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Original Research Article

Naloxone supplementation during vitrification of equine in vitro matured oocytes after overnight holding: insights from a comparative study with the bovine model

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ABSTRACT

Cryopreservation of equine mature oocytes remains a major challenge in assisted reproduction, mainly due to the limited availability of material and logistical constraints requiring a holding phase. To address these issues, this study evaluated whether naloxone (NX), an opioid receptor antagonist with reported antioxidant properties, could improve vitrification outcomes and whether bovine oocytes could serve as a suitable preliminary and supportive model. Two experiments were conducted. In experiment 1, immature (bGV) and mature (bMII) bovine oocytes were vitrified without (VIT) or with NX (VIT-NX) to assess protocol efficiency and evaluate whether results obtained in bovine MII oocytes (without holding) could serve as a counterpart to equine oocytes subjected to overnight holding. In experiment 2, equine in vitro matured oocytes after overnight holding (eMII) were vitrified with or without NX, and analysed for viability, reactive oxygen species (ROS), glutathione (GSH), high mitochondrial membrane potential (HMMP), and developmental competence following intracytoplasmic sperm injection (ICSI). Expression of apoptosis-related genes (BCL2, BAX, p53, survivin) was assessed by qRT-PCR. In bovine oocytes, NX did not affect vitrification efficiency or maturation rates, whereas oocytes vitrified at the MII stage yielded a higher proportion of viable mature oocytes after warming (bGV 13.5 %, vs. bMII 46.1 %) ($P < 0.05$). In equine oocytes, NX negatively affected post-warming viability (eMII-VIT-NX 64.7 ± 17.3) compared with non-vitrified oocytes (eMII 91.1 ± 16.9), with intermediate results observed for vitrification without NX (eMII-VIT 74.9 ± 25.0). NX did not significantly affect GSH or HMMP ($P > 0.05$), but prevented the increase in intracellular ROS levels induced by vitrification. Overall, vitrification reduced cleavage rates and increased degeneration ($P < 0.05$), with no differences between vitrification protocols. qRT-PCR revealed stable BCL2 expression, inconsistent detection of pro-apoptotic genes, and no significant differences among groups in the BAX:BCL2 ratio, indicating limited transcriptional activation of apoptosis. In conclusion, naloxone supplementation did not improve equine MII oocyte survival or developmental competence, although demonstrated antioxidant activity. Bovine oocytes confirmed the value of a preliminary model to test oocyte vitrification protocol efficiency but could not reliably predict equine responses to naloxone.

1. Introduction

In human medicine, oocyte cryopreservation is widely applied, whereas in veterinary species, outcomes remain poor [1], limiting its application. To date, only three foals have been produced from vitrified oocytes. In the first case, oocytes were matured in vivo, vitrified, and subsequently transferred into the oviducts of inseminated mares [2]. More recently, two studies reported the births of foals from oocytes matured in vitro and fertilized by ICSI [3,4]. Despite numerous attempts to improve outcomes, no vitrification protocol has yet proven sufficiently reliable or efficient for the commercial application of in vitro embryo production from cryopreserved equine oocytes. Most recently, satisfactory outcomes were achieved only with in vivo-matured oocytes, reaching a blastocyst rate of 40 % and a pregnancy rate of 67 % [5].

Cryopreservation at the immature stage is theoretically less harmful to the meiotic spindle, which is not yet fully formed. Although spindle

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repolarization has been observed after warming in some human oocytes vitrified at the mature stage [6], a similar recovery does not appear to occur in vitrified equine oocytes [7]. Vitrification affects the cytoskeleton, spindle formation, calcium ion transport, and homeostasis [8]. At the mitochondrial level, swelling and reduced matrix density have been observed in equine oocytes following vitrification [9,10].

Like the oocytes of many other species, equine oocytes are rich in lipids, which can exacerbate oxidative stress during freezing, particularly during the lipid phase. Promising results in other species have been achieved by culturing oocytes in media that reduce lipid accumulation and supplementing both culture and vitrification media with antioxidants [11,12]. In equine mature oocytes, melatonin supplementation improved cleavage rates, although blastocyst formation remained limited [4].

Naloxone, an antagonist of the μ-opioid receptor (MOR), exhibits concentration-dependent effects and can act as a partial agonist at higher concentrations [13]. MOR expression has been detected in the mare oviduct [14,15] and in equine cumulus-oocyte complexes (COCs) [16]. At the COC level, a high concentration naloxone acted as a MOR agonist, impairing meiosis and increasing chromosomal abnormalities, whereas at a low concentration it functioned as a MOR antagonist, enhancing maturation and reducing chromatin defects [16]. Similarly, supplementation of IVM medium with high-dose naloxone reduced maturation rates in both canine [17] and porcine [18] oocytes, while a low concentration improved oocyte maturation and increased the inner cell mass to total cell ratio in porcine blastocysts [18]. Beyond its receptor-mediated activity, naloxone has also been investigated for potential antioxidant properties [19]. In a preliminary study on equine oocytes, low-dose naloxone supplementation during vitrification improved blastocyst rates for both immature and mature COCs; however, the limited sample size precluded definitive conclusions [20]. More recently, a deeper investigation into its role during vitrification of equine immature COCs revealed a dual antioxidant effect depending on vitrification timing and the presence of a holding phase [21]. Specifically, naloxone enhanced meiotic competence in oocytes vitrified immediately after collection, with less consistent effects when combined with overnight holding, whereas it exhibited antioxidant activity in oocytes vitrified after holding but an opposing effect in those vitrified immediately [21].

Holding immature oocytes allows not only for easier transport but also for more flexible timing of laboratory procedures [22], considering that in vitro maturation (IVM) usually requires approximately 30 h. Moreover, IVM rates are variable (44–61 %), especially when oocytes are collected post-mortem [23]. Therefore, a major limitation in equine oocyte research, particularly with mature oocytes, is their scarce availability. To overcome this, bovine embryos have been used as model for testing new freezing protocols for equine embryos [24], owing to the greater availability and ease of collection of bovine oocytes. Although both bovine and equine oocytes are susceptible to cryodamage, bovine oocytes demonstrated greater robustness in vitrification, making them an ideal model for preliminary studies [25]. Additionally, the greater availability of in vitro culture and fertilization techniques for bovines [26], highlight cattle as a valuable model for advancing reproductive practices in equines.

The objective of this study was to investigate the effect of naloxone supplementation during the vitrification of equine mature oocytes, using bovine oocytes as a preliminary and supportive model.

2. Materials and methods

All chemicals were purchased from Sigma–Aldrich (Merck, Milan, Italy) unless otherwise stated. Plasticware was purchased from Thermo Fisher Scientific (Monza, Italy).

2.1. Study experimental design

The study was structured into two experiments, as illustrated in Fig. 1. The first experiment aimed to validate the use of bovine oocytes as a supportive model for equine oocytes, given the limited availability of the latter and the logistical constraints associated with vitrifying mature equine oocytes, especially under non-holding conditions. In this phase, both immature (bGV-VIT, bGV-VIT-NX) and mature (bMII-VIT, bMII-VIT-NX) bovine oocytes were vitrified using a protocol originally developed for equine oocytes, with or without naloxone supplementation. GV oocytes were evaluated for their ability to resume meiosis and reach the MII stage, while post-warming viability was assessed for mature oocytes. Immature bovine oocytes were included to verify whether the effects of naloxone previously observed in immature equine oocytes could be reproduced in the bovine model, since bovine mature oocytes would have been particularly useful to simulate non-holding conditions for the equine oocytes. The second experiment focused on equine in vitro matured oocytes after overnight holding (eMII-VIT, eMII-VIT-NX), which were vitrified using the same protocol to directly assess the effect of naloxone, with non-vitrified oocytes (eMII) serving as controls in subsequent analyses. Viability, reactive oxygen species (ROS) levels, intracellular glutathione (GSH) content, and high mitochondrial membrane potential (HMMP) levels were evaluated in warmed equine oocytes using fluorescent staining. To further assess the developmental competence, intracytoplasmic sperm injection (ICSI) was performed post-warming, and embryo development was evaluated 48 h after fertilization. Not all warmed oocytes were used for every analysis; oocytes were randomly allocated to viability, oxidative status (ROS/GSH/HMMP), ICSI, or qRT-PCR assessments, depending on the experimental replicates.

2.2. Bovine oocyte collection and maturation

Bovine ovaries were collected from 16- to 17-month-old Limousine heifers at a local slaughterhouse (Inalca S.p.A, Modena) and transported to the laboratory within 2–3 h after slaughter at 25 °C in a thermos case. In the lab, the ovaries were washed with demineralized water, and cumulus-oocyte complexes (COCs) were harvested by aspirating follicular fluid using a 21-gauge butterfly infusion set connected to a vacuum pump (KMAR-5100, Cook, Australia). The collected oocytes were then classified according to the IETS (International Embryo Technologies Society) grading system [27]. Oocytes with compact cumulus oophorous with at least 4 layers of cells and homogeneous cytoplasm were selected. Part of the collected oocytes was vitrified immediately after collection at the GV stage (10–20 oocytes per treatment), while the remaining oocytes underwent IVM before vitrification.

For IVM, groups of 50 COCs were washed with HSOF (modified synthetic oviductal fluid [28] supplemented with 10 mM HEPES, essential and non-essential amino acids, and 6 mg/ml fraction-V bovine serum albumin; 280–290 mOSm, pH 7.4) and cultured for 20 h in 2 ml maturation medium at 38.5 °C, in a humidified atmosphere of 5 % CO_2 in air (Heracell 150i CO_2 incubator, Thermo Scientific). Maturation medium consisted of Tissue Culture Medium (TCM 199) supplemented with 10 ng/ml epidermal growth factor (EGF), 100 ng/ml insulin-like growth factor (IGF-I), 0.1 IU/ml porcine FSH-LH (Pluset, Calier, Italy), 1.2 mM L-cysteine, 1 mM Na-pyruvate, 75 μg/ml kanamycin, and 10 % of foetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Italy). At the end of the maturation period, oocytes were denuded using a fine pipette. Denuded oocytes with a normal appearance, and a visible extruded polar body (PB), were considered suitable for vitrification.

2.3. Equine oocyte collection and maturation

Equine ovaries were collected from routinely slaughtered mares at a commercial abattoir (Zerbini e Ragazzi S.n.c, Reggio Emilia). Animals were not slaughtered for the purpose of this study, and no procedures

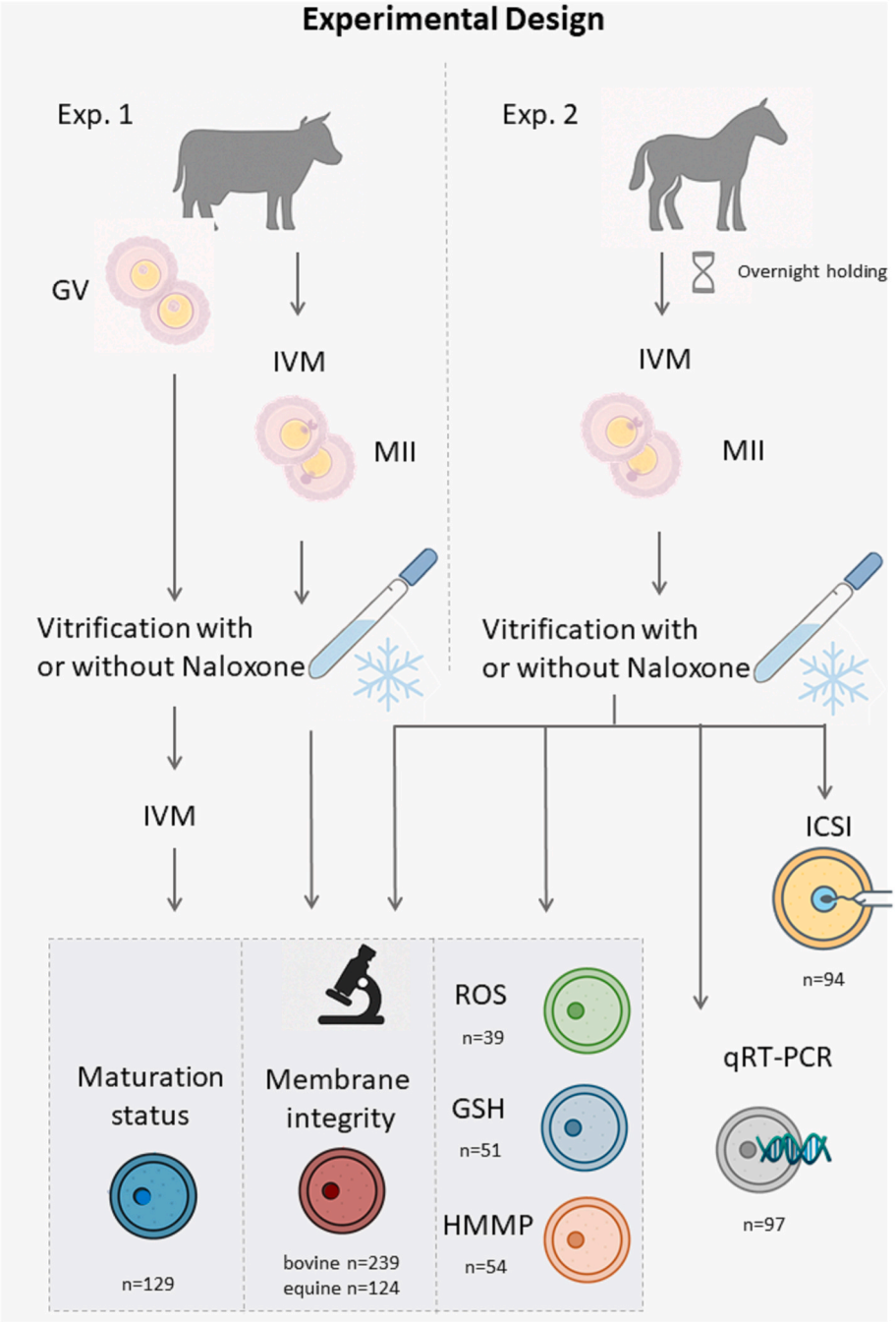

Fig. 1. Schematic illustration of the distribution of bovine and equine oocytes into experimental groups based on meiotic status (immature-GV or mature-MII) and vitrification protocol, either with or without naloxone supplementation. Maturation status was assessed in bovine GV oocytes after warming and in vitro maturation (IVM), while membrane integrity was evaluated in both warmed bovine and equine MII oocytes. In equine MII oocytes, levels of reactive oxygen species (ROS), intracellular glutathione (GSH), and high mitochondrial membrane potential (HMMP) were measured, along with the expression of apoptosis-related genes by quantitative real-time PCR (qRT-PCR). Developmental competence was further assessed in equine MII oocytes by intracytoplasmic sperm injection (ICSI).

were performed on live animals. The ovaries were transported to the laboratory within 2–3 h after slaughter at 25 °C in an insulated container. Oocytes were collected as previously described [29]. Briefly, ovaries were rinsed with demineralized water, and oocytes were harvested by aspirating the content of 10–30 mm follicles using a 19-gauge butterfly infusion set connected to a vacuum pump (about 100 mmHg). The aspirated follicular fluid was collected into 250 mL glass flasks and filtered through a 65 μm mesh nylon filter (EmSafe, Minitube, Germany). COCs were then searched at a stereomicroscope (Wild Heerbrugg, Switzerland). The oocytes were subjected to overnight holding, kept in HSOF at room temperature in the dark for 20–22 h.

For IVM, groups of 20–25 COCs were cultured in 500 μl maturation medium in four-well plates at 38.5 °C, in a humidified atmosphere of 5 % CO_2 in air. Maturation medium consisted of Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12, Gibco, Life Technologies, Italy) supplemented with 10 % (v/v) FBS, 50 ng/ml EGF, 100 ng/ml IGF-1, and 0.1 IU/ml porcine FSH-LH.

At the end of the maturation period, oocytes were denuded in trypsin-EDTA solution (0.25 % w/v trypsin, 1 mM disodium EDTA, 0.120 M NaCl, 0.88 mM Na_2HPO_4, 1.84 mM KH_2PO_4, 5 mM KCl, 5.5 mM D-glucose, 20 mM HEPES) for 60 s, washed once in HSOF plus 10 % FBS to inactivate trypsin, and washed twice in HSOF. Denuded oocytes

showing a normal appearance and a visible extruded PB, were considered suitable for vitrification.

2.4. Vitrification and warming

Immature COCs or matured denuded oocytes were vitrified after a 3 steps exposure to cryoprotectants on a cryotop (Cryotop, Kitazato Supply, Japan) and immediately immersed in liquid nitrogen. Briefly, 4–5 COCs were exposed for 30 s to the first vitrification solution (V1) (HSOF containing 5 % ethylene glycol (EG) and 5 % dimethyl sulfoxide (DMSO), with or without 10^{-8} M Nx), 30 s in V2 (HSOF containing 10 % EG and 10 % DMSO, with or without 10^{-8} M Nx), and finally 30 s in V3 (HSOF containing 20 % EG, 20 % DMSO, sucrose 0,65 M, and Ficoll 10 mg/ml, with or without 10^{-8} M Nx). Oocytes were stored in liquid nitrogen for at least 3 days before warming. For warming, they were exposed to decreasing sucrose-containing solutions (0.250 M, 0.188M, and 0.125 M in HSOF) for 30 s each. The number of recovered COCs or oocytes was recorded (recovery rate after warming). Naloxone powder (Sigma-Aldrich N7758) was dissolved in sterile distilled water and added to the vitrification medium (10 μl/ml) immediately before use.

2.5. Evaluation of oocyte meiotic competence

A thin glass pipette was used to remove cumulus cells from bovine vitrified/warmed COCs after IVM. Oocytes were then morphologically evaluated under a stereomicroscope, and those exhibiting a disrupted plasma membrane were classified as degenerate and excluded from further analyses. For staining, bisbenzimide fluorescent dye (Hoechst 33342) 10 μg/ml in phosphate-buffered saline (PBS; 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.137 M NaCl, 8.1 mM Na_2HPO_4) supplemented with 0.1 % w/v polyvinyl alcohol (PVA) was used. Oocytes were incubated for 15 min in the dark at room temperature and finally evaluated under a Nikon Eclipse E400 epifluorescence microscope (Nikon Europe BV, The Netherlands) equipped with an UV-2A 330–380 nm excitation filter to assess nuclear configuration. Oocytes were classified as mature (metaphase II, MII), immature (from germinal vesicle, GV, to metaphase I, MI), or degenerate (disrupted membranes or undefined nuclear configuration). The analysis was done in 3 replicates, with 17–33 oocytes per treatment in each replicate.

2.6. Evaluation of oocyte viability

Warmed mature oocytes from both species (6 replicates with 9–36 oocytes per treatment for bovine and 9 replicates with 1–21 oocytes per treatment for equine) were incubated for 2 h in HSOF at 38.5 °C in humidified air with 5 % CO_2. Viability was then evaluated using Hoechst 33342/propidium iodide (PI, 10 μg/ml in PBS + PVA) staining. Only oocytes with a visibly intact plasma membrane were selected for staining; the others were directly counted as non-viable. Oocytes were incubated for 30 min at room temperature in the dark and then evaluated under an epifluorescence microscope equipped with UV-2A (330–380 nm) and TRITC (540/25 nm) excitation filters. Those with a damaged cell membrane fluoresced red, and were classified as non-viable. All samples were evaluated under the same settings by the same operator to ensure consistency.

2.7. Detection of reactive oxygen species (ROS) and glutathione (GSH) levels

Intracellular ROS and GSH levels were determined using 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen™, Thermo Fisher Scientific, Italy) and 4-chloromethyl-6.8-difluoro- 7-hydroxycoumarin (CellTracker Blue, CMF2HC, Invitrogen™, Thermo Fisher Scientific, Italy) respectively. Oocytes were incubated 20 min in the dark at room temperature in PBS + PVA and 10 μM H2DCFDA or 10 μM CellTracker Blue. After staining, oocytes were washed once in PBS + PVA and examined under an epifluorescence microscope equipped with TRITC (540/25 nm) and FITC (465–495 nm) excitation filters. Images of fluorescent oocytes were acquired (Nikon Digital Sight DS-U3 with NIS-Element software, Nikon, Japan), keeping the same acquisition parameters for all groups, and subsequently analysed with an open-source software (ImageJ). For each image, oocyte fluorescence intensity was measured and normalized to the background, as previously described [21]. Final values are expressed as arbitrary units.

2.8. Mitochondrial membrane potential detection

Mitochondrial membrane potential of oocytes was assessed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen, Italy). Denuded oocytes were incubated at 38.5 °C for 30 min in the dark in 30 μl microdrops of HSOF supplemented with 0,2 μg of JC-1. Then, the oocytes were immediately examined under an epifluorescence microscope. Only one fluorescent image (red channel) was acquired for each oocyte, to detect the mitochondria with high membrane potential (HMMP, orange stained). Images were analysed as previously reported for ROS and GSH.

2.9. Intracytoplasmic sperm injection (ICSI)

Control and vitrified/warmed equine oocytes displaying an intact plasma membrane and an extruded PB were injected with frozen/thawed spermatozoa from a stallion of proven fertility after simple washing. ICSI was first performed on control oocytes, which had to be injected immediately. For each warming of vitrified oocytes, a batch of fresh controls was included, resulting in 7 replicates for controls (1–6 oocytes per replicate) and 5 for vitrified oocytes (1–7 oocytes per treatment in each replicate).

Conventional ICSI was performed at 37 °C using a micromanipulator (Narishige Co. Ltd, Tokyo, Japan) mounted on an inverted microscope (Nikon TE 300, Nikon, Kawasaki, Japan). Following ICSI, oocytes were cultured in 20 μl droplets of SOF-IVC (SOF supplemented with MEM amino acids and 16 mg/ml fatty acid free-bovine serum albumin, FAF-BSA) under mineral oil at 38.5 °C in a modified atmosphere (5 % CO_2, 7 % O_2, and 88 % N_2) for 48 h, before cleavage assessment. After 48 h, cleaved embryos were stained with Hoechst 33342 (10 μg/ml bisbenzimide in PBS + PVA) for 30 min at room temperature, washed in PBS and observed using an epifluorescence microscope to assess the number of nuclei. ICSI was performed in 7 replicates (5 for vitrified oocytes).

2.10. Quantitative real-time PCR (qRT-PCR) gene expression analysis for Bcl2, Bax, p53 and survivin

The experiment was done in 3 replicates with 9–13 in vitro matured oocyte per group (total oocytes: eMII n = 32; eMII-VIT n = 33; eMII-VIT-NX n = 32). Oocytes were denuded by digestion of zona pellucida in pronase solution (0.5 % w/v in PBS), washed twice in PBS, and snap-frozen in molecular grade water (20 μl) before storage at −80 °C. The RNA extraction, retrotranscription and RT-PCR were performed according to Gugole et al., 2025 [21]. Briefly, pool of MII oocytes were added with 2 μl of SideStep lysis and Stabilization buffer (Agilent Technologies, Santa Clara, CA, USA). To avoid genomic DNA contamination, the lysed samples (14 μl) were added with 2 μl of the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). After the DNase reaction protocol, the entire volume (16 μl) was mixed by pipetting and then retrotranscribed with cDNA using 5X iScript RT Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA), following the manufacturer's instructions, in a final volume of 20 μl to obtain cDNA. Quantitative PCR was carried out using a CFX96 thermal cycler (Bio-Rad). All the primers used for interest genes (Bcl2, Bax, p53 and survivin) and reference genes (GAPDH, ACTB) was reported, and the RT-PCR experiments were reported in our previous paper [21]. The specificity of the amplified PCR products was confirmed by agarose gel

electrophoresis and melting curve analysis. The relative mRNA expressions of tested genes were normalized by using the ΔCt method (ΔCt = Ct mean reference genes–Ct interest gene) and then the relative expression was calculated as fold of change (2-ΔΔCt method) in respect to the MII oocyte control group obtained under different condition (VIT or VIT-NX).

2.11. Statistical analysis

Data are expressed as percentage or mean ± standard deviation. Maturation and viability results in different treatments were compared using a binomial Generalized Linear Model (GLM) with logit function. Fluorescence intensity results were checked for normality using the Shapiro-Wilk test and then compared using a GLM for gamma log distribution and Wald pairwise comparison. Recovery rate after warming, the proportion of intact COCs/oocytes based on morphological assessment, and comparison between immature versus mature oocytes or different species was assessed using a contingency table Chi square test. Data were analysed using IBM SPSS Statistics 29.0 (IBM Corporation, Milan, Italy), with significance assessed at $P < 0.05$.

For gene expression data (ΔCt values), normal distributions were evaluated by means of Shapiro–Wilk and Kolmogorov-Smirnov tests, and, according to the results, statistic parametric test was performed (one-way ANOVA with significance level of $P \leq 0.05$; GraphPad Prism software, version 9.1, La Jolla, CA).

3. Results

3.1. Experiment 1

A total of 510 bovine COCs were used, of which 200 were vitrified before IVM, while 310 were first matured and then vitrified at the MII stage (n = 263).

For both immature and mature bovine oocytes, no significant differences were observed between the two vitrification protocols ($P > 0.05$) (Tables 1 and 2).

Given the absence of significant effects of naloxone supplementation on vitrification outcomes, data were pooled to compare the vitrification efficiency of immature versus mature bovine oocytes. A lower proportion of oocytes was vitrified when using mature oocytes (263/310, 84.8 %) compared to immature ones (200/200, 100 %) ($P < 0.05$). The presence of cumulus cells in immature oocytes did not significantly affect the recovery rate after warming (183/200 COCs, 91.5 % vs. 250/263 MII oocytes, 95.1 %) ($P > 0.05$) but it reduced the number of oocytes with intact membranes at stereomicroscopic evaluation after IVM (bGV 159/183, 86.9 %) compared to mature vitrified oocytes 2 h after warming (bMII 239/250, 95.6 %) ($P < 0.05$). Vitrified mature oocytes showed higher overall efficiency in terms of viable MII oocytes (143/310, 46.1 %) compared to immature oocytes (27/200, 13.5 %) ($P < 0.05$).

3.2. Experiment 2

Out of 906 in vitro matured equine COCs, a total of 536 MII oocytes were used, of which 392 were vitrified and 144 served as controls. Similar to bovine mature oocytes, 374 (95.4 %) were recovered after warming, and 357 (90.8 %) remained morphologically intact 2 h after warming ($P > 0.05$).

Table 2
Efficiency of vitrification and viability of bovine mature oocytes (mean ± SD), assessed by Hoechst 33342/propidium iodide staining 2 h after warming, using vitrification solutions without (bMII-VIT) or with (bMII-VIT-NX) naloxone.

Group	Vitrified oocytes	Recovered oocytes (%)	Intact oocytes (%)	Viable oocytes (%)
bMII-VIT	128	123 (96.1)	118 (95.3)	70 (56.9)
bMII-VIT-NX	135	127 (94.1)	121 (95.3)	73 (57.4)

No differences between protocols were observed in recovery rate and in the number of oocytes with intact membranes at stereomicroscopic evaluation ($P > 0.05$) (Table 3). However, equine oocyte viability was significantly higher ($P < 0.05$) in the control group compared with the vitrified group with naloxone, whereas the vitrified group without naloxone showed intermediate results ($P > 0.05$) (Table 3).

The results of staining for ROS and GSH intracellular levels, as well as HMMP, are graphically summarised (Fig. 2). Intracellular ROS levels tended to differ among groups ($P = 0.060$), mainly due to a significant increase in eMII-VIT group compared with the control ($P < 0.05$) in the pairwise comparison (Fig. 2A). Conversely, no statistically significant differences ($P > 0.05$) were observed in intracellular GSH levels (Fig. 2B) or HMMP (Fig. 2C).

Oocyte developmental competence after ICSI was reduced by cryopreservation ($P < 0.05$), leading to a higher rate of oocyte degeneration ($P < 0.05$), with no significant differences between vitrification protocols ($P > 0.05$) (Table 4). In the eMII group, a total of 9 embryos were obtained (3 at the 2-cell stage, 5 at 4-cell stage, and 1 at the 8-cell stage); in the eMII-VIT group, 2 embryos (1 at the 2-cell stage, and 1 at the 8 cell stage); and in the eMII-VIT-NX group, 1 embryo (at the 4 cell stage).

qRT-PCR analysis on eMII, eMII-VIT and eMII-VIT-NX groups showed the detection for both reference genes (Table 5). Regarding interest genes, BCL2 was detectable in all replicates for each group (9/9), BAX in two replicates per group (6/9), and p53 only in two replicates of the control group and in one replicate for each vitrification group (4/9) (Table 5). Survivin was not detectable in any sample. To calculate fold changes relative to control group, undetectable samples were assigned a Cq value of 40, corresponding to the maximum cycle of the PCR protocol. Samples exhibited high variability, particularly for BCL2 and BAX, and no statistically significant differences were observed among groups (Fig. 3A). BAX:BCL2 ratio was calculated and did not differ significantly among groups (eMII 0.88, eMII-VIT 0.59, and eMII-VIT-NX 0.42)

Table 3
Recovery, protocol efficiency (intact/recovered), and percentage (mean ± SD) of viable equine mature oocytes before (eMII) or after vitrification without (eMII-VIT) or with (eMII-VIT-NX) naloxone, assessed by Hoechst 33342/propidium iodide (H/PI) stain 2 h after warming.

Group	Vitrified oocytes	Recovered oocytes (%)	Overall intact oocytes (%)	H/PI stained oocytes (%)	Viable oocytes
eMII	/	/	/	32	91.1 ± 16.9 a
eMII-VIT	194	188 (96.9)	174 (92.5)	60	74.9 ± 25.0 ab
eMII-VIT-NX	198	186 (93.9)	173 (93.0)	64	64.7 ± 17.3 b

Table 1
Efficiency of vitrification and meiotic competence of immature bovine oocytes using solutions without (bGV-VIT) or with (bGV-VIT-NX) naloxone supplementation.

Group	Vitrified COCs	Recovered COCs (%)	Intact COCs (%)	Intact oocytes after IVM (%)	MII oocytes (%)	IMM oocytes (%)	DEG oocytes (%)
bGV-VIT	100	90 (90.0)	78 (86.7)	58 (64.4)	14/90 (15.6)	6/90 (6.7)	70/90 (77.8)
bGV-VIT-NX	100	93 (93.0)	88 (94.6)	71 (76.3)	13/93 (14.0)	13/93 (14.0)	67/93 (72.0)

COCs = cumulus-oocyte complexes; IVM = in vitro maturation, MII = mature oocytes; IMM = immature oocytes; DEG = degenerate oocytes.

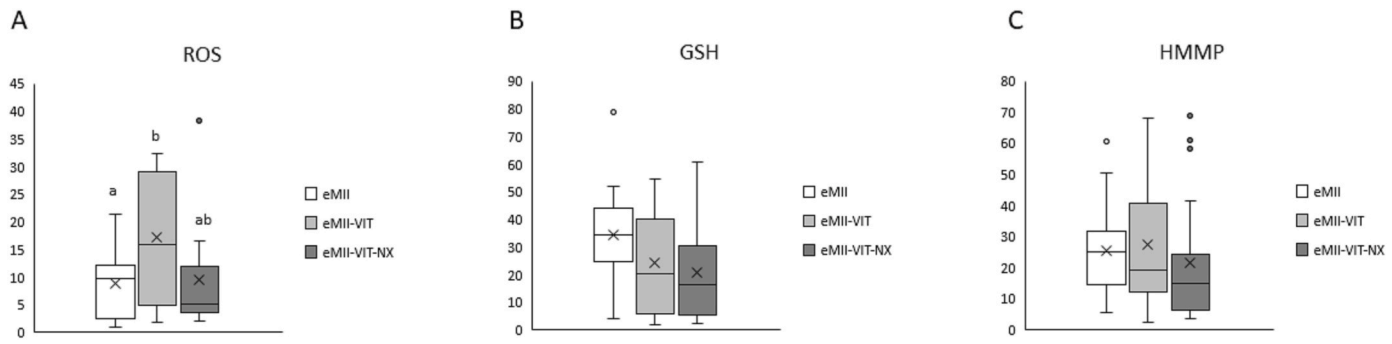

Fig. 2. Quantification of oxidative stress, intracellular glutathione levels, and mitochondrial activity in equine vitrified in vitro matured oocytes after overnight holding, determined by fluorescent staining 2 h after warming. (A) Reactive oxygen species (ROS) levels measured using H2DCFDA; (B) Intracellular glutathione (GSH) levels assessed using CellTracker Blue; (C) Mitochondrial membrane potential evaluated by JC-1 staining. Three experimental groups are shown: control oocytes (eMII), vitrified oocytes without naloxone (eMII-VIT), and vitrified oocytes with naloxone (eMII-VIT-NX). Different letters (a, b) indicate significant differences ($P < 0.05$).

Table 4
Percentage (mean ± SD) of cleaved and degenerate equine mature oocytes before (eMII) or after vitrification without (eMII-VIT) or with (eMII-VIT-NX), as assessed by Hoechst 33342 staining 48 h after ICSI.

Group	ICSI (n)	Lysed	Cultured oocytes (n)	Cleaved	Degenerate
eMII	36	8	28	39,3 ± 32,2 a	10,7 ± 19,7 a
eMII-VIT	30	3	27	7,3 ± 10,1 b	32,7 ± 44,7 b
eMII-VIT-NX	28	2	26	2,9 ± 6,4 b	37,1 ± 51,1 b

Table 5
Number of samples in which each gene was detected, relative to the triplicates in each experimental group. The range of Cycle quantity (Cq) detected by RT-PCR is reported. ND= Not Detectable.

gene	eMII	eMII-VIT	eMII-VIT-NX
GAPDH	3/3 (range Cq 25.9–32.8)	3/3 (range Cq 26.8–31.6)	3/3 (range Cq 29.5–30.7)
BACT	3/3 (range Cq 25.4–35.7)	3/3 (range Cq 28.4–33.7)	3/3 (range Cq 30.3–34.5)
BCL2	3/3 (range Cq 24.8–33.8)	3/3 (range Cq 26.6–32.3)	3/3 (range Cq 28.9-2.8)
BAX	2/3 (range Cq 29.9–34.2)	2/3 (range Cq 31.6–36.4)	2/3 (range Cq 35.6)
P53	2/3 (range Cq 33.3–36)	1/3 (Cq 35.8)	1/3 (Cq 36.6)
Survivin	ND	ND	ND

(Fig. 3B).

4. Discussion

In the present study, NX did not affect the vitrification efficiency of bovine immature or mature oocytes. Conversely, equine immature oocytes displayed greater meiotic competence when vitrified under the same conditions (i.e., immediately vitrified in the presence of NX) [21]. This suggests a species-specific interaction between naloxone and COCs, independent of exposure to a holding period.

Nevertheless, in both bovine and equine mature oocytes, NX supplementation did not influence vitrification efficiency or oocyte viability 2 h after warming. However, in equine oocytes, when vitrified samples were compared with non-vitrified controls, NX negatively affected viability. In this context, bovine oocytes served as the immediately vitrified counterpart, whereas equine oocytes underwent overnight holding. Overnight holding at uncontrolled room temperature may have contributed to these differences, as already reported for equine immature oocytes [21].

It has been shown that bovine oocytes can be held at room temperature for 6–10 h without detrimental effects on maturation or embryo development [30], but overnight holding in the absence of maturation inhibitors has not been described. In horses, overnight holding is considered useful for oocyte shipment without impairing developmental competence [31]. On the other hand, although uncontrolled (22–27 °C) overnight holding did not compromise viability or energy/redox status in equine oocytes, it did increased apparent cytoplasmic maturation before IVM, as reflected in a more mature mitochondrial distribution in condensed chromatin configuration oocytes [31]. It has also been suggested that overnight holding prior to IVM may act as a pre-selection step, thereby enhancing the developmental competence of surviving oocytes [32].

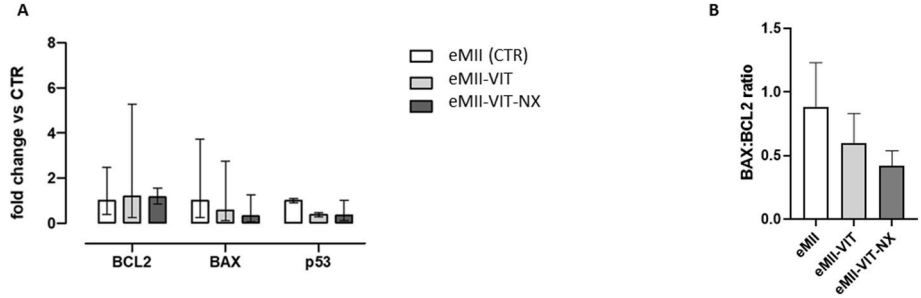

Fig. 3. Expression of apoptosis-related genes evaluated by qRT-PCR in equine in vitro matured oocytes after overnight holding before (eMII) and after vitrification without (eMII-VIT) or with naloxone (eMII-VIT-NX). (A) Expression levels of BCL2, BAX, and p53 in eMII-VIT and eMII-VIT-NX relative to eMII. (B) BAX:BCL2 expression ratio. Error bars represent the range of relative gene expression.

Given the discrepancies observed between immature equine and bovine COCs vitrified immediately with or without NX, some concerns arise regarding the suitability of bovine non-held oocytes as a model for equine ones.

Our findings confirm that, in the bovine model, the equine vitrification protocol performs more efficiently at the MII than at the GV stage. This observation is consistent with what has been reported in horses, considering the outcomes obtained with immature equine oocytes [21]. These results reinforce the notion that the maturation stage at the time of cryopreservation is a critical determinant of oocyte survival and subsequent competence, and that GV-stage vitrification still represents a major biological challenge. It has long been argued that GV oocytes represent the meiotic stage most resistant to cryodamage, particularly due to the absence of the meiotic spindle, a delicate structure present in mature oocytes [33]. However, bovine immature oocytes show lower water permeability than mature oocytes and higher permeability for ethylene glycol at low concentrations; this inverse relationship with CPA concentration is not observed in mature oocytes [34]. Nonetheless, recent findings suggest that damage to other delicate structures, such as the cumulus cell compartment or transzonal processes, may impair the maturation, fertilization, and embryonic development of oocytes vitrified at the germinal vesicle stage [35]. Conversely, MII oocytes contain higher levels of polyunsaturated fatty acids and lower levels of saturated fatty acids [36,37] which may allow their membranes to be more flexible and resistant to low temperatures due to their lipid composition [38,39]. Moreover, the lipid content in oocytes matured in vitro is higher than those matured in vivo, resembling the levels found in immature oocytes [40].

Equine mature oocytes subjected to vitrification showed lower survival rates compared to the non-vitrified group. The results obtained in this study agree with previous reports in domestic animals, in which oocyte vitrification leads to a loss of gametes in terms of survival after warming [41]. Although vitrification is an effective preservation method that surpassed slow freezing decades ago [42], the outcomes achieved still do not fully match those of unfrozen gametes.

Beyond its role as an opioid antagonist, naloxone has been reported to exert protective effects against oxidative stress [19]. Cryopreservation procedures are known to provoke oxidative stress, via ROS production, lipid peroxidation, and related damage to membranes, DNA, and organelles. Other studies have highlighted the positive impact of antioxidant molecules, such as melatonin, on cryopreserved equine oocytes [4]. In this study, ROS levels in the NX-treated group remained comparable to controls, suggesting that NX may help prevent the oxidative stress induced by vitrification, although these results should be interpreted with caution given the limited sample size and intrinsic variability of oocytes. However, this lack of ROS elevation was not associated with improved oocyte survival, suggesting that degenerative and apoptotic pathways may still be involved. Notably, the absence of a ROS increase does not necessarily indicate preserved mitochondrial function, as other factors, such as altered redox balance [43] or calcium homeostasis [44], can still impact mitochondrial dynamics and membrane potential. This may explain the apparent dissociation between ROS levels and the trend toward lower HMMP observed in oocytes vitrified with naloxone compared to those vitrified without it, although the difference was not significant, likely due to the variability among ovary batches. MOR receptors regulate calcium entry into oocytes [16], and further investigation of how calcium homeostasis interacts with mitochondrial function in this context would be worthwhile.

Vitrification and naloxone supplementation did not appear to alter GSH content in equine MII oocytes, although their developmental competence was reduced. As for HMMP, the high variability among oocyte batches may have masked the significance of the GSH reduction in vitrified oocytes. Glutathione, a thiol tripeptide (γ-glutamyl cysteinyl glycine), is the primary non-enzymatic defence against oxidative stress due to the reducing properties of its sulfhydryl group [45]. Its synthesis during oocyte maturation is crucial for sperm chromatin decondensation and male pronucleus formation [46].

Indeed, ICSI outcomes at 72 h were poorer in cryopreserved oocytes, consistent with previous reports indicating that ICSI of vitrified equine oocytes yields lower fertilization rates than fresh controls [5,7,20,47,48]. Vitrification not only increased the degeneration rate but also directly reduced the developmental competence of non-degenerated oocytes, as evidenced by their failure to cleave after ICSI. The relatively low cleavage rate of non-vitrified oocytes observed in this study may be attributable to the use of a ICSI technique without piezo assistance [49] and to the involvement of a newly trained operator. Furthermore, it must be noted that the use of oocytes obtained from slaughterhouse ovaries introduces inherent variability in gamete quality due to unknown animal history and factors related to tissue transport, handling, and preservation. This variability, while unavoidable, is a recognized challenge in studies of this nature and, together with factors such as advanced age, known to alter meiotic spindle morphology and chromosome alignment, may further influence embryo viability and developmental competence, irrespective of vitrification [5].

The balance between pro- and anti-apoptotic factors is crucial for oocyte survival. Among these, p53 acts as a key pro-apoptotic regulator that promotes the expression of cell-death mediators such as BAX, while anti-apoptotic proteins like BCL2 and survivin counteract these pathways and support cell viability. Our RT-PCR results revealed stable detection of BCL2 and low or inconsistent detection of BAX and p53 across treatments, with no differences in relative expression. This pattern contrasts with findings in bovine oocytes, where vitrification has been reported to increase BAX mRNA levels and decrease BCL2 mRNA levels, contributing to mitochondrion-mediated apoptosis [50]. Interestingly, our data more closely resemble those observed in vitrified canine oocytes, where BCL2 expression was increased whereas BAX was undetectable, suggesting limited transcriptional activation of pro-apoptotic pathways [51]. Moreover, while an increased BAX:BCL2 ratio is a hallmark of metabolic stress in equine embryos exposed to high glucose concentrations [52], no such increase was observed here. Future studies should clarify whether the BAX:BCL2 ratio in oocytes is affected by vitrification. Taken together, these interspecies comparisons suggest that equine MII oocytes may possess a species-specific resilience to vitrification-induced apoptotic signalling, despite the observed reduction in viability and developmental competence, or alternatively activate these pathways at post-transcriptional or delayed stages, warranting further temporal and protein-level investigations.

5. Conclusion

In conclusion, this study demonstrates that, despite the widespread use of vitrification, equine in vitro matured oocytes after overnight holding remain highly sensitive to cryopreservation and do not benefit from naloxone supplementation. Naloxone failed to improve post-warming survival, mitochondrial membrane potential, or developmental competence, and it did not significantly alter the expression of key apoptosis-related genes, although its prevention of the ROS increase suggests an antioxidant action. Comparative trials using bovine oocytes confirmed that this species can serve as a preliminary model for vitrification protocols but cannot reliably predict the equine response, particularly regarding the interaction of naloxone with the holding phase. Overall, our findings indicate that naloxone does not provide meaningful protection during vitrification of equine in vitro matured oocytes after overnight holding, and that the mechanisms underlying species- and stage-specific responses remain to be clarified. Future studies should focus on both temporal and protein-level analyses of apoptosis and calcium homeostasis to better understand the interplay between cryoprotectants, holding, naloxone, and oocyte physiology, and to develop more effective strategies for equine oocyte cryopreservation. Finally, it cannot be excluded that a more comprehensive deciphering of naloxone-induced antioxidative defence-related and cytoprotective pathways may help in developing efficient and

repeatable strategies for the cryogenic protection of equine, bovine and other mammalian nuclear recipient oocytes in assisted reproductive technologies such as standard in vitro fertilization [53–55], intracytoplasmic sperm injection [5,56,57] and somatic cell cloning [58–61].

CRediT authorship contribution statement

Penelope Maria Gugole: Writing – original draft, Project administration, Investigation, Data curation, Conceptualization. **Augusta Zannoni:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Eleonora Iacono:** Writing – review & editing, Resources. **Barbara Merlo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Data curation, Conceptualization.

Declaration of interest

I have nothing to declare.

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