

SHORT COMMUNICATION OPEN ACCESS

# Extracellular Vesicles From Heat- and Oxidative-Stressed Granulosa Cells Improve Redox Balance and Embryo Development in Bovine Oocytes

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## ABSTRACT

In vitro culture (IVC) exposes oocytes to suboptimal conditions that may disrupt redox homeostasis and compromise developmental competence. Extracellular vesicles (EVs) mediate intercellular communication, and their cargo reflects the physiological state of donor cells. We hypothesized that EVs derived from heat-stressed (HS) or oxidative-stressed (OS) granulosa cells (GCs) differentially modulate oocyte redox status and embryo development. GCs were exposed to 42°C for 24 h or to 5 µM H<sub>2</sub>O<sub>2</sub> for 40 min, and EVs were subsequently isolated from conditioned media. GC-EVs (1 × 10<sup>9</sup> particles/mL) were supplemented during in vitro maturation (IVM). Redox parameters were assessed using triple fluorescence staining for reactive oxygen species (ROS), glutathione (GSH) and mitochondrial activity, combined with 3D confocal segmentation. EV-treated oocytes showed reduced ROS/GSH ratios compared with controls, indicating an improved redox balance. HS-EVs specifically enhanced mitochondrial activity. Cleavage rates were unaffected, whereas OS-EVs increased Day 7 blastocyst rates and HS-EVs improved hatching rates. These findings demonstrate that stress-conditioned GCs-EVs differentially regulate oocyte redox status, mitochondrial activity and developmental competence, supporting their potential to optimize IVC systems.

## 1 | Introduction

Oocyte maturation and developmental competence involve a delicate crosstalk between the oocyte and the surrounding somatic cells, forming the cumulus-oocyte complex (COC) (Wang 2025). This communication can be disrupted during IVC, where the absence of physiological fluids and antioxidant defences, together with stressors such as temperature or oxygen fluctuations, can generate OS. OS occurs when the balance between ROS and antioxidant defences is disrupted, damaging lipids, proteins and nucleic acids, ultimately affecting embryo development (Agarwal et al. 2022).

Recently, EVs have emerged as key communicators within the ovarian follicles (Ávila et al. 2019). Their supplementation improves oocyte competence and embryo development (Da Silveira et al. 2017). Under suboptimal conditions, such as metabolic stress, follicular fluid (FF)-EVs from small follicles improve maturation and embryo development (Lipinska et al. 2025). Furthermore, FF-EVs from large follicles enhance bovine oocyte vitrification outcomes, a major stressor in assisted reproductive technologies (Diaz-Muñoz et al. 2025).

Moreover, EV cargo reflects the physiological state of donor cells; thereby, stress exposure can generate stress-responsive EVs

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that help recipient cells withstand subsequent stress (Alharbi et al. 2021), as reported for OS and HS GCs-EVs (Gebremedhn et al. 2020; Saeed-Zidane et al. 2017). Notably, HS GCs-EVs have been reported to protect bovine oocytes from HS during IVM (Menjivar et al. 2023).

However, the comparative effects of EVs from HS- and OS-GCs on bovine oocytes under standard IVM conditions remain unclear. We hypothesized that stress-derived GCs-EVs differentially modulate oocyte quality by regulating ROS accumulation, GSH levels and mitochondrial activity, thereby influencing embryo developmental competence.

## 2 | Materials and Methods

### 2.1 | Granulosa Cell Culture, Stress Induction and EVs Isolation and Characterization

Bovine GCs from 3 to 6 mm follicles were cultured at a density of  $3.75 \times 10^5$  viable cells per dish in TCM-199 supplemented with 10% exosome-depleted foetal bovine serum and antibiotic-antimycotic (100×) until subconfluence. Cells were then exposed to  $5 \mu\text{M}$   $\text{H}_2\text{O}_2$  in PBS for 40 min, followed by washing and a further 24 h in culture medium or  $42^\circ\text{C}$  for 24 h. Controls were cultured at  $38.5^\circ\text{C}$  ( $n = 3$  replicates). Culture EVs were isolated from conditioned media by size-exclusion

chromatography (HBM-PEV; Hansa BioMed) concentrated with Amicon filters and characterized by nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM) and flow cytometry (FC). EV uptake by COCs was assessed using PKH26 staining as described by Diaz-Muñoz et al. (2025).

### 2.2 | In Vitro Maturation, Fertilization and Embryo Culture

Methodologies for IVM, in vitro fertilization (IVF) and IVC have been previously described by Diaz-Muñoz et al. (2025). Briefly, COCs were IVM for 24 h in medium supplemented with  $1 \times 10^9$  particles/mL of EVs derived from non-treated GCs (CT-EVs), OS-treated cells (OS-EVs) or HS-treated cells (HS-EVs). Oocytes matured without EVs served as controls. After IVF with frozen-thawed semen, embryos were cultured to the blastocyst stage. Cleavage (48 h) and Day 7 (D7) blastocyst rates were recorded. D7 embryos were graded by blastocoel expansion ( $n = 78-81/3$  replicates).

### 2.3 | Mitochondrial Activity, ROS Levels, and GSH Content Triple Staining and 3D Segmentation

At 24 h, mitochondrial activity, ROS levels and GSH content were simultaneously evaluated in individual oocytes using a

A)

| Group  | Particles/mL $\pm$ SD                                      | Mean $\pm$ SD (nm)           |
|--------|------------------------------------------------------------|------------------------------|
| CT-EVs | $2.07 \times 10^{11} \pm 1.60 \times 10^{10}$ <sup>a</sup> | $237.3 \pm 4.9$ <sup>a</sup> |
| OS-EVs | $1.98 \times 10^{11} \pm 2.20 \times 10^{10}$ <sup>a</sup> | $223.2 \pm 7.0$ <sup>a</sup> |
| HS-EVs | $2.79 \times 10^{10} \pm 3.10 \times 10^8$ <sup>b</sup>    | $171.7 \pm 4.2$ <sup>b</sup> |

B)

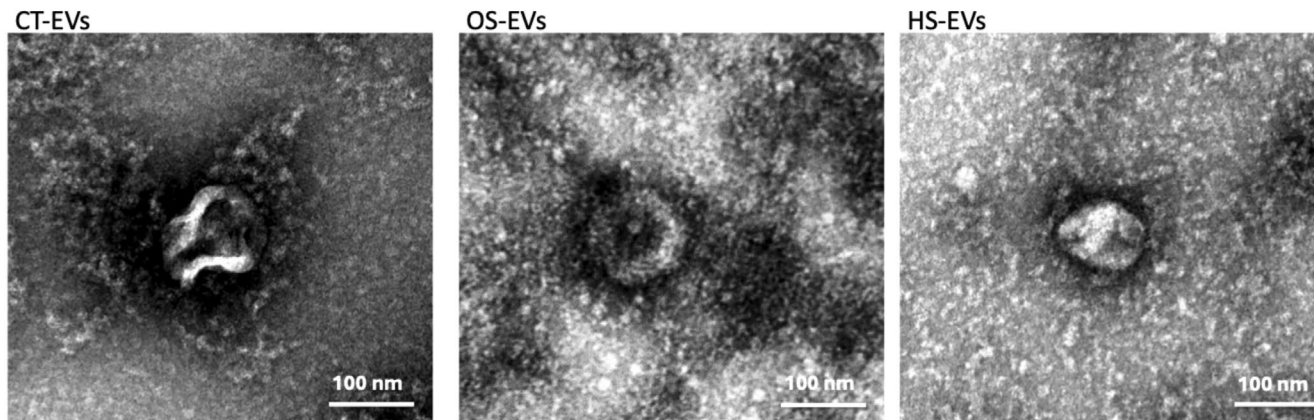


**FIGURE 1** | Characterization of EV samples derived from control, oxidative-stressed and heat-stressed granulosa cells. (A) Concentration and mean size of GC-EVs. Different superscript letters indicate significant differences between columns. (B) Representative transmission electron microscopy (TEM) images from EVs. Scale bar = 100 nm.

newly optimized triple fluorescent staining protocol combined with confocal microscopy and 3D image segmentation. To this end, COCs were denuded and incubated for 30 min in PBS with 100 nM MitoTracker Red CMXRos (mitochondria, excitation/emission: 561/566–707 nm; ThermoFisher, Cat. No. M7512), 20  $\mu$ M CellTracker (GSH, excitation/emission: 405/410–460 nm;

ThermoFisher, Cat. No. C12881) and 50  $\mu$ M CellRox (ROS, excitation/emission: 488/507–583 nm; ThermoFisher, Cat. No. C10444), added simultaneously from stock solutions according to the manufacturer's instructions. Then, oocytes were fixed for 15 min in 2% paraformaldehyde and mounted in 5  $\mu$ L Vectashield ( $n = 14$ –15/3 replicates). All steps were performed at 38.5°C.

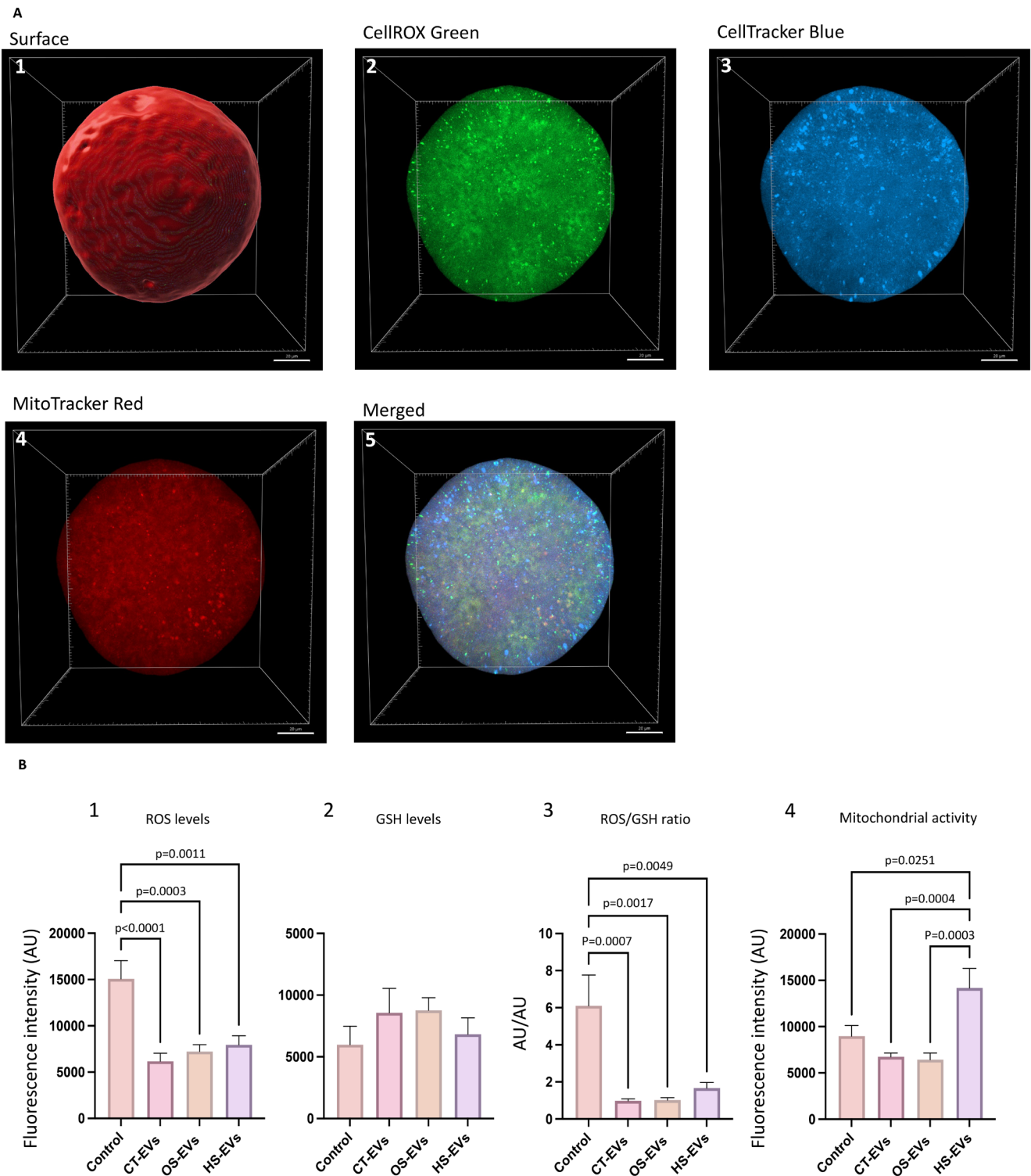


**FIGURE 2** | (A) Representative images of the (1) surface representing the oocyte plasma membrane, (2) CellRox (ROS), (3) CellTracker (GSH), (4) MitoTracker (mitochondria) and (5) merged. (B) Quantification of (1) ROS, (2) GSH level, (3) ROS/GSH ratio and (4) mitochondrial activity in IVM bovine oocytes in the presence of GC-EVs. Data are presented as mean  $\pm$  SEM. Scale bar: 20  $\mu$ m.

The same day, full 3D oocyte volumes were acquired using a Zeiss LSM 980 confocal microscope (0.77  $\mu\text{m}$  z-steps, 0.25  $\mu\text{m}$  pixel size and 0.77  $\mu\text{s}$  dwell time) under constant settings. Imaris 9.5 was used to segment oocyte surfaces and quantify mean fluorescence intensity (AU).

## 2.4 | Statistical Analysis

One-way ANOVA with the Tukey post hoc test was used for group comparisons ( $p \leq 0.05$ ) after ensuring normality and variance homogeneity. Results are shown as mean  $\pm$  SEM.

## 3 | Results

### 3.1 | Characterization of EVs From Control and Stressed Granulosa Cells

NTA showed EV concentration and size for each group, with HS-EVs exhibiting significantly lower concentration and smaller particle size than CT-EVs and OS-EVs (Figure 1A). TEM confirmed the characteristic shape (Figure 1B), and FC detected CD81, CD9 and CD63 expression, with no detectable signal for BSA or calnexin, indicating the absence of cellular contaminants. Furthermore, EV uptake was confirmed after 24 h of IVM.

### 3.2 | Mitochondrial Activity, ROS Levels and GSH Content

Figure 2B–1–3 reveals significantly higher ROS levels in control oocytes relative to all EV-treated groups, whereas GSH levels did not differ among groups. Accordingly, the ROS/GSH ratio was significantly higher in the control group than in the EV groups. Figure 2B–4 shows higher mitochondrial activity in HS-EV-treated oocytes than in the other groups.

### 3.3 | Developmental Rates

As shown in Figure 3, supplementation with stressed GC-EVs during IVM did not affect cleavage rates among groups. Day 7 blastocyst rates were similar in the Control, CT-EVs and HS-EVs groups. However, OS-EVs significantly increased blastocyst rates. Despite no differences in non-expanded or expanded blastocysts, HS-EVs enhanced hatching rates.

## 4 | Discussion

Our results showed that supplementation with EVs isolated from GCs during IVM reduced the ROS/GSH ratio in all groups, indicating a common antioxidant effect regardless of

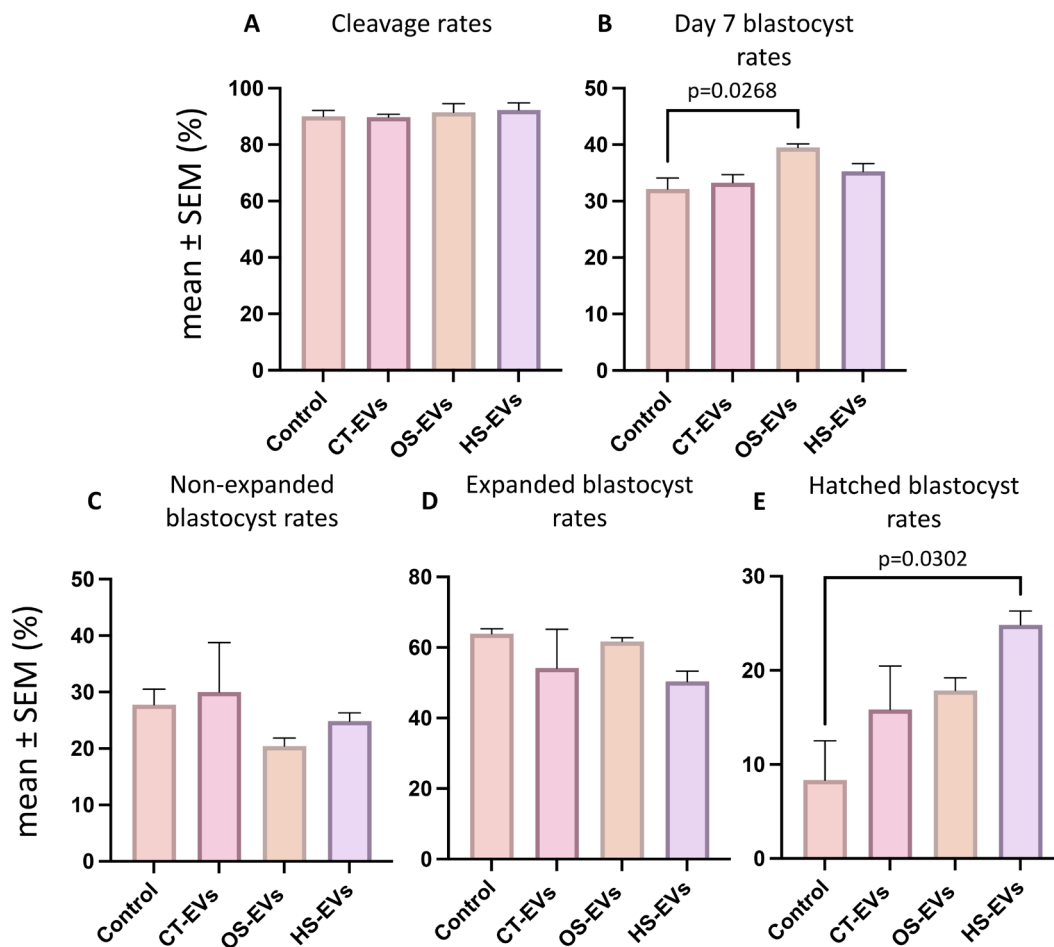


**FIGURE 3** | Embryo development outcomes following IVM in the presence of GC-EVs. (A) Cleavage rate at 48 h post-fertilization, (B) Day 7 blastocyst rate, (C) non-expanded, (D) expanded and (E) hatched blastocyst rate at Day 7. Data are presented as mean  $\pm$  SEM.

the GC stress condition. Consistent with the improved redox balance, OS-EVs increased blastocyst yield, supporting that OS-EVs cargo may be capable of enhancing oocyte competence and subsequent embryonic development. In addition, OS-EVs have been shown to enhance Nrf2 signalling and upregulate antioxidant genes in GCs (Saeed-Zidane et al. 2017). However, neither CT-, HS-, nor OS-EVs were reported to reduce intracellular ROS levels in GCs in the absence of stress (Gebremedhn et al. 2020; Saeed-Zidane et al. 2017), suggesting that their effect relies on modulation of antioxidant pathways rather than ROS scavenging. Notably, previous studies have shown that EVs from FF can reduce ROS levels in oocytes without an increase in blastocyst rates (Schneberger et al. 2026), indicating that additional cargo components or mechanisms may influence developmental outcomes. This is consistent with our findings in CT- and HS-EVs, which improved redox balance without a corresponding increase in blastocyst yield. Although HS-EVs did not affect blastocyst yield, they improved hatching rates, indicating a potential effect on embryo quality. Furthermore, only HS-EVs enhanced mitochondrial activity, suggesting that HS induces specific adaptive signals in GC-EVs cargo. This aligns with reports describing miRNA enrichment in HS-EVs associated with metabolic and glutathione-related pathways (Gebremedhn et al. 2020), as well as reduced expression of stress-related genes in oocytes exposed to HS (Menjivar et al. 2023) that could potentially explain the increased mitochondrial activity. Stress-conditioned GC-EVs appear to carry distinct molecular signals capable of modulating oocyte redox balance, mitochondrial activity and ultimately influencing developmental competence, supporting the role of GC-EV-mediated communication in transmitting adaptive responses to environmental stress to the oocyte within the ovarian follicle.

## 5 | Conclusions

In the present study, EV supplementation during IVM improved oocyte redox balance, supporting the role of EVs as regulators of the intracellular oxidative environment during maturation. EVs derived from differently stressed GCs exerted distinct functional effects: OS-EVs enhanced embryo yield, whereas HS-EVs increased mitochondrial activity and improved hatching rates, suggesting a differential modulation of oocyte metabolic competence and embryo quality. These findings indicate that the physiological status of donor GCs determines the functional properties of their EVs and highlight the potential of stress-conditioned GC-EVs as biological modulators for improving bovine IVC systems.

### Author Contributions

Conceptualization: J.D.-M., Y.N.C., K.C.-B., D.R. and T.M.; Formal analysis: J.D.-M.; Funding acquisition: M.L.-B., D.R. and T.M.; Investigation: J.D.-M. and S.G.; Methodology: J.D.-M., K.C.-B. and Y.N.C.; Project administration: M.L.-B. and T.M.; Resources: C.O.H., M.L.-B., D.R. and T.M.; Supervision: T.M. and D.R.; Validation: M.L.-B., D.R. and T.M.; Writing – original draft: J.D.-M., Y.N.C. and K.C.-B.; Writing – review and editing: D.R. and T.M. All authors have read and agreed to the published version of 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 request.

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