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Original Research Article

Effects of holding and the addition of naloxone on vitrification of equine immature oocytes

Penelope Maria Gugole [a,*], Augusta Zannoni [a,b], Monica Forni [b,c], Eleonora Iacono [a,b], Filippo Zambelli [d], Barbara Merlo [a,b]

[a] *Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, Ozzano Emilia, 40064, Bologna, Italy*
[b] *Health Science and Technologies Interdepartmental Center for Industrial Research (CIRI-SDV), University of Bologna, Bologna, Italy*
[c] *Department of Medical and Surgical Sciences, University of Bologna, via Massarenti 9, 40138, Bologna, Italy*
[d] *Eugin, Barcelona, Spain*

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ABSTRACT

This study investigates the effects of overnight holding and naloxone (Nx) supplementation on the vitrification outcomes of equine immature oocytes. Oocytes were divided into six experimental groups based on treatment combinations: fresh (F) and held (H) control oocytes, oocytes vitrified with or without Nx (10^{-8} M) (VIT and VIT-Nx), oocytes vitrified after overnight holding with or without Nx (10^{-8} M) (H-VIT and H-VIT-Nx). They were assessed for survival, meiotic competence, intracellular oxidative stress, mitochondrial activity and distribution, apoptosis, and apoptotic gene expression. At survival rate determination, the degeneration rate was higher in VIT and VIT-Nx compared to F ($P < 0.05$). The highest maturation rate was observed in VIT-Nx. A significant reduction in ROS levels was observed in H compared to F ($P < 0.05$). ROS levels were similar between F and VIT, while the Nx supplementation tended to increase them (VIT-Nx vs F: $P = 0.053$; VIT-Nx vs VIT: $P = 0.069$). Conversely, in oocytes vitrified after overnight holding, vitrification induced an increase in ROS levels (H vs VIT: $P < 0.05$), which was not observed in H-VIT-Nx. GSH intracellular levels showed significant differences only in held oocytes, with higher GH levels in H compared to H-VIT and H-VIT-Nx ($P < 0.05$). All treatments induced an increase in HMMP levels compared to F ($P < 0.05$). In H oocytes, mitochondria were distributed throughout the entire oolemma (TOMM20) and active mitochondria (D-LAT) were detected in the outermost region. Incontrast, in H-VIT-Nx, potentially active mitochondria were spread throughout the cytoplasm. AnnexinV/PI staining revealed that the percentage of viable oocytes was higher ($P < 0.05$) in F and H than in all vitrified/warmed oocytes, and H-VIT-Nx had the highest degeneration rate ($P < 0.05$). RT-PCR analysis confirmed the detection for both reference genes, and target genes *BCL2* and *Survivin* in all samples. In contrast, *BAX* and *p53* transcripts were consistently undetectable. No significant differences were observed in the expression of *BCL2* and *Survivin* between groups. In conclusion, overnight holding at uncontrolled room temperature can alter oocyte characteristics and lead to variable results after vitrification. Nx demonstrated contrasting antioxidant effects depending on the vitrification timing, but it appeared to improve IVM outcomes in oocytes vitrified immediately after collection.

1. Introduction

In horses, cryopreservation of sperm can be considered successful and is routinely used for both commercial and research purposes. On the contrary, preservation of genetic material from mares is still a challenge.

Despite oocyte cryopreservation is routinely practiced in humans and laboratory animals, the efficiency is low in domestic animals [1]. The methods available for oocyte cryopreservation are slow-freezing, based on relatively low concentrations of cryoprotectants (CPAs) and long equilibration periods, and vitrification, based on high concentrations of CPAs and direct plunging into liquid nitrogen [2]. The large size of the oocyte, its high water content, and the peculiar intracellular structure, make this specialized cell very susceptible to cryoinjury [3]. Nonetheless, the advent of vitrification represented a milestone in

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* Corresponding author. Department of Veterinary Medical Sciences, University of Bologna, via Tolara di Sopra 50, Ozzano Emilia, 40064, BO, Italy.
E-mail addresses: penelopemaria.gugol2@unibo.it (P.M. Gugole), augusta.zannoni@unibo.it (A. Zannoni), monica.forni@unibo.it (M. Forni), eleonora.iacono2@unibo.it (E. Iacono), zambelli.filippo@gmail.com (F. Zambelli), barbara.merlo@unibo.it (B. Merlo).

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human IVF. Vitrification was demonstrated to be superior over slow-freezing protocols and its efficiency in terms of embryo development and pregnancy outcomes was not different compared to fresh counterparts [4].

In horses, oocyte vitrification is still considered experimental, and to date, only three foals have been born from cryopreserved oocytes. The first foal born from vitrified oocytes was obtained from *in vivo* matured oocytes transferred into the oviduct of inseminated mares (*in vivo* fertilized) [5]. Almost two decades later, the birth of two foals from vitrified/warmed *in vitro* matured oocytes fertilized by ICSI was reported [6,7]. Many factors have been investigated trying to optimize the cryopreservation protocols for horse oocytes vitrification, including nuclear maturation status and cumulus morphology [8,9], CPAs combinations [10–12], the effect of vitrification on the DNA fragmentation of cumulus cells [13], the effect of melatonin as antioxidant [7], and the maternal age [14]. However, the reason why developmental competence of equine vitrified oocytes decreases after vitrification has not been fully elucidated.

Endogenous opioid peptides are neuromodulators also playing a regulatory role in the reproductive system, influencing processes such as hormone release, ovulation, and reproductive behaviour [15]. Naloxone (Nx) is an opioid receptor antagonist which have opposite effects depending on concentration, indicating it may act as a partial agonist at higher concentrations [16] and counteract the modulatory action of opioid agonists on the μ-opioid receptor (MOR), influencing ion channels and second messenger effectors [17]. The dual effect of Nx on *in vitro* maturation (IVM) was initially demonstrated on bovine oocytes [18]. The presence of μ-opioid receptor (MOR) was then demonstrated in the mare oviduct [19,20], and in the cumulus-oocyte complex (COC) [21], with a different seasonal expression and a role in regulating meiotic competence. At a high concentration (10^{-3} M), Nx acted as an agonist at MOR, similar to β-endorphins, reducing the rate of MII oocytes and increasing the incidence of oocytes with incomplete or incorrect chromosome migration [21]. In contrast, at a low concentration (10^{-8} M), Nx exhibited antagonist activity, improving maturation rates and decreasing the occurrence of abnormal chromatin patterns [21]. Naloxone's action might be related to mitogen-activated protein kinase (MAPK) signalling pathway, as endogeneous opiods are known to affect MAPK in oocytes [18]. The expression of MOR has been studied also in canine, human, and porcine oocytes [22–24]. Similarly to what observed in bovine and equine oocytes, the addition of Nx during IVM at high concentrations reduced the rate of MII oocytes in canine [22] and porcine [24] oocytes. Conversely, at low concentration, it had a beneficial effect on maturation rate and ratio of inner cell mass to total cells in blastocysts in pig [24]. Indeed, in porcine oocyte IVM, Nx at a low concentration synergistically increased the MII rate with cyclic adenosine monophosphate (cAMP), likely due to Nx's antagonistic action at MOR, which mediates the process of the opioid inhibition of cAMP production [24]. Moreover, a recent study [25] testing the hypothesis that Nx could prevent oxidative stress in PC12 cells (derived from a pheochromocytoma cell line) treated with H_2O_2, revealed that it protects cells from reactive oxygen species (ROS) production by acting as an antioxidant agent. It counteracted intracellular ROS production, reduced H_2O_2-induced apoptosis levels, and prevented the oxidative damage-dependent increases of the percentage of cells in G2/M phase [25]. In addition, a preliminary study investigating embryo development after ICSI of vitrified equine oocytes pre- and post-IVM, with or without the addition of Nx, found that blastocysts were obtained only in groups treated with Nx [26]. However, due to the low number of oocytes used, it was not possible to draw definitive conclusions about the effect of Nx.

Even though oocyte vitrification can be easily learned by relatively inexperienced technicians [27], it might be more practical to transport immature oocytes intended for cryopreservation to ICSI laboratories at room temperature, as for commercial equine OPU/ICSI programs, and vitrify them at the lab, rather than transporting them in liquid nitrogen after field vitrification. Overnight holding of equine immature oocytes may induce a pre-selection of the most competent oocytes [28]. Foals have been obtained after vitrification of oocytes matured immediately [6] and those matured after overnight holding [7], but there is only a brief preliminary study about the effect of overnight holding on the maturation rate of vitrified immature horse oocytes [10].

The aims of this study were to evaluate the effect of holding on vitrification of equine immature oocytes and the possible beneficial action of Nx when added to vitrification solutions.

2. Materials and methods

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

2.1. Oocyte collection and study design

Ovaries were collected from slaughtered mares and transported to the laboratory within 2–3 h at 25 °C in an insulated container. Oocytes were collected as previously described (Merlo et al., 2018). Briefly, ovaries were rinsed with demineralized water, and oocytes were recovered 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). Cumulus-oocyte-complexes (COCs) were then searched at a stereomicroscope and classified as previously reported [29]. Briefly, COCs with at least 3 to 5 layers of cumulus cells attached were classified as compact (having a tight, complete compact cumulus with a distinct, smooth hillock), expanded (having a granular or expanded cumulus), and corona radiata (having only corona radiata present). The COCs were evenly distributed into six groups: 1) fresh control (F) (n = 118); 2) overnight holding (H) (n = 119); 3) vitrified (VIT) (n = 137); 4) vitrified with the addition of Nx 10^{-8} M (VIT-Nx) (n = 137); 5) vitrified after overnight holding (H-VIT) (n = 121); 6) vitrified with the addition of Nx 10^{-8} M after overnight holding (H-VIT-Nx) (n = 139). The overnight holding groups were kept in HSOF (Hepes Synthetic Oviductal Fluid) at room temperature (range 21–26 °C) in the dark for 20–22 h. The study design is illustrated in Fig. 1.

2.2. Oocyte vitrification/warming

COCs were vitrified after a 3 steps exposure to cryoprotectants on a cryotop (Cryotop, Kitazato Supply, Japan) and immediately immerse 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 [18]), 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, COCs were exposed to decreasing sucrose-containing solutions (0.250 M, 0.188M, and 0.125 M in HSOF) for 30 s each. Subsequently, COCs intended for various analyses, except for IVM, were incubated at 38.5 °C in humidified air with 5 % CO_2 for 2 h in HSOF before being denuded in a 0.25 % trypsin solution for 60 s, washed once in HSOF supplemented with 10 % (v/v) fetal bovine serum (FBS) (Gibco®, Thermo Fisher Scientific, Italy) to inactivate trypsin, and finally washed twice in HSOF. Control COCs were similarly denuded after collection (F) or overnight holding (H). All oocytes were evaluated under a stereomicroscope for survival. Those exhibiting a disrupted plasma membrane were classified as degenerate (see Table 2) and excluded from further analyses.

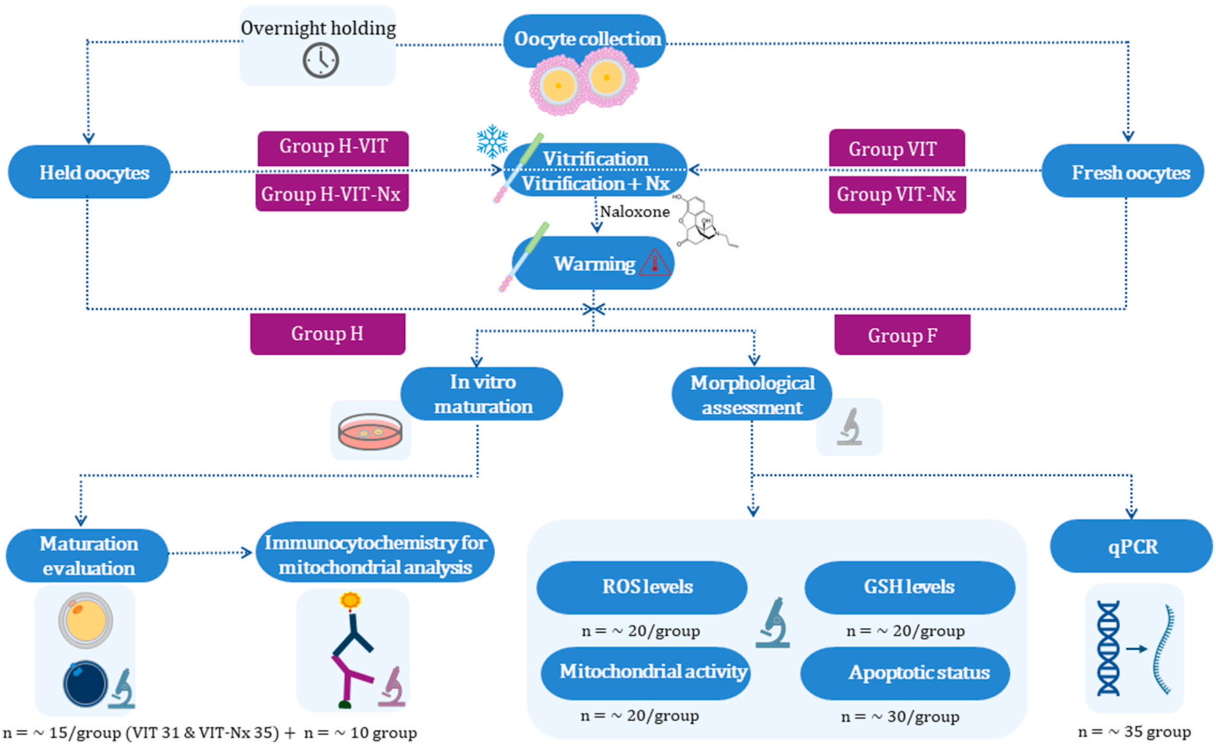

Fig. 1. Experimental Design. Schematic illustration of the distribution of equine immature COCs into six experimental groups based on immediate or delayed (after overnight holding) vitrification, with or without naloxone (Nx), compared to fresh and held control groups. The following parameters were assessed: meiotic competence, reactive oxygen species (ROS) and glutathione (GSH) levels, mitochondrial activity, mitochondrial analysis post-maturation, apoptosis, and the expression of apoptosis-related genes. Experimental groups: F = fresh control; VIT = immediately vitrified; VIT-Nx = immediately vitrified with Nx (10^{-8} M); H = held control; H-VIT = vitrified after overnight holding; H-VIT-Nx = vitrified with Nx (10^{-8} M) after overnight holding.

Table 1
Primer sequences for RT-PCR, values of PCR Efficiency (%), range Cycle quantity (Cq).

Gene	Accession number	Primer sequence	% PCR Efficiency	Range Cq	Reference
BCL2	XM_001499714.1	F 5′-GCGTGGAAAGCGTAGACAAGGAGATG-3′ R 5′-AGGCTCTAGGTGGTCATTCAGGTAAGTG-3′	94.7	26.79–33.89	[Present paper]
BAX	XM_001489207.1	F 5′-ATCGGAGATGAGCTGGACAGTAAC-3′ R 5′-GGCAAAGTAGAAAAGGGCAACAAC-3′	94.5	ND	[Present paper]
p53	XM_001918153.1	F 5′-CTCACTATCATCACCCTGGAAGAC-3′ R 5′-GTGTTACTGGACAATACTCGCTTAG-3′	89.2	ND	[Present paper]
Survivin	XM_001915400.1	F 5′-TTCATCCACTGTCCCACTGA-3′ R 5′-GTTCCTCTATGGGGTCGTCA-3′	92.3	27.24–33.84	[30]
GAPDH	NM_001163856	F 5′- TGGTGAAGGTCGGAGTAAAC -3′ R 5′- TGTAGTTGAGGTCAATGAAGGG -3′	84.1	22.8–32.9	[31]
ACTB	AF035774.1	F 5′- ATCGTGCGTGACATCAAGGA -3′ R 5′- AGGAAGGAGGGCTGGAAGAG -3′	92.1	25–33.5	[31]

ND= Not Detectable.

Table 2
Survival rate of equine immature oocytes, vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by morphological evaluation 2 h after warming.

Groups	N oocytes	Survived (%)	Degenerate (%)
F	118	107 (90.7)^{a}	11 (9.3)^{a}
VIT	137	110 (80.3)^{b}	27 (19.7)^{b}
VIT-Nx	137	106 (77.4)^{b}	31 (22.6)^{b}
H	119	100 (84.0)^{a,b}	19 (16.0)^{a,b}
H-VIT	121	104 (86.0)^{a,b}	17 (14.0)^{a,b}
H-VIT-Nx	139	111 (79.9)^{b}	28 (20.1)^{b}

Different superscript letters in columns indicate statistical significance ($P < 0.05$). F = fresh; H = holding; VIT = vitrified; Nx = naloxone.

2.3. *Evaluation of oocyte meiotic competence*

Fresh and held control COCs, and vitrified/warmed COCs were *in vitro* matured for 30 h in Dulbecco Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12) supplemented with 10 % (v/v) FBS, 50 ng/ml epidermal growth factor, 100 ng/ml insulin-like growth factor 1, 0.1 IU/mL porcine FSH-LH (Pluset, Calier, Italy) at 38.5 °C, in humidified air at 5 % CO_2.

At the end of the maturation period, oocytes were denuded as previously described and stained with Hoechst 33342 (bisbenzimide, 10 μg/ml in phosphate buffered saline (PBS) supplemented with 0.1 % polyvinyl alcohol (PVA)) for 15 min in the dark at room temperature. They were then washed once in PBS + PVA, mounted on glass slides, and evaluated under an epifluorescence microscope (Nikon Europe BV, The Netherlands) equipped with UV-2A (330–380 nm) excitation filter. Only oocytes displaying an extruded polar body and a visible metaphase plate

(MII) were considered mature, those presenting nuclear configurations ranging from germinal vesicle to the metaphase I stage were categorized as immature, while those with disrupted membranes or an undefined nuclear configuration were classified as degenerate. The experiment was conducted in 3 replicates with 5–6 oocytes per group. An additional replicate was performed for the VIT (n = 17) and VIT-Nx (n = 20) groups to confirm the results.

2.4. 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. H2DCFDA is used as an indirect measure of ROS activity in cells, since when ROS levels increase, the H2DCFDA is oxidized to its fluorescent form (2′,7′-dichlorofluorescein - DCF). CMF2HC dye is a cell-permeable, non-fluorescent compound that becomes fluorescent upon reaction with intracellular thiols. Four replicates were performed for each staining with 4–5 immature oocytes per group. Oocytes were incubated 30 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, mounted on glass slides with vaseline anticompression layer, sealed with cover slips and examined under a Nikon Eclipse E400 epifluorescence microscope equipped with UV-2A (330–380 nm) and FITC (465–495 nm) excitation filters. Each group of oocytes was mounted and immediately imaged (Digital Sight camera, DS-U3, Nikon Europe BV, The Netherlands), using the oftware NI-Elements D3.2 Laboratory Imaging, Nikon Europe BV, The Netherlands), keeping the same acquisition parameters for all groups. Images of fluorescent oocytes were analysed with a widely used open source software (FIJI ImageJ) that allows users to visualize, inspect, quantify, and validate scientific image data. The area of interest was selected using the selection tool. From the Analyze menu "set measurements" was selected and we made sure to have area integrated intensity and mean grey value selected. Then, we selected "Measure" from the analyze menu. It was important to select a region next to the cell that has no fluorescence, the background. Then, area, mean, minimum and maximum, integrated density, raw integrated density and length were calculated. These parameters are calculated from the pixel values along the line. CTCF (corrected total cell fluorescence) formula (CTCF (pixel) = Integrated Density – [Area of selected cell x Mean fluorescence of background readings]) was used to calculate cell fluorescence [32]. Final values are expressed s arbitrary units.

2.5. Detection of mitochondrial activity

Mitochondrial activity of equine immature oocytes was assessed by 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Invitrogen, Italy), which stains mitochondria depending on mitochondrial membrane potential (MMP). Four replicates were performed with 4–5 oocytes per group. JC-1 is a dye, naturally exhibit green fluorescence, which is able to enter and accumulate into the mitochondria, and form reversible complexes called J aggregates (yellow-orange fluorescence) when the inner membranes are hyperpolarized [33]. Denuded oocytes were incubated in 25 μl of HSOF supplemented with 2 μl of a 150 μM JC-1 solution [34] for 30 min in the dark at room temperature, then they were washed once in PBS + PVA, mounted on glass slides with vaseline anticompression layer, sealed with cover slips, and examined under an epifluorescence microscope equipped with TRITC (540/25 nm) and FITC (465–495 nm) excitation filters. Each group of oocytes was mounted and immediately imaged using the same acquisition parameters for all groups. Only one fluorescent image (red channel) was acquired for each oocyte to detect mitochondria with high membrane potential (HMMP). Green fluorescence was not clearly detectable, and attempts to enhance it up to the autofluorescence limit of negative controls were considered neither worthwhile nor reliable. Such adjustment could be misleading in assessing the red/green signal ratio, especially given the weak and poorly distributed green fluorescence observed in confocal microscopy images of equine oocytes stained with JC-1 [35]. Images were analysed as previously described for ROS and GSH.

2.6. Immunocytochemistry for mitochondrial analysis

Mitochondrial localization and activity were analysed in equine oocytes after IVM. Oocytes were denuded, and those with a clearly visible extruded polar body were fixed in 4 % paraformaldehyde for 15 min and stored in PBS at 4 °C (~10 oocytes per group). Before staining, the zona pellucida was removed with an acidic Tyrode's solution, then oocytes were permeabilized in 4 % Triton-X for 1 h, with a subsequent blocking with goat serum for 3 h. The oocytes were then stained overnight at 4 °C with primary antibodies for a subunit of the pyruvate dehydrogenase (dihydrolipoamide S-acetyltransferase, D-LAT, 1:100, mouse monoclonal, Thermo Fisher Scientific, Spain) and a mitochondrial membrane protein (translocase of outer mitochondrial membrane, TOMM20, 1:100, recombinant rabbit monoclonal, Thermo Fisher Scientific, Spain), to assess potential mitochondrial activity and localization, respectively. The expression of DLAT is indicative of mitochondrial activity because it is an essential enzyme within the pyruvate dehydrogenase complex, located in the mitochondrial matrix. Oocytes were then incubated with secondary antibodies, goat anti-mouse Alexa Fluor 488 (1:500, Thermo Fisher Scientific, Spain) and goat anti-rabbit Alexa Fluor 568 (1:500, Thermo Fisher Scientific, Spain) for D-LAT and TOMM20, respectively. The stained oocytes were mounted on glass slides with cover slips using Rapid Clear 1.47 mounting medium (Thermo Fisher Scientific, Spain) and DAPI. Each oocyte was visualized under a microscope, morphology and staining conditions were evaluated, the stage of the oocytes was checked, to confirm the previous MII morphological assessment, and only MII oocytes were analysed. Sample visualization with 60X oil objective was facilitated using the Dragonfly High Speed Confocal Microscope System (Oxford Instruments, UK) equipped with Fusion program. Dragonfly is an advanced imaging platform with high contrast and multi-dimensional capabilities encompassing four key imaging modalities. It operates as a multi-point confocal system, enabling high-speed and high-sensitivity imaging. This microscope is characterized by a capture speed of at least 10 times faster than conventional confocal technology. Using a confocal microscope, it was possible to visualize inside the oocytes in order to visualize all its "layers". With the Fusion program, it was possible to acquire images of each sample and each stack. A specific light source was chosen for each antibody: 488 GFP Sona1 for DLAT (color: green), 561 mCHERRY Sona1 for TOMM20 (color: red), and 405 Dapi Sona1: for DAPI (color: blue). Afterwards, the image processing program FIJI ImageJ was used. Images were analysed as previously described. An area of 30 μm inward from the oocyte cortex, to exclude the area without active mitochondria, was quantified.

2.7. Evaluation of the apoptotic status

Annexin V/propidium iodide (PI) staining was performed according to the manufacturer's instructions (Dead Cell Apoptosis Kit, Invitrogen™, Thermo Fisher Scientific, Italy). The experiment was done in 7 replicates with 4–5 immature oocytes per group. Samples were incubated for 30 min at 4 °C in the dark for staining with Annexin V, a phospholipid-binding protein that detects the translocation of phospholipid phosphatidylserine from the inner to the outer cytoplasmic membrane, which is known to occur during the early stages of apoptosis, and PI to distinguish live cells from dead cells. Then oocytes were washed twice in buffer solution and observed at an epifluorescence microscope. Oocytes were classified into three groups: viable oocytes without annexin staining in the membrane; early apoptotic oocytes with a homogeneous positive annexin signal in the membrane; and dead oocytes with PI-positive red nuclei, which is indicative of membrane damage [34].

2.8. Quantitative real-time PCR (RT-PCR) gene expression analysis for BCL2, BAX, p53 and survivin

The experiment was done in 3 replicates with 10–13 immature oocytes per group. 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 lysis of the oocytes was performed by using SideStep lysis and Stabilization buffer (Agilent Technologies, Santa Clara, CA, USA), as described according to Galeati et al., 2016 [36].

Briefly, pool of oocytes were added with 2 μl of SideStep lysis and Stabilization buffer and mixed very well by pipetting. To avoid DNA genomic 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 DNase reaction protocol, all the volume (16 μl) were retrotranscribed with cDNA using 5X RT Supermix (BioRad) following the manufacturer's instructions, in a 20 μl final volume to obtain cDNA. The kit used for retrotranscription was primed both oligo d (T) and random examers. Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. Primers sequence for *BCL2*, *BAX*, *p53*, and *Survivin* were designed by using Beacon Designer 2.07 (Premier Biosoft International, Palo Alto, CA, USA). Regarding to the reference genes, *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), and *ACTB* (Actin B), and *HPRT* (Hypoxanthine Phosphoribosyltransferase 1) were selected. The *HPRT* transcripts resulted not detectable in any sample. Then, the stability of the two reference genes (*GPDH* and *ACTB*) was evaluated through M and CV mean values (0.8035 and 0.322 respectively) by the CFX software (BioRad). All the primers used (*BCL2*; *BAX*; *p53*; *Survivin*; *GAPDH* and *ACTB*) were located on different exons and they were reported in Table 1.

A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 10 μl of iTaq Universal SYBR Green Supermix (Bio-RAD), 0.8 μl of the forward and reverse primers (5 mM each) of each target gene, 2 μl cDNA, and 7.2 μl of water. The qPCR protocol used for the transcriptional characterization was: 10 min at 95 °C, 40 cycles at 95 °C for 15 s and at 60 °C for 30 s, followed by a melting step from 55 °C to 95 °C (80 cycles of 0.5 °C increase/cycle). The specificity of the amplified PCR products was confirmed by agarose gel electrophoresis and melting curve analysis. Real-time efficiency was evaluated by amplification of a standardized amount of cDNA, derived from cDNA of equine corpus luteum, starting from 150 ng with subsequent 5-fold dilutions (75, 15, 3, 0.6, and 0.12 ng). RT-PCR efficiency showed a values between 94.7 % and 84.1 % (*BCL2*- 94.7 %; *BAX*-94.5 %; *p53*-89.2 %; *Surivin*-92.3 %; *GAPDH*-84.1 %; *ACTB*-92.1 %) (Table 1). The relative mRNA expressions of tested genes were normalized by using the ΔCt method (ΔCt = Ct geometric mean reference genes–Ct interest gene) [37] and then the relative expression was calculated as fold of change ($2^{-\Delta\Delta Ct}$ method) [38] in respect to the oocyte control group obtained under different condition (F, H, or VIT).

2.9. Statistical analysis

Data from survival evaluation, Annexin V/PI staining, and maturation ability are expressed as percentages and were compared using the Chi Square test. Data on the intensity of different stainings are expressed as mean ± standard deviation, as well as for maturation rates in the additional experiment comparing only VIT and VIT-Nx (including all replicates for these groups). Data were checked for normality using the Shapiro-Wilk test. Then a Generalized Linear Model (GLM) for a gamma distribution and log link function was used, with Wald pairwise comparisons. When analysing the overall effect of vitrification, the comparison was made between non-vitrified (F and H) and vitrified/warmed (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) oocytes. Data were analysed using IBM SPSS Statistics 29.0 (IBM Corporation, Milan, Italy), with significance assessed at $P < 0.05$. Only data obtained from at least five oocytes per group were included in the analysis. Consequently, the immunocytochemistry data for mitochondrial analysis from the F, VIT, and VIT-Nx groups were excluded.

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

3. Results

A total of 771 equine immature oocytes were used for IVM and various staining procedures, while 214 oocytes were stored for qPCR. The survival rate, as determined by evaluation at the stereomicroscope after cumulus removal, was similar among vitrification groups ($P > 0.05$). However, the degeneration rate was higher in oocytes vitrified immediately after collection compared to fresh control ($P < 0.05$), while no difference was observed between held control (H) and oocytes vitrified after overnight holding (H-VIT, and H-VIT-Nx) ($P > 0.05$) (Table 2).

Analysing the ability of vitrified oocytes to resume meiosis and reach the MII stage (Table 3), no significant differences were observed among the vitrification groups ($P > 0.05$), likely due to the low number of oocytes. However, only VIT-Nx achieved a maturation rate not significantly different from F and H ($P > 0.05$). To confirm the positive effect of Nx on oocytes vitrified immediately after collection, additional oocytes were vitrified in the VIT and VIT-Nx groups, with a total of 31 and 35 oocytes, respectively. The presence of Nx significantly improved the maturation rate ($P < 0.05$; VIT: 37.8 ± 13.1 % vs. VIT-Nx: 59.2 ± 8.2), primarily due to a lower degeneration rate ($P < 0.05$; VIT: 58.6 ± 8.0 % vs. VIT-Nx: 38.3 ± 11.8), as the percentage of non-matured oocytes was similar between groups ($P > 0.05$; VIT: 3.6 ± 5.1 % vs. VIT-Nx: 2.5 ± 3.5).

When comparing all groups for ROS intracellular levels, significant differences were observed (Fig. 2A) ($P < 0.05$). Specifically, a significant reduction in ROS levels was observed in held oocytes (H) compared to fresh (F) controls ($P < 0.05$). In oocytes vitrified immediately after collection, vitrification (VIT vs F) did not affect ROS levels ($P > 0.05$), while the addition of Nx (VIT-Nx) tended to increase ROS levels (VIT-Nx vs F: $P = 0.053$; VIT-Nx vs VIT: $P = 0.069$). Conversely, in oocytes vitrified after a holding period, vitrification (H-VIT vs H) induced a significant increase in ROS levels ($P < 0.05$), whereas the presence of Nx during vitrification (H-VIT-Nx) did not lead to a significant increase compared to the held control group (H-VIT-Nx vs H: $P > 0.05$). No differences in ROS levels were observed among the vitrification groups ($P > 0.05$). Additionally, intracellular ROS levels were higher in vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) oocytes compared to non-vitrified

Table 3
Maturation rate of equine immature oocytes, vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by Hoechst 33342 staining.

Groups	N oocytes	Mature (%)	Immature (%)	Degenerate (%)
F	18	12 (77.8)^{a}	1 (5.6)^{a}	3 (16.7)^{a}
VIT	14	4 (28.6)^{b}	1 (7.1)^{a}	9 (64.3)^{b}
VIT-Nx	15	8 (53.3)^{a,b}	0 (0.0)^{a}	7 (46.7)^{a,b}
H	16	12 (75.0)^{a}	1 (6.3)^{a}	3 (18.8)^{a}
H-VIT	16	5 (31.3)^{b}	3 (18.8)^{a,b}	8 (50.0)^{b}
H-VIT-Nx	16	5 (31.3)^{b}	6 (37.5)^{b}	5 (31.3)^{a,b}

Different superscript letters in columns indicate statistical significance ($P < 0.05$). F = fresh; H = holding; VIT = vitrified; Nx = naloxone.

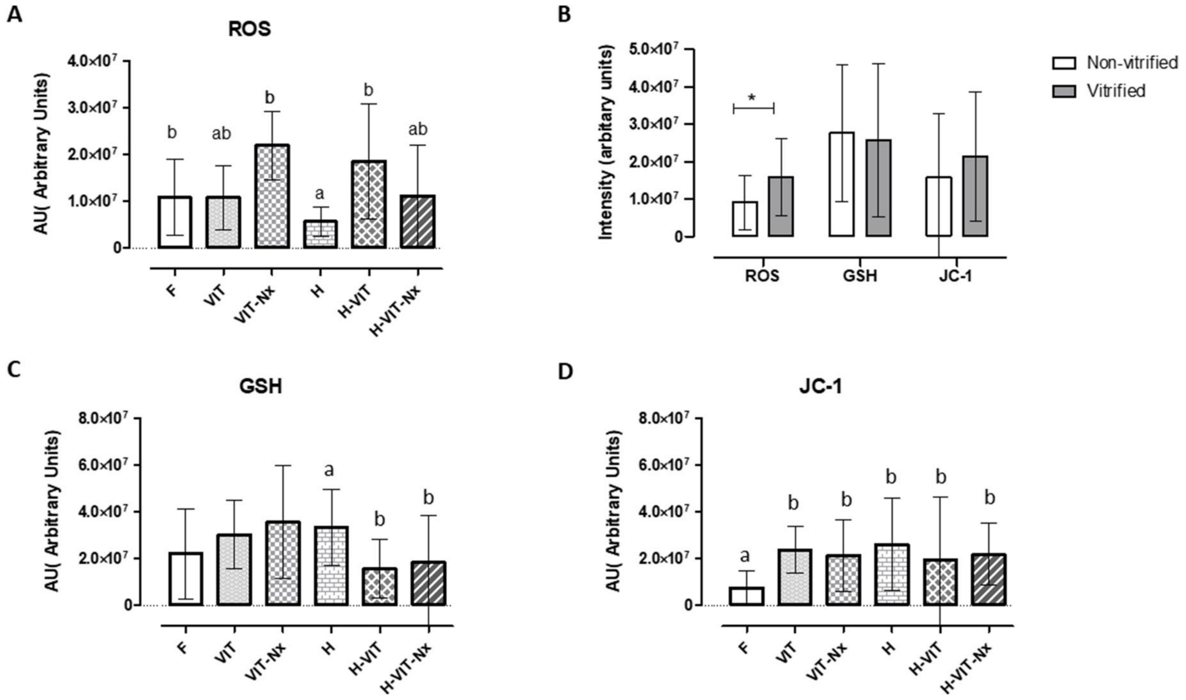

Fig. 2. Evaluation of equine immature oocytes, vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed 2 h after warming by A) H2DCFDA for reactive oxygen species (ROS) levels determination in various experimental groups. Different letters indicate statistically significant differences among groups ($P < 0.05$) B) H2DCFDA for ROS levels, CellTracker Blue for glutathione (GSH) levels, JC-1 for high mitochondrial membrane potential (HMMP) in vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) and non-vitrified (F and H) equine immature oocytes. The symbol * indicates statistically significant differences among groups ($P < 0.05$); C) CellTracker Blue for GSH levels determination in various experimental groups. Different letters indicate statistically significant differences among held groups ($P < 0.05$); D) JC-1 for HMMP determination in various experimental groups. Different letters indicate statistically significant differences among groups ($P < 0.05$).

(F and H) ones ($P < 0.05$) (Fig. 2B).

GSH intracellular levels were not significantly different when comparing all groups, vitrification groups, or fresh control group and immediately vitrified oocytes ($P > 0.05$) (Fig. 2C). On the other hand, considering held oocytes, GSH levels were higher in the control group compared to vitrified oocytes ($P < 0.05$) (Fig. 2C). Overall, intracellular GSH levels were similar in vitrified oocytes compared to non-vitrified ones ($P > 0.05$) (Fig. 2B).

High mitochondrial membrane potential, assessed by JC-1 (orange fluorescence), showed significant differences ($P < 0.05$) between groups (Fig. 2D). All treatments (holding and vitrification with or without naloxone) induced an increase in HMMP levels compared to fresh control oocytes. However, no significant difference in HMMP levels were observed between vitrified and non-vitrified oocytes ($P < 0.05$) (Fig. 2B). In held matured oocytes, mitochondria were distributed throughout the entire oolemma, as shown by TOMM20 staining (Fig. 3A). The presence of DLAT, used as a marker for active mitochondria, was detected in the outermost region of the oocytes, with no signal in the centre (Fig. 3A). This same pattern was predominantly observed in both control (4/5) (Fig. 3B) and vitrified (5/7) oocytes (Fig. 3C), but not in oocytes vitrified in presence of Nx (3/7) (Fig. 3D). In these oocytes, the signal of potentially active mitochondria was spread

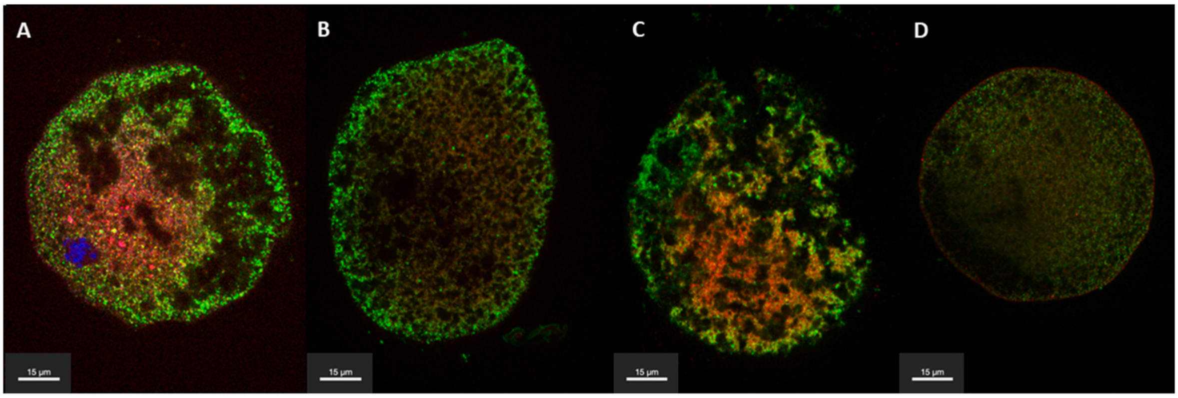

Fig. 3. Confocal microscope images of equine oocyte mitochondrial activity (stained in green by D-LAT) and localization (stained red by TOMM20) after IVM. A) Control oocyte from the overnight holding group (H) at maturation stage confirmation. Note the MII plate (stained in blu by DAPI), and the absence of the first polar body, which was lost during zona pellucida removal; B) Another control oocyte of the overnight holding group (H); C) Oocyte vitrified after overnight holding (H-VIT); D) Oocyte vitrified after overnight holding in presence of naloxone (H-VIT-Nx). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

throughout the cytoplasm (4/7). Moreover, when comparing the amount of DLAT (active mitochondria), no significant differences were observed between groups, nor between vitrified and non-vitrified oocytes ($P > 0.05$) (Fig. 6).

Results from AnnexinV/PI staining are showed in Table 4. The percentage of viable oocytes was higher ($P < 0.05$) in non-vitrified than in all vitrified/warmed oocytes, and H-VIT-Nx protocol was the less efficient protocol ($P < 0.05$). Early apoptotic oocytes were more present ($P < 0.05$) in H-VIT compared to non-vitrified groups (F and H), while the other vitrification protocols showed intermediate results. No dead oocytes were found in H group, so the rate was significantly lower than in all groups ($P < 0.05$). The percentage of dead oocytes was lower in VIT compared to H-VIT-Nx ($P < 0.05$), and intermediate for VIT-Nx and H-VIT. Considering oocytes treated immediately after collection, the rate of dead oocytes was significantly increased compared to control only in presence of Nx ($P < 0.05$).

Comparing total non-vitrified and vitrified oocytes (Fig. 4), the vitrification process significantly reduced the rate of viable oocytes, while simultaneously increasing the rates of early apoptotic and dead oocytes ($P < 0.05$).

RT-PCR analysis confirmed the detection for both reference genes, and target genes *BCL2* and *Survivin* in all samples. In contrast, *BAX* and *p53* transcripts were consistently undetectable. When comparing fresh and held groups separately, no significant differences were observed in the expression of *BCL2* and *Survivin* (Fig. 5A). However, the expression of both genes was significantly increased in vitrified oocytes compared to non-vitrified ones ($P < 0.05$) (Fig. 5C).

4. Discussion

In the present study, equine immature oocytes were vitrified using Cryotop® following a previously described protocol [5,26]. The research aimed to investigate the effects of adding naloxone to the vitrification solutions and to explore the impact of overnight holding on vitrification outcomes.

Survival rates, assessed morphologically 2 h after warming, were similar across various treatments, ranging from 77.4 % to 86.0 %. However, the degeneration rate appeared higher for oocytes vitrified immediately after collection compared to non-vitrified control, while it was similar among groups of oocytes held overnight. Previous studies [28,39] have observed that oocytes already partially compromised may be less tolerant to overnight holding, resulting in an increased degeneration rate after maturation. This may explain why the holding period appeared to minimize the differences in outcomes between vitrified and control held oocytes. During the holding period, any partially compromised oocytes likely degenerated, while in the fresh control group such compromised oocytes were not detectable at morphological evaluation.

Furthermore, as previously observed in canine oocytes [40], morphological evaluation under the stereomicroscope is a reliable method for detecting oocytes with ruptured plasma membranes, but it does not allow for the identification of oocytes with damaged membrane. Indeed, in our study, dead oocytes were detected using Annexin V/PI staining, even though only oocytes that appeared to have intact plasma membranes during morphological evaluation were stained. Moreover, although the relatively low number of oocytes analysed may limit the robustness of the result interpretation, no dead oocytes were found in the held control group, confirming that partially compromised oocytes may degenerate during overnight holding.

Table 4

Viable, early apoptotic, and dead rates of equine immature oocytes, vitrified either immediately or after overnight holding, with or without the addition of naloxone to vitrification solutions, as assessed by AnnexinV/PI staining.

Groups	N oocytes	Viable (%)	Early apoptotic (%)	Dead (%)
F	30	24 (80.0)^{a}	2 (6.7)^{a}	4 (13.3)^{b}
VIT	29	15 (51.7)^{b}	6 (20.7)^{a,b}	8 (27.6)^{b,c}
VIT-Nx	26	9 (34.6)^{b}	5 (19.2)^{a,b}	12 (46.2)^{c,d}
H	29	27 (93.1)^{a}	2 (6.9)^{a}	0 (0.0)^{a}
H-VIT	28	9 (32.1)^{b}	9 (32.1)^{b}	10 (35.7)^{c,d}
H-VIT-Nx	32	7 (21.9)^{c}	7 (21.9)^{a,b}	18 (56.3)^{d}

Different superscript letters in columns indicate statistical significance ($P < 0.05$). F = fresh; H = holding; VIT = vitrified; Nx = naloxone.

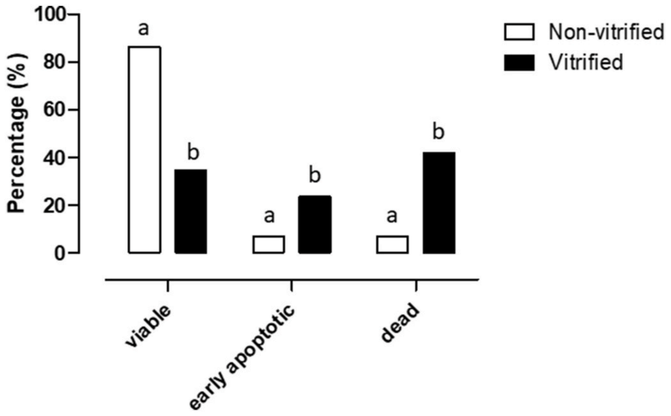

Fig. 4. Evaluation of apoptotic status of equine non-vitrified (F and H) and vitrified (VIT, VIT-Nx, H-VIT, and H-VIT-Nx) immature oocytes as assessed by Annexin V/PI staining 2 h after warming. Different letters indicate statistically significant differences among groups ($P < 0.05$).

After IVM of vitrified/warmed immature oocytes, approximatively one-third successfully reached the MII stage, with 55.3 % achieving MII when oocytes were vitrified immediately after collection in the presence of naloxone (compared to 77.8 % MII for fresh control oocytes). Similar maturation rates have been reported [9,14]. However, direct comparison between studies is challenging because the success of vitrification depends on both oocyte quality and the specific techniques employed. Significant variability arises when working with oocytes recovered from ovaries sourced at slaughterhouses, where critical factors such as time from slaughter to oocyte recovery, tissue handling, and mare reproductive status and age are unknown. Despite these differences, and considering that the low number of oocytes may limit result interpretation, naloxone appeared to have a positive effect on meiotic competence of oocytes vitrified immediately after collection but not on those held prior to vitrification. Holding at room temperature maintains meiotic arrest in horse oocytes [41], which may have interfered with Nx's action.

A recent study revealed that Nx has protective effects on oxidative stress [25]. In the present study, Nx exhibited opposite effects depending on the timing of vitrification. It tended to increase ROS levels in oocytes vitrified immediately after collection, while it prevented the rise in ROS levels in held oocytes following vitrification. Additionally, overnight holding reduced ROS levels in non-vitrified oocytes. This suggests that the holding period influences the activity of Nx on equine immature oocytes. Oocytes physiologically utilize oxygen for energy production through mitochondrial oxidative phosphorylation, and ROS production increases during IVM [42]. It can be hypothesized that overnight holding not only maintains meiotic arrest, but also reduces the metabolic activity of the oocyte, thereby decreasing the physiological ROS levels. The effect of holding on ROS production was investigated in one study that assessed mitochondrial energy/redox potential in both immature and matured equine oocytes [43]. ROS levels were measured in relation to nuclear chromatin configuration, and although the mean intracellular ROS levels were numerically lower in held oocytes than in those immediately stained across all configurations, the difference was not statistically significant [43]. Additionally, different holding media were used (Earle's/Hank's' M199-based medium [43] vs HSOF in the present study), which may have played an important role in influencing the observed differences. ROS levels were evaluated in another study

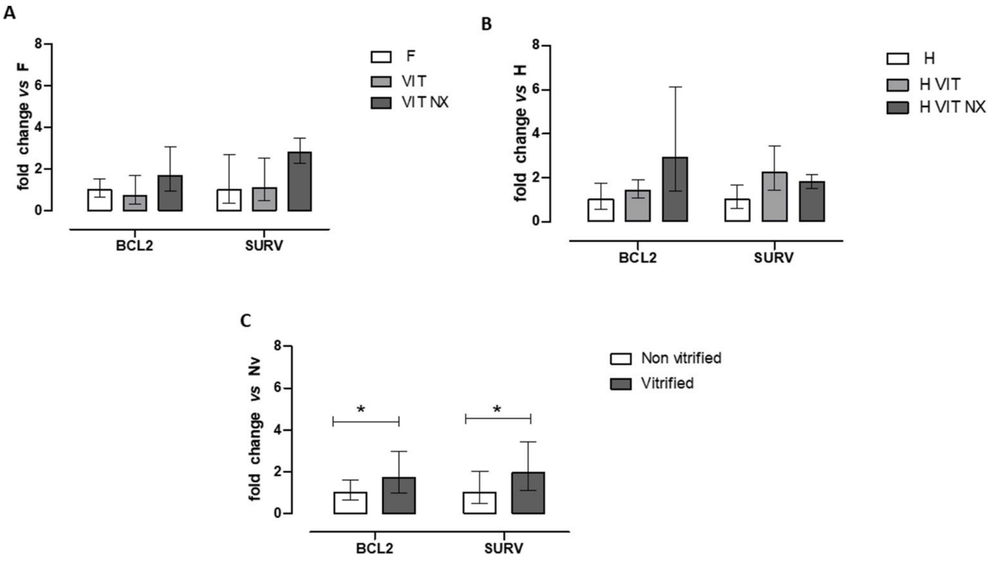

Fig. 5. Relative expression levels of BCL2 and *Survivin* genes evaluated by qPCR in equine immature oocytes vitrified under different experimental conditions: A) expression was normalized to fresh control oocytes (F); B) expression was normalized to held control oocytes (H); C) expression was normalized to non-vitrified oocytes (F and H). The symbol * indicates statistically significant differences among groups ($P < 0.05$).

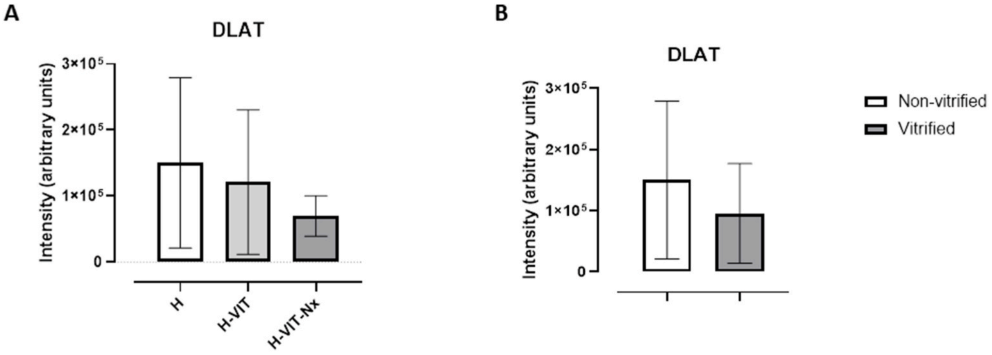

Fig. 6. Amount of DLAT (active mitochondria) in equine *in vitro* matured held oocytes before and after vitrification with or without naloxone (10^{-8} M): A) comparison between held control oocytes (H: $n = 5$) and oocytes vitrified after overnight holding without (H-VIT: $n = 7$) or with naloxone (H-VIT-Nx: $n = 7$); B) comparison between non-vitrified (H) and vitrified (H-VIT, and H-VIT-Nx) oocytes.

involving immature vitrified/warmed equine oocyte matured in presence of melatonin [7], where oocytes were held overnight prior to vitrification. In that study, the vitrification and warming process increased intracellular oxidative stress, as evidenced by higher ROS levels [7]. Further studies are needed to better understand the modifications induced by overnight holding in equine immature oocytes, particularly to explain the different responses to Nx, which may be linked to changes in MOR expression.

Glutathione plays a crucial role in protecting cells from the detrimental effects of ROS [44], modulates protein and DNA synthesis by affecting redox status, and is involved in the assembly of microtubules [45]. However, an inverse correlation between GSH and ROS levels was not observed in this study. This phenomenon has already been reported for some antioxidants in cattle [46,47]. In horses, there are no reports of GSH determination in oocytes using CellTracker Blue staining. Nevertheless, it has been demonstrated that GSH concentrations are lower in germinal vesicle oocytes compared to post-maturation oocytes, both *in vivo* and *in vitro* [48]. The synthesis of GSH during oocyte maturation is crucial for sperm chromatin decondensation and male pronuclear formation [49], playing a pivotal role in subsequent steps of *in vitro* embryo production [50]. Our overall results could suggest that vitrification does not affect GSH content in equine immature oocytes, which contrast with findings in immature cat [51] and silver fox [52] oocytes, where vitrification has been shown to impact GSH levels adversely. However, a decrease in GSH levels, regardless the presence of Nx, was observed in oocytes vitrified after overnight holding. It would be valuable to investigate whether these oocytes can attain optimal GSH levels following maturation, or if GSH supplementation during post-warming recovery culture [53] could alleviate the detrimental effects of vitrification on GSH levels in held equine immature oocytes.

Regarding active mitochondria, both holding and vitrification increased HMMP levels, whereas the addition of Nx did not influence this rise. The higher MMP observed in held oocytes compared to freshly collected ones in this study contrasts with findings from another study [43]. In that study, holding oocytes at un-controlled room temperature (22–27 °C), similar to our conditions, affected chromatin configuration, increasing the proportion of meiotically competent oocytes (condensed chromatin (CC) and prometaphase I/metaphase I configurations), but did not alter mitochondrial activity as detected by MitoTracker Orange CMTM Ros [43]. Nonetheless, oocytes in the CC configuration exhibited a more mature mitochondrial distribution and higher mitochondrial activity compared to less advanced stages [43]. Since CC configuration was most frequent in held oocytes [43], it is plausible that our holding conditions primarily selected CC oocytes, enabling JC-1 staining to detect a significant increase in HMMP. Moreover, the rise in HMMP observed in immediately vitrified oocytes is likely attributable to a different mechanism. ROS tipically induce mitochondrial permeability, which reduces MMP [54]. Vitrification has been shown to decrease MMP in human [55], porcine [56], bovine [57], and sheep [58] oocytes. However, it was observed that this reduction in MMP in human MII oocytes is temporary; within 4 h of warming, MMP levels spontaneously recover to those observed in fresh oocytes [55]. In the present study, vitrified-warmed oocytes were incubated for 2 h at 38.5 °C prior to staining, thus it is not possible to determine if MMP decreased after vitrification. In our experimental conditions, as reported for human MII oocytes, the MMP of immediately vitrified equine oocytes increased after warming followed by incubation, potentially in preparation for meiosis resumption stimulated by the increased temperature [43].

As observed in human [59] and bovine [60] species, equine mature oocytes are characterized by numerous mitochondria distributed throughout the cytoplasm [61]. The abundance of inactive mitochondria is thought to support the oocyte's prolonged quiescence, protecting it from damage caused by ROS generated by active mitochondria [62]. A distinctive feature of these cells is the presence of a layer of active mitochondria in the subcortical area, with no mitochondrial activity detectable in the central region. The accumulation of active mitochondria in the peripheral cytoplasm and/or around the nucleus has been identified as a marker of full cytoplasmic maturation in equine oocytes [63]. Notably, considering the low number of analysed oocytes, it appears that vitrification did not alter this pattern in held oocytes, whereas the addition of Nx resulted in an even distribution of active mitochondria throughout the cytoplasm. This alteration could indicate an immature cytoplasmic condition [63].

In general, ROS are associated with induction of death in apoptotic process [64]. The increase of ROS after oocyte vitrification has been associated with a higher rate of early apoptotic oocytes (assessed using Annexin V/PI staining) in cattle [65]. In porcine oocytes, vitrification induced a rise in the percentage of early apoptotic oocytes (Annexin V + and PI-) and a contemporaneous increase of dead oocytes (PI+) [34]. On the other hand, in prepubertal goat oocytes, even if vitrified oocytes showed higher ROS levels, no differences were found in the number of early apoptotic oocytes (Annexin V + and PI-), while a decrease in live oocytes and an increase in dead oocytes was observed [66]. In the present study, the vitrification process reduced the rate of viable oocytes and, similarly to what observed in the pig [34], increased early apoptotic and dead oocytes. Although the number of analysed oocytes was relatively limited, the poorest outcome was observed for held oocytes vitrified in presence of naloxone, and the rate of dead oocytes was increased by the addition of Nx in oocytes vitrified immediately after collection. While early apoptosis, characterized by mitochondrial depolarization and phosphatidylserine externalization, is typically a precursor to cell death, it is not always irreversible. When the stressor is removed and favorable conditions are provided, early apoptotic processes can sometimes be halted or reversed [67]. For instance, oocytes recovering from changes induced by cryopreservation have demonstrated restored functionality when subjected to antioxidant supplementation and culture techniques that mitigate oxidative stress [7,53,65,68,69]. In this context, the potential antioxidant effect of Nx was not evident, particularly in freshly vitrified oocytes, as it actually tended to increased ROS levels post-warming.

The increased expression of anti-apoptotic genes such as *BCL2* and *Survivin* observed in vitrified-warmed oocytes, alongside the absence of consistent or significant levels of the pro-apoptotic gene *BAX* and the tumor suppressor gene *p53*, suggests a possible protective mechanism against mitochondrial-mediated apoptosis during cryopreservation. Given that fully grown oocytes at the GV stage are transcriptionally dormant, these changes likely result from post-transcriptional regulation, where previously stored mRNAs are stabilized or degraded in response to vitrification-induced stress, rather than new mRNA synthesis. The *BCL2* family plays a pivotal role in outer mitochondrial membrane permeabilization, with *BAX* promoting cytochrome *c* release and apoptosis, while *BCL2* inhibits this release, thus preventing apoptotic progression [65]. *Survivin*, recognized as a marker for developmental potential, further contributes to anti-apoptotic mechanisms by binding to and inhibiting caspases, particularly caspase-3, thereby preventing apoptosome formation [70]. The lack of *p53* expression, typically associated with stress-induced apoptosis, suggests that vitrification-induced apoptosis in oocytes may proceed via alternative, *p53*-independent pathways. This aligns with previous findings indicating that *p53* expression does not correlate with the morphological quality of bovine embryos, suggesting its limited involvement in oocyte cryopreservation responses [71]. These observations, combined with the modulation of *BCL2* and *BAX* under various oocyte maturation and stress conditions [72,73], emphasize the critical role of oxidative stress and mitochondrial integrity in regulating apoptotic gene expression during cryopreservation. The post-transcriptional activation of stored mRNAs encoding anti-apoptotic proteins, likely triggered by vitrification and warming stress, could serve as a protective response to maintain oocyte viability. However, these findings should be interpreted with caution, as they may reflect differential stabilization or degradation of mRNA transcripts rather than active transcriptional changes. Future studies focusing on the expression of these proteins should be conducted. Additionally, in this study, oocyte for qPCR were stored after enzymatic removal of the zona pellucida, a procedure that likely selected for higher-quality oocytes, excluding from the analysis necrotic ones and those with compromised plasma membranes.

Finally, there are some limitations to consider when interpreting the qPCR results regarding the selection of reference genes, a critical factor for accurate and consistent quantification. Unfortunately, the most reliable reference genes in equine oocytes have not been studied previously. Therefore, we select three potential reference genes (*HPRT*, *ACTB*, and *GAPDH*) from different functional classes to reduce the change of co-regulation. Before performing the normalization, we evaluated the gene stability parameter (M value) for the detectable transcripts of the reference genes (*GAPDH* and *ACTB*), as lower values indicate higher expression stability. The observed M value ($M = 0.8035$) was higher than the accepted cut-off value ($M = 0.5$). However, we consider a range between 0.6 and 0.9 to reflect relatively good stability according to Smits et al. [74], based on a study conducted on equine *in vivo*, fresh and frozen *in vitro* blastocysts.

5. Conclusion

Vitrification increased oxidative stress, apoptosis, and the proportion of mitochondria with high MMP in equine immature oocytes. Overnight holding at uncontrolled room temperature reduced oxidative stress. However, this approach also reduced GSH levels after vitrification, potentially compromising oocyte developmental competence, and modified the oocyte's response to Nx. In fact, while naloxone supplementation positively influenced meiotic competence in oocytes vitrified immediately after collection, its effects were less consistent when combined with overnight holding. Conversely, Nx exhibited antioxidant activity in oocytes vitrified after holding but had the opposite effect in those vitrified immediately. This consideration is essential when evaluating the effects of antioxidants or other molecules to improve vitrification outcomes for immature equine oocytes, as overnight holding at uncontrolled room temperature may alter oocyte characteristics and lead to variable results. Overall, Nx displayed contrasting antioxidant effects depending on the vitrification timing, but it appeared to improve IVM outcomes in oocytes vitrified immediately after collection. The relatively low number of oocytes used in each analysis might limit the interpretation of the study results. Nevertheless, these findings emphasize the importance of tailoring vitrification protocols to the condition of the oocytes and suggest the need for further investigation into Nx's properties. Its potential application during the post-warming period warrants exploration to enhance cryopreservation outcomes for equine immature oocytes.

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. **Monica Forni:** Writing – review & editing. **Eleonora Iacono:** Writing – review & editing. **Filippo Zambelli:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Barbara Merlo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

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