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Oxidative and Mitochondrial Status of Vitrified Equine In Vitro Matured Oocytes Using Temperature-Specific Equilibration Protocols

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ABSTRACT

Cryopreservation of equine in vitro matured (IVM) oocytes remains a major challenge in assisted reproduction due to their high susceptibility to cryoinjury, including oxidative stress and mitochondrial dysfunction. The aim of this study was to evaluate the physiological status of vitrified/warmed equine IVM oocytes following vitrification using two temperature-specific equilibration protocols by assessing reactive oxygen species (ROS), intracellular glutathione (GSH) content and mitochondrial oxidative activity and distribution patterns. Oocytes were vitrified after 28 h of IVM either at 25°C (in VIT25) or at 38.5°C (in VIT38.5) and cultured for an additional 2 h after warming. No significant differences were detected among groups in ROS levels, GSH content, mitochondrial oxidative activity or distribution patterns ($p > 0.05$). These results indicate that the vitrification protocols evaluated did not induce detectable alterations in redox balance or mitochondrial function in equine IVM oocytes. This suggests that vitrified/warmed oocytes may maintain characteristics compatible with fertilisation and subsequent embryonic development, although their developmental competence was not directly evaluated and warrants further investigation.

1 | Introduction

Despite advances in vitrification techniques, the cryopreservation of equine in vitro matured (IVM) oocytes remains challenging, with limited post-thaw survival and developmental competence (De Coster et al. 2020). Cryopreservation may induce several cellular alterations, including oxidative stress, mitochondrial dysfunction and cytoskeletal damage, which can compromise oocyte quality (Cao et al. 2022). Consequently, although vitrified equine IVM oocytes can occasionally develop into blastocysts or pregnancies (Angel et al. 2020), overall developmental outcomes remain low. Most vitrification protocols used for equine oocytes rely on fixed exposure times to equilibration and vitrification solutions, without considering the osmotic response of the oocyte to cryoprotectant addition and removal. Mathematical modelling approaches can help predict the osmotic behaviour of oocytes and identify exposure conditions minimising osmotic stress during cryopreservation (Caliskan et al. 2024). Based on previous modelling of equine oocyte osmotic response (Gago et al. 2026), two vitrification protocols with temperature-specific equilibration times were established. The equilibration times were mathematically derived from membrane permeability parameters obtained through osmotic modelling, considering the temperature-dependent kinetics of water and cryoprotectant transport in equine IVM oocytes. The aim of this study was to evaluate the physiological status of vitrified/warmed equine IVM oocytes following these protocols by assessing reactive oxygen species (ROS), intracellular glutathione (GSH) content and mitochondrial oxidative activity and distribution patterns after warming.

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2 | Materials and Methods

2.1 | Oocyte Collection and IVM

Ovaries were collected from two local abattoirs and transported to the laboratory in pre-warmed water (35°C–37°C). Cumulus–oocyte complexes (COCs) were recovered within 2–3 h post-mortem by incising and scraping follicular walls. COCs were held overnight in holding medium consisting of TCM-199 Hanks–HEPES, TCM-199 Earle's, 20% fetal bovine serum (FBS), 0.3% sodium pyruvate and 0.25% gentamicin at room temperature without light exposure for 17–18 h. Oocytes were then transferred to IVM medium containing TCM-199 Earle's, 10% FBS, 5 mU/mL FSH and 50 ng/mL EGF and in vitro matured for 30 h at 38.5°C in a humidified atmosphere containing 6% CO_2.

2.2 | Oocyte Vitrification and Warming

At 28 h of IVM, oocytes were denuded and those displaying a polar body were selected for vitrification. Oocytes were exposed to equilibration solution (ES; 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (Me_2SO) in HM (TCM-199 HEPES supplemented with 20% FBS)) at 25°C for 17 min 8 s (VIT25) or at 38.5°C for 7 min 42 s (VIT38.5). Oocytes were then transferred to vitrification solution (VS; 15% (v/v) EG, 15% (v/v) Me_2SO and 0.5 M sucrose in HM) for < 1 min, loaded onto a Cryotop device and plunged into liquid nitrogen. Exposure times were chosen based on optimised vitrification/warming protocols developed by mathematical modelling by García-Martínez et al. (2020) with species-specific modifications (Gago et al. 2026). Warming was performed by sequential exposure of the oocytes to 1 M sucrose for 1 min, 0.5 M sucrose for 4 min and HM for 5 min. After warming, oocytes were cultured for two additional hours. Oocytes IVM for 30 h served as fresh controls. Oocytes from all groups were collected at 30 h of IVM to assess ROS production ($n = 55$), intracellular GSH levels ($n = 52$) and mitochondrial oxidative activity and distribution patterns ($n = 55$). A total of 55 oocytes were analysed across eight independent replicates (3 to 10 oocytes/replicate).

2.3 | ROS, GSH and Mitochondrial Oxidative Activity Levels

At 30 h of IVM, oocytes from all groups were washed in phosphate-buffered saline and incubated for 30 min at 38.5°C in a drop containing 50 μM CellROX Green, 20 μM CellTracker Blue and 100 nM MitoTracker Red CM-H_2XRos to assess ROS levels, intracellular GSH levels, and mitochondrial oxidative activity and distribution patterns, respectively. Mitochondrial distribution patterns were quantitatively determined as described in Luis-Calero et al. (2024) (see Figure 1). After incubation,

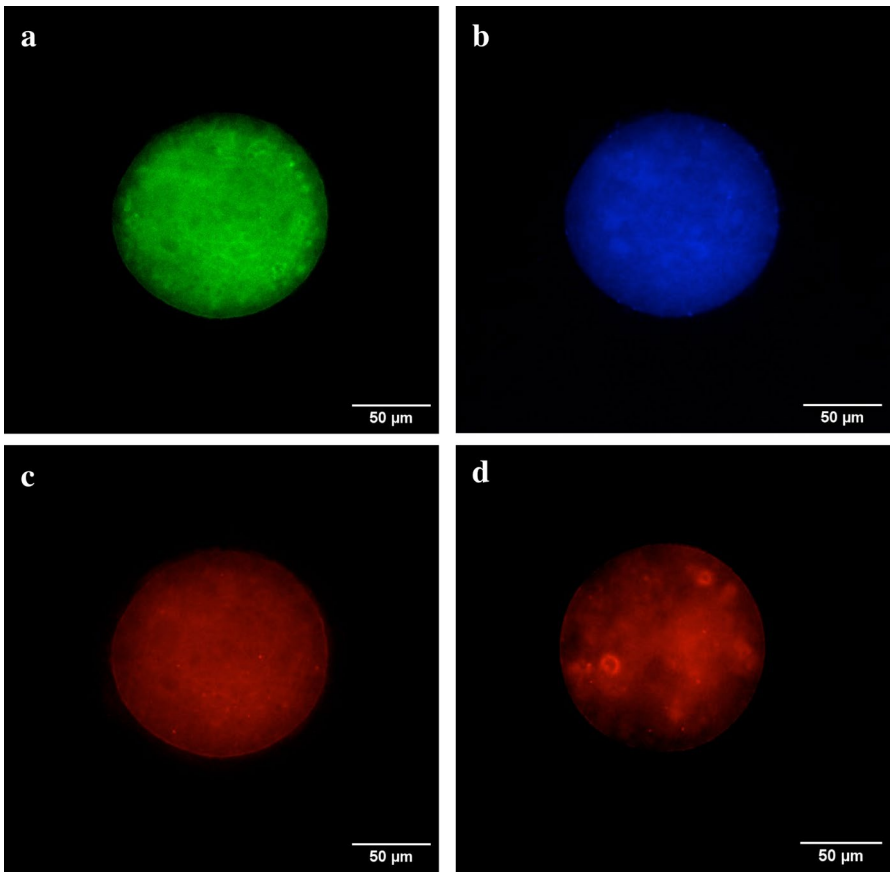


FIGURE 1 | Representative epifluorescence images of reactive oxygen species (ROS), intracellular glutathione (GSH) and mitochondrial distribution patterns of equine IVM oocytes. (a) ROS positive staining (CellROX Green); (b) GSH positive staining (CellTracker Blue); (c, d) Mitochondrial oxidative activity intensity; (c) homogeneous or; (d) heterogeneous mitochondrial distribution patterns (MitoTracker Red CM-H_2XRos). Scale bar: 50 μm.

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oocytes were fixed in 4% paraformaldehyde for 30 min at 38.5°C and were mounted on coverslips using self-adhesive reinforcement rings with Vectashield mounting medium. Fluorescence images were acquired using a SLIDEVIEW VS200 slide scanner with appropriate excitation/emission filters (ex.352–404, em.414–450; ex.460–488, em.500–530; ex.542–566, em.579–611). Images were captured using a UPLXAPO20X objective to evaluate fluorescence intensity and mitochondrial distribution patterns.

2.4 | Statistical Analysis

Data normality was assessed using the Shapiro–Wilk test and outliers were identified using the ROUT test. Normally distributed data were analysed by one-way ANOVA followed by Dunnett's post hoc test, whereas non-normal data were analysed using the Kruskal–Wallis test followed by Dunn's post hoc test. Data are presented as mean ± SEM. Statistical significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 9.

3 | Results and Discussion

Cryopreservation has been associated with increased oxidative stress and mitochondrial dysfunction in mammalian oocytes, which can compromise cytoplasmic quality and developmental competence (Cao et al. 2022; García-Martínez et al. 2020; Mateo-Otero et al. 2021). However, under the present conditions, vitrification did not significantly affect ROS production (Control vs. VIT25, $p = 0.57$; Control vs. VIT38.5, $p = 0.99$, Figure 2a), intracellular GSH levels (Control vs. VIT25, $p = 0.99$; Control vs. VIT38.5, $p = 0.99$, Figure 2b) or mitochondrial oxidative activity (Control vs. VIT25, $p = 0.79$; Control vs. VIT38.5, $p = 0.99$, Figure 2c) in equine IVM oocytes, independently of the vitrification temperature. One possible explanation is that oocytes were maintained overnight in holding medium prior to IVM. Previous studies in equine oocytes have shown that this holding step can reduce intracellular ROS levels in equine oocytes and improve oocyte competence after vitrification, suggesting a protective effect against oxidative stress during subsequent manipulations (Gugole et al. 2025). In agreement with these observations, intracellular GSH levels were also maintained after

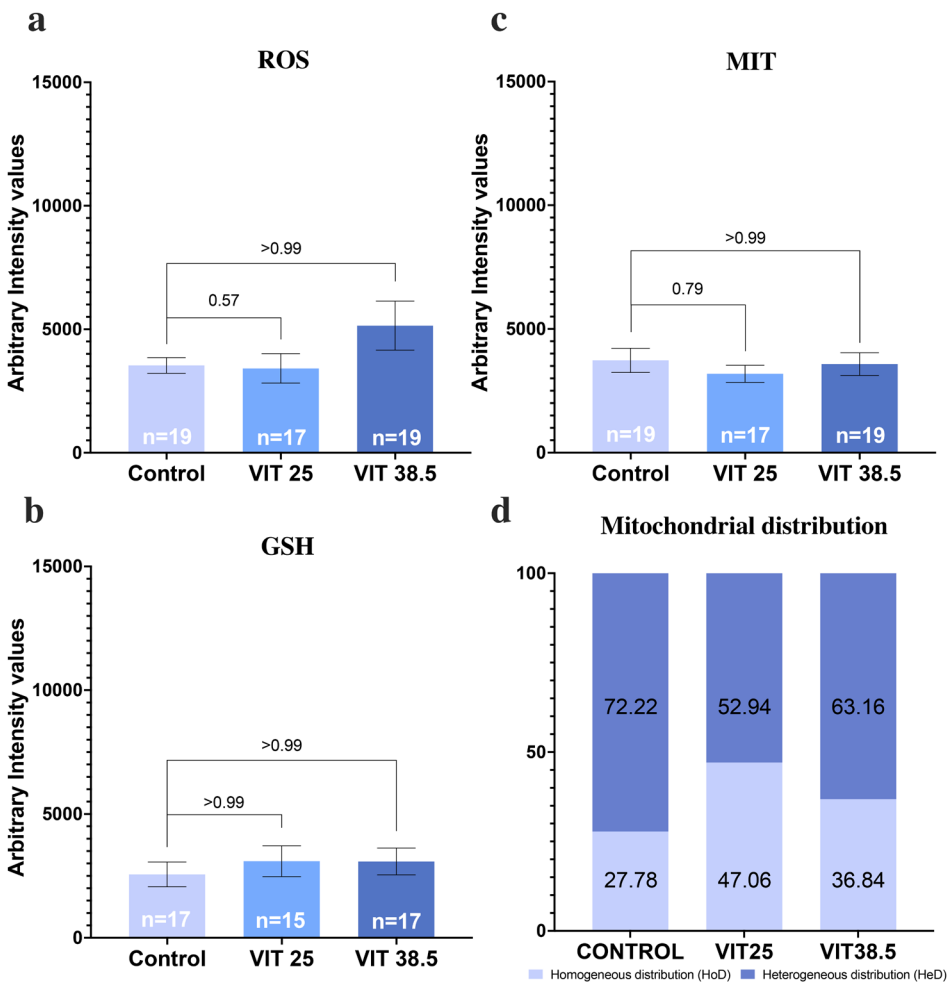


FIGURE 2 | Physiological status of equine IVM oocytes after vitrification and warming. (a) Reactive oxygen species (ROS); (b) Intracellular glutathione (GSH) levels; (c) Mitochondrial oxidative activity (MIT) (mean ± SEM); (d) Proportion of oocytes displaying homogeneous (HoD) or heterogeneous (HeD) mitochondrial distribution. CONTROL: Oocytes IVM for 30 h; VIT25: Oocytes vitrified at 25°C after 28 h of IVM and cultured for 2 additional hours after warming; VIT38.5: Oocytes vitrified at 38.5°C after 28 h of IVM and cultured for 2 additional hours after warming. No statistically significant differences were detected among groups ($p > 0.05$).

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vitrification, indicating that the intracellular antioxidant capacity (primarily represented by glutathione (GSH), a major non-enzymatic antioxidant involved in cellular redox homeostasis (Luberda 2005)) was not significantly depleted under the conditions evaluated. These findings are consistent with previous studies reporting no differences in mitochondrial-specific ROS production between vitrified and non-vitrified equine oocytes (Clérico et al. 2021) and comparable intracellular GSH levels in vitrified and fresh equine oocytes (Gugole et al. 2025). In contrast, mitochondrial distribution patterns showed some variability among groups (Figure 2d). In the control group, 72.22% of oocytes displayed a heterogeneous mitochondrial distribution pattern (HeD), whereas 27.78% showed a homogeneous mitochondrial distribution pattern (HoD), consistent with previous reports indicating that 69.64% of mature equine oocytes exhibit a HeD (Luis-Calero et al. 2024). In vitrified oocytes, mitochondrial distribution appeared more balanced between patterns (VIT25: 47.06% HoD, 52.94% HeD; VIT38.5: 36.84% HoD, 63.16% HeD). Although these differences were not statistically significant, they may reflect subtle alterations in cytoplasmic organisation following vitrification, as previously suggested in mammalian oocytes (Cao et al. 2022). Although no significant differences were detected among groups, the limited availability of equine mature oocytes may have constrained the statistical power to detect subtle but biologically relevant differences.

Overall, the vitrification protocols evaluated did not induce significant changes in oxidative stress, mitochondrial oxidative activity, or intracellular GSH levels in equine IVM oocytes at 2 h post-warming although subtle alterations in mitochondrial organisation cannot be excluded. These findings indicate that vitrification under the evaluated conditions preserves key indicators of cellular homeostasis in equine oocytes and provides additional insight into their tolerance to cryopreservation procedures. Functional outcomes such as fertilisation and blastocyst formation should be evaluated in future studies.

Author Contributions

S.G., T.M. and N.C.-B. conceived and designed the experiments; S.G., M.V.-F., J.D.-M. and M.S.-M. performed the experiments; S.G., M.A. and E.M. performed the experiments and data analysis; T.M. and N.C.-B. provided the resources and S.G. T.M. and N.C.-B. wrote the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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