terms of cleavage (75 vs 77%) or blastocyst rate (29 vs 26%; melatonin (+) and (-) respectively). However, vitrified, melatonin (+) oocytes reached similar cleavage (62%) and blastocyst (15%) rates than non-vitrified oocytes. No differences either in cleavage or blastocyst rate were observed among vitrification groups (Table 2, Figure 3). Also, when analyzing the effect of vitrification procedure, a decrease in embryo development of vitrified oocytes compared to non-vitrified groups was observed (P= 0.0168 for cleavage; P= 0.0265 for blastocyst rate).

Two pregnancies were established from vitrified/warmed oocytes; one from melatonin (+) and the other from melatonin (-) treatment groups. A 17 and 33% pregnancy rate (pregnancy/transferred embryo) was obtained from vitrified, melatonin (+) and (-) oocytes, respectively. In addition, pregnancies were also established from non-vitrified, melatonin (+) and (-) oocyte groups (50% and 83% pregnancy rate, respectively), indicating the ICSI embryos produced were viable. The lack of available recipients precluded the transfer of all the blastocysts produced in the study; however, embryos in all treatment groups continued to increase in diameter and hatch (partially or totally) *in vitro* (Figure 4).

The two pregnancies established from vitrified/warmed oocytes were allowed to progress to term; the remaining pregnancies from non-vitrified oocytes were terminated after fetal sexing on day 60 due to the need to free those recipients for the commercial operation. Two healthy foals, one colt from melatonin (+) and one filly from melatonin (-) treatment, were born from vitrified/warmed oocytes . Gestation lengths (considering day 0 = day of ICSI) were 338 days for the colt and 329 days for the filly, respectively (Figure 5).

4. Discussion

Equine oocyte vitrification is still considered an experimental procedure due to its low success, with only three live foals reported to date [5,6]. Several contributing factors have been associated to the lack of success; among these, oocyte maturation status at vitrification [12], presence or absence of cumulus cells [6], media cryoprotectant composition and exposure to

vitrification solutions [8]. Clearly, a better understanding of the effects of the vitrification/warming process is needed in order to make improvements to the current procedures in the horse.

Reactive oxygen species (ROS) are metabolism by-products generated during the intermediate steps of oxygen reduction. Superoxide anion radical, hydrogen peroxide, and hydroxyl radical have been implicated in oxidative stress of gametes and embryos. Our work demonstrated that in the horse, as in other species, the intracellular oxidative status is affected by the vitrification and warming process, resulting in an increase in ROS activity. These results are in agreement with previous observations reported for human [39] mouse [40], cattle [20,41] and pigs [42]. Increased ROS level in oocytes can lead to mitochondrial disfunction [43], alterations in calcium oscillations, reductions in ATP content and fertilization failure [44,45]. The increased ROS activity of vitrified/warmed oocytes could, in part, explain the reduced developmental rates observed in equine studies [6,8,46].

The superoxide anion reactive oxygen species is generated as a by-product of mitochondrial oxidative phosphorylation (mROS). Oxidative stress has been associated with mitochondrial damage [47] and mitochondrial DNA (mtDNA) is particularly prone to mutations due to its lack of histones which normally contribute to ROS control [48]; therefore, the generation of superoxide has been described as one of the most damaging sources of toxicity and mitochondrial dysfunction. In the present study, we found no difference in the mROS levels after IVM of equine oocytes in absence of melatonin. However, when oocytes were supplemented with melatonin during IVM we found a significant decrease in mitochondrial-specific ROS, for both non-vitrified and vitrified oocytes. These results are consistent with previous observations in other species, indicating that melatonin may have an action as a mitochondrial-specific antioxidant [49] and adds the body of evidence regarding the antioxidant and free radical scavenging properties of melatonin and its metabolites [25,50].

In cattle, El-Raey et al. [28] reported improved oocyte maturation and reduced ROS in presence of melatonin. In pigs, melatonin supplementation during maturation resulted in higher glutathione content and inhibited apoptosis when oocytes were exposed to heat stress [51]. Although in our present study maturation rate of non-vitrified oocytes supplemented with melatonin was only marginally improved, some studies have indicated that melatonin's effect might be dose-dependent [52]. In addition, it must me noted the reduced number of equine oocytes precluded a preliminary dose-response curve; therefore, it is possible the melatonin concentration

selected for our study based on previous publications for pigs [27,37] may not be adequate for the horse; further experiments should evaluate the optimal concentration for the equine species. To our knowledge, this is the first report of melatonin addition to maturation medium of equine oocytes.

In addition, there are no reports of melatonin supplementation for the improvement of vitrification outcome in the horse and only a few reports are available for other animal species. In the mouse, a 1-hour of exposure of metaphase-II oocytes to melatonin during post-warming stabilization period did not result in improvements to embryo developmental competence [53]. On the contrary, Zhang et al., [54] reported vitrified metaphase-II oocytes reached similar blastocyst formation than non-vitrified control when melatonin supplementation was conducted for the complete post-warming and IVC period (2-hour stabilization, 5-hour activation and complete IVC), indicating the length of exposure to antioxidant could be a contributing factor. Similar findings were also reported for mature bovine vitrified oocytes supplemented with melatonin both during IVM and vitrification procedure, suggesting a relationship between developmental potential, ROS reduction and the consequent inhibition of apoptotic events due to melatonin addition [55]. In our present study, vitrified melatonin (+) oocytes reached similar cleavage (61, 75 and 77%, respectively) and blastocyst rate (15, 29 and 26%, respectively) than non-vitrified, melatonin (+) and (-) oocytes; this combined with our observations regarding melatonin's ability to reduce mROS indicates the beneficial role of melatonin on mitochondrial health may be related to the improved embryo development from equine vitrified oocytes.

Although the mechanism of melatonin action is still unclear, some reports have suggested a relationship between melatonin, mitochondrial function and lipid metabolism. In 2017, Jin et al. [56] reported higher number of lipid droplets (LDs) and upregulated gene expression related to lipogenesis after maturation of porcine oocytes with melatonin supplementation. In another study, He et al. (2017) [57] showed pig oocytes supplemented with melatonin had higher maturation rates, more lipid droplets and higher triglyceride content. Interestingly, they reported reduced mitochondrial reactive oxygen species (mROS) content and a significant down-regulation of mtDNA-encoded genes after melatonin treatment. This reduction in mROS levels is in agreement with our present study in horses. Because both porcine and equine oocytes are characterized by relatively high lipid content, a possible mechanism of action regarding melatonin-mitochondria-lipid should be analyzed in the future.

Although the lack of available recipients precluded the transfer of every blastocyst produced, the transferred embryos from non-vitrified oocytes resulted in 50 and 83% pregnancy rates while embryos from vitrified oocytes resulted in 17 and 33% pregnancy rates, from melatonin (+) and (-) treatments respectively. Noteworthy, this study was limited by the lack of available recipients which precluded the transfer of every grade 1 and 2 embryo produced. Although no statistical difference on pregnancy rates was observed, they tended to be lower with melatonin supplementation and this aspect should be further analyzed in future studies. Also, when compared to non-vitrified groups, vitrified/warmed oocytes had lower pregnancy rates although no statistical differences were observed probably due to the low number of observations (P= 0.32). These results are similar to those previously reported by Ortiz-Escribano et al. [6] for vitrified oocytes, where 1 out of 4 transfers resulted in pregnancy establishment, indicating that embryo viability from vitrified oocytes could be compromised.

Previously, our laboratory reported ICSI blastocyst development from immature, abattoir-derived vitrified and non-vitrified oocytes [9]. Noteworthy, we described an asynchrony in blastocyst development between vitrified and non-vitrified groups; embryos from vitrified oocytes reached the same developmental stage (early blastocyst) approximately 24 h later than embryos from non-vitrified controls, possibly indicating compromised viability [9]. In the present study, although the same dispersion in developmental rate was noticed, the large number of oocytes available for injection allowed a better selection of embryos at more advanced stages on day 7 post-injection, thus resulting in potentially better quality embryos (with more organized trophectoderm and incipient blastocoel cavity) being transferred to day 5 recipients. In this regard, we hypothesize quality selection may be a fundamental aspect to the success in establishing pregnancies from vitrified oocytes, as their darker color (particularly around the morula stage) makes morphological evaluation difficult. Noteworthy, some of early developing embryos from vitrified oocytes were kept in culture and were observed to hatch by day 10.

Our study resulted in two pregnancies from vitrified oocytes, one each from melatonin (-) and melatonin (+) groups and the birth of the second and third live foals reported in the scientific literature for vitrified oocytes followed by ICSI, IVC and transfer. This represents an advancement since, prior to our present study, a total of three live births had been informed [5,6] and only one [6] was the result of vitrification / ICSI procedure of immature oocytes similar to the present work.

5. Conclusion

We demonstrated intracellular reactive oxygen species are increased by the vitrification process of immature oocytes. Melatonin supplementation reduced mitochondrial-related ROS, improved ICSI embryo development and resulted in the establishment of an ongoing pregnancy from immature, vitrified oocytes. To our knowledge this is the first report of use of melatonin in an equine ICSI/in vitro culture system and a first attempt to improve embryo development from vitrified oocytes, resulting in live foals.

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References

[1] Fuku, E., Kojima, T., Shioya, Y., Marcus, G. J., and Downey, B. R. 1992. In vitro fertilization and development of frozen-thawed bovine oocytes. *Cryobiology*, *29*(4), pp. 485-492. doi:10.1016/0011-2240(92)90051-3

[2] Oktay, K, Pelin Cil, A., and Bang, H. 2006. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil steril*, 86(1), pp. 70-80. doi:10.1016/j.fertnstert.2006.03.017

[3] Luyet, B. J., and Gibbs, M. C. 1937. On the mechanism of congelation and of death in the rapid freezing of epidermal plant cells. *Biodynamica*, *1*(25), pp.1-18. doi:10.1016/S0011-2240(66)80029-9

[4] Kuleshova, L., Gianaroli, L., Magli, C., Ferraretti, A., and Trounson, A. 1999. Birth following vitrification of a small number of human oocytes: case report. *Human Reprod*, *14*(12), pp.3077-3079. doi:10.1093/humrep/14.12.3077

[5] Maclellan, L.J., Carnevale, E.M., Da Silva, M.C., Scoggin, C.F., Bruemmer, J.E. and Squires, E.L., 2002. Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. *Theriogenology*, *58*(5), pp.911-919. doi:10.1016/S0093-691X(02)00920-2

[6] Ortíz-Escribano, N., Bogado Pascottini, O., Woelders, H., Vandenberghe, L., De Schauwer, C., Govaere, J., et al. (2018). An improved vitrification protocol for equine immature oocytes, resulting in a first live foal. *Equine Vet J*, *50*(3), pp.391-397. doi:10.1111/evj.12747

[7]. Tharasanit T., Colenbrander B., and Stout T. A. E. (2006a). Effect of maturation stage at cryopreservation on post-thaw cytoskeleton quality and fertilizability of equine oocytes. *Mol Reprod Dev*, 73, 627–637. doi:10.1002/mrd.20432

[8]. Canesin, H.S., Brom-de-Luna, J.G., Choi, Y.H., Pereira, A.M., Macedo, G.G. and Hinrichs, K., 2018. Vitrification of germinal-vesicle stage equine oocytes: Effect of cryoprotectant exposure time on in-vitro embryo production. *Cryobiology, 81*, pp.185-191. doi:10.1016/j.cryobiol.2018.01.001

[9]. Clérico, G., Rodríguez, M.B., Taminelli, G., Butteri, A., Veronesi, J.C., Fernández, S., et al., 2018. Vitrification of Immature Oocytes for the Production of Equine Embryos by ICSI: Protocol Effect on Maturation, Embryo Development, Mitochondrial Distribution and Functionality. *J Equine Vet Sci, 66*, pp.192-193. doi:10.1016/j.jevs.2018.05.083

[10]. Tharasanit T., Colleoni S., Lazzari G., Colenbrander B., Galli C., and Stout T. A. E., 2006b. Effect of cumulus morphology and maturation stage on the cryopreservability of equine oocytes. *Reproduction*, 132, 759–769. doi:10.1530/rep.1.01156

[11] Maclellan, L.J., Stokes, J.E., Preis, K.A., McCue, P.M. and Carnevale, E.M., 2010. Vitrification, warming, ICSI and transfer of equine oocytes matured in vivo. *Anim Reprod Sci, 121*, pp.S260-S261. doi:10.1016/j.jevs.2018.05.083

[12]. Angel, D., Canesin, H.S., Brom-de-Luna, J.G., Morado, S., Dalvit, G., Gomez, D., et al., 2020. Embryo development after vitrification of immature and in vitro-matured equine oocytes. *Cryobiology*, In press. doi:10.1016/j.cryobiol.2020.01.014

[13]. Tharasanit T., Colleoni S., Galli C., Colenbrander B., and Stout T. A. E., 2009. Protective effects of the cumulus-corona radiata complex during vitrification of horse oocytes. *Reproduction*, 137, 391–401. doi:10.1530/REP-08-0333

[14] Ducheyne K. D., Rizzo M., Daels P. F., Stout T. A. E., and de Ruijter-Villani M., 2019. Vitrifying immature equine oocytes impairs their ability to correctly align the chromosomes on the MII spindle. *Reprod Fertil Dev*, doi:10.1071/RD18276

[15] De Coster, T., Velez, D.A., Van Soom, A., Woelders, H. and Smits, K., 2020. Cryopreservation of equine oocytes: looking into the crystal ball. *Reprod Fertil Dev*, *32*(5), pp.453-467**.** doi:10.1071/RD19229

[16] Nagai, S., Mabuchi, T., Hirata, S., Shoda, T., Kasai, T., Yokota, S., and Hoshi, K., 2004. Oocyte mitochondria: strategies to improve embryogenesis. *Hum cell, 17(4)*, pp.195-201. doi: 10.1111/j.1749-0774.2004.tb00043.x.

[17] Mishra, P., and Chan, D. C., 2014. Mitochondrial dynamics and inheritance during cell division, development and disease. *Nat Rev Mol Cell Biol*, *15*(10), pp.634-646. doi:10.1038/nrm3877

[18] Babayev, E., and Seli, E., 2015. Oocyte mitochondrial function and reproduction. *Curr Opin Obstet & Gynecol*, *27*(3), pp.175. doi:10.1097/GCO.0000000000000164

[19] Dai, J., Wu, C., Muneri, C. W., Niu, Y., Zhang, S., Rui, R., and Zhang, D., 2015. Changes in mitochondrial function in porcine vitrified MII-stage oocytes and their impacts on apoptosis and developmental ability. *Cryobiology*, *71*(2), pp.291-298. doi:10.1016/j.cryobiol.2015.08.002

[20] Gutnisky, C., Morado, S., Gadze, T., Donato, A., Alvarez, G., Dalvit, G., et al. 2020. Morphological, biochemical and functional studies to evaluate bovine oocyte vitrification. *Theriogenology, 143,* pp.18-26. doi:10.1016/j.theriogenology.2019.11.037

[21] Gao, C., Han, H.B., Tian, X.Z., Tan, D.X., Wang, L., Zhou, G.B., et al., 2012. Melatonin promotes embryonic development and reduces reactive oxygen species in vitrified mouse 2-cell embryos. *J Pineal Res*, *52*(3), pp.305-311. doi:10.1111/j.1600-079X.2011.00944.x

[22] Nazmara, Z., Salehnia, M., and Hossein Khani, S., 2014. Mitochondrial distribution and ATP content of vitrified, in vitro matured mouse oocytes. *Avicenna J Med Biotechnol*, *6*(4), pp.210. ISSN: 20084625

[23] Nohales-Córcoles, M., Sevillano-Almerich, G., Di Emidio, G., Tatone, C., Cobo, A. C., Dumollard, R., et al., 2016. Impact of vitrification on the mitochondrial activity and redox homeostasis of human oocyte. *Hum Reprod*, *31*(8), pp.1850-1858. doi:10.1093/humrep/dew130

[24] Leon J, Acuna-Castroviejo D, Escames G, Tan OX, Reiter RJ. Melatonin mitigates mitochondrial malfunction. 2005. *J Pineal Res*, 38:1–9. doi:10.1111/j.1600-079X.2004.00181.x

[25] Reiter, R.J., Rosales-Corral, S., Tan, D.X., Jou, M.J., Galano, A., et al., 2017. Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cell Mol Life Sci*, *74*(21), pp.3863-3881. doi:10.1007/s00018-017-2609-7

[26] Acuña-Castroviejo D, G. Escames Rosa, J. León López, A. Carazo Gallego and H. Khaldy, 2003. Mitochondrial regulation by melatonin and its metabolites. *Adv Exp Med Biol,* 527, pp.549-557. doi:10.1007/978-1-4615-0135-0_63

[27] Shi, J.M., Tian, X.Z., Zhou, G.B., Wang, L., Gao, C., Zhu, S.E., Zeng, S.M., et al., 2009. Melatonin exists in porcine follicular fluid and improves in vitro maturation and parthenogenetic development of porcine oocytes. *J Pineal Res*, *47*(4), pp.318-323. doi:10.1111/j.1600-079X.2009.00717.x

[28] El-Raey, M., Geshi, M., Somfai, T., Kaneda, M., Hirako, M., Abdel-Ghaffar, A.E., et al., 2011. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. *Mol Reprod Dev*, *78*(4), pp.250-262. doi:10.1002/mrd.21295

[29] Zhao, X., Min, J., Du, W., Hao, H., Liu, Y., Qin, T., et al., 2015. Melatonin enhances the in vitro maturation and developmental potential of bovine oocytes denuded of the cumulus oophorus. *Zygote, 23*(4), 525-536. doi:10.1017/S0967199414000161

[30] Lord, T., Nixon, B., Jones, K. T., and Aitken, R. J., 2013. Melatonin prevents postovulatory oocyte aging in the mouse and extends the window for optimal fertilization in vitro. *Biol Reprod, 88*(3), pp.67-1. doi:10.1095/biolreprod.112.106450

[31] Tamura, H., Takasaki, A., Miwa, I., Taniguchi, K., Maekawa, R., Asada, H., et al., 2008. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res, 44*(3), pp.280-287. doi:10.1111/j.1600-079X.2007.00524.x

[32] De Pauw, I.M.C., Van Soom, A., Mintiens, K., Verberckmoes, S. and de Kruif, A., 2003. In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. *Theriogenology, 59*(5-6), pp.1093-1107. doi:10.1016/S0093-691X(02)01207-4

[33] Lane, M., Bavister, B.D., Lyons, E.A. and Forest K.T., 1999. Containerless vitrification of mammalian oocytes and embryos. *Nat Biotechnol, 17*(12):1234-6. doi:10.1038/70795

[34] Lane, M., Schoolcraft, W.B., Gardner, D.K. and Phil, D., 1999. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril, 72*(6), pp.1073-1078. doi:10.1016/S0015-0282(99)00418-5

[35] Morado, S.A., Cetica, P.D., Beconi, M.T. and Dalvit, G.C., 2009. Reactive oxygen species in bovine oocyte maturation in vitro. *Reprod Fertil Dev, 21*(4), pp.608-614. doi:10.1071/RD08198

[36] Salavati, M., Ghafari, F., Zhang, T. and Fouladi-Nashta, A.A., 2012. Effects of oxygen concentration on in vitro maturation of canine oocytes in a chemically defined serum-free medium. *Reproduction, 144*(5), p.547. doi:10.1530/REP-12-0176

[37] Rodriguez-Osorio, N., Kim, I.J., Wang, H., Kaya, A. and Memili, E., 2007. Melatonin increases cleavage rate of porcine preimplantation embryos in vitro. *J Pineal Res, 43*(3), pp.283-288. doi:10.1111/j.1600-079X.2007.00475.x

[38] Tremoleda, J.L., Stout T.A., Lagutina I., Lazzari G., Bevers M.M., Colenbrander B., et al., 2003. Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biol Rprod, 1;69(6)*:1895-906. doi.org/10.1095/biolreprod.103.018515

[39] Gupta, M.K., Uhm, S.J. and Lee, H.T., 2010. Effect of vitrification and beta-mercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. *Fertil Steril, 93*(8), pp.2602-2607. doi:10.1016/j.fertnstert.2010.01.043

[40] Tatone, C., Di Emidio, G., Barbaro, R., Vento, M., Ciriminna, R. and Artini, P.G., 2011. Effects of reproductive aging and postovulatory aging on the maintenance of biological

competence after oocyte vitrification: insights from the mouse model. *Theriogenology*, *76*(5), pp.864-873. doi:10.1016/j.theriogenology.2011.04.017

[41] Zhao, X.M., Du, W.H., Wang, D., Hao, H.S., Liu, Y.A.N., Qin, T, et al., 2011. Recovery of mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture. *Mol Reprod Dev*, *78*(12), pp.942-950. doi:10.1002/mrd.21389

[42] Ren, L., Fu, B., Ma, H. and Liu, D., 2015. Effects of mechanical delipation in porcine oocytes on mitochondrial distribution, ROS activity and viability after vitrification. *Cryoletters*, *36*(1), pp.30-36. ISSN: 01432044

[43] Ahn HJ, Sohn IP, Kwon HC, Jo DH, Park YD, Min CK., 2001. Characteristics of the cell membrane fluidity, actin fibers, and mitochondrial dysfunctions of frozen-thawed two-cell mouse embryos. *Mol Reprod Dev* 61, pp.466–76. doi:10.1002/mrd.10040

[44] Somfai T, Ozawa M, Noguchi J, Kaneko H, Kuriani Karja NW, Farhudin M, 2007. Developmental competence of in vitro–fertilized porcine oocytes after in vitro maturation and solid surface vitrification: effect of cryopreservation on oocyte antioxidative system and cell cycle stage. *Cryobiology* 55, pp.115–26. doi:10.1016/j.cryobiol.2007.06.008

[45] Yoneda A, Suzuki K, Mori T, Ueda J, Watanabe T., 2004. Effects of delipidation and oxygen concentration on in vitro development of porcine embryos. J Reprod Dev 2004;50:287–95. doi:10.1262/jrd.50.287

[46] Canesin, H.S., Brom-de-Luna, J.G., Choi, Y.H., Ortiz, I., Diaw, M. and Hinrichs, K., 2017. Blastocyst development after intracytoplasmic sperm injection of equine oocytes vitrified at the germinal-vesicle stage. *Cryobiology*, *75*, pp.52-59. doi:10.1016/j.cryobiol.2017.02.004

[47] Kowaltowski, A. J., and Vercesi, A. E., 1999. Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med*, *26*(3-4), pp.463-471. doi:10.1016/S0891-5849(98)00216-0

[48] Lin, M. T., and Beal, M. F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), pp.787-795. doi:10.1038/nature05292

[49] Mansouri, A., Gaou, I., de Kerguenec, C., Amsellem, S., Haouzi, D., Berson, A., et al., 1999. An alcoholic binge causes massive degradation of hepatic mitochondrial DNA in mice. *Gastroenterology*, *117*(1), pp.181-190. doi:10.1016/S0016-5085(99)70566-4

[50] Reiter, R. J., Tan, D. X., and Maldonado, M. D., 2005. Melatonin as an antioxidant: physiology versus pharmacology. *J Pineal Res*, *39*(2), pp.215-216. doi:10.1111/j.1600-079X.2005.00261.x

[51] Li, Y., Zhang, Z., He, C., Zhu, K., Xu, Z., Ma, T., et al., 2015. Melatonin protects porcine oocyte in vitro maturation from heat stress. *J Pineal Res*, *59*(3), pp.365-375. doi:10.1111/jpi.12268

[52] Adriaens, I., Jacquet, P., Cortvrindt, R., Janssen, K. and Smitz, J., 2006. Melatonin has dose-dependent effects on folliculogenesis, oocyte maturation capacity and steroidogenesis. *Toxicology, 228(2-3),* pp.333-343. doi:10.1016/j.tox.2006.09.018

[53] Li, W., Cheng, K., Zhang, Y., Meng, Q., Zhu, S.e., and Zhou, G., 2015. No effect of exogenous melatonin on development of cryopreserved metaphase II oocytes in mouse, J Anim Sci Biotechno, 6(1), 42. doi:10.1186/s40104-015-0041-0

[54] Zhang, Y., Li, W., Ma, Y., Wang, D., Zhao, X., Zeng, C., et al., 2016. Improved development by melatonin treatment after vitrification of mouse metaphase II oocytes. *Cryobiology, 73(3),* pp.335-342. doi.org/10.1016/j.cryobiol.2016.09.171

[55] Zhao, X.M., Hao, H.S., Du, W.H., Zhao, S.J., Wang, H.Y., Wang, N., et al., 2016. Melatonin inhibits apoptosis and improves the developmental potential of vitrified bovine oocytes. *J Pineal Res*, *60*(2), pp.132-141. doi:10.1111/jpi.12290

[56] Jin, J. X., Lee, S., Taweechaipaisankul, A., Kim, G. A., and Lee, B. C., 2017. Melatonin regulates lipid metabolism in porcine oocytes. *J Pineal Res, 62*(2), e12388. doi:10.1111/jpi.12388

[57] He, B., Yin, C., Gong, Y., Liu, J., Guo, H. and Zhao, R., 2018. Melatonin-induced increase of lipid droplets accumulation and in vitro maturation in porcine oocytes is mediated by mitochondrial quiescence. *J Cellular Physiol*, *233*(1), pp.302-312. doi:10.1002/jcp.25876

Tables and figures

Table 1. Composition of solutions and exposure times to cryoprotectants.

Vitrification		**DMSO**	**EG**	**Trehalose**	**Volume**	**Exposure time**
	VS1 (23ºC)	5% (0.7 M)	5% (0.9 M)	---	100 µL	45”
	VS2 (23ºC)	10% (1.4 M)	10% (1.8 M)	---	100 µL	45”
	VS3 (23ºC)	20% (2.8 M)	20% (3.6 M)	0.65 M	100 µL	30”
Warming						
	WS1 (38ºC)	---	---	0.25 M	1800 µL	1’
	WS2 (38ºC)	---	---	0.19 M	100 µL	1’
	WS3 (38ºC)	---	---	0.125 M	100 µL	1’

The same base medium was used for all the solutions: Medium 199 (Hank’s salts) with 20% FBS; VS: vitrification solution; WS: warming solution; DMSO: dimethyl sulfoxide; EG: ethylene glycol. In VS3 the exposure time includes the loading onto the vitrification device. Exposure time of WS1 starts when the last oocyte is realeased from the device in the warming solution.

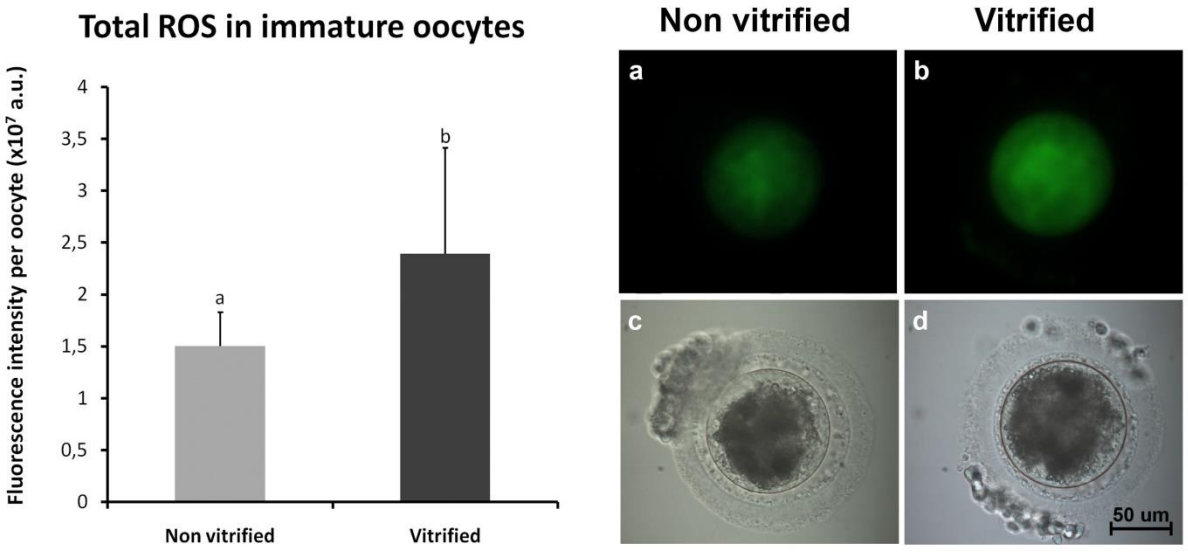

Figure 1. Oxidative status of vitrified and non-vitrified immature equine oocytes indicated by fluorescence intensity of total reactive oxygen species after DCHF-DA staining (Experiment 1). Total ROS production was higher in vitrified/warmed oocytes ($P < 0.05$). Bars in the diagram represent Mean fluorescence intensity + SEM. Different superscripts indicate significant differences. Pictures show representative oocytes after staining with DCHF-DA of non-vitrified (a) and vitrified (b) groups, including the corresponding bright field image (c and d respectively).

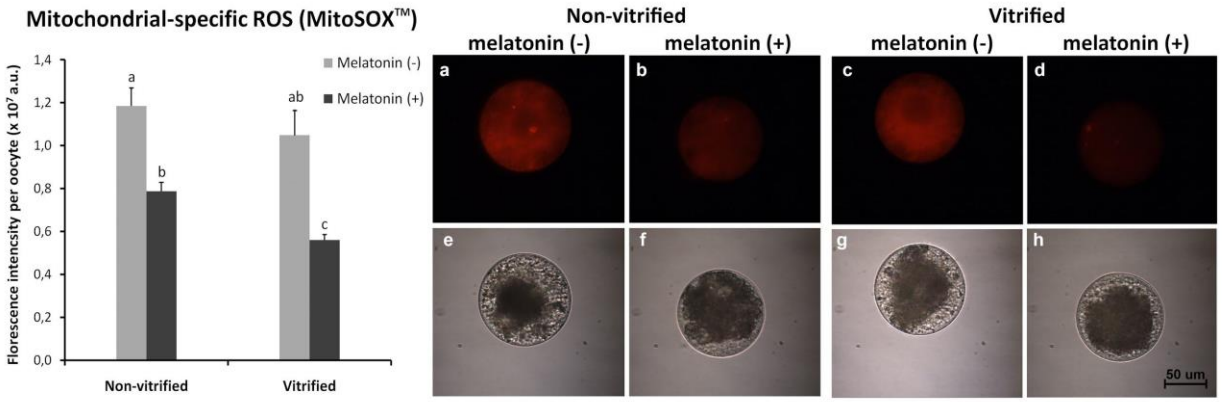

Figure 2. MitoSOX™ fluorescence indicating mitochondrial-specific reactive oxygen superoxide of non-vitrified and vitrified equine oocytes matured with or without melatonin supplementation. Melatonin supplementation significantly decreased mROS in both non-vitrified and vitrified groups. Each bar represents Mean fluorescence intensity + SEM. Different superscripts indicate significant differences. Pictures show representative images of MitoSOX™ staining and its corresponding bright field caption in a,e) non-vitrified melatonin (–) group; b,f) non-vitrified melatonin (+) group; c,g) vitrified melatonin (-) group; d,h) vitrified melatonin (+) group.

Table 2. ICSI development, embryo transfer and pregnancies of non-vitrified and vitrified oocytes, with or without melatonin supplementation.

Oocyte group	N	Maturation rate	Cleavage rate	Blastocyst rate	No. Embryos grade 1-2	No. Embryos grade 3-4	No. Transf.	Pregnancy rate	♂:♀
Non-vitrified melatonin +	100	65±4.8 (65/100)	75±5.3 (49/65)[a]	29±5.7 (19/65)[a]	17	2	5[*]	50% (2/4)	1 : 1
Non-vitrified melatonin -	137	53±4.2 (73/137)	77±5.0 (56/73)[a]	26±5.1 (19/73)[a]	16	3	7[*]	83% (5/6)	2 : 3
Vitrified melatonin +	110	54±4.8 (59/110)	61±6.3 (36/59)[ab]	15±4.9 (9/59)[ab]	6	3	8[**]	17% (1/6)	1 : 0
Vitrified melatonin -	106	54±4.7 (57/106)	46±6.6 (26/57)[b]	9±3.8 (5/57)[b]	4	1	3	33% (1/3)	0 : 1

Data are presented as Mean % ± SEM. Cleavage and Blastocyst rate was calculated from matured oocytes. No differences were observed in embryo grading or pregnancy rate between treatments.

[*] Two of the blastocysts were transferred together to the same recipient.

[**] Two of the embryos were classified as grade 3 due to an enlarged periviteline space (although a distinctive trophectoderm was observed) and transferred in pairs with two, grade 1 blastocysts to the same recipients.

[a,b] Different superscripts within a column indicate significant differences, generalized linear model, $P<0.05$.

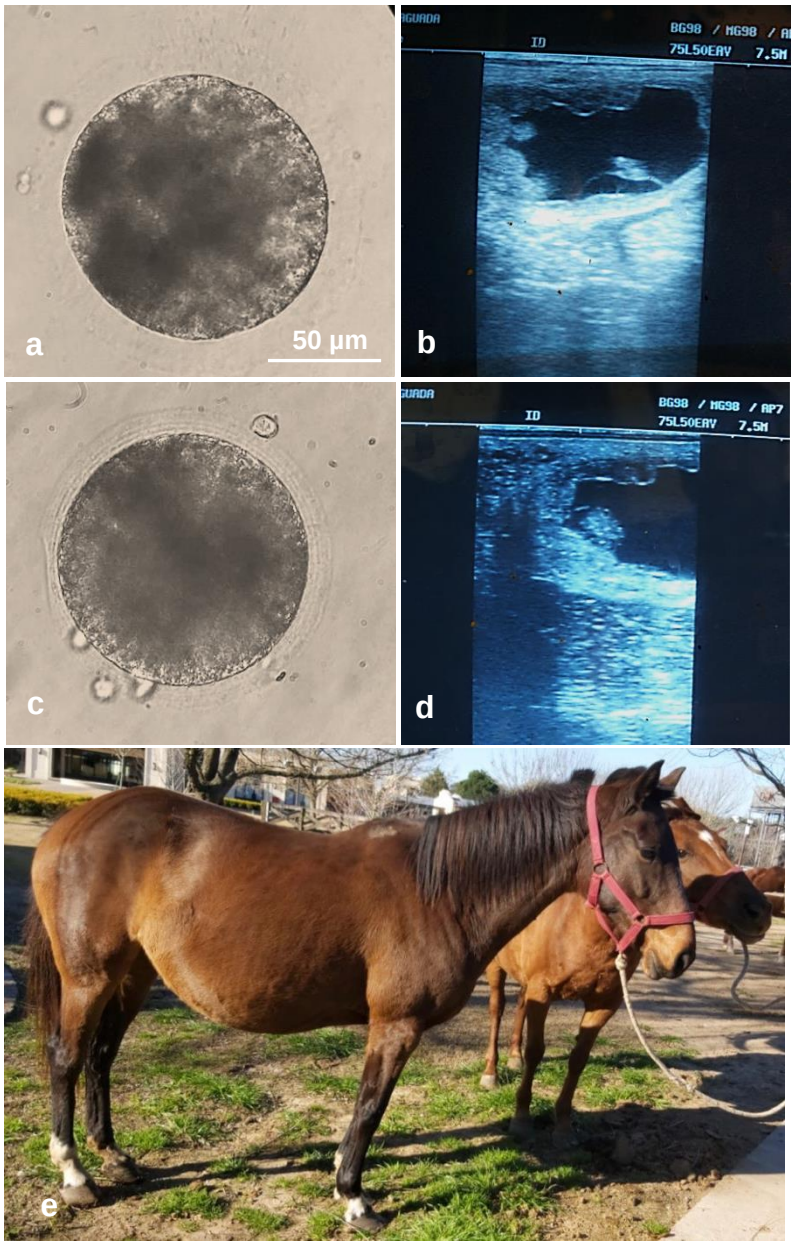

Figure 3. Day 7 ICSI blastocysts from vitrified oocytes transferred to recipients, resulting in the establishment of pregnancies. a, b). Vitrified, melatonin (+), grade 2 blastocyst and ultrasound confirmation of pregnancy after transfer of the same embryo to a synchronized recipient. c, d) Vitrified, melatonin (-), grade 2 blastocyst and confirmation of pregnancy. e) Recipient mare, day 270 of pregnancy, melatonin (+) embryo from vitrified oocyte.

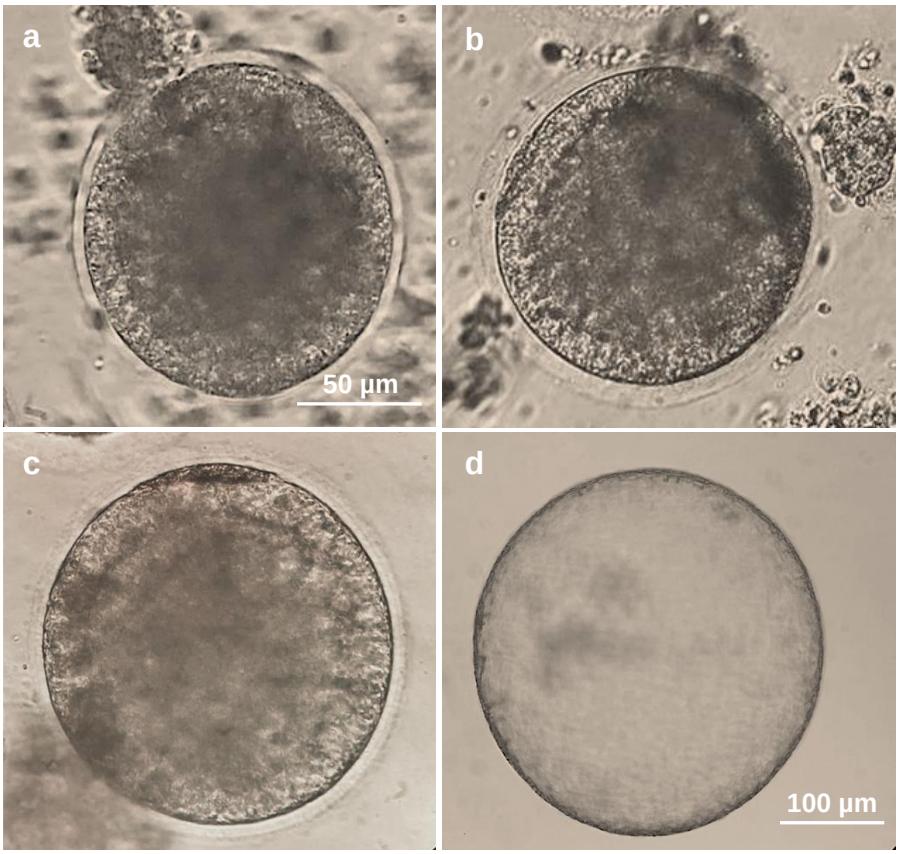

Figure 4. Day 10, non-transferred ICSI blastocysts. a) Vitrified, melatonin (+), grade 1 blastocyst. b) Vitrified, melatonin (-), grade 2 blastocyst. c) Non-vitrified, melatonin (+) grade 1 blastocyst. a-c) 40X magnification. d) Zona-thinned vitrified, melatonin (+) blastocyst (20X magnification).

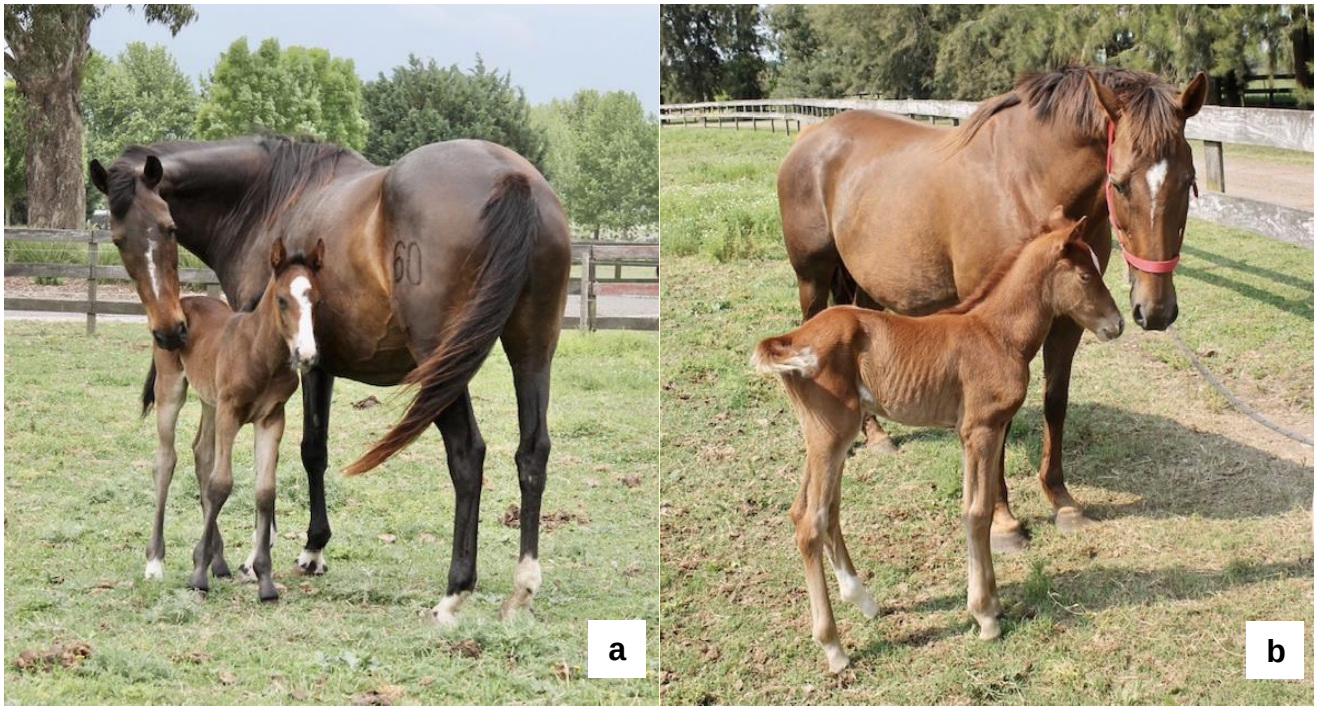

Figure 5. Live foals produced from vitrified/warmed oocytes. a. A healthy colt born from melatonin (+) treatment and b. A healthy filly from melatonin (-) treatment. Gestation lengths (considering day 0 = day of ICSI) were 338 days for the colt and 329 days for the filly, respectively.