DISSERTATION

TARGETING THE NRF2 SIGNALING PATHWAY TO IMPROVE OOCYTE QUALITY AND EMBRYO DEVELOPMENT UNDER STRESS CONDITIONS

Submitted by

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ABSTRACT

TARGETING THE NRF2 SIGNALING PATHWAY TO IMPROVE OOCYTE QUALITY AND EMBRYO DEVELOPMENT UNDER STRESS CONDITIONS

The increasing frequency and severity of environmental stressors associated with global climate change pose significant challenges to livestock production systems worldwide. Among these, heat stress has emerged as a leading cause of reproductive inefficiency in cattle, compromising ovarian function, oocyte quality, and preimplantation embryonic development. In addition to environmental thermal stress, embryos produced through assisted reproductive technologies are frequently exposed to suboptimal in vitro culture conditions that further exacerbate cellular stress. Although these insults arise from different sources, a common consequence is excessive accumulation of reactive oxygen species (ROS), leading to oxidative stress, mitochondrial dysfunction, metabolic disturbances, and reduced developmental competence. Despite the recognized importance of oxidative stress in reproductive failure, the molecular mechanisms that enable reproductive cells and embryos to adapt to these challenges remain incompletely understood. The work presented in this dissertation investigated the role of oxidative stress as a common mediator of reproductive dysfunction across multiple stages of female reproduction, from the ovarian follicle to the preimplantation embryo. Particular emphasis was placed on the Nuclear factor erythroid 2-related factor 2 (NRF2) pathway, a master regulator of cellular redox homeostasis that coordinates antioxidant, cytoprotective, and metabolic responses to oxidative challenges. Through a combination of literature synthesis and experimental studies utilizing

bovine granulosa cells, oocytes, and embryos, this dissertation examined how activation of NRF2-mediated antioxidant defenses influences cellular resilience under conditions of heat stress and oxidative stress induced by in vitro culture. Collectively, the findings demonstrate that oxidative stress represents a central mechanism through which heat stress and suboptimal culture environments compromise reproductive performance. Exposure to thermal and oxidative challenges disrupted redox homeostasis, increased ROS accumulation, impaired mitochondrial function, altered metabolic activity, and reduced developmental competence in granulosa cells, oocytes, and embryos. Conversely, activation of NRF2 signaling through naturally occurring antioxidant compounds enhanced cellular antioxidant capacity, preserved mitochondrial integrity, improved metabolic efficiency, and restored developmental potential. Importantly, the beneficial effects of NRF2 activation extended beyond immediate protection against oxidative damage, contributing to improvements in embryo quality and in molecular indicators of embryonic competence. This dissertation further establishes the NRF2–KEAP1 signaling axis as a critical regulator of cellular adaptation to environmental and culture-induced stress during early reproductive development. By demonstrating that targeted activation of endogenous antioxidant pathways can mitigate the detrimental consequences of both heat stress and oxidative stress, these studies provide mechanistic insight into how reproductive cells maintain functionality under adverse conditions. Moreover, the findings identify NRF2-mediated antioxidant signaling as a promising target for improving ovarian function, oocyte quality, embryo competence, and the overall efficiency of assisted reproductive technologies.

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CHAPTER I— Molecular and developmental consequences of heat stress on the bovine oocyte and embryo competence [1]

1. Overview

Seasonal heat stress (HS) is a pervasive environmental challenge with profound consequences for female reproductive physiology, affecting ovarian function, oocyte maturation, and early embryonic development. At the ovarian level, HS disrupts follicular growth, impairs steroidogenesis, and compromises granulosa cell function, thereby creating a suboptimal microenvironment that reduces oocyte competence. In oocytes, HS induces oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, spindle abnormalities, chromosomal missegregation, and persistent epigenetic alterations. These disruptions extend into early embryonic development, where redox imbalance, apoptosis, ER stress, and altered lineage allocation reduce cleavage and blastocyst formation, compromise trophectoderm and inner cell mass integrity, and impair implantation potential. Maternal heat exposure further exacerbates embryonic vulnerability by altering the oviductal and uterine environment, reducing embryotrophic factors and antioxidant defenses, and ultimately influencing offspring phenotype and fertility, potentially across generations. Accordingly, this review aims to synthesize current knowledge on the physiological and molecular impacts of heat stress on ovarian function, oocyte maturation, and early embryonic development. To this end, we consider studies conducted under both in vivo and in vitro conditions, highlighting shared and distinct mechanisms of thermal stress at the organ and cellular levels to identify potential targets for intervention.

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[1]Ramírez G, Menjivar N, Gebremedhn S, Gad A, Held-Hoelker E, Hoelker M, Zewdie G, Tesfaye D. Molecular and developmental consequences of heat stress on oocyte and embryo competence. *Submitted to Animal Reproduction Science.*

2. Introduction

Global warming induced seasonal thermal stress is continuing to rise (Hansen et al., 2006) with an exponential increase in the number of heat days in different regions of the world (Davariashtiyani et al., 2023). Extreme HS during summer disrupts various reproductive processes ranging from ovarian functionality to pronounced depression of conception rate in beef and dairy cows worldwide (Davariashtiyani et al., 2023). HS exerts a significant impact on animal productivity, health, reproduction, and overall welfare. The situation is aggravated for dairy cows, as the sector is under tremendous pressure to increase milk production amid rising demand worldwide for milk and milk products. The environmental HS and endogenous heat production due to high metabolic rate in dairy cow's compromise animal reproductive performance, which is an essential component for farm productivity (Tao et al., 2020).

The impact of seasonal thermal stress on mammalian female reproduction is multifaceted. One of the most affected dynamic organs is the ovary, and its follicular microenvironment. Ovaries are responsible for safeguarding female gametes and secreting ovarian steroids that ultimately regulate reproductive functions. HS disrupts reproductive function through physiological, behavioral, and endocrine alterations that directly affect the hypothalamic–pituitary–ovarian (HPO) axis. Ultrasonographic studies have demonstrated that HS alters ovarian follicular dynamics and impairs follicular growth rate and prolongs follicular wave duration, indicating compromised follicular function (Dovolou et al., 2023). HS increased the number of midsize follicles thereby reducing follicular dominance, which is accompanied by decreased estradiol and inhibin secretions and increased circulating FSH levels (Parker et al., 2003; Wolfenson et al., 1995a). As the functional unit of the ovary, the impact of HS on the follicle has a far-reaching effect on the oocyte and the functionality of the surrounding somatic cells (granulosa and theca cells).

In addition to seasonal measurements on the impact of thermal stress on oocyte maturation, several in vitro experiments using temperatures up to 43°C to induce HS in oocytes indicated a significant impact on oocyte growth (Kawano et al., 2022), reduced mitochondrial membrane potential (MMP), increased accumulation of reactive oxygen species (ROS), oxidative stress, and induced apoptosis (Feng et al., 2024a; Lee et al., 2023; Roth & Hansen, 2005; Wrzecińska et al., 2023). Taken together, the detrimental effects of HS are observed across in vivo and in vitro models, which significantly impair oocyte quality by disrupting both nuclear and cytoplasmic maturation, increasing chromosomal and spindle abnormalities, elevating oxidative stress and apoptosis, and compromising subsequent embryo development.

Several studies have demonstrated the carryover effects of HS exposure during in vitro oocyte maturation on the developmental competence and molecular architecture of the resulting blastocysts. Specifically, altered MMP, reduced ATP levels, and decreased mtDNA copy number have been reported in blastocysts derived from HS-exposed oocytes (Feng et al., 2024b; Miętkiewska et al., 2022). HS is closely associated with reduced reproductive performance in cattle, in part due to its detrimental effects on blastocyst formation, implantation rates, and increased embryonic mortality. The severity of these impairments is largely stage-specific, with early developmental stages, particularly cleavage and blastocyst formation, being most susceptible. In this literature review, we systematically summarize findings from both in vitro and in vivo studies that evaluate the molecular, functional, and developmental impacts of environmental thermal stress on ovarian and follicular function, oocyte maturation, and early embryonic development.

3. The impact of thermal stress on ovarian function

3.1 Impact of heat stress on follicular growth and dominance

HS adversely affects animal performance and health, disrupting follicular growth (Wolfenson et al., 1997), corpus luteum (CL) function, preimplantation embryo development, and the uterine environment and receptivity. A particularly pronounced effect of HS occurs during follicular development. HS impairs the growth and function of small antral follicles, thereby disrupting the formation of the preovulatory dominant follicle and compromising both ovulation and CL formation (Roth et al., 2000; Wolfenson et al., 1995b). The vulnerability of ovarian follicles to heat stress depends on the developmental stage. Preantral, primary, and secondary follicles are susceptible to elevated temperatures under in vitro conditions, with secondary follicles exhibiting the highest sensitivity, followed by early secondary follicles (L. H. de Aguiar et al., 2020b). Notably, dairy cows exposed to elevated temperatures exhibited reduced diameters of dominant follicles during the first and second follicular waves, resulting in disrupted follicular dominance (Reddy & Anitha, 2024; Badinga et al., 1993; Wilson et al., 1998). This disruption is characterized by an increased number of mid-sized dominant follicles and the absence of a single dominant follicle, leading to a significant reduction in plasma inhibin concentration and an increase in follicle-stimulating hormone (FSH) levels (Roth et al., 2000). These alterations may explain the higher incidence of abnormal ovulation patterns and twinning observed during summer (Ryan & Boland, 1991). The impact of HS on follicular development is long-lasting; cows exposed to HS require at least four to six follicular waves to recover and produce developmentally competent oocytes after HS exposure (Hulshof et al., 1994; Roth et al., 2001).

3.2 Impact of heat stress on the function of follicular cells

Granulosa cells are considered the most important follicular somatic cells that support the growth and development of oocytes, provide a suitable microenvironment that ensures the maturation and

ovulation of developmentally competent oocytes, and secrete hormones (Edson et al., 2009). Granulosa cells play a key role in the synthesis of estradiol and progesterone. During the early growing stage of follicular development, FSH stimulates the conversion of androgen to estradiol through aromatase, and after ovulation, granulosa cells are converted into progesterone-producing luteal cells (Albertini et al., 2001; Senthilkumaran et al., 2004). Thus, it is critical to maintain the proper function of granulosa cells, as any disruption in their quality and function could result in negative consequences in the maturation of the enclosed oocytes and the subsequent embryonic development, leading to reduced fertility outcomes (Sammad et al., 2022). Under higher temperatures, granulosa cells showed lower production of estradiol, accompanied by silent estrous signs, leading to lower progesterone production (Sammad et al., 2022).

Recently, a field experiment on the transcriptomic profile of granulosa cells derived from beef cows obtained during summer and winter seasons revealed distinct transcriptomic profiles and genes involved in pathways including cell adhesion molecules, chemokine signaling, cytokine-cytokine receptor interaction were enriched by genes down regulated in granulosa cells derived from summer season, while genes upregulated are involved in protein processing and metabolic pathways (Joyce et al., 2024). This signifies the seasonal alteration in the gene expression profile of granulosa cells in beef cows, which can potentially reflect seasonal variability in pregnancy success. Under in vitro conditions, several reports have shown adverse effects of HS on granulosa cell survival and the expression of key genes. Bovine granulosa cells subjected to high temperatures (41°C) showed increased ROS at 24h, activating NRF2 and its antioxidant targets CAT and PRDX1. It also induced strong endoplasmic reticulum stress, marked by elevated GRP78 and GRP94 expression and increased GRP78 protein levels, along with upregulation of apoptotic genes BAX and CASPASE-3 (Wondie Alemu et al., 2018) (Figure 1.1). Concurrently, HS reduced

cell proliferation, reflected by decreased PCNA expression. Overall, elevated temperature triggered oxidative stress, ER stress, apoptosis, and reduced proliferation, prompting NRF2-mediated protective responses in bovine granulosa cells (Alemu et al., 2018). Similarly, granulosa cells subjected to in vitro HS showed increased ROS, oxidized proteins, apoptosis, and elevated expression of heat shock proteins and antioxidants, alongside reduced cell viability (Gebremedhn et al., 2020). Interestingly, in the same study, exposure to higher temperature altered the expression of cellular miRNAs in granulosa cells and the extracellular vesicles-mediated release of miRNAs in response to HS. The study further revealed that supplementing granulosa cell cultures with EVs from heat-stressed cells promoted an adaptive response during subsequent heat exposure. However, this effect was minimal at normal temperatures. Overall, EVs released from heat-stressed granulosa cells can transfer bioactive molecules that help recipient cells better withstand exposure to recurrent thermal stress.

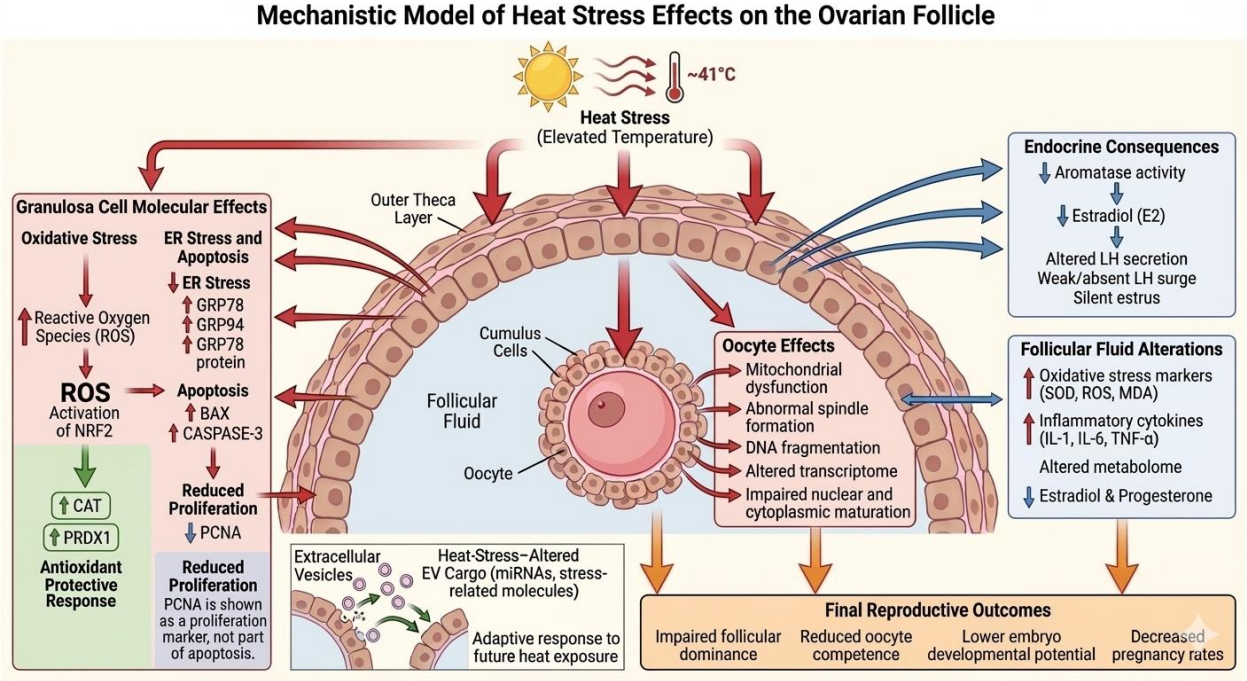

Figure 1.1 A model illustrating the molecular consequences of environmental heat stress (HS) on ovarian function and its subsequent impact on reproductive outcomes. Reduced fertility reflects

the cumulative effects of HS on follicular cells (granulosa and theca cells), including disrupted steroidogenesis, increased oxidative stress, mitochondrial dysfunction, and altered gene expression, which collectively compromise follicular homeostasis. In parallel, HS perturbs extracellular vesicle (EV)-mediated molecular signaling, impairing the transfer of regulatory molecules (e.g., miRNAs, proteins, and lipids) and disrupting intercellular communication within the follicular microenvironment. These alterations, together with impaired oocyte growth, meiotic competence, and cytoplasmic maturation, ultimately result in reduced developmental competence, compromised embryo quality, impaired implantation potential, and decreased pregnancy rates.

3.3 Impact of heat stress on follicular microenvironment

A study performed in dairy cows to determine the impact of HS on the metabolic characteristics of the follicular fluid revealed that HS significantly changes the abundance of metabolites that are associated with oxidative stress, including SOD, GSH-Px, CAT, MDA, T-AOC, ROS, inflammatory factors including IL-1, IL-6, TNF-α, heat shock proteins, HSP70, and HSP90, and steroid hormones estradiol and progesterone (Li et al., 2025). The study further reported that 1,544 differential metabolites in the follicular fluid of the heat-stressed group, which were mainly enriched in pathways such as steroid hormone biosynthesis, neuroactive ligand-receptor interactions, D-amino acid metabolism, tyrosine metabolism, phenylalanine metabolism, and tryptophan metabolism. Molecular analysis revealed that mural granulosa cells from heat-stressed cows had lower expression of key steroidogenic proteins, including StAR, LHR, aromatase, and lower follicular estradiol levels, along with increased oxidative stress markers, including SOD1 and SOD2, and the apoptosis effector caspase-3, which were positively correlated with oxidative stress-induced apoptosis (Kim et al., 2026). These results demonstrated that thermal stress induces coordinated disruptions in molecular, hormonal, and oxidative pathways within the ovarian

microenvironment, ultimately impairing oocyte maturation and reducing fertility. The molecular consequences of thermal stress on ovarian follicular development, endocrine function, cellular proliferation, EV cargo composition, and subsequent impact on reproductive outcomes are summarized in Figure 1.2.

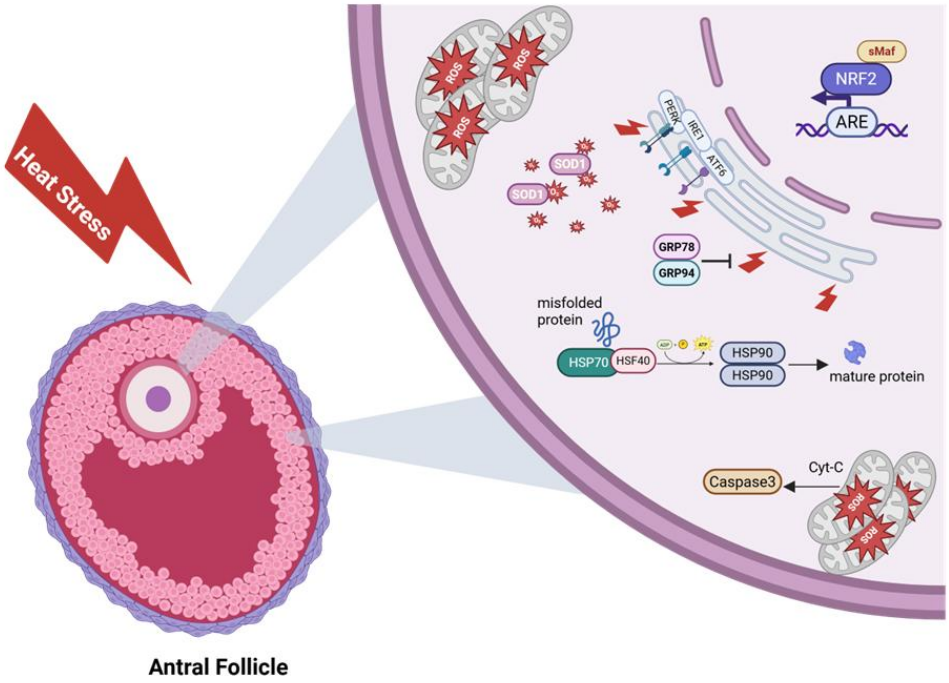

Figure 1.2. Molecular mechanisms underlying the response of antral follicle–enclosed somatic cells and oocytes to thermal stress. Heat stress induces oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction in both somatic (granulosa and theca) cells and the oocyte. The primary line of defense, and among the most extensively studied pathways, includes the NRF2-mediated antioxidant response and the unfolded protein response (UPR), which act coordinately to restore cellular homeostasis by regulating redox balance, protein folding, and stress adaptation. When these protective mechanisms are insufficient or overwhelmed, persistent stress leads to apoptosis, impaired cellular communication, and compromised oocyte competence,

ultimately affecting follicular integrity and developmental potential.

4. Molecular impact of heat stress on oocyte maturation

HS during oocyte maturation reduces bovine oocyte developmental competence, cleavage rates, and blastocyst formation (de Barros & Paula-Lopes, 2018; Feng et al., 2024b). It triggers oxidative damage, ER dysfunction, and altered gene expression in oocytes as well as surrounding cumulus cells (Miętkiewska et al., 2022; Naranjo-Gómez et al., 2021). These changes have far-reaching consequences affecting the pre- and post-implantation phases of embryonic development (Feng et al., 2024b; Naranjo-Gómez et al., 2021). Thermal stress adversely affects both nuclear and cytoplasmic maturation processes, frequently leading to aberrant spindle architecture, impaired mitochondrial function, promoting DNA fragmentation, and contributing to diminished embryo developmental potential (Aroyo et al., 2007).

4.1 Redox imbalance in oocytes under heat stress

At the cellular level, HS is commonly modeled at 40–41.5°C during in vitro maturation (IVM) and has been shown to disrupt mitochondrial distribution and alter the expression of mitochondrial genes involved in the respiratory chain in metaphase II (MII) oocytes (Gendelman & Roth, 2012a). Furthermore, elevated temperatures increase mitochondrial workload and electron transport chain leakage, elevating mitochondrial ROS in germinal vesicle and MII oocytes, overwhelming antioxidant defenses like SOD and GSH-Px (Feng et al., 2024b; Lang et al., 2024). In bovine IVM at 41°C, mitochondrial ROS levels in oocytes reach those observed following direct oxidant exposure, while glutathione buffering capacity becomes compromised (Held-Hoelker et al., 2025; Payton et al., 2018). Several studies have shown that ROS surges during IVM at 41°C, with NRF2 pathway activation as a compensatory response, though insufficient to prevent apoptosis.

Moreover, this leads to lipid peroxidation, protein oxidation, and DNA damage, reducing mitochondrial membrane potential, ATP levels, and mtDNA copy number (Payton et al., 2018; Tariq et al., 2025). Redox imbalance has been shown to go along with an increased expression of pro-apoptotic genes, while the expression of anti-apoptotic genes decreases (Held-Hoelker et al., 2025; Itami et al., 2018). If adaptive antioxidant responses like SOD and GSH-Px fail, this pushes oocytes towards apoptosis (Cao & Kaufman, 2014; Latham, 2015; Zhao et al., 2017). Consequently, the accumulation of excessive ROS and chronic ER stress caused by elevated temperatures leads to spindle abnormalities, errors in chromosome segregation, and defects in the cytoskeleton in oocytes, thereby reducing their developmental competence (Payton et al., 2018; Song et al., 2024).

4.2 Endoplasmic reticulum stress response in oocytes exposed to thermal stress

In bovine and other mammalian oocytes, elevated mitochondrial ROS disturbs protein folding in the ER and calcium homeostasis, triggering the UPR and, if unresolved, apoptosis (Cao & Kaufman, 2014; Held-Hoelker et al., 2025; Payton et al., 2018; Zhao et al., 2017). The ER and mitochondria physically interact via mitochondria-associated membranes (MAMs), which regulate Ca^{2+} transfer and apoptotic signaling in oocytes (W. Lin et al., 2021; Zhao et al., 2017). In mouse oocytes, increased MAM abundance and higher IP3R1 and PACS-2 expression enhance ER–mitochondria coupling, sensitizing mitochondria to ER Ca^{2+} release and promoting ROS-linked dysfunction (Zhao et al., 2017). These contacts create microdomains in which mitochondrial ROS and Ca^{2+} flux can rapidly affect the ER redox state and vice versa (Cao & Kaufman, 2014; W. Lin et al., 2021). Furthermore, redox imbalance fuels ER stress through mitochondrial-ER contacts, amplifying UPR and epigenetic disruptions. In agreement with this, antioxidant interventions (e.g., HO-1) or chaperone supplementation (HSP70) have been shown to

partially rescue maturation rates (Divvela et al., 2025; Wang et al., 2019). Disturbed UPR mechanisms cause misfolded protein buildup, which, in turn, triggers ER stress markers, such as GRP78/BiP sequestration, in bovine oocytes and cumulus cells, compromising Ca^{2+} homeostasis and maternal mRNA storage. Resulting effects include spindle abnormalities and impaired blastocyst formation (Alemu et al., 2018; T. Lin et al., 2019). Elevated temperatures increase mitochondrial ROS, further oxidizing ER luminal and membrane proteins, disrupt Ca^{2+} pumps and channels, and exacerbate ER Ca^{2+} depletion, ultimately leading to the accumulation of misfolded proteins (Cao & Kaufman, 2014; Latham, 2015; Zhao et al., 2017).

4.3 Thermal stress-induced epigenetic alterations in oocytes

Beyond the immediate impact of elevated temperatures on the intrinsic quality of oocytes and embryos, HS induces epigenetic modifications, including alterations in chromatin remodeling and DNA methylation patterns, in both oocytes and early embryos (de Barros & Paula-Lopes, 2018; Diaz et al., 2021b; Zhu et al., 2021). HS has been shown to downregulate histone modifications (H1, H2A, H2B, and H4) and DNA methylation in both bovine oocytes and embryos (Diaz et al., 2021b; Feng et al., 2024b). Exposure to HS before fertilization induced the accumulation of H3K9me3 and HP1 in early embryos. Specifically, an increased accumulation of H3K9me3 was observed in the nuclei of four-cell and eight-cell stage embryos derived from heat-shocked oocytes (Diaz et al., 2025). Furthermore, a higher proportion of four-cell-stage embryos derived from heat-shocked oocytes showed increased HP1 fluorescence, suggesting abnormal chromatin compaction (Camargo et al., 2019). Recent reports indicate that both in vivo and in vitro HS altered DNA methylation patterns and reduced developmental competence in bovine embryos, evidencing stage-dependent differences and highlighting susceptibility at early cleavage stages (Diaz et al., 2025). Feng and colleagues showed that HS significantly reduced global DNA methylation (5mC)

and hydroxymethylation (5hmC) levels at all stages from oocyte to blastocyst, with a minimum at the 8-cell stage and persistently lower 5hmC in thermal-stressed embryos (Feng et al., 2024b). This might be explained by reduced mRNA levels of DNMT1, DNMT3A, DNMT3B, and histone H2A in blastocysts derived from heat-stressed oocytes, indicating an impaired active and maintenance methylation machinery. Consequently, HS during bovine oocyte maturation might leave a persistent molecular "imprint" that is clearly detectable in early embryos up to the blastocyst stage, particularly in terms of redox homeostasis, ER stress/UPR signaling, and epigenetic programming (de Barros & Paula-Lopes, 2018; Feng et al., 2024b; Kawano et al., 2025).

4.4 Embryonic consequences of heat stress during maturation

It is important to understand that HS effects during maturation affect the vitality of the resulting embryos. Zygotes and cleavage-stage embryos derived from heat-stressed oocytes show increased ROS levels, along with impaired antioxidant capacity and reduced developmental competence (Divvela et al., 2025). In agreement with findings in oocytes, altered mitochondrial membrane potential, reduced ATP, and mtDNA copy number were also detected in the resulting blastocysts (Feng et al., 2024b; Miętkiewska et al., 2022). Furthermore, antioxidant systems (SOD, CAT, GSH-Px, peroxiredoxins) are downregulated or functionally overwhelmed in heat-stressed ovaries, follicles, and embryos, resulting in increased apoptotic index and TUNEL-positive nuclei in morulae and blastocysts derived from heat-stressed oocytes or early embryos (de Barros & Paula-Lopes, 2018; Feng et al., 2024b; Naranjo-Gómez et al., 2021). Reduction of inner cell mass and compromised trophectoderm integrity, as well as altered expression of stress-related and developmentally important genes (e.g., HSPs, BAX/BCL2, antioxidant enzymes, pluripotency markers) in blastocysts, was reported previously (de Barros & Paula-Lopes, 2018; Held-Hoelker

et al., 2025; Kawano et al., 2025; Lang et al., 2024). Several studies revealed that blastocysts derived from heat-stressed bovine oocytes exhibit increased expression of ER stress markers (e.g., GRP78/BiP, XBP1, and ATF6 pathway genes), as well as genes associated with apoptosis and mitochondrial dysfunction (Feng et al., 2024b; Held-Hoelker et al., 2025; Lang et al., 2024). Together, heat exposure during maturation disrupts the cytoskeleton, mitochondrial distribution, and calcium homeostasis and promotes apoptosis in the developing embryo (de Barros & Paula-Lopes, 2018; Lang et al., 2024; Naranjo-Gómez et al., 2021).

Taken together, the effects of HS during oocyte maturation have long-term consequences in early embryo development, resulting in a combination of phenotypes including redox imbalance, ER stress/UPR activation, and abnormal epigenetic reprogramming. These findings support the mechanistic chain of events in bovine reproduction, in which HS during oocyte maturation triggers mitochondrial dysfunction and ER stress, resulting in oxidative damage and associated epigenetic alterations in later stages of embryonic development.

5. Physiological and molecular consequences of heat stress on early embryonic development

HS, detailed extensively in the preceding sections, is interlinked with lowered reproductive performance in cattle, owing in part to impaired blastocyst formation, reduced implantation rates, and increased embryonic mortality. The severity of impediments to embryonic development at the helm of HS is largely stage-specific and primarily occurs during the pre-attachment stage, affecting cleavage and blastocyst formation, although its impact dwindles as the embryo continues in development (Ealy et al., 1993). The greatest detrimental effects to the early developing embryo occur during the pre-genome activation period (1–8 cell stage), whilst the embryo relies heavily on maternally inherited RNAs and proteins, lacking a robust stress response. For instance, early embryonic sensitivity to HS has been shown through the exposure of two-cell embryos and their

subsequent rapid transcriptional increase of HSP70 Member 1A (HSPA1A), Heat Shock Protein beta-1 (HSPB1), and the synthesis of HSP70 (AKhan et al., 2020; Rivera & Hansen, 2001). Additionally, embryos exposed to elevated temperatures (e.g., 40–41 °C) during early cleavage frequently exhibit reduced progression through successive mitotic divisions and significantly lowered rates of blastocyst formation, indicating compromised developmental competence and viability relative to embryos maintained at physiological temperatures (~38.5 °C) (Rivera & Hansen, 2001). Short- to moderate HS exposure during the zygote or two-cell stage of development has also been shown to disrupt cytoskeletal integrity, increase ROS, and alter mitochondrial function, all of which hinder the progression of early cleavage stage embryos through blastulation (Abdelnour et al., 2020; Al-Katanani et al., 2002b; Edwards et al., 2005; Ferreira et al., 2016; Li et al., 2015; Menjivar, Gad, Gebremedhn, et al., 2023; Nabenishi et al., 2012; Pavani et al., 2016; Rodrigues et al., 2019; Roth & Hansen, 2004, 2005b).

Early embryonic stage-dependent sensitivity is further underscored by evidence that embryos become increasingly thermotolerant to HS concomitant with the embryonic genome activation (8-16 cell stage) (P. J. Hansen, 2006). Nevertheless, even embryos exposed to HS amid the early cleavage window are less likely to form morphologically ‘normal’ blastocysts, often exhibiting epigenetic and apoptotic changes that reflect cellular stress and reduced developmental potential, as shown to be partially mitigated by our group via practical abatement strategies such as extracellular vesicles (Menjivar et al., 2026; Menjivar, Gad, Gebremedhn, et al., 2023) and the use of antioxidants (Ramírez et al., 2026) cultured in vitro.

HS alters cell-fate decisions and subsequent implantation potential during early embryonic development via perturbations in lineage allocation, cellular survival pathways, and blastocyst quality, ultimately reducing the embryo's competence for attachment and pregnancy establishment.

Exposure of early embryos to elevated temperatures induces cellular damage and oxidative stress that are known to decrease blastocyst total cell number, with particularly pronounced reductions in trophectoderm (TE) cells (Sakatani et al., 2004, 2007, 2008, 2012), the lineage responsible for placental formation and implantation (Sakatani, 2017). Additionally, HS-induced apoptosis via caspase-dependent pathway activation duly alters the balance between surviving blastomeres, undermining lineage allocation in a developmentally regulated manner (Paula-Lopes & Hansen, 2002). In addition, HS exposure during early embryogenesis has also been shown to induce abnormal chromatin remodeling and epigenetic modifications (e.g., altered H3K9me3 accumulation and heterochromatin organization), which have the capacity to reprogram gene expression patterns that govern differentiation of the inner cell mass (ICM) and TE lineages, thereby affecting downstream developmental trajectories and embryonic competence for implantation (Camargo et al., 2019). In early cleavage heat shock, it has also been shown that past a reduced blastocyst yield, further alterations in mitochondrial function and DNA integrity persist, suggesting selective survival of metabolically altered embryos with potential impacts on their post-transfer viability (Carvalheira et al., 2024). Collectively, such efforts to reshape cell-fate decisions, affecting embryonic lineage composition and the molecular programming of the early embryo, are determinants that, in part, compromise pregnancy establishment, increasing early embryonic loss in cattle.

Extending beyond the immediate impediment of HS on cleavage and blastocyst formation, long-term and potentially transgenerational consequences persist that are believed to be mediated through epigenetic reprogramming and altered phenotypic programming, rather than immediate changes in DNA sequence alone. For instance, in dairy cattle, maternally exposed environmental HS has been shown to alter an embryo's epigenome (DNA methylation, histone modifications,

stress-responsive gene expression, etc.), affecting fertility, growth, metabolism, and reproduction across multiple generations (F_2–F_3) via the direct fetal germline exposure during early embryonic development (Weller et al., 2021). In the same multigenerational study, it was shown that seasonal HS exposure during development at F_0 correlates with milk production, dystocia, stillbirth, and maturation traits in F_2–F_3 females, indicating plausible epigenetic effects persisting across multiple generations independent of direct HS exposure (Weller et al., 2021). While multigenerational epigenetic inheritance remains highly debated, such evidence suggests that maternal HS exposure during critical windows of embryonic and fetal development in part shapes offspring outcomes spanning multiple generations.

A prime determinant governing the earliest stages of embryonic development is the supraphysiological milieu within the oviduct and uterine environments. Particularly, HS disrupts the composition of oviductal and uterine secretions (histotroph), reducing concentrations of key embryotrophic factors such as insulin-like growth factor-1 (IGF-1), colony-stimulating factor-2 (CSF2), amino acids, and antioxidants that aid in sustaining embryo metabolism and survival (Sakatani, 2017). Additionally, HS increases ROS production in reproductive tract epithelial cells, leading to oxidative damage, impaired mitochondrial function, and decreased antioxidant capacity in luminal fluids, thereby indirectly exposing developing embryos to cellular stress (Sakatani, 2017; Wolfenson & Roth, 2019). Additionally, thermoregulatory blood flow redistribution reduces uterine and ovarian perfusion, limiting tissue oxygenation and impairing oviductal transport and endometrial preparation for implantation (Wolfenson & Roth, 2019). However, mechanisms to study such undertakings in vitro are largely stunted due to the inaccessibility of physiological organs and inherent drawbacks, including critical restrictions on the formation of vivo tissue-like structures and long-term physiological function when cultured in 2D. Thus, our group specifically

has made strides in using organoid models to better understand the molecular undertakings of the bovine oviduct to HS (Menjivar, Gad, Thompson, et al., 2023), mapping the architecture of in vitro organoid-derived EVs with those collected in vivo and from in vitro cells in 2D and elucidating their subsequent impact on embryos developed under conditions of HS (Menjivar et al., 2026).

6. Conclusions

HS is an escalating environmental challenge with profound consequences for female reproductive performance, particularly through its detrimental effects on ovarian and follicular function, oocyte maturation, and early embryonic development. This review summarizes the current state of the art on the physiological and molecular impacts of HS on ovarian function, oocyte competence, and early embryo development. Moreover, it highlights key mechanisms underlying HS-induced damage at the organ, cellular, and molecular levels, enabling the identification of potential targets for intervention. In addition to various pharmacological strategies, emerging molecular approaches, including EV-mediated signaling, offer promising avenues to enhance cellular resilience in oocytes and embryos.

Incorporating assisted reproductive technologies, such as artificial insemination, embryo transfer, and cryopreservation, provides practical alternatives to maintain profitability during HS seasons. This review integrates findings from both in vivo and in vitro studies, revealing shared and distinct molecular mechanisms to HS. However, technical limitations remain in in vitro models, particularly in accurately assessing embryonic viability and mimicking the maternal environment. Future research should continue integrating in vivo and advanced in vitro models, such as organoid systems, to better elucidate maternal-embryo interactions under HS.

As global temperatures continue to rise, advancing our understanding of the molecular mechanisms underlying HS will be critical for developing effective mitigation strategies.

Addressing this challenge is essential to safeguard reproductive efficiency and ensure the sustainability of livestock production under increasingly adverse environmental conditions.

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CHAPTER II—Enhancing the antioxidant capacity of bovine granulosa cells to mitigate the impact of heat stress via NRF2 pathway activation[2]

1. Overview

Global warming-induced thermal stress is an escalating threat to livestock fertility, disrupting ovarian follicular function through excessive accumulation of reactive oxygen species (ROS) and oxidative damage. Granulosa cells (GCs), which are essential for maintaining follicular homeostasis and supporting oocyte competence, are particularly vulnerable to heat stress (HS)-induced oxidative injury. Although antioxidant defense mechanisms exist in vivo, in vitro systems lack many endogenous protective factors, rendering follicular cells highly susceptible to oxidative stress. Therefore, this study aimed to investigate whether supplementation with the plant-derived antioxidants quercetin (QUE), carnosol (CAR), and sulforaphane (SFN) could mitigate HS-induced oxidative damage in bovine GCs during in vitro culture. Antioxidant supplementation, either individually or in combination, was evaluated under normothermic (NT) and HS conditions. Across GC culture models, QUE and SFN supplementation promoted nuclear NRF2 activation, reduced ROS accumulation, and restored mitochondrial function and apoptosis levels under HS conditions. These findings demonstrate that antioxidant supplementation during GC culture effectively alleviates HS-induced cellular dysfunction by restoring redox balance and preserving mitochondrial integrity. Collectively, this study provides mechanistic evidence supporting the use of NRF2-activating antioxidants as a strategy to protect ovarian follicular cells against HS.

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[2]Ramírez G, Gad A, Menjivar N, Burlingham S, Cheng M, Chen T, Ghosh S, Tesfaye D. Enhancing antioxidant capacity via NRF2 pathway activation to mitigate heat stress–induced oxidative damage in bovine granulosa cells, oocytes, and embryos. *Published at Frontiers in Cell and Developmental Biology*

2. Introduction

Heat stress (HS), defined as the inability to adequately dissipate body heat, exerts well-documented detrimental effects on cattle reproductive physiology and productivity (Khan et al., 2023; Lovarelli et al., 2024; Wolfenson et al., 2000; Wolfenson and Roth, 2019). Within the ovarian follicular microenvironment, HS induces the accumulation of reactive oxygen species (ROS), triggering oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and apoptosis (Amaral et al., 2020). Granulosa cells (GCs), which play a central role in supporting follicular growth and oocyte maturation, are particularly susceptible to oxidative damage. ROS accumulation in the ovary has been shown to induce GC apoptosis and accelerate corpus luteum degeneration, thereby compromising follicular function and female fertility (Liu et al., 2020; Zhang et al., 2023). Since GCs regulate steroidogenesis, metabolic support, and the follicular microenvironment, HS-induced dysfunction in these cells can profoundly impair ovarian competence.

To counteract thermal and oxidative stress, GCs activate endogenous antioxidant defense pathways aimed at maintaining cellular homeostasis, including the induction of heat shock proteins (HSPs) and the unfolded protein response (UPR) (Marei and Leroy, 2022). Among these protective systems, Nuclear factor erythroid 2-related factor 2 (NRF2) represents a key regulator of cellular redox balance. NRF2 controls the transcription of more than 100 oxidative stress-associated genes involved in detoxification and antioxidant defense (Chen, 2022; Lu et al., 2016; Thiruvengadam et al., 2021). Under physiological conditions, NRF2 activity is tightly regulated by Kelch-like ECH-associated protein 1 (KEAP1), which promotes NRF2 ubiquitination and proteasomal degradation. However, under oxidative stress conditions, NRF2 dissociates from KEAP1, translocates to the nucleus, and activates antioxidant response element (ARE) dependent genes that mitigate cellular damage.

Previous studies have demonstrated that pharmacological activation of NRF2 using natural plant-

derived compounds may represent a promising strategy to alleviate oxidative damage in reproductive cells (Khadrawy et al., 2019; Taqi et al., 2021). Among these compounds, quercetin (QUE), carnosol (CAR), and sulforaphane (SFN) have well-characterized interactions with KEAP1 (Hong et al., 2005; Ji et al., 2023; Luo et al., 2022). QUE, a natural antioxidant polyphenol, binds KEAP1 at Arg483 through hydrogen bonding and additionally promotes NRF2 phosphorylation through protein kinase C (PKC), thereby enhancing NRF2 activity and reducing oxidative stress (Luo et al., 2022). CAR forms hydrogen bonds with Serine602 and Glutamine530, as well as π-π stacking interactions with Tyr572 of KEAP1, leading to NRF2 activation and amelioration of oxidative stress-related phenotypes in KGN cells (Ji et al., 2023). SFN, abundant in cruciferous vegetables, modifies KEAP1 cysteine residues through a reversible reaction that mimics ROS-induced KEAP1 modification (Hong et al., 2005). Functional studies further demonstrate that SFN promotes NRF2 nuclear translocation, increases antioxidant enzyme expression, and rescues GCs from oxidative damage in vitro (Sohel et al., 2017).

Despite growing evidence supporting NRF2 activation as a protective strategy, it remains unclear whether exogenous antioxidant supplementation can effectively enhance the antioxidant capacity and metabolic competence of GCs under HS conditions. Therefore, this study investigated whether supplementation with QUE, CAR, and SFN during GC culture under normothermic and HS conditions could enhance NRF2-mediated cellular defense mechanisms and mitigate oxidative stress-induced dysfunction.

3. Materials and Methods

3.1 Ovarian collection

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 1 h. Ovaries were placed in an insulated container filled with physiological saline solution (0.9%

NaCl) at 37 °C. Upon arrival, ovarian samples were immediately washed three times with a warmed 0.9% NaCl solution, followed by a rinse with 70% ethanol.

3.2 Granulosa cells isolation

Follicular fluid containing GCs and COCs was aspirated from ovarian follicles ranging between 3 and 8 mm in diameter using a vacuum pump (GenX International; Guelph, ON, Canada) calibrated to 50 mmHg. The pumping system was connected to a standard 18-gauge hypodermic needle (Covidien Monoject™; Mansfield, MA, United States) and paired with a sterile 50 mL collection tube (CELLTREAT® Scientific Products; Pepperell, MA, United States). The aspirated follicular fluid was left to settle undisturbed for 10 min, allowing a visible cellular pellet containing COCs to form at the bottom of the tube. The supernatant containing GCs was then carefully transferred to a sterile 15 mL tube (Thermo Fisher Scientific; Waltham, MA, United States) with 3 mL of warm Dulbecco's Phosphate Buffered Saline (DPBS (1x)) without calcium and magnesium chloride (Sigma-Aldrich; St. Louis, MO, United States).

3.3 Antioxidant dilutions and preparation

Quercetin (QUE; Sigma-Aldrich; St Louis, MO, United States), Carnosol (CAR; MedChemExpress; Monmouth Junction, NJ, United States), and Sulforaphane (SFN; Selleck Chemicals; Houston, TX, United States) were prepared at 10 mM stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich; St Louis, MO, United States). To ensure consistency across treatments, the final DMSO concentration was standardized and maintained constant for all experimental groups, including the vehicle controls. Working dilutions were freshly prepared immediately before use.

3.4 Granulosa cells culture, antioxidant supplementation, and heat stress exposure

The follicular fluid suspension containing GCs was centrifuged at 500 × g for 7 min, after which

the GC pellet was collected and the supernatant discarded. The pellet was resuspended in red blood cell (RBC) lysis buffer (Sigma-Aldrich; St Louis, MO, United States) and incubated for 3 min. The reaction was stopped by washing the cells in Dulbecco's Modified Eagle's Media/Ham's Nutrient Mixture F12 (DMEM/F12) (Sigma-Aldrich; St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich; St. Louis, MO, United States), 1% Penicillin-Streptomycin (Sigma-Aldrich; St. Louis, MO, United States), and 1% Amphotericin B solution (Sigma-Aldrich; St. Louis, MO, United States). The cells were then centrifuged again at 500 × g for 5 min. This process was repeated until a pure pellet was obtained. The supernatant was discarded, and the pellet was washed with DPBS (1x) before being resuspended in the DMEM/F12 media described above. Quantity and viability of GCs were assessed using a hemocytometer (Hausser Scientific, Horsham, PA, United States) and the trypan blue exclusion method (Sigma-Aldrich; St. Louis, MO, United States). Depending on the assay, GCs were seeded at 2×10^5 cells per well in 24-well plates or at 1×10^4 cells per well in 96-well tissue-culture-treated plates (United States Scientific, Inc., Ocala, FL) in DMEM/F12 media as described above.

Bovine GCs were initially cultured for 24 h until reaching subconfluency. Antioxidant supplementation was performed following a brief wash a with DPBS (1x) for QUE (5 μM), CAR (10 μM), SFN (1 μM), a combination of three (MIX), vehicle (DMSO), and an untreated control for 24 h. Subsequently, control groups were maintained under normal temperature (NT, 38.5 °C) for 24 h, while the HS-treated groups were transferred for 8 h to a different incubator programmed at 42 °C and then returned to normal temperature (NT) for the remaining 16 h, a method to mimic daylight conditions, and summing in a 72 h total length of culture. The 42 °C temperature is commonly used in vitro to induce a robust and reproducible heat-stress response without causing excessive cell death in bovine GCs (Roth and Hansen, 2004). A schematic overview of the

experimental design is shown in Figure 2.1.

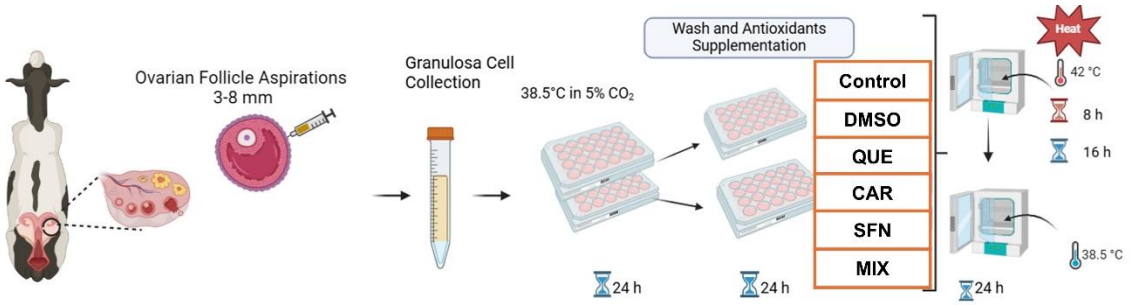

Figure 2.1 Experimental models for Bovine granulosa cell in vitro culture.

3.5 Antioxidant dose optimization and cell viability assay

Bovine GC collection, culture, HS treatment, and antioxidant supplementation were performed as indicated above. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technology, Japan). Doses tested were 1, 5, 10, and 20 µM for QUE, CAR, and SFN under NT and HS conditions. For this, 10 µL of the reagent was added to each well and incubated for 3 h at 38.5 °C in a 5% CO2 incubator. Optical density of the formazan dye was measured at 450 nm using a microplate reader (BioTek Instruments, Germany). Wells containing only media with the antioxidant, but no cells, served as blanks for background correction. All assays were performed in quadruplicate. Based on these results, the highest non-toxic doses were selected: 1, 5, and 10 µM for SFN, QUE, and CAR, and an antioxidant mix was based on these doses (Figure 2.2). Additionally, these doses were used for further experiments.

3.6 Active NRF2 binding assay on nuclear extracts

Nuclear activation of NRF2 in antioxidant-supplemented bovine GC nuclear extracts under NT and HS was assessed using the commercial Trans-AM® NRF2 kit (Active Motif, Carlsbad, CA, United States). Following culture, the NEPER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific; Waltham, MA, United States) was employed to separate nuclear and cytoplasmic

protein fractions from bovine GCs from four biological replicates. Protein concentrations in the resulting nuclear extract suspensions were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, United States) following the manufacturer's instructions. Subsequently, the TransAM® NRF2 ELISA was used to analyze the nuclear protein extracts from individual and combined antioxidant-supplemented groups under NT and HS conditions. Optical density was measured at 450 nm with a reference wavelength of 655 nm using a microplate reader (BioTek Instruments, Germany). Wells containing all reactive agents were used as a blank for background correction.

3.7 Intracellular reactive oxygen species accumulation assay

The impact of thermal stress on intracellular ROS accumulation was assessed using 10 μM 2′, 7′-Dichlorofluorescein Diacetate (H2DCFDA) (Sigma Aldrich; St. Louis, MO, United States) on GCs, bovine denuded oocytes, and blastocysts. GCs were seeded at 2 × 10^5 cells per well in 24-well plates containing 600 μL of media as described above. Following the culture (washing, antioxidant supplementation, and heat exposure), the media was removed, and cells from both NT and HS treatments were washed twice with DPBS (1x) (Sigma-Aldrich; St. Louis, MO, United States). Cells were then incubated with 10 μM H2DCFDA diluted in 200 μL of non-supplemented F12 culture media for 30 min in a CO2 incubator. After incubation, cells were washed three times using DPBS (1x) (Sigma Aldrich; St. Louis, MO, United States), and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Fluorescence intensity was quantified from. jpg images from different biological replicates, taken from two specific locations per well, across four wells per treatment group per replicate (n = 16 images per group). All images were acquired with identical exposure and light settings, avoiding signal saturation, using a green fluorescent filter (excitation 494 nm, emission 518 nm). Fluorescence intensity was analyzed using

ImageJ software (National Institute of Health, Bethesda, MD, United States).

3.8 Mitochondrial Membrane Potential δΨm assay

The detrimental effect of heat stress on mitochondrial depolarization was analyzed using the cationic dye 5,5′,6,6′-tetrachloro1,1′,3,3′ tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, United States) on GCs, denuded oocytes, and blastocysts. GCs were seeded at 2 × 10^5 cells per well in 24-well plates containing 600 µL of media as described above. Following the culture (washing, antioxidant supplementation, and heat exposure), the media was removed, and cells from both NT and HS treatments were washed twice with DPBS (1x) (Sigma-Aldrich; St. Louis, MO, United States). Cells were then incubated with 2 µM JC-1 staining diluted in 200 µL media culture for 30 min in a CO2 incubator. After incubation, cells were washed three times using DPBS (1x) (Sigma Aldrich; St. Louis, MO, United States) and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Images were captured at two defined locations per well, across four wells per treatment group per replicate (n = 16 total per treatment). Fluorescence intensity was quantified from. jpg images obtained from different biological replicates. Imaging for J-monomers was conducted using a wavelength of 488 nm for J-monomers and excitation/emission settings at 490/525 nm (FITC filter, green fluorescence), indicative of low MMP. Similarly, images for J-aggregates were carried out using a wavelength of 561 nm and excitation/emission at 596/615 nm (TRITC filter/red fluorescence), indicating high MMP. Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, United States). The ratio of red to green fluorescence was subsequently calculated to determine MMP, expressed as an arbitrary unit.

3.9 Annexin V/propidium iodide staining

To assess the apoptotic index (early apoptosis + late apoptosis) in bovine GCs, co-staining of annexin V and propidium iodide (PI) was performed using the Cell Meter APC-Annexin V Binding Apoptosis Assay Kit (Biomol International; Plymouth Meeting, PA, United States), according to the manufacturer's recommendations with minor modifications. GCs were analyzed using the Cytek 4 laser-Aurora™ (Cytek Biosciences; Fremont, CA, United States). Assays were performed in duplicate, and the percentages of different cell populations within the treatment groups (live and healthy, early apoptotic, late apoptotic, necrotic) were recorded for analysis.

3.10 Gene expression analysis of antioxidant and stress-related markers

The impact of antioxidants on modulating stress response genes in bovine GCs was investigated using qRT-PCR. Total RNA from GCs was isolated from three replicates using the miRNeasy® mini kit (Qiagen; Hilden, Germany). Total RNA sample concentrations and integrity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, United States). RNA samples were stored at −80 °C until further use.

Following this, cDNA was synthesized by reverse transcription using oligo(dT)18 primers and the First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, United States). For each sample type, cDNA synthesis was performed using the maximum amount of high-quality RNA available per biological replicate, resulting in different RNA input amounts for granulosa cells (33.2 ng). Gene expression analyses were conducted to normalize relative expression levels to the geometric mean of the reference genes β-ACTIN and GAPDH. Gene-specific primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the list of primers is indicated in Table 2.1. Formal qRT-PCR analysis was performed using the CFX96 Touch Real-Time PCR

Detection System (Bio-Rad; Hercules, CA, United States). The following thermocycling conditions were applied for amplification: initial denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 15 s, and 60 °C for 60 s. The specificity of the amplification was determined by melting curve analysis generated at the end of each run, and the data were analyzed using the comparative C(T) (2^−ΔΔCt) method (Schmittgen and Livak, 2008). Statistical analyses were performed on the ΔΔCt values, while fold-change values (2^−ΔΔCt) were used for data presentation in the figures.

3.11 Statistical analysis

Data were analyzed using GraphPad Prism version 8.4.2 (GraphPad Software, San Diego, CA, USA). For experiments in which all treatments were tested at both temperatures, a two-way analysis of variance (ANOVA) was performed with temperature and treatment as fixed factors, followed by Tukey's multiple-comparison test. For qRT-PCR analyses, statistical tests were performed on ΔΔCt values, whereas fold-change values (2^−ΔΔCt) were used for graphical presentation. Each target gene was analyzed independently; therefore, no additional correction for multiple testing across different genes was applied. Data are presented as mean ± SEM of biological replicates. Statistical significance was declared at $P \leq 0.05$.

Table 2.1 Sequence-specific primers used for qRT-PCR analysis.

Gene name	Accession number	Primer sequences
NRF2	NM_001011678	F5′-cccagtcttcactgctcctc-3′ R5′-tcagccagcttgtcattttg-3′

SOD1	NM_174615	F5′-agaggcatgttggagacctg-3′ R5′-cagcgttgccagtctttgta-3′
HSP70	NM_001038505	F5′-aatgccagttgccaatgctg-3′ R5′-atcgagagttcctccaccca-3′
HSP90	NM_001012670	F5′-tcactgaggaaatgccaccc-3′ R5′-atggagacagagcgctgaac-3′
GRP78	NM_001075148	F5′-tgcgaagccctatagctgac-3′ R5′-agtaggtggtacccaggtcg-3′
GRP94	NM_174700	F5′-tgctgtgtggagagggaatg-3′ R5′-tcctgtgaccacaatcccaa-3′
β-ACTIN	NM_173979	F5′-tgtccaccttccagcagat-3′ R5′-tcaccttcaccgttccagt-3′
GAPDH	NM_001034034	F5′-aatggagccatcaccatc-3′ R5′-gtggttcacgcccatcaca-3′
HMOX1	NM_001014912.1	F5′-gcaaggtgcaagacttggct-3′ R5′-cacatggcgtaaagccccac-3′
PRDX1	NM_174431.1	F5′-tcctatttcagtggaactgat-3′ R5′-aagcaatgatctccgtgggg-3′
CAT	NM_001035386.2	F5′-tgggacccaactacttccag-3′ R5′-aagtgggtcctgtgttccag-3′

4. Results

4.1 Dose optimization for antioxidant supplementation

To determine the optimal antioxidant concentrations, we first evaluated doses of 1, 5, 10, and 20μM for QUE, CAR, and SFN. As indicated in Figure 2.2, most of the doses tested did not compromise cell viability, confirming their safety and efficacy under HS conditions. Based on

these results, we then selected the highest non-toxic, biologically active concentrations,1μM SFN, 5μM QUE, and 10μM CAR, for all subsequent experiments. In addition, a combinatorial treatment (MIX) was also included to assess potential synergistic effects of antioxidants. For all endpoints evaluated, two-way ANOVA revealed a significant temperature × antioxidant treatment interaction ($p<0.05$), indicating that antioxidant effects were dependent on the presence or absence of thermal stress conditions.

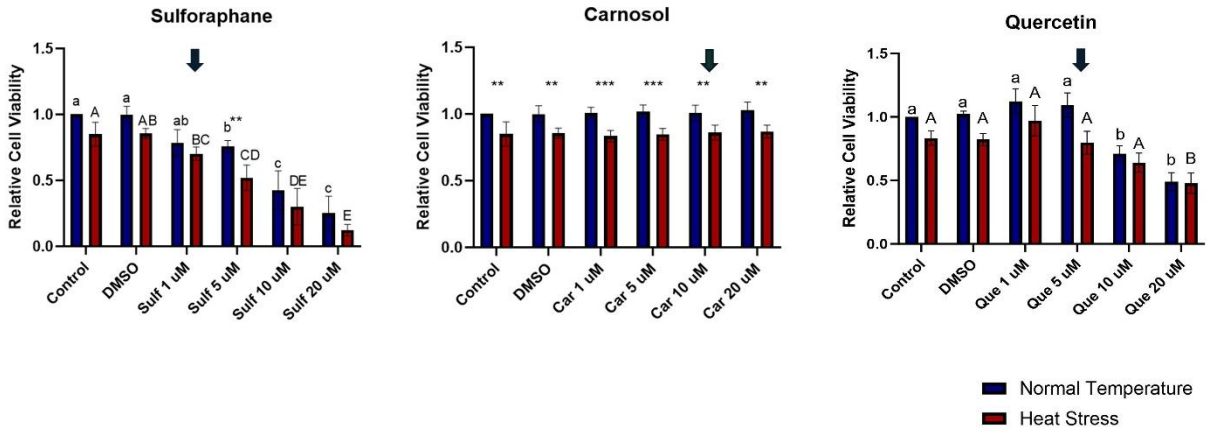

Figure 2.2 Dose-dependent impact of antioxidant supplementation on bovine granulosa cell viability. Cell Viability assay CCK8 for each antioxidant under heat stress (HS) and normal temperature (NT). **p < 0.01; ***p < 0.001 between NT and HS conditions within the treatment group; different lowercase letters show significant differences between NT treatment groups and uppercase letters for HS treatment groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

4.2 Impact of QUE, CAR, and SFN on the cellular NRF2-mediated oxidative stress response pathway

Gene expression analysis revealed that NRF2 transcript levels were upregulated in all antioxidant-

treated groups under HS conditions compared to the respective control, with statistical significance for only HS QUE (p=0.0260) and HS SFN (p=0.0163) compared to HS control (Figure 2.3). Moreover, increased expression of downstream antioxidant genes SOD1, HO1, and PRDX1 *(p<0.05*) was observed in the SFN-treated group compared with the control and vehicle groups under HS. Quantification of nuclear NRF2 protein in cultured GCs demonstrated an increase in QUE (*p=0.0002*) and SFN (*p=0.0007*) treated groups under HS conditions compared to the non-supplemented HS control and vehicle groups (Figure 2.4).

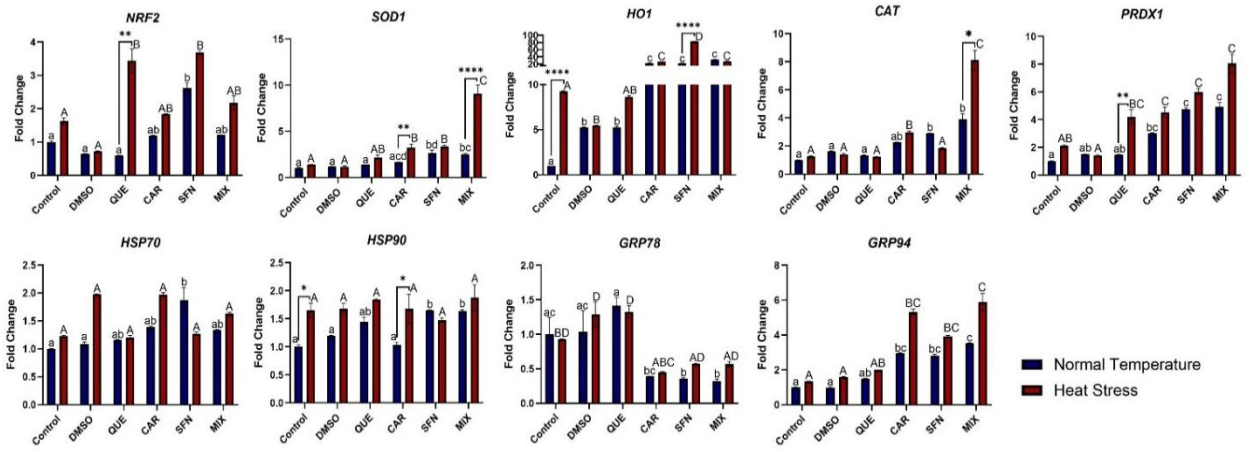

Figure 2.3 The impact of antioxidant supplementation on stress-related gene expression in bovine GCs. qRT-PCR was performed to quantify selected stress-related genes *NRF2, SOD1, HO1, PRDX1, CAT, HSP70, HSP90, GRP78*, and *GRP94*. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001 between normal temperature (NT) and heat stress (HS) conditions within the treatment group. Different lowercase letters indicate significant differences between treatment groups under NT, and uppercase letters indicate significant differences between treatment groups under the HS condition.

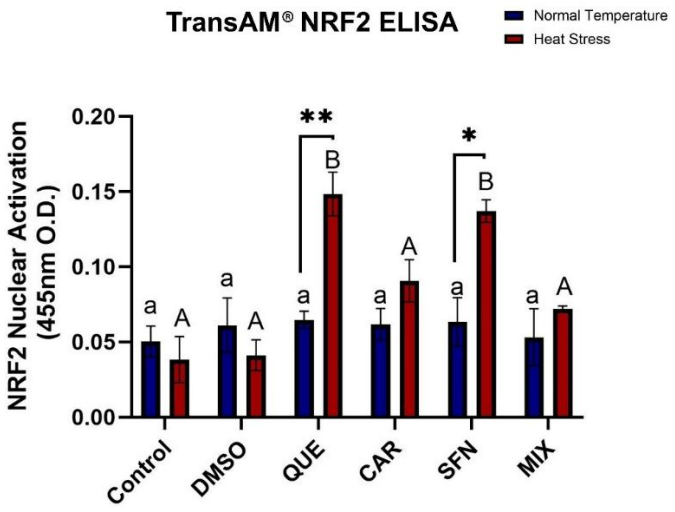

Figure 2.4 The beneficial effect of antioxidant supplementation on NRF2 protein nuclear expression in bovine GCs. TransAM® NRF2 ELISAs were used for nuclear protein extracts from antioxidant-supplemented bovine granulosa cells under Normal Temperature (NT) and Heat Stress (HS). Blue bars represent NT groups, and red bars represent HS treatments. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *$*p < 0.05$, **$p < 0.01$, ***$p < 0.001$* between NT and HS conditions within the treatment group. Different lowercase letters indicate significant differences between NT treatment groups, and uppercase letters indicate significant differences between HS treatment groups.

ROS accumulation was reduced for QUE (*$p<0.0001$*), CAR (*$p<0.0001$*), SFN (*$p<0.0001$*), and MIX (*$p<0.0001$*) compared to the control and vehicle under HS conditions (Figure 2.5). Additionally, MMP was increased in GCs treated with QUE (*$p=0.0130$*), CAR (*$p=0.0048$*), SFN (*$p=0.0382$*), and MIX (*$p<0.0001$*) under conditions of HS compared to the control and vehicle-treated groups (Figure 2.5), indicating improved mitochondrial function and maintenance of cellular homeostasis.

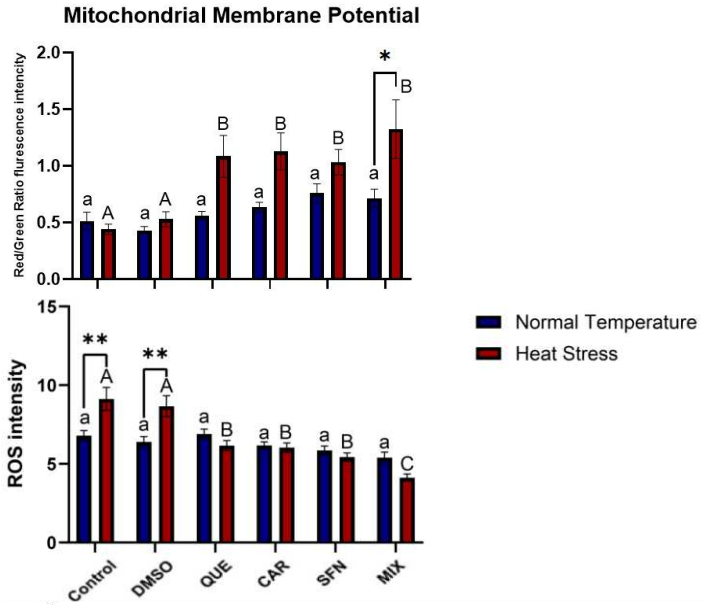

Figure 2.5 The beneficial effect of antioxidant supplementation on ROS accumulation and Mitochondrial membrane potential (MMP) in bovine GCs. ROS accumulation assay 2′,7′-DCFH-DA quantification of the relative fluorescence intensity of ROS in bovine GCs. Quantitative red/green fluorescence intensity ratio using 5,5′,6,6′tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocynanine iodide (JC-1) for the measurement of MMP (ΔΨm), an indicator of mitochondrial activity in bovine GCs. Green fluorescence represents JC-1 monomers, whereas red fluorescence represents JC-1 aggregates. Blue bars represent Normal Temperature (NT) groups, and red bars represent Heat Stress (HS) treatments. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between NT and HS conditions within the treatment group. Different lowercase letters indicate significant differences between NT treatment groups, and uppercase letters indicate significant differences between HS treatment groups.

Reductions in the total rate of apoptosis in GCs were also observed for QUE (*p=0.0187*), CAR (*p=0.0234*), SFN (*p=0.0165*), and MIX (*p=0.0228*) compared to the control group under HS

conditions (Figure 2.6). While all antioxidant treatments improved oxidative balance, QUE and SFN supplementation robustly activated NRF2 signaling as confirmed by nuclear extracts ELISA assays (Figure 2.4). Given the central role of NRF2 in regulating antioxidant defense, these findings provided a mechanistic basis for narrowing oocyte maturation experiments.

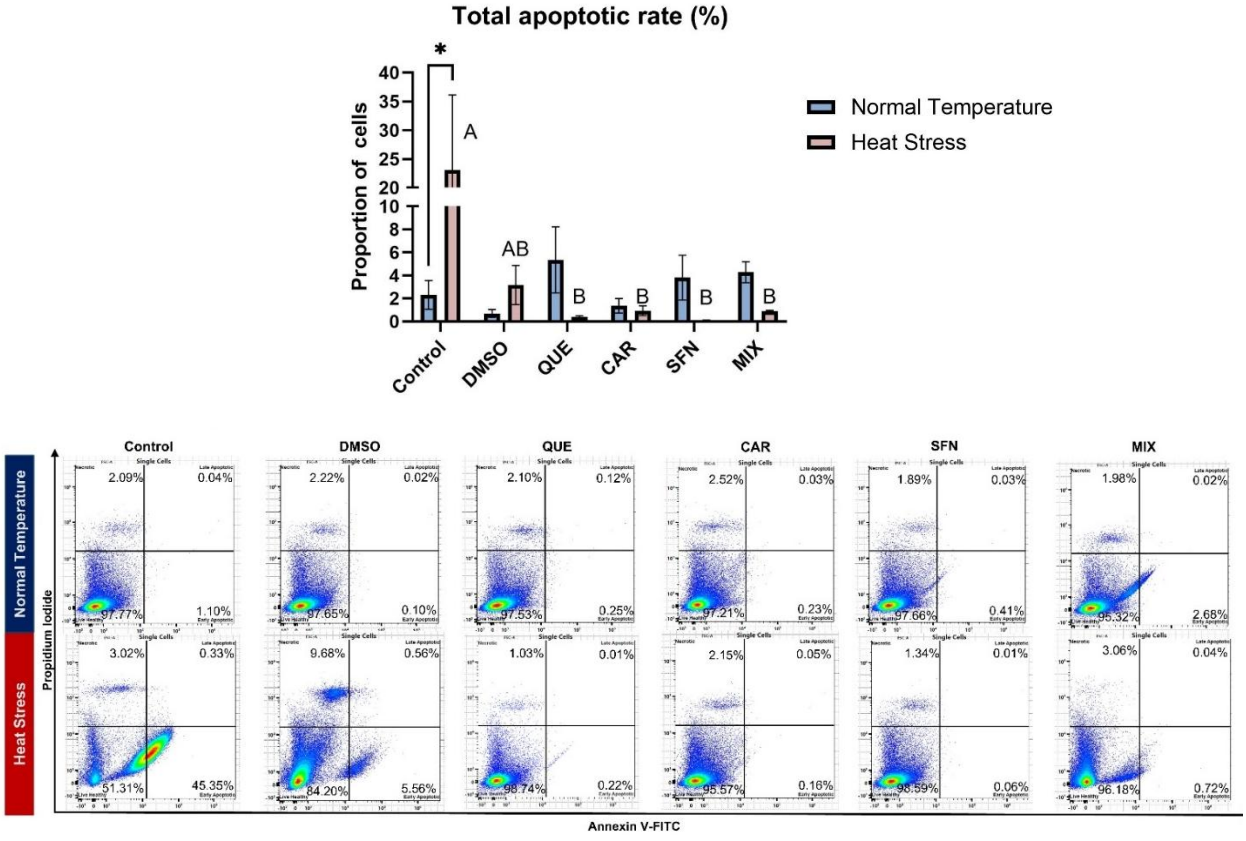

Figure 2.6 The beneficial effect of antioxidant supplementation on the apoptosis index in bovine GCs. Annexin V/PI staining was evaluated using flow cytometry to determine the total apoptotic rate, including the proportion of early and late apoptotic cells. Representative panels shown. Blue bars represent Normal Temperature (NT) groups, and red bars represent Heat Stress (HS) treatments. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between NT and HS conditions within the treatment group.

Different lowercase letters indicate significant differences between NT treatment groups, and uppercase letters indicate significant differences between HS treatment groups.

5. Discussion

Environmental thermal stress remains one of the most significant factors compromising reproductive efficiency in livestock, particularly in dairy cattle exposed to increasingly frequent periods of elevated ambient temperatures. Within the ovarian follicle, HS disrupts cellular homeostasis by promoting excessive generation of ROS, which subsequently induces oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis (Amaral et al., 2020; Gaskins et al., 2021; Sammad et al., 2020). GCs play a critical role in maintaining follicular development and oocyte competence by providing metabolic substrates, steroid hormones, growth factors, and antioxidant support. Therefore, damage to GCs caused by thermal stress may compromise the follicular microenvironment long before detrimental effects become evident in the oocyte itself. The objective of the present study was to determine whether supplementation with plant-derived NRF2 activators could enhance the antioxidant capacity of bovine GCs and mitigate the detrimental effects of HS.

As an initial screening step, we evaluated three antioxidants (QUE, CAR, SFN, and their combination) in GCs exposed to HS, assessing cellular function, oxidative homeostasis, and their ability to activate NRF2 signaling. Although all antioxidants improved redox homeostasis, only QUE and SFN significantly promoted nuclear NRF2 activation, as confirmed by nuclear extract ELISA assays. Since NRF2 is a central regulator of cellular antioxidant defense, these findings provided a mechanistic basis for selecting QUE and SFN for subsequent experiments. This selection strategy ensured that only compounds capable of both improving cellular function and activating the NRF2-KEAP1 antioxidant pathway were advanced for further investigation.

The NRF2-KEAP1 pathway serves as a major cellular defense mechanism against oxidative stress. Under basal conditions, NRF2 is retained in the cytoplasm through its interaction with KEAP1 and is continuously targeted for proteasomal degradation. However, oxidative insults or electrophilic compounds such as SFN disrupt this interaction, allowing NRF2 to translocate into the nucleus and activate antioxidant response element (ARE)-dependent genes involved in ROS detoxification, redox regulation, and cellular survival (Lu et al., 2016). Consistent with this mechanism, QUE and SFN supplementation significantly enhanced NRF2 nuclear translocation in HS-exposed GCs and increased the expression of several antioxidant-related genes, including SOD1, HO1, and PRDX1. These findings suggest the successful activation of endogenous antioxidant defense pathways that enhance GCs' ability to cope with oxidative insults.

HS-induced oxidative stress is known to disrupt GC function by promoting ROS accumulation, mitochondrial dysfunction, apoptosis, and impaired follicular support capacity (Prasad et al., 2016; Yang et al., 2021). Consistent with these observations, our results demonstrated that HS increased intracellular ROS accumulation, whereas antioxidant supplementation significantly reduced ROS levels. Excessive ROS production is recognized as a primary mediator of heat-induced cellular damage because it promotes lipid peroxidation, protein oxidation, DNA damage, and disruption of intracellular signaling pathways. Previous studies have demonstrated that elevated ROS concentrations impair GC viability and compromise their ability to support normal follicular development. Therefore, the reduction in ROS observed following antioxidant supplementation likely represents a key mechanism underlying the improved cellular resilience observed in the present study.

The improvements in oxidative balance observed following antioxidant supplementation were accompanied by significant preservation of mitochondrial function and reductions in apoptosis.

Mitochondria represent both a major source and a primary target of ROS, and excessive oxidative stress can trigger mitochondrial membrane depolarization, disruption of ATP production, and activation of apoptotic signaling pathways. Consistent with this mechanism, HS reduced mitochondrial membrane potential and increased the proportion of apoptotic GCs, indicating that thermal stress compromised cellular viability via oxidative damage-mediated mitochondrial dysfunction. Importantly, supplementation with QUE, CAR, SFN, and the antioxidant mixture restored mitochondrial membrane potential while significantly reducing apoptosis. These findings suggest that attenuation of oxidative stress preserved mitochondrial integrity and prevented activation of cell death pathways. Because GC survival is essential for maintaining follicular homeostasis, steroidogenesis, and metabolic support of the oocyte, reducing apoptosis may be a critical mechanism by which antioxidant supplementation preserves ovarian function under HS.

The present findings are further supported by previous work from our laboratory demonstrating the central role of NRF2 in regulating oxidative stress responses in bovine GCs. Khadrawy et al. (2019) reported that both endogenous and exogenous modulation of NRF2 significantly influence the expression of antioxidant defense genes and cellular responses to oxidative stress in bovine GCs. In that study, activation of NRF2 increased the expression of key antioxidant enzymes that maintain redox homeostasis, whereas disruption of NRF2 signaling impaired the cellular capacity to counteract oxidative damage. These findings established NRF2 as a critical regulator of GC function and survival under stress conditions. The results of the present study further extend these observations by demonstrating that activation of NRF2 through phytochemical supplementation remains effective under HS conditions, a physiologically relevant challenge that simultaneously disrupts redox balance, mitochondrial function, and cell viability. The increased NRF2 nuclear translocation observed following QUE and SFN supplementation, together with reduced ROS

accumulation, restored mitochondrial membrane potential, and significantly reduced apoptotic cell death, further supporting the interconnected relationship between oxidative stress, mitochondrial dysfunction, and GC survival (Khadrawy et al., 2019).

The protective effects of SFN observed in the present study are highly consistent with previous findings from our laboratory demonstrating the efficacy of SFN as an activator of the NRF2-ARE pathway in bovine GCs. Sohel et al. (2019) reported that SFN supplementation protected GCs against H_2O_2-induced oxidative stress by enhancing NRF2 signaling, increasing the expression of antioxidant genes including CAT, SOD1, PRDX1, and TXN1, reducing intracellular ROS accumulation, and preserving mitochondrial activity.

A notable finding of the present study was that although all antioxidant treatments improved oxidative homeostasis and reduced apoptosis, only QUE and SFN significantly enhanced NRF2 nuclear translocation. This distinction highlights the importance of evaluating both functional and mechanistic endpoints when screening antioxidant compounds. While CAR and the antioxidant mixture improved cellular homeostasis, QUE and SFN demonstrated the additional capacity to activate endogenous stress-response pathways. Interestingly, the combination treatment did not further enhance NRF2 activation beyond that achieved by individual supplementation, suggesting that additive activation of the pathway may not necessarily occur when multiple compounds are administered simultaneously. These findings emphasize the importance of mechanistic screening approaches when selecting antioxidant interventions for reproductive applications and suggest that activation of endogenous defense pathways may be more beneficial than simple ROS scavenging alone.

Collectively, these findings demonstrate that HS compromises GC function through a cascade involving ROS accumulation, mitochondrial dysfunction, and apoptosis. Supplementation with

QUE and SFN effectively enhanced NRF2-mediated antioxidant defense mechanisms, resulting in increased expression of antioxidant genes, reduced oxidative stress, preserved mitochondrial function, and improved cellular survival. These findings provide mechanistic evidence that activation of the NRF2 pathway can enhance GC resilience to thermal stress and support maintenance of the follicular microenvironment. Moreover, the identification of QUE and SFN as the most effective NRF2-activating compounds established the foundation for the subsequent chapters of this dissertation, which investigated whether similar cytoprotective mechanisms could be leveraged to improve oocyte quality and embryonic developmental competence under oxidative stress conditions.

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CHAPTER III—Mitigation of heat stress-induced oxidative damage in bovine oocytes, and embryos via NRF2 pathway activation[2]

1. Overview

This study aimed to investigate whether supplementation with the antioxidants quercetin (QUE), and sulforaphane (SFN) during in vitro maturation (IVM) could mitigate HS-induced oxidative damage and improve embryonic developmental competence. Antioxidant supplementation was performed during oocyte maturation under normothermic (NT) and HS conditions. QUE and SFN supplementation activated nuclear NRF2 signaling, reduced ROS accumulation, and restored mitochondrial function under HS conditions. In thermally stressed oocytes, antioxidant supplementation also improved blastocyst development and. Furthermore, single-embryo metabolic profiling revealed reduced oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in blastocysts derived from antioxidant-treated oocytes, indicating enhanced metabolic efficiency. Quantitative analysis of embryo competence-associated genes further demonstrated restoration of the embryo competence index (ECI) following QUE and SFN supplementation. Collectively, these findings demonstrate that antioxidant supplementation during oocyte maturation alleviates HS-induced reproductive dysfunction by restoring redox homeostasis, preserving embryonic metabolic efficiency, and re-establishing embryo competence, thereby providing a mechanistically grounded strategy to mitigate climate-driven fertility decline in cattle.

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[2]Ramírez G, Gad A, Menjivar N, Burlingham S, Cheng M, Chen T, Ghosh S, Tesfaye D. Enhancing antioxidant capacity via NRF2 pathway activation to mitigate heat stress–induced oxidative damage in bovine granulosa cells, oocytes, and embryos. *Published at Frontiers in Cell and Developmental Biology.*

2. Introduction

Heat stress (HS), defined as the inability to adequately dissipate body heat, exerts significant detrimental effects on cattle reproductive physiology and fertility (Khan et al., 2023; Lovarelli et al., 2024; Wolfenson et al., 2000; Wolfenson and Roth, 2019). Within the ovarian follicular microenvironment, HS promotes the accumulation of reactive oxygen species (ROS), leading to oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and apoptosis (Amaral et al., 2020). Oocytes are especially vulnerable to oxidative damage because their maturation occurs synchronously with follicular development and depends heavily on bidirectional communication with surrounding granulosa and cumulus cells. Cumulus cells (CCs) support oocyte competence through the transfer of metabolites and mRNA cargo via trans-zonal projections (TZPs), contributing to the oocyte maternal mRNA reserve essential for subsequent embryonic development (Macaulay et al., 2016). Disruption of this finely coordinated follicular communication by HS can compromise oocyte quality and developmental competence.

The detrimental effects of oxidative stress during oocyte maturation extend beyond fertilization and influence early embryonic development. Previous studies have demonstrated that HS exposure during oocyte maturation alters embryonic developmental potential and molecular architecture, partly through epigenetic modifications (Amaral et al., 2020; Bie et al., 2017; Kono et al., 1996). Failure to adequately control ROS accumulation during these early developmental stages can impair mitochondrial function, reduce developmental competence, and compromise blastocyst formation (Aglan et al., 2020; Amin et al., 2014; Khadrawy et al., 2019; Khadrawy et al., 2020; Sohel et al., 2018; Taqi et al., 2021). Therefore, understanding the molecular mechanisms governing oxidative stress responses in oocytes and embryos is critical for improving reproductive outcomes under thermal stress conditions.

In response to oxidative and thermal stress, oocytes and embryos activate endogenous antioxidant defense systems, including heat shock proteins (HSPs), the unfolded protein response (UPR), and NRF2-mediated antioxidant signaling pathways (Marei and Leroy, 2022). Nuclear factor erythroid 2-related factor 2 (NRF2) is considered a master regulator of cellular redox homeostasis and controls the expression of numerous antioxidant and cytoprotective genes (Chen, 2022; Lu et al., 2016; Thiruvengadam et al., 2021). Under basal conditions, NRF2 is negatively regulated by Kelch-like ECH-associated protein 1 (KEAP1), which facilitates its ubiquitination and degradation. However, oxidative stress disrupts the NRF2-KEAP1 interaction, enabling NRF2 nuclear translocation and activation of antioxidant response element- (ARE-) dependent genes. Previous work from our group demonstrated that inadequate activation of the NRF2-mediated oxidative stress response results in excessive ROS accumulation, mitochondrial dysfunction, and impaired embryonic development (Aglan et al., 2020; Amin et al., 2014; Khadrawy et al., 2019; Khadrawy et al., 2020; Sohel et al., 2018; Taqi et al., 2021). Natural antioxidant compounds capable of activating NRF2 signaling have emerged as promising therapeutic candidates for protecting reproductive cells against oxidative stress (Khadrawy et al., 2019; Taqi et al., 2021). Among these, quercetin (QUE), carnosol (CAR), and sulforaphane (SFN) exhibit well-characterized interactions with KEAP1 (Hong et al., 2005; Ji et al., 2023; Luo et al., 2022). QUE enhances NRF2 signaling through hydrogen bonding with KEAP1 at Arg483 and through protein kinase C-mediated NRF2 phosphorylation (Luo et al., 2022). CAR activates NRF2 signaling via interactions with KEAP1 residues Serine602, Glutamine530, and Tyr572 (Ji et al., 2023). SFN modifies KEAP1 cysteine residues, thereby mimicking ROS-induced KEAP1 disruption and promoting NRF2 activation (Hong et al., 2005). Functional studies have shown that SFN increases antioxidant enzyme expression and enhances cellular resistance to oxidative damage (Sohel et al.,

2017). Although NRF2-activating compounds have shown promising antioxidant effects, their ability to improve oocyte competence and embryonic developmental outcomes under HS conditions remains incompletely understood. Therefore, this study investigated whether supplementation with QUE, CAR, and SFN during oocyte culture under normothermic and HS conditions could enhance NRF2-mediated antioxidant defense mechanisms and improve metabolic and embryonic competence.

3. Materials and Methods

3.1 Ovarian collection

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 1 h. Ovaries were placed in an insulated container filled with physiological saline solution (0.9% NaCl) at 37° C. Upon arrival, ovarian samples were immediately washed three times with a warmed 0.9% NaCl solution, followed by a rinse with 70% ethanol.

3.2 Cumulus-oocyte-complex isolation

Follicular fluid containing cumulus-oocyte-complex (COCs) was aspirated from ovarian follicles ranging between 3-8 mm in diameter using a vacuum pump (GenX International; Guelph, ON, Canada) calibrated to 50 mmHg. The pumping system was connected to a standard 18-gauge hypodermic needle (Covidien Monoject™; Mansfield, MA, USA) and paired with a sterile 50 mL collection tube (CELLTREAT® Scientific Products; Pepperell, MA, USA). The aspirated follicular fluid was left to settle undisturbed for 10 min, allowing a visible cellular pellet containing COCs to form at the bottom of the tube.

3.3 Antioxidant dilutions and preparation

Quercetin (QUE; Sigma-Aldrich; St Louis, MO, USA), and Sulforaphane (SFN; Selleck Chemicals; Houston, TX, USA) were prepared at 10 mM stock solution in dimethyl sulfoxide (DMSO; Sigma-

Aldrich; St Louis, MO, USA). To ensure consistency across treatments, the final DMSO concentration was standardized and maintained constant for all experimental groups, including the vehicle controls. Working dilutions were freshly prepared immediately before use.

3.4 In vitro maturation, antioxidant supplementation, and heat stress exposure

COCs were collected from abattoir ovaries as described above. Retrieved COCs were grouped (50 per well) and placed into 4-well culture dishes (Thermo Fisher Scientific, Waltham, MA, USA) containing 1 mL of chemically defined media for in vitro maturation (IVM) of oocytes pre-equilibrated at 38.5° C in 5% CO2 (De La Torre-Sanchez et al., 2006). At the time of equilibration, all IVM media were supplemented with 15 ng/mL NIDDK-oFSH-20, 1 µg/mL USDA-LHB-5, 1 µg/mL estradiol 17β, 50 ng/mL epidermal growth factor, and 0.1 mM cysteamine (De La Torre-Sanchez et al., 2006). Four treatment groups were selected based on the prior initial assays in GCs related to NRF2 nuclear activation: 5µM QUE, 1µM SFN, and a vehicle control group (DMSO). IVM lasted 23 ± 1 h and began with four groups incubated under normothermic conditions (NT, 38.5° C) for the first 8 h. Subsequently, the HS QUE, HS SFN, and HS control groups were transferred to a different incubator programmed at 41° C for the remaining 15 h, while the NT control group remained at NT conditions. Previous studies have demonstrated that exposure of bovine oocytes to 41 °C during IVM is sufficient to impair developmental competence, supporting its use as a physiologically relevant HS model (Paula-Lopes and Hansen, 2002). A schematic overview of the experimental design is shown in Figure 3.1.

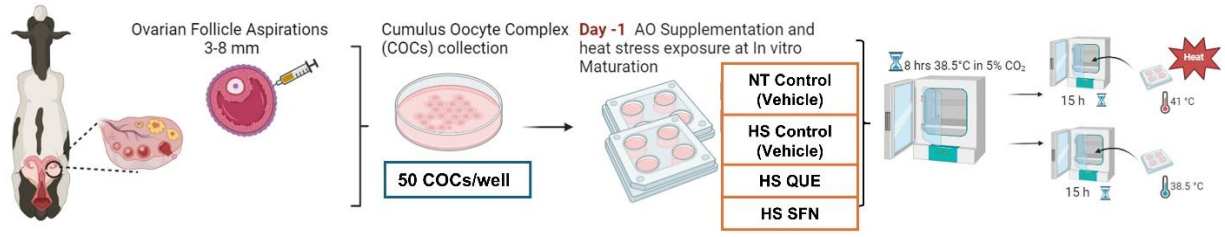


Figure 3.1 Experimental model for Bovine in vitro oocyte maturation (IVM).

3.5 In vitro fertilization and embryo culture

For in vitro fertilization (IVF), COCs were transferred (in <20 μL of maturation media) into 4-well culture dishes containing 430 μL of equilibrated CSU chemically defined media for IVF (F-CDM) (De La Torre-Sanchez, Jose Fernando et al., 2006) and maintained at 38.5° C and 5% CO2 in humidified air. Frozen-thawed bull sperm from a single ejaculation were processed to separate viable and motile sperm using a multilayer 45%/90% Percoll® (Sigma-Aldrich; St. Louis, MO, USA) gradient and centrifuged at 800 × g for 20 min. Following this, the supernatant was discarded, and the remaining sperm pellet was washed with 2 mL of CSU chemically defined media for the handling of early embryos (HCDM-1) (De La Torre-Sanchez et al., 2006) and centrifuged at 300 × g for 5 min. Sperm concentration was determined using a hemocytometer, and the suspension was adjusted to 5×10^6 sperm/mL using equilibrated F-CDM. Diluted sperm was then added to COCs at a final concentration of 0.5×10^6 sperm/mL and co-incubated for 18 h at 38.5° C in 5% CO2 in humidified air. Following fertilization, presumptive zygotes were vortexed for 90 s at maximum speed to remove cumulus cells (CCs). Denuded zygotes were then transferred in groups of 50 to pre-equilibrated 4-well dishes containing 500 μL of CSU chemically defined media for in vitro culture of early embryos (CDM-1). Embryos were cultured for 56 h in a mixed gas incubator maintained at 38.5° C in 5% CO_2, 5% O_2, and 90% N_2. Embryos with ≥ 4 blastomeres were then transferred in groups of 35 to pre-equilibrated 4-well dishes containing 500 μL of CSU chemically defined media for in vitro culture of late embryos (CDM-2). Embryos were cultured for 120 h in a mixed-gas incubator maintained at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 (De La Torre-Sanchez et al., 2006). Blastocyst developmental rates were evaluated on day 7 (168 h) and day 8 (192 h) post-IVF. A schematic overview of the experimental design is shown in Figure 3.2.

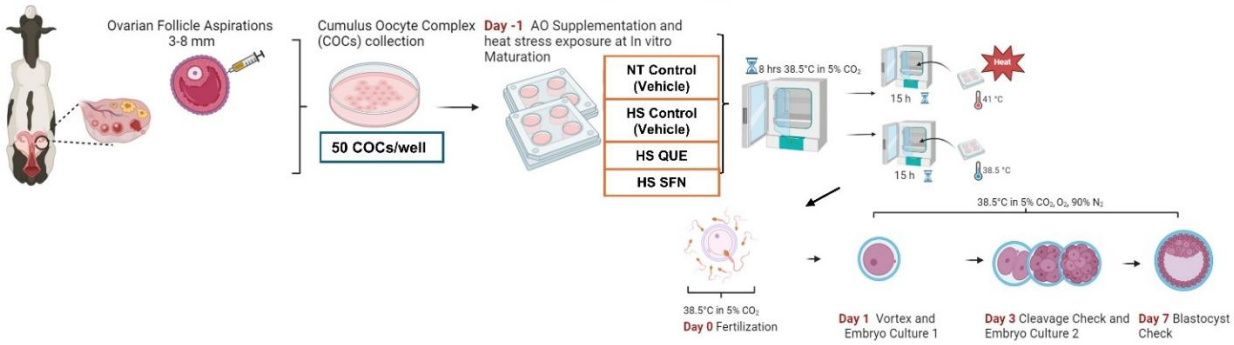


Figure 3.2 Experimental model for Bovine in vitro fertilization (IVF) and in vitro embryo culture (IVC).

3.6 Gene expression analysis of antioxidant and stress-related markers

The impact of antioxidants on modulating stress response genes in denuded oocytes, and CCs was investigated using qRT-PCR. Total RNA was extracted from four pooled replicates of denuded oocytes and CCs (50 COCs) and from blastocysts (10 blastocysts) using the RNeasy® plus micro kit (Qiagen; Hilden, Germany). Total RNA sample concentrations and integrity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). RNA samples were stored at −80° C until further use.

Following this, cDNA was synthesized by reverse transcription using oligo(dT)18 primers and the First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, USA). For each sample type, cDNA synthesis was performed using the maximum amount of high-quality RNA available per biological replicate, resulting in different RNA input amounts for denuded oocytes (22.2 ng), cumulus cells (29.4 ng), and blastocysts (16.8 ng). These differences reflect biological and technical limitations in RNA yield inherent to each sample type, rather than differences in experimental normalization. Gene expression analyses were conducted for each sample type, and relative expression levels were normalized to the geometric mean of the reference genes β-ACTIN and GAPDH. The geometric mean of expression levels of β-ACTIN and GAPDH was used to normalize

the expression of candidate genes. Gene-specific primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the list of primers is indicated in Table 3.1. Formal qRT-PCR analysis was performed using the CFX96 Touch Real-Time PCR Detection

Table 3.1. Sequence-specific primers used for qRT-PCR analysis.

Gene name	Accession number	Primer sequences
PTGS2	NM_174445	F5`-cgatgagcagttgttccaga-3′ R5′-gaaagacgtcaggcagaagg-3′
EGFR	XM_002696890	F5`-gacccgaaagaactggacat-3′ R5′-tgttatatccaggccgacaa-3′
PTX3	NM_001076259	F5`-acctgggattcaaagaaagg-3′ R5′-caccctcccagatattgaag-3′
NRF2	NM_001011678	F5′-cccagtcttcactgctcctc-3′ R5′-tcagccagcttgtcattttg-3′
SOD1	NM_174615	F5′-agaggcatgttggagacctg-3′ R5′-cagcgttgccagtctttgta-3′
HSP70	NM_001038505	F5′-aatgccagttgccaatgctg-3′ R5′-atcgagagttcctccaccca-3′
HSP90	NM_001012670	F5′-tcactgaggaaatgccaccc-3′ R5′-atggagacagagcgctgaac-3′
GRP78	NM_001075148	F5′-tgcgaagccctatagctgac-3′ R5′-agtaggtggtacccaggtcg-3′
GRP94	NM_174700	F5′-tgctgtgtggagagggaatg-3′ R5′-tcctgtgaccacaatcccaa-3′

β-ACTIN	NM_173979	F5′-tgtccaccttccagcagat-3′ R5′-tcaccttcaccgttccagt-3′
GAPDH	NM_001034034	F5′-aatggagccatcaccatc-3′ R5′-gtggttcacgcccatcaca-3′
CHSY1	NM_001191157.1	F5′-catggcgaggcccaggat-3′ R5′-tggaccacgtcctgaaggc-3′
EIF4A3	NM_001046188.2	F5′-tttgtggctgtggaacggga-3′ R5′-gtcagccagtcaaccttcctct-3′
CDK7	NM_001075715.1	F5′-aggccttggaacaaggaggatt-3′ R5′-tattgtgccttttcactgtttcct-3′
GSTO1	NM_001075214.2	F5′-accccggtgccagctttatc-3′ R5′-tgttgatgacttcatgccgga-3′
TPI1	NM_001013589.3	F5′-ccgcatcatttatgggggttct-3′ R5′-agctacctagtccctggctca-3′
YWHAG	NM_174793.2	F5′-gccccggatcatcctcgtcc-3′ R5′-ttcagctccgtcacgttcttca-3′
CCNA2	NM_001075123.2	F5′-taagacgcgacgggttgcac-3′ R5′-ggccaagacatcttcactttctga-3′
LSM4	NM_001035436.2	F5′-gccgggcgaggtgtgttc-3′ R5′-tcgtctcgggaatggaggga-3′
HMOX1	NM_001014912.1	F5′-gcaaggtgcaagacttggct-3′ R5′-cacatggcgtaaagccccac-3′
PRDX1	NM_174431.1	F5′-tcctatttcagtggaactgat-3′ R5′-aagcaatgatctccgtgggg-3′
CAT	NM_001035386.2	F5′-tgggacccaactacttccag-3′ R5′-aagtgggtcctgtgttccag-3′

System (Bio-Rad; Hercules, CA, United States). The following thermocycling conditions were applied for amplification: initial denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 15 s, and 60 °C for 60 s. The specificity of the amplification was determined by melting curve analysis generated at the end of each run, and the data were analyzed using the comparative C(T) (2^−ΔΔCt) method (Schmittgen and Livak, 2008). Statistical analyses were performed on the ΔΔCt values, while fold-change values (2^−ΔΔCt) were used for data presentation in the figures.

3.7 Proximity ligation assay of KEAP1-NRF2 protein-protein interactions

Following the manufacturer's NaveniFlex proximity ligation assay (PLA) protocol (Navinci Diagnostics, Uppsala, Sweden), the embryos were counterstained using two primary antibodies (NRF2 polyclonal antibody, Invitrogen; Carlsbad, CA, USA, unconjugated, rabbit polyclonal antibody, PA138312; KEAP1 (G-2), Santa Cruz Biotechnology; Dallas, TX, USA, mouse monoclonal antibody, sc-365626) and mounted on a poly-L-lysine-coated slide (Newcomer Supply; Middleton, WI, United States) with 5–10 μL of ProLong Diamond Antifade Mountant (Invitrogen; Carlsbad, CA, USA) and a coverslip, allowing them to dry flat (>24 h). PLA/DAPI-labeled embryos were imaged using the LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective with fitted blue (345/455) and red (596/615) fluorescent filters. For the analysis, fluorescent images were converted to grayscale, puncta counts and total cell counts were obtained using ImageJ software (National Institute of Health, Bethesda, MD, USA). The ratio of PLA puncta count normalized to total cell number from individual blastocysts was subsequently calculated. For each treatment group, 6–11 blastocysts were analyzed.

3.8 Mitochondrial function assays

3.8.1 Intracellular Reactive Oxygen Species accumulation assay

The impact of thermal stress on intracellular ROS accumulation was assessed using 10 μM 2', 7'-Dichlorofluorescein Diacetate (H2DCFDA) (Sigma Aldrich; St. Louis, MO, USA) on bovine denuded oocytes, and blastocysts. ROS accumulation in denuded oocytes (n = 20 per treatment) and blastocysts (n = 14 per treatment,) was assessed as described in Chapter 2. Briefly, denuded oocytes and blastocysts from each treatment group were washed twice in phosphate-buffered saline supplemented with 0.1% polyvinylpyrrolidone (DPBS (1x)-PVP) (Sigma Aldrich; St. Louis, MO, USA). Samples were then incubated for 30 min at 38.5°C in 50 μL drops of 10 μM H2DCFDA dissolved in H-CDMM (for oocytes) or HCDM-2 (for blastocysts). Following incubation, oocytes and blastocysts were washed three times in DPBS (1x) containing PVP (0.1%) and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Fluorescence intensity was quantified from .jpg images by manually selecting the oocyte or blastocyst area. All images were acquired with identical exposure and light settings, avoiding signal saturation, using a green fluorescent filter (excitation 494 nm, emission 518 nm). Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

3.8.2 Mitochondrial Membrane Potential δΨm Assay

The detrimental effect of heat stress on mitochondrial depolarization was analyzed using the cationic dye 5,5',6,6'-tetrachloro1,1',3,3' tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, USA) on denuded oocytes, and blastocysts. MMP (δΨm) in oocytes (duplicates; a total of 20 oocytes per treatment) and blastocysts (duplicates; a total of 14-15 blastocysts per treatment) was evaluated using the cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen, Carlsbad, CA, USA), dissolved in H-CDMM for oocytes and HCDM-2 media for blastocysts, as an indicator of mitochondrial activity, as previously described (Pasquariello et al., 2019). Briefly, denuded mature oocytes and blastocysts

from the respective treatment groups were incubated with 2 μM JC-1 for 30 min in the dark at 38.5 °C under their optimal culture conditions. Following incubation, samples were washed three times with DPBS (1×) supplemented with 0.1% PVP and immediately imaged using an inverted laser scanning confocal microscope. Oocytes were imaged using the ZEISS LSM800 equipped with a 20× air objective, while blastocysts were imaged using the ZEISS LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective.

Fluorescence intensity was quantified from .jpg (for GCs) and .czi (for oocytes and blastocysts) images obtained from different biological replicates. Imaging for J-monomers was conducted using a wavelength of 488 nm for J-monomers and excitation/emission settings at 490/525 nm (FITC filter, green fluorescence), indicative of low MMP. Similarly, images for J-aggregates were carried out using a wavelength of 561 nm and excitation/emission at 596/615 nm (TRITC filter/red fluorescence), indicating high MMP. Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA). The ratio of red to green fluorescence was subsequently calculated to determine MMP, expressed as an arbitrary unit.

3.8.3 Oxygen consumption rate and extracellular acidification rate assays

Metabolic analyses of individual day 7 blastocysts (n=4-17) were performed using a microchamber with electrochemical-based oxygen and pH sensors, as described (Y. Obeidat et al., 2019; Y. M. Obeidat et al., 2019). Briefly, the microchamber was filled with 150 μL of CSU chemically defined media (CDM-2). Then individual embryos were placed in the middle of each of the six channels containing CDM-2 media. The three-electrode oxygen sensor was connected to a potentiostat (Quadstat EA 164H, eDAQ Inc., Colorado Springs, CO) that applied a voltage to the sensor and monitored the decrease in oxygen-reduction current over time. The applied potential for all amperometric oxygen assays was set to −0.6 V. The pH sensor was connected to a custom-made

Ina333 instrumentation amplifier circuit that measured the change in voltage. The starting (room air-saturated) oxygen concentration and pH of the media were measured as baseline values for 2 min and calibrated as previously described in detail (Obeidat et al. 2019).

3.9 Statistical analysis

Data were analyzed in GraphPad Prism version 8.4.2 (GraphPad; San Diego, CA, United States). For experiments in which all treatments were tested at both temperatures, a two-way Analysis of Variance (ANOVA) was performed with temperature and treatment as fixed factors, followed by Tukey's multiple-comparison test. When treatments were applied at only one temperature (HS), data were analyzed using one-way ANOVA, and Tukey's post hoc test was used to compare treatment groups with the corresponding temperature-matched control. Data are presented as the Mean ± SEM of biological replicates. Statistical significance was identified at $p \leq 0.05$.

4. Results

4.1 Antioxidant supplementation effectively mitigates the detrimental effects of heat stress during oocyte maturation via modulating the physiology of the surrounding cumulus cells

Transcript levels in denuded oocytes remained largely unaltered across all treatment groups, with variability observed only for *GRP78*, which was significantly increased in the HS control group compared to both the NT control and antioxidant-treated groups (Figure 3.3). CCs displayed distinct transcriptional responses to antioxidant supplementation under HS conditions (Figure 3.4A). Our results showed that *HSP90* expression was reduced in the QUE-treated group compared to the HS control (*p=0.04*), while *GRP94* expression was also lower in the QUE-treated group (*p=0.008*). Similarly, *SOD1* expression was up-regulated in CCs from both the QUE group (*p=0.003*), and the SFN group (*p=0.0001*). Also, HS SFN showed increased expression of *CAT* (*p=0.02*) compared to

the HS control group. ROS accumulation assay revealed that both antioxidants successfully reduced ROS accumulation (Figure 3.4B). Consistently, the MMP assay assessed by JC-1 staining was enhanced in both the QUE (*p=0.0004*) and SFN-treated (*p<0.0001*) groups compared to the HS control group (Figure 3.4C).

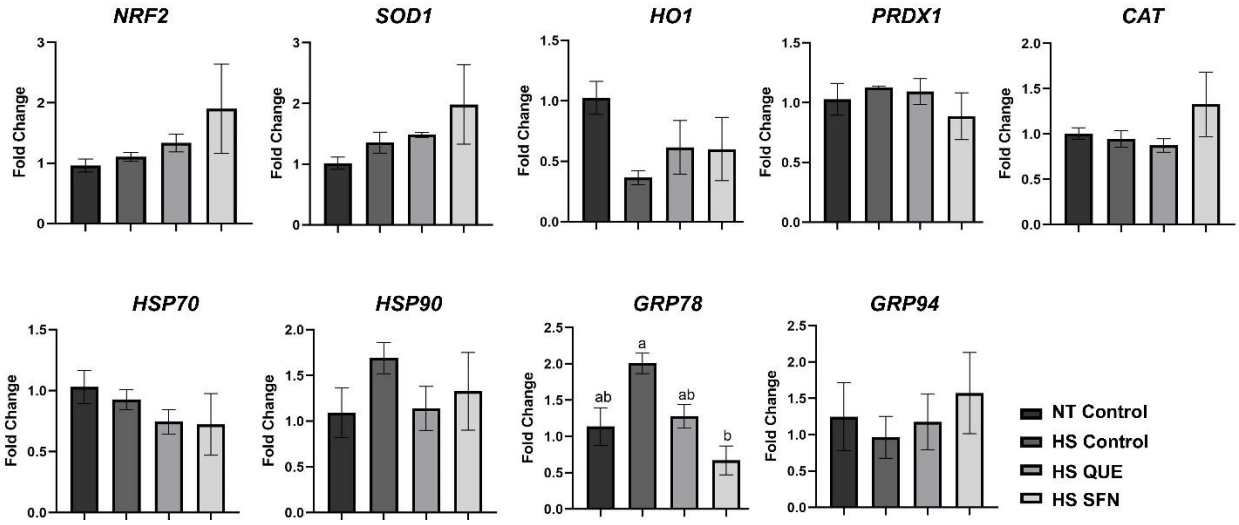
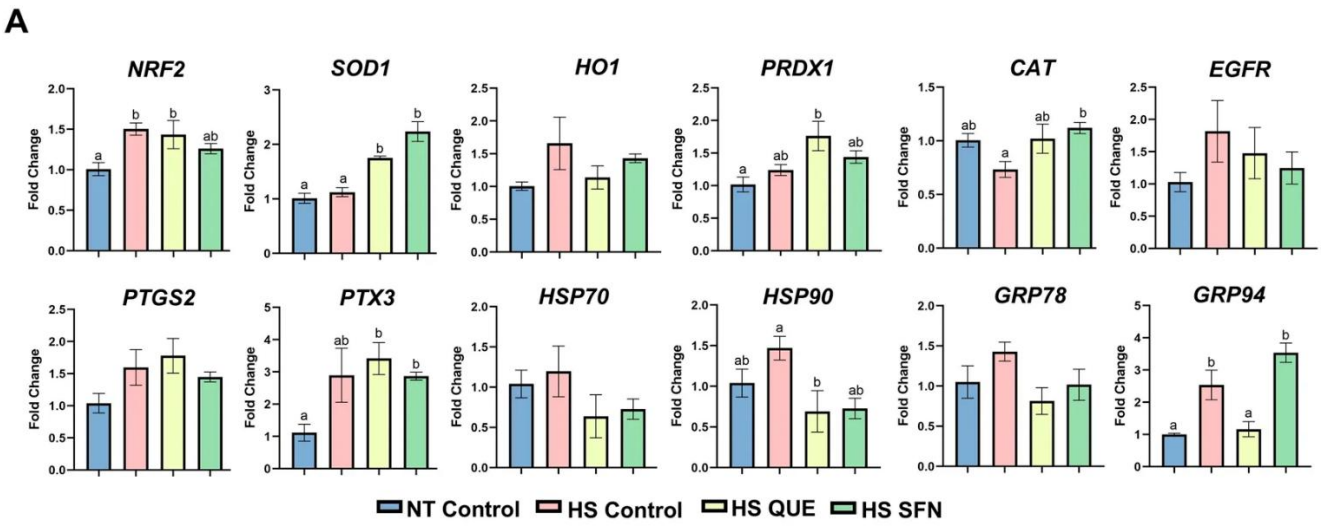
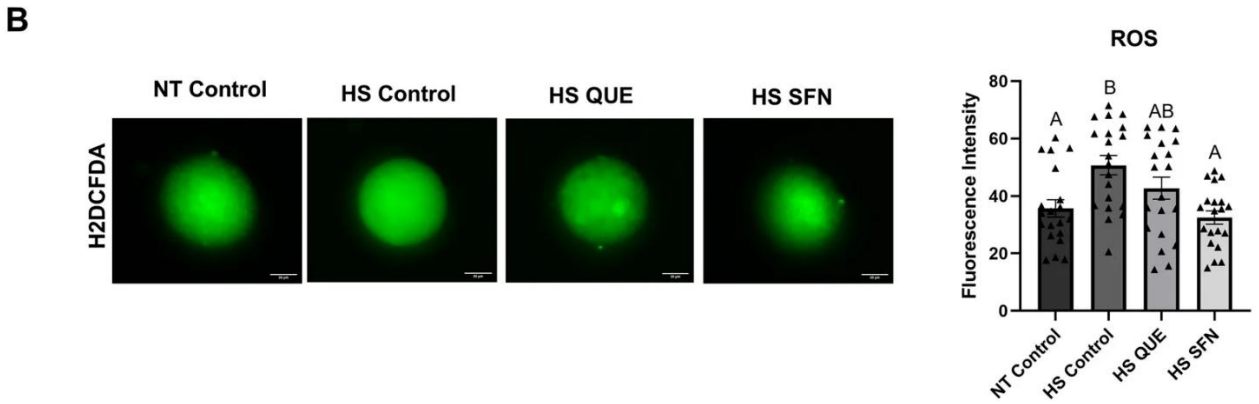


Figure 3.3 The effect of antioxidant supplementation on mitigating HS effects on denuded oocyte transcripts. Stress-related gene expression analysis in cumulus cells, qRT-PCR was performed to quantify selected stress-related genes *NRF2, SOD1, HO1, CAT, PRDX1, HSP70, HSP90, GRP78*, and *GRP94*. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Different lowercase letters indicate significant differences between treatment groups.


A
NRF2
SOD1
HO1
PRDX1
CAT
EGFR
PTGS2
PTX3
HSP70
HSP90
GRP78
GRP94
Fold Change
NT Control
HS Control
HS QUE
HS SFN


B
NT Control
HS Control
HS QUE
HS SFN
H2DCFDA
ROS
Fluorescence Intensity
A
B
AB
A

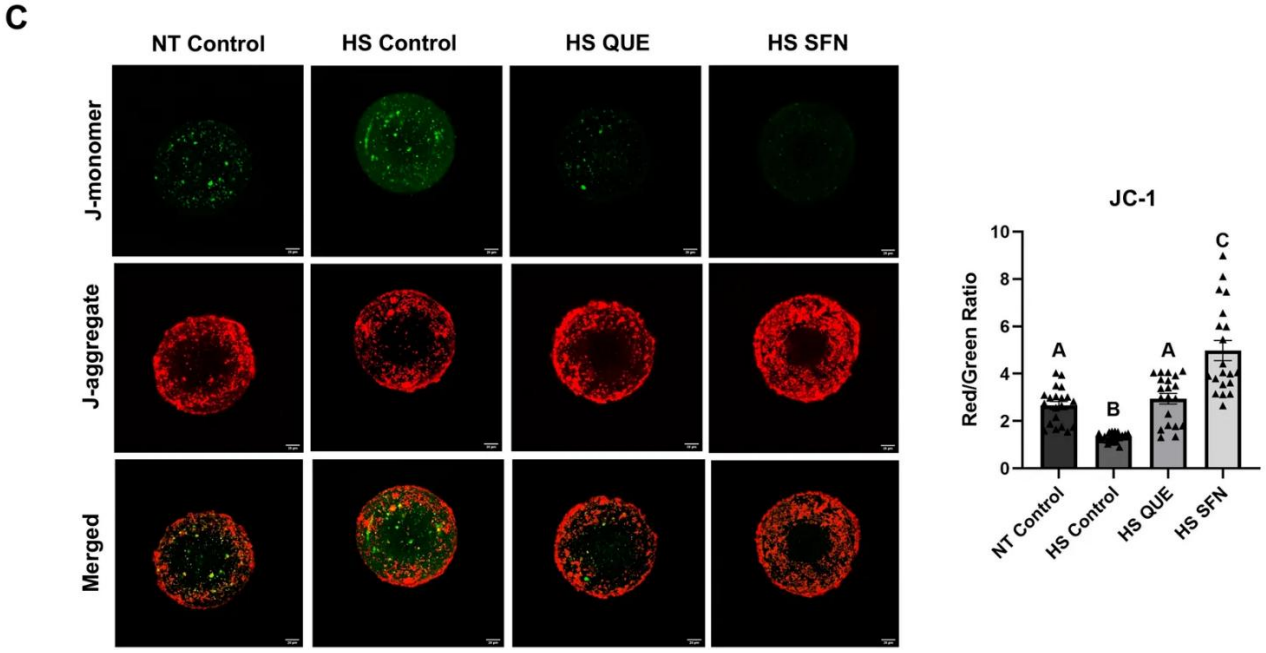

C
NT Control
HS Control
HS QUE
HS SFN
J-monomer
J-aggregate
Merged
JC-1
Red/Green Ratio
A
B
A
C

Figure 3.4. The protective effect of antioxidant supplementation mitigating HS effects on cumulus cells transcripts and oocyte function following maturation. (**A**) Relative mRNA abundance of stress-related genes (*NRF2, SOD1, HO1, CAT, PRDX1, HSP70, HSP90, GRP78, GRP94*) and cumulus expansion markers (*PTGS2, EGFR, PTX3*) in cumulus cells, determined by qRT-PCR. (**B**) Intracellular reactive oxygen species (ROS) levels in single oocytes were measured by H2DCFDA fluorescence. Quantification was performed in triplicate (15–20 oocytes per treatment). Representative fluorescence images are shown. Scale bar = 20 μm. (**C**) Mitochondrial membrane potential (δΨm) assessed by JC-1 staining and expressed as the red/green fluorescence ratio. Quantification was performed in duplicate (15–20 oocytes per treatment). Representative fluorescence images are shown. Scale bar = 20 μm. Data are presented as mean ± SEM. Different uppercase letters indicate significant differences among treatment groups (one-way ANOVA followed by Tukey's test; $p \leq 0.05$).

4.2 The effect of antioxidant supplementation during oocyte maturation on the developmental capacity of oocytes following in vitro fertilization

As shown in Figure 3.5A, compared to those cultured under thermoneutral conditions, exposure of oocytes to HS results in a drastic reduction in the number of oocytes that develop to the blastocyst stage following IVF ($p<0.0001$). However, priming oocytes with both QUE and SFN resulted in higher blastocyst rates compared to the non-supplemented HS group with $p<0.0001$ and $p=0.0002$, respectively, following the same trend in blastocyst rate from cleaved embryos (Figure 3.5B). Similarly, while HS reduced total cell number per blastocyst compared to the NT group ($p=0.0166$; Figure 3.5C), supplementation with QUE and SFN restored the cell number to the level comparable to the NT group.

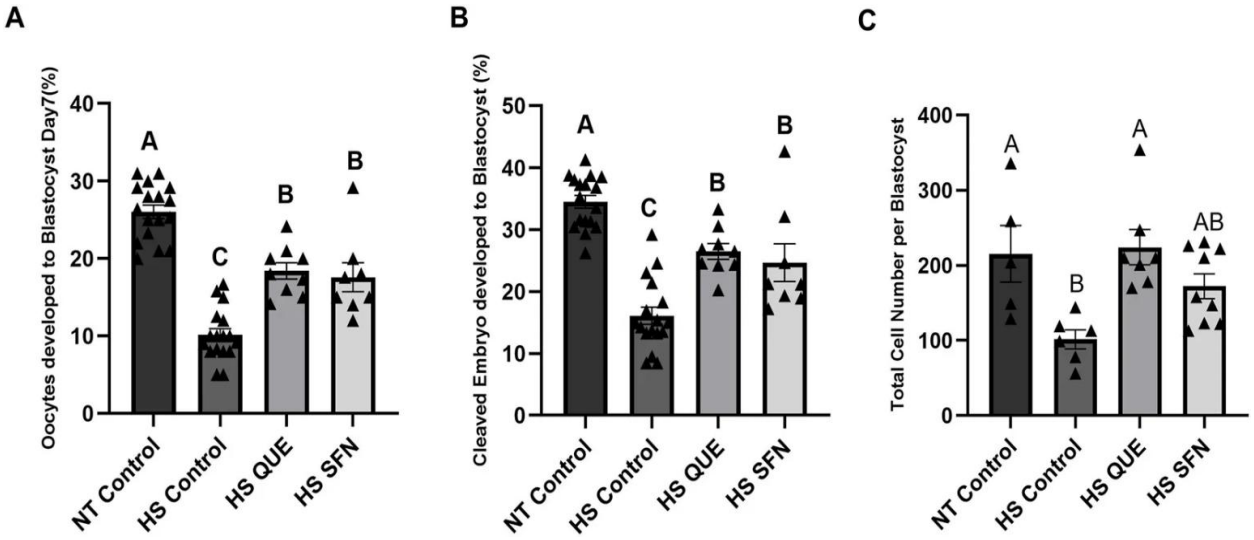


Figure 3.5 The effect of antioxidant supplementation on the developmental capacity of oocytes matured under Heat Stress. Results are from a total of 8-9 replicates (780–900 total oocytes per treatment). (**A**) Percentage of oocytes developed into blastocysts. (**B**) Percentage of cleaved embryos developed to blastocyst. (**C**) Total cell number per single blastocyst: quantification was carried out in quadruplicate (a total of 5–10 blastocysts per treatment). Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters indicate a statistically significant difference between treatment groups ($p \leq 0.05$).

4.3 Antioxidant supplementation under heat conditions during in vitro maturation modulates ROS accumulation and mitochondrial membrane potential of the resulting blastocysts

To elucidate the long-term impact of antioxidant priming and HS exposure during oocyte maturation on the molecular architecture and viability of the resulting blastocysts, various metabolic and molecular parameters were investigated. As shown in Figure 3.6A, although IVF and IVC were conducted under thermoneutral conditions without antioxidant supplementation, priming oocytes

with QUE or SFN effectively restored ROS levels in blastocysts to those observed under thermoneutral conditions, compared with HS groups (HS QUE, $p=0.0024$; HS SFN, $p=0.0002$). Furthermore, both antioxidant treatments successfully recovered MMP of blastocysts compared with the non-supplemented HS control (HS QUE, $p=0.0008$; HS SFN, $p<0.0001$) (Figure 3.6B).

To further investigate the impact of antioxidant supplementation on the activity of the NRF2-KEAP1 signaling pathway in the resulting blastocysts, the highly sensitive Proximity Ligation Assay (PLA) was used to assess protein-protein interactions between KEAP1 and NRF2 (Figure 3.6C). In representative images of Naviflex Puncta (red fluorescence), the cytoplasmic binding between NRF2 and KEAP1 across different treatment groups was visualized as puncta. Antioxidant supplementation during IVM promoted dissociation of KEAP1-NRF2 complexes, as evidenced by reduced puncta count per blastomere compared with the HS control group (HS QUE $p=0.0007$; HS SFN $p<0.0001$). Puncta counts were normalized to the total cell number of each corresponding embryo.

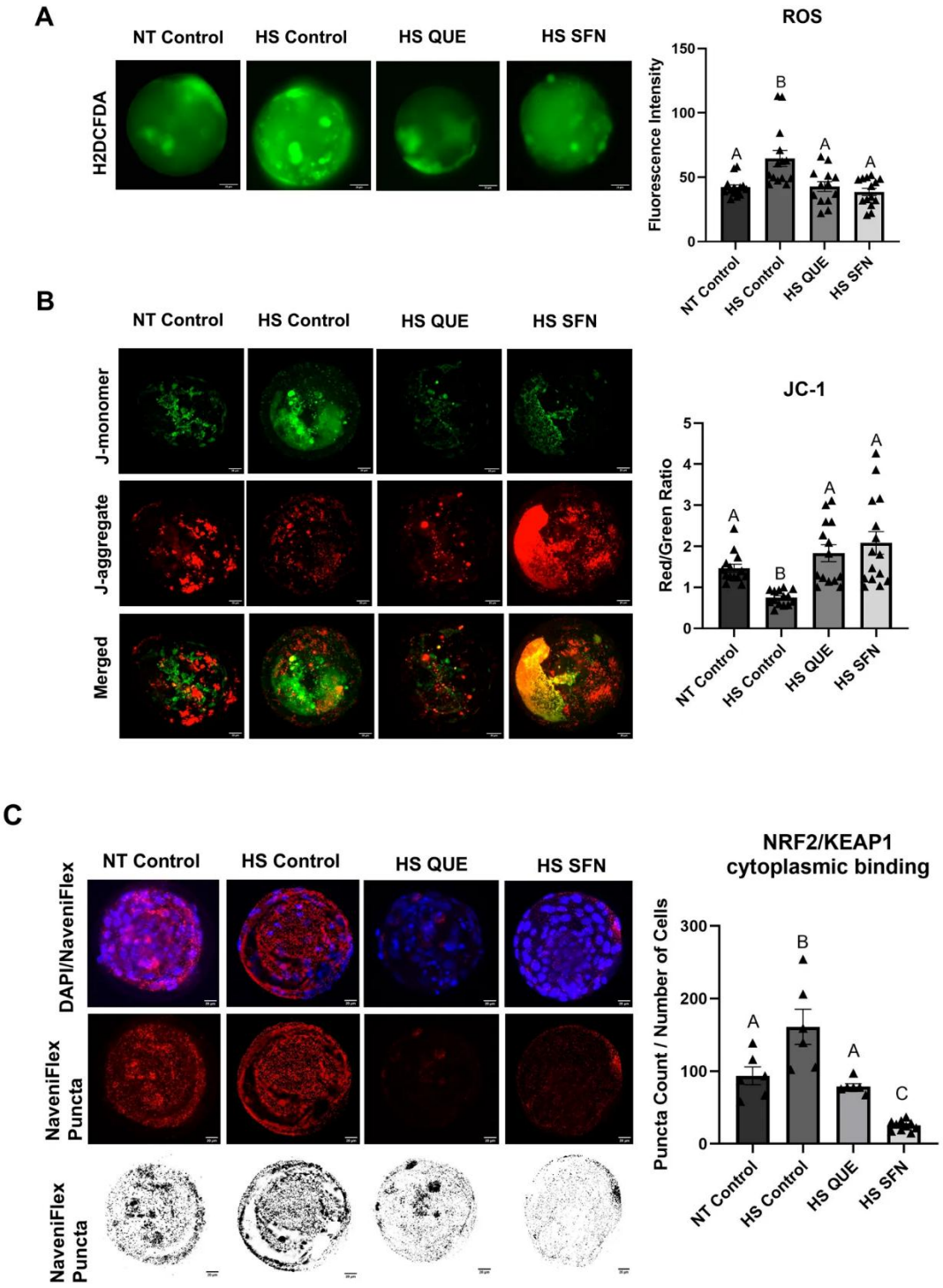


Figure 3.6 Functional alterations of antioxidant supplementation under HS conditions during in vitro maturation on blastocyst quality and NRF2-KEAP1 Protein-Protein Interaction. (**A**) Intracellular reactive oxygen species (ROS) levels in single blastocysts measured by H2DCFDA

fluorescence. Quantification was performed in triplicate (12–15 blastocysts per treatment). Representative fluorescence images are shown. Scale bar = 20 μm. (**B**) Mitochondrial membrane potential (δΨm) assessed by JC-1 staining and expressed as the red/green fluorescence ratio. Quantification was performed in duplicate (12–15 blastocysts per treatment). Representative fluorescence images are shown. Scale bar = 20 μm. (**C**) KEAP1–NRF2 protein-protein interaction was evaluated by Proximity Ligation Assay (PLA) and confocal microscopy. Representative images show PLA puncta (red fluorescence), indicating cytoplasmic KEAP1–NRF2 binding. Puncta counts were normalized to total cell number, with 6–10 embryos analyzed per treatment across four replicates. Fewer puncta indicate increased availability of free, active NRF2. Data are presented as mean ± SEM. Different uppercase letters indicate significant differences among treatment groups (one-way ANOVA followed by Tukey's test; $p \leq 0.05$).

4.4 Metabolic function of preimplantation embryos derived from antioxidant primed oocytes matured under heat stress conditions

In the present study, we integrated a metabolic multi-sensor platform capable of monitoring a single embryo oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real-time, by applying amperometric methods described in (Obeidat, et al. 2019). The use of this microchamber enables monitoring of single-embryo metabolism during development in a small volume of media. OCR, which is an indicator of aerobic metabolism, was measured in fmol per second per embryo. ECAR is an indirect marker of anaerobic metabolism, and it was measured in mpH per minute per embryo. As shown in Figure 3.7A, supplementation with both QUE and SFN reduced OCR to the thermoneutral level, compared with HS groups (HS QUE, *p=0.0078*; HS SFN, *p=0.0048*). Similarly, ECAR values were reduced to levels comparable to the thermoneutral embryos when compared to

the HS control group (HS QUE *p=0.0009*; HS SFN *p=0.0067*) (Figure 3.7B).

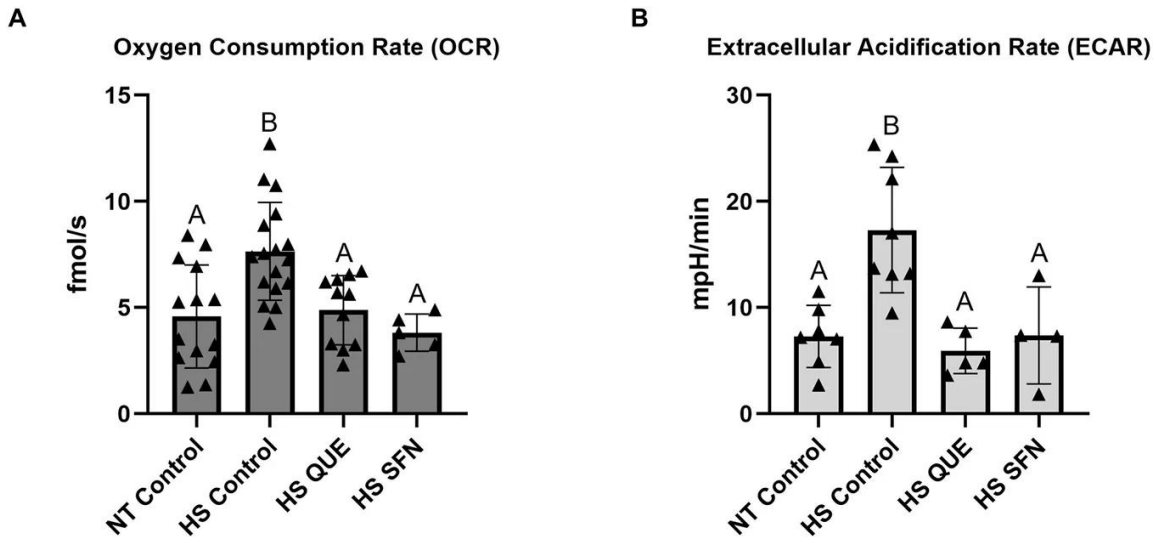


Figure 3.7 Metabolic function of preimplantation embryos from antioxidant-treated oocytes matured under HS conditions. The Metabolic multisensor platform was used on single pre-implantation embryos to quantify: (**A**) Oxygen Consumption Rate (OCR) in quercetin and sulforaphane treatments under HS conditions measured in single embryos (n = 4–17 per group). (**B**) Extracellular Acidification Rate (ECAR) in quercetin and sulforaphane treatments under HS conditions measured in single embryos (n = 4-7 per group). Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters show a statistically significant differences between treatment groups with $p \leq 0.05$.

4.5 Antioxidant supplementation during oocyte maturation restored the embryonic competence index in resulting blastocysts

As shown in Figure 3.8C, the ECI values were reduced for blastocysts derived from thermal-stressed oocytes compared with the thermoneutral group (*p=0.0070*). However, supplementation with QUE and SFN during oocyte maturation under thermal stress conditions rescues the ECI of the resulting

blastocysts compared with non-treated HS controls (*p=0.0052* and *p=0.0054*, respectively). Among the competence-associated genes (Figure 3.8A), CHSY1 was significantly reduced in the nontreated HS control group compared to the thermoneutral control group (*p=0.0339*). However, the supplementation of SFN increased CHSY1 expression compared to the HS control group (*p=0.0008*). Among the non-competence-associated genes (Figure 3.8B), CCNA2 was upregulated in the HS control group compared to the NT control (*p=0.0149*), while supplementation of QUE and SFN downregulated CCNA2 expression as compared to the HS control (*p=0.0464* and *0.0047*, respectively). Moreover, QUE supplementation reduced EIF4A3 expression compared to the HS control group (*p=0.0180*). Collectively, supplementation with QUE and SFN during oocyte maturation under thermal stress conditions rescues the ECI of the resulting blastocysts compared with their non-treated thermal-stressed counterparts (Figure 3.8C).

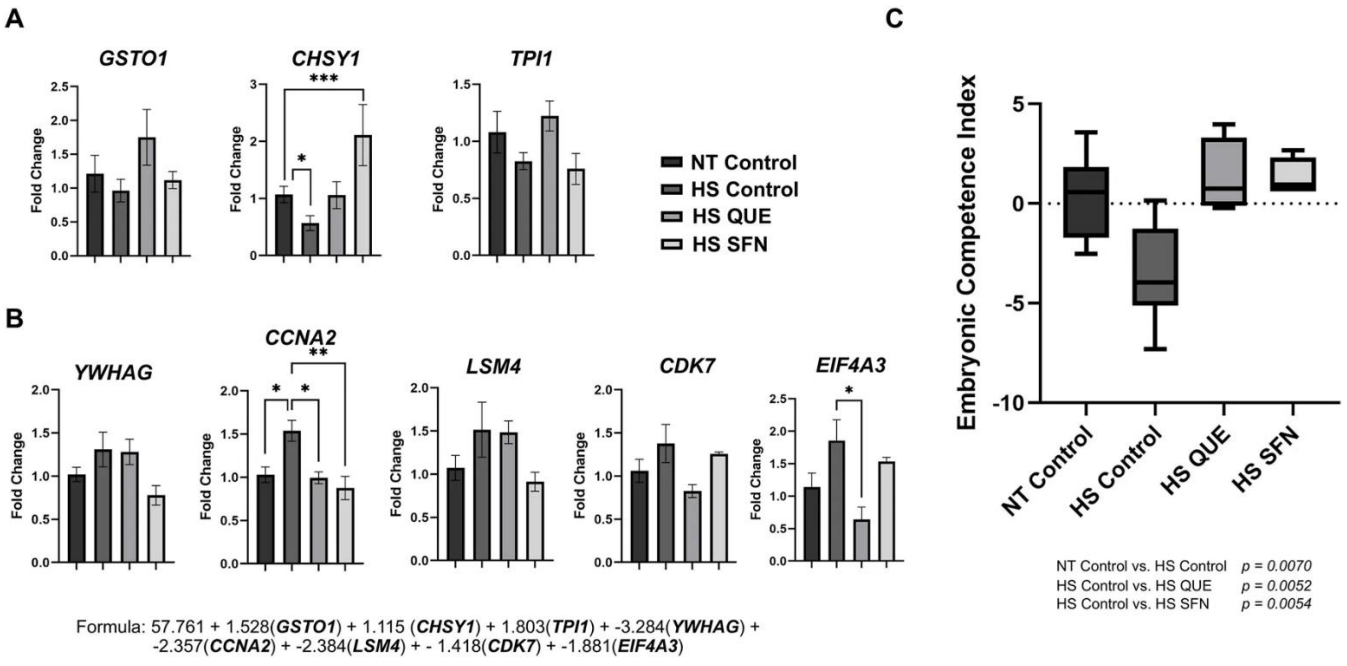


Figure 3.8 The Embryonic Competence Index (ECI) is restored by antioxidant supplementation during IVM under HS. The ECI is a predictive model based on a Bayesian multiple regression formula using transcriptomic biomarkers associated with embryo viability. qRT-

PCR was performed to quantify (**A**) competence-associated genes (*GSTO1, CHSY1*, and *TPI1*) and (**B**) noncompetence-associated genes (*YWHAG, CCNA2, LSM4, CDK7,* and *EIF4A3*). (C) ECI values were calculated using the validated eight-gene predictive formula ($R^2 = 0.96$). Data are presented as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons. For individual genes, significance is denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. P-values for ECI comparisons are indicated in the graph.

5. Discussion

Environmental thermal stress is a major factor compromising oocyte quality, embryonic development, and fertility in cattle through oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis (Amaral et al., 2020; Gaskins et al., 2021; Sammad et al., 2020). Oocytes are particularly susceptible to thermal and oxidative insults, with sensitivity reported as early as 105 days before ovulation and persisting through ovulation and fertilization (Torres-Júnior et al., 2008). Given this prolonged window of susceptibility, impaired developmental outcomes under HS conditions are largely attributed to compromised oocyte quality rather than sperm-associated factors (Rahman et al., 2018; Roth, 2017). Therefore, the objective of this chapter was to determine whether supplementation with plant-derived antioxidants during in vitro maturation (IVM) under HS conditions could mitigate oxidative and mitochondrial damage in oocytes and improve the developmental competence of the resulting embryos.

Excess ROS accumulation within the follicular microenvironment deteriorates oocyte competence by inducing granulosa and cumulus cell apoptosis, disrupting cumulus–oocyte communication, altering spindle assembly and cytoskeletal dynamics, and accelerating ovarian aging through mitochondrial dysfunction, telomere shortening, inflammation, and apoptosis (Prasad et al., 2016;

Yaacobi-Artzi et al., 2022; Yang et al., 2021). Consistent with these reports, HS in our model significantly increased ROS accumulation and reduced mitochondrial membrane potential (MMP) in oocytes (Figure 2B, C), despite minimal changes in transcript abundance within the oocyte itself (Suppl. Figure 4). In contrast, cumulus cells exhibited clear activation of antioxidant pathways, as both QUE and SFN increased the expression of NRF2 downstream genes (Figure 2A). Importantly, antioxidant supplementation reduced ROS levels to values comparable to non-heat-stressed controls while restoring MMP, demonstrating preservation of mitochondrial function during IVM. These findings align with previous studies showing that SFN alleviates oxidative stress in mouse oocytes (Li et al., 2023) and protects bovine oocytes against paraquat-induced toxicity through NRF2-mediated mechanisms (Feng et al., 2023). Together, these observations reinforce the critical role of the cumulus compartment in mediating antioxidant protection during oocyte maturation and support the use of QUE and SFN as targeted interventions against HS-induced oxidative stress.

Thermal stress exposure during oocyte maturation also induced a pronounced carryover effect on subsequent embryo development, resulting in reduced blastocyst development (Figure 3A, B). This developmental impairment was accompanied by elevated ROS levels and decreased MMP in blastocysts (Figure 4A, B), consistent with our observations in oocytes. To further evaluate whether antioxidant supplementation during IVM influenced NRF2 signaling in embryos, we assessed KEAP1–NRF2 interactions using a highly sensitive protein–protein interaction assay (Figure 4C). Embryos derived from heat-stressed oocytes exhibited abundant KEAP1–NRF2 puncta, indicating cytoplasmic sequestration of NRF2. In contrast, QUE and SFN supplementation significantly reduced puncta counts per blastomere, suggesting increased availability of free, active NRF2. These findings parallel our observations during oocyte maturation and are consistent with previous studies demonstrating that QUE activates NRF2 signaling and improves mitochondrial activity and

developmental outcomes in bovine embryos (Khadrawy et al., 2020b).

HS is known to exert particularly detrimental effects during early embryonic development prior to zygotic genome activation (ZGA), contributing significantly to early pregnancy loss in cattle (de Barros and Paula-Lopes, 2018). Recent advances in embryo biology have identified molecular markers predictive of embryonic competence, leading to development of the Embryonic Competence Index (ECI), which estimates the likelihood of embryo survival and pregnancy establishment (Rabaglino et al., 2023; Rabaglino and Hansen, 2024). In the present study, antioxidant supplementation during IVM restored ECI values under HS conditions and was accompanied by reduced oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) compared with HS controls (Figure 5A, B). These findings indicate that embryo competence is closely associated with metabolic efficiency and reduced energetic expenditure (Leese et al., 2022). The normalization of OCR and ECAR following antioxidant supplementation is consistent with the "Quiet Embryo Hypothesis," which proposes that developmentally competent embryos exhibit lower metabolic activity than less viable counterparts (Leese, 2002; Leese et al., 2022). Thus, QUE and SFN not only stabilized embryonic metabolism but also positively influenced molecular pathways associated with embryonic competence and pregnancy establishment.

Collectively, our findings demonstrate that supplementation with QUE and SFN during oocyte maturation under HS conditions attenuates oxidative damage, preserves mitochondrial function, stabilizes embryonic bioenergetics, and restores embryonic competence through activation of the NRF2-mediated antioxidant response pathway in oocytes, cumulus cells, and resulting blastocysts. To our knowledge, this is the first report demonstrating that QUE and SFN can effectively reverse the long-term consequences of HS during oocyte maturation, including impaired metabolic efficiency and altered embryonic competence in preimplantation embryos. These findings provide a

strong mechanistic foundation for integrating NRF2-activating antioxidant strategies into assisted reproductive technologies (ARTs) aimed at improving reproductive outcomes under environmental stress conditions in livestock species.

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CHAPTER IV—Modulation of the NRF2 pathway mitigates oxidative damage and enhances metabolic competence in bovine preimplantation embryos

1. Overview

Assisted reproductive technologies (ARTs) are essential tools for improving genetic gain in livestock and for overcoming infertility associated with ovulation failure, impaired fertilization, and compromised early embryonic development. Among these technologies, in vitro embryo production (IVP) systems are widely used; however, embryos generated in vitro are highly susceptible to oxidative stress due to the absence of key in vivo protective components, particularly endogenous antioxidant defenses. Excessive accumulation of reactive oxygen species (ROS) during embryo culture disrupts mitochondrial function, impairs developmental competence, and compromises embryo viability. In response to oxidative stress, embryos activate cytoprotective pathways, including the Nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway, a master regulator of the cellular antioxidant response. Antioxidants such as quercetin (QUE) can disrupt KEAP1-mediated inhibition, thereby promoting NRF2 activation and induction of downstream antioxidant defense mechanisms. The objective of this study was to determine whether transient activation of NRF2 through QUE supplementation during the in vitro culture (IVC) period could improve embryo survival, mitochondrial function, and developmental competence under suboptimal culture conditions. Using a bovine IVP model, embryos were cultured under either physiological oxygen tension (5% O_2) or atmospheric oxygen tension (20% O_2) to induce oxidative stress, with or without supplementation of 5 μM QUE during early embryo culture. Developmental competence was assessed through blastocyst development rates, mitochondrial membrane potential (MMP), ROS accumulation, and embryo metabolic profiling

using oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements. In addition, NRF2 activation dynamics were evaluated through proximity ligation assay (PLA) analysis to elucidate KEAP1–NRF2 protein interactions, while embryo sex was determined through PCR amplification of the SRY and BSP genes. Exposure to 20% oxygen significantly impaired embryo developmental competence, increased ROS accumulation, reduced MMP, and elevated metabolic activity compared with embryos cultured under physiological oxygen conditions of 5%. In contrast, QUE supplementation restored mitochondrial function, reduced oxidative stress, normalized OCR and ECAR profiles, and improved developmental outcomes in both male and female embryos. Furthermore, PLA analysis showed that QUE supplementation reduced KEAP1–NRF2 protein interactions under oxidative stress conditions, suggesting enhanced NRF2 activation and antioxidant signaling. These findings provide mechanistic evidence on the role of QUE in modulating the NRF2-mediated antioxidant defenses during preimplantation development and suggest the use of targeted antioxidant strategies to improve IVP embryo quality and ART outcomes in livestock species.

2. Introduction

Sustainable agriculture is essential for ensuring global food security, a central objective of the United Nations Sustainable Development Goals. According to the Food and Agriculture Organization (FAO), global food production must increase by approximately 60% by 2050 to meet the demands of a population expected to exceed nine billion people in the same year (Nations, 2009). The cattle industry plays a critical role in achieving this goal, relying heavily on reproductive efficiency to sustain the production of milk, meat, and other animal-derived products. However, fertility remains a major challenge in both dairy and beef production systems. Early embryonic mortality accounts for a substantial proportion of reproductive losses in cattle despite fertilization rates exceeding 90%

(Diskin et al., 2006). Consequently, assisted reproductive technologies (ARTs) have become indispensable tools for improving reproductive efficiency and accelerating genetic gain. In particular, the combination of in vitro embryo production (IVP), genomic selection, and sexed semen technologies has transformed modern cattle breeding programs by enabling the rapid dissemination of superior genetics (Sirard, 2018; Ferré et al., 2020). The widespread adoption of these technologies has resulted in in vitro-produced embryos representing the majority of bovine embryos transferred worldwide. Despite these advances, IVP systems remain substantially less efficient than natural reproduction. Blastocyst development rates generally range between 30 and 40%, and pregnancy outcomes following embryo transfer are often inferior to those achieved using in vivo-derived embryos (Hasler, 2000; Daly et al., 2020). Furthermore, embryos produced under in vitro conditions frequently exhibit altered gene expression profiles, reduced cryotolerance, impaired developmental competence, and compromised pregnancy outcomes compared with their in vivo counterparts (Cagnone and Sirard, 2016; Ramos-Ibeas et al., 2019). These observations indicate that the in vitro environment imposes physiological stressors capable of disrupting normal embryonic development and limiting the overall efficiency of ARTs.

Among the various stressors associated with IVP systems, oxidative stress is considered one of the principal factors compromising embryo development (Guerin et al., 2001; Takahashi, 2012; Agarwal et al., 2022). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the antioxidant capacity of the cell, resulting in oxidative damage to DNA, proteins, lipids, and organelles (Dennery, 2010; Agarwal et al., 2012). Although controlled ROS production is necessary for normal cellular signaling and developmental processes, excessive ROS accumulation disrupts cellular homeostasis and negatively affects embryo viability (Dennery, 2010; Takahashi, 2012). During in vivo development, oocytes and embryos are protected by an extensive antioxidant network

present within the follicular, oviductal, and uterine environments (El Mouatassim et al., 2000; Castillo et al., 2003; Gupta et al., 2008). In contrast, embryos cultured in vitro lack many of these endogenous protective mechanisms and are exposed to environmental conditions that promote ROS generation (Martin-Romero et al., 2008; Agarwal et al., 2022). Factors such as oxygen tension, media composition, pH fluctuations, temperature changes, and routine laboratory handling procedures have all been shown to contribute to oxidative stress during embryo culture (Goto et al., 1993; Harvey, 2007; Ramos-Ibeas et al., 2019). Consequently, oxidative stress is now recognized as a major contributor to defective embryo development in both livestock and human ART systems (Agarwal et al., 2022; Ramos-Ibeas et al., 2019).

Among the environmental factors influencing oxidative stress, oxygen concentration is particularly important. Physiological oxygen levels within the female reproductive tract range between approximately 2 and 8%, whereas atmospheric oxygen concentrations commonly used during embryo culture are approximately 20% (Harvey, 2007; Ciray et al., 2009). Exposure to atmospheric oxygen has consistently been associated with increased ROS production, impaired blastocyst development, altered cellular metabolism, and reduced embryo quality (Ciray et al., 2009; Li et al., 2016). Although low-oxygen culture systems are increasingly recommended, many IVF laboratories continue to expose embryos to atmospheric oxygen concentrations during at least part of the culture period (Christianson et al., 2014). Such conditions create an oxidative environment that differs markedly from those encountered during normal development and may adversely affect embryonic competence. Mitochondria, as the powerhouses of the cell, represent both a major source and a primary target of ROS during early embryonic development. Excessive ROS accumulation disrupts mitochondrial membrane potential (MMP), impairs ATP production, damages mitochondrial DNA, and ultimately compromises developmental competence (Takahashi, 2012; Agarwal et al., 2022).

Because mitochondrial activity is essential for supporting cleavage, compaction, blastulation, and lineage specification, maintenance of mitochondrial integrity is critical for successful embryo development. Beyond direct cellular damage, oxidative stress profoundly influences embryonic metabolism. According to the Quiet Embryo Hypothesis, embryos with the highest developmental competence maintain lower metabolic activity and utilize energy more efficiently than embryos experiencing cellular stress (Leese et al., 2022). To counteract oxidative stress, embryos activate several endogenous defense mechanisms, among which the Nuclear factor erythroid 2-related factor 2 (NRF2) signaling pathway serves as a central regulator of cellular redox homeostasis (Amin et al., 2014; Bellezza et al., 2018; Yamamoto et al., 2018). NRF2 controls the transcription of more than one hundred genes involved in antioxidant defense, detoxification, mitochondrial function, metabolism, and cellular survival (Malhotra et al., 2010; Holmström et al., 2013; Chen, 2022). Under basal conditions, NRF2 is bound by Kelch-like ECH-associated protein 1 (KEAP1), which promotes its ubiquitination and proteasomal degradation, thereby maintaining low NRF2 activity (Zhang and Hannink, 2003; Kansanen et al., 2013). During oxidative stress, modification of critical cysteine residues within KEAP1 disrupts the KEAP1–NRF2 complex, allowing NRF2 accumulation and nuclear translocation, where it binds antioxidant response elements (AREs) and activates the transcription of cytoprotective genes (Nguyen et al., 2009; Bellezza et al., 2018; Yamamoto et al., 2018). Once in the nucleus, NRF2 forms heterodimers with small musculoaponeurotic fibrosarcoma proteins (sMAFs), further enhancing ARE-mediated transcription of antioxidant and detoxification enzymes (Hayes and Dinkova-Kostova, 2014; Sengoku et al., 2022). Through these mechanisms, NRF2 plays a pivotal role in restoring redox balance, preserving mitochondrial integrity, and preventing oxidative damage-induced apoptosis. Importantly, NRF2 has emerged not only as a regulator of antioxidant defense but also as an important modulator of mitochondrial bioenergetics

and metabolic homeostasis, influencing substrate utilization, respiratory efficiency, and mitochondrial quality-control pathways (Holmström et al., 2013). Previous studies have demonstrated that activation of NRF2 is closely associated with improved embryo survival under oxidative stress conditions and enhanced developmental competence during bovine preimplantation development (Amin et al., 2014; Taqi et al., 2019). Collectively, these findings identify NRF2 as a promising therapeutic target for improving embryo resilience during IVP.

Pharmacological activation of NRF2 using naturally occurring plant-derived compounds has emerged as an attractive strategy for mitigating oxidative stress during assisted reproduction. Among these compounds, quercetin (QUE), a naturally occurring flavonoid abundant in fruits and vegetables, has received considerable attention because of its potent antioxidant and cytoprotective properties (Heim et al., 2002; Kelly, 2011). In addition to directly scavenging ROS, quercetin activates NRF2 signaling through disruption of the KEAP1–NRF2 complex and induction of ARE-dependent gene expression (Tanigawa et al., 2007). Molecular studies have demonstrated that quercetin interacts with KEAP1 through hydrogen-bond formation at Arg483 while simultaneously promoting protein kinase C-mediated NRF2 phosphorylation, thereby enhancing NRF2 activity and antioxidant responses (Luo et al., 2022). Previous investigations have shown that quercetin supplementation protects mammalian embryos from oxidative injury and improves developmental outcomes under stressful culture conditions (Yu et al., 2014). In bovine embryos, quercetin treatment enhanced blastocyst development and activated NRF2-dependent antioxidant pathways during oxidative stress (Khadrawy et al., 2020). Nevertheless, despite these promising findings, the effects of transient NRF2 activation during the early cleavage stages on mitochondrial function, metabolic activity, and developmental competence of female and male embryos remain poorly understood.

Therefore, the objective of the present study was to determine whether transient activation of the

NRF2 pathway through quercetin supplementation during in vitro embryo culture improves embryo survival and developmental competence under oxidative stress conditions induced by atmospheric oxygen exposure. We hypothesized that quercetin supplementation would reduce oxidative stress by disrupting KEAP1-mediated NRF2 inhibition, thereby enhancing antioxidant defenses, preserving mitochondrial function, stabilizing embryonic metabolism, and ultimately improving blastocyst developmental competence in male and female blastocysts.

3. Materials and Methods

3.1 Ovarian collection

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 1 h. Ovaries were placed in an insulated container filled with physiological saline solution (0.9% NaCl) at 37° C. Upon arrival, ovarian samples were immediately washed three times with a warmed 0.9% NaCl solution, followed by a rinse with 70% ethanol.

3.2 Cumulus-oocyte-complex isolation

Follicular fluid containing COCs was aspirated from ovarian follicles ranging between 3-8 mm in diameter using a vacuum pump (GenX International; Guelph, ON, Canada) calibrated to 50 mmHg. The pumping system was connected to a standard 18-gauge hypodermic needle (Covidien Monoject™; Mansfield, MA, USA) and paired with a sterile 50 mL collection tube (CELLTREAT® Scientific Products; Pepperell, MA, USA). The aspirated follicular fluid was left to settle undisturbed for 10 min, allowing a visible cellular pellet containing COCs to form at the bottom of the tube.

3.2.1 Antioxidant dilutions and preparation

Quercetin (QUE; Sigma-Aldrich; St Louis, MO, USA), was prepared at 10 mM stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich; St Louis, MO, USA). To ensure consistency across treatments, the final DMSO concentration was standardized and maintained constant for all

experimental groups, including the vehicle controls. Stocks and working dilutions were freshly prepared immediately before use.

3.2.2 In vitro maturation and in vitro fertilization

COCs were collected from abattoir ovaries as described above. Retrieved COCs were grouped (50 per well) and placed into 4-well culture dishes (Thermo Fisher Scientific, Waltham, MA, USA) containing 1 mL of chemically defined media for in vitro maturation of oocytes (IVM) pre-equilibrated at 38.5° C in 5% CO2 (De La Torre-Sanchez et al., 2006). At the time of equilibration, all IVM media were supplemented with 15 ng/mL NIDDK-oFSH-20, 1 μg/mL USDA-LHB-5, 1 μg/mL estradiol 17β, 50 ng/mL epidermal growth factor, and 0.1 mM cysteamine (De La Torre-Sanchez et al., 2006). IVM lasted 23 ± 1 h. For in vitro fertilization (IVF), COCs were transferred (in <20 μL of maturation media) into 4-well culture dishes containing 430 μL of equilibrated CSU chemically defined media for IVF (F-CDM) (De La Torre-Sanchez, Jose Fernando et al., 2006) and maintained at 38.5° C and 5% CO2 in humidified air. Frozen-thawed bull sperm from a single ejaculation were processed to separate viable and motile sperm using a multilayer 45%/90% Percoll® (Sigma-Aldrich; St. Louis, MO, USA) gradient and centrifuged at 800 × g for 20 min. Following this, the supernatant was discarded, and the remaining sperm pellet was washed with 2 mL of CSU chemically defined media for the handling of early embryos (HCDM-1) (De La Torre-Sanchez et al., 2006) and centrifuged at 300 × g for 5 min. Sperm concentration was determined using a hemocytometer, and the suspension was adjusted to 5×10^6 sperm/mL using equilibrated F-CDM. Diluted sperm was then added to COCs at a final concentration of 0.5×10^6 sperm/mL and co-incubated for 18 h at 38.5° C in 5% CO2 in humidified air.

3.3 In vitro embryo culture, antioxidant supplementation and oxygen tension exposure

Following fertilization, presumptive zygotes were vortexed for 90 s at maximum speed to remove

cumulus cells (CCs). Denuded zygotes were then randomly assigned to one of four experimental groups according to oxygen tension and quercetin (QUE) supplementation during in vitro culture (IVC): (1) physiological oxygen control (5% O_2), (2) physiological oxygen supplemented with 5 µM QUE (5% O_2 + QUE), (3) atmospheric oxygen control (20% O_2), and (4) atmospheric oxygen supplemented with 5 µM QUE (20% O_2 + QUE). Zygotes were transferred in groups of 50 to pre-equilibrated 4-well dishes containing 500 µL of (with or without QUE) CSU chemically defined medium for in vitro culture of early embryos (CDM-1). Embryos were cultured for 56 h at 38.5°C in 5% CO_2 and either 5% O_2 (90% N_2 balance) or 20% O_2, depending on the assigned treatment group. For the QUE treatment groups, quercetin was supplemented at a final concentration of 5 µM exclusively during the CDM-1 culture period, corresponding to the early cleavage stage of development. After 56 h of culture, embryos reaching the ≥4-cell stage were transferred in groups of 35 to pre-equilibrated 4-well dishes containing 500 µL of CSU chemically defined medium for in vitro culture of late embryos (CDM-2). No quercetin was added during the CDM-2 culture period. Embryos were cultured in CDM-2 for an additional 120 h at 38.5°C under the same oxygen conditions assigned during the early culture period (5% or 20% O_2) (De La Torre-Sanchez et al., 2006). Blastocyst developmental rates were evaluated on Day 7 (168 h) and Day 8 (192 h) post-IVF. A schematic overview of the experimental design is shown in Figure 4.1.

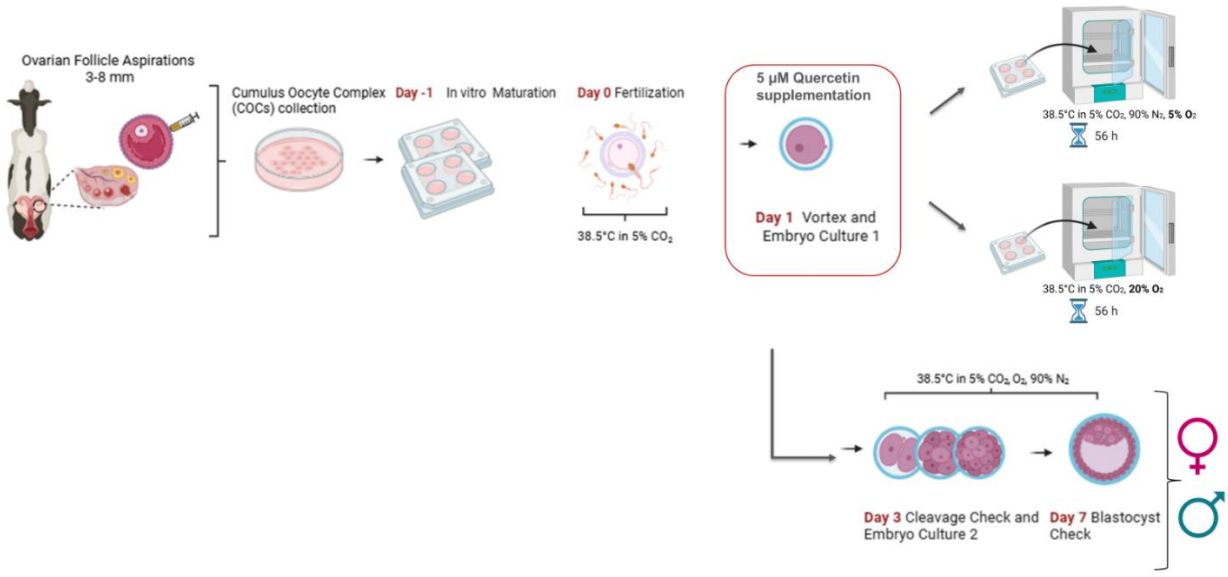

Figure 4.1 Experimental design for investigating the impact of quercetin supplementation during embryo culture and subsequent collection of embryos after exposure to high and low oxygen tension.

3.5 Proximity ligation assay of KEAP1-NRF2 protein-protein interactions in preimplantation embryos

Following the manufacturer's NaveniFlex proximity ligation assay (PLA) protocol (Navinci Diagnostics, Uppsala, Sweden), the embryos were counterstained using two primary antibodies (NRF2 polyclonal antibody, Invitrogen; Carlsbad, CA, USA, unconjugated, rabbit polyclonal antibody, PA138312; KEAP1 (G-2), Santa Cruz Biotechnology; Dallas, TX, USA, mouse monoclonal antibody, sc-365626) and mounted on a poly-L-lysine-coated slide (Newcomer Supply; Middleton, WI, United States) with 5–10 µL of ProLong Diamond Antifade Mountant (Invitrogen; Carlsbad, CA, USA) and a coverslip, allowing them to dry flat (>24 h). PLA/DAPI-labeled embryos were imaged using the LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective with fitted blue (345/455) and red (596/615) fluorescent filters. For the analysis, fluorescent images were converted to grayscale, puncta counts and total cell counts were obtained

using ImageJ software (National Institute of Health, Bethesda, MD, USA). The ratio of PLA puncta count normalized to total cell number from individual blastocysts was subsequently calculated. For each treatment group, 12–32 blastocysts were analyzed.

3.6 Mitochondrial function assays

3.6.1 Intracellular Reactive Oxygen Species accumulation assay

The impact of oxidative stress on intracellular ROS accumulation was assessed using 10 μM 2', 7'-Dichlorofluorescein Diacetate (H2DCFDA) (Sigma Aldrich; St. Louis, MO, USA) on bovine blastocysts. ROS accumulation in preimplantation embryos was assessed as described in Chapter 2. Briefly, denuded blastocysts from each treatment group were washed twice in phosphate-buffered saline supplemented with 0.1% polyvinylpyrrolidone (DPBS (1x)-PVP) (Sigma Aldrich; St. Louis, MO, USA). Samples were then incubated for 30 min at 38.5°C in 50 μL drops of 10 μM H2DCFDA dissolved in HCDM-2. Following incubation, blastocysts were washed three times in DPBS (1x) containing PVP (0.1%) and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Fluorescence intensity was quantified from .jpg images by manually selecting the oocyte or blastocyst area. All images were acquired with identical exposure and light settings, avoiding signal saturation, using a green fluorescent filter (excitation 494 nm, emission 518 nm). Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA).

3.6.2 Mitochondrial Membrane Potential δΨm Assay

The detrimental effect of oxidative stress on mitochondrial depolarization was analyzed using the cationic dye 5,5',6,6'-tetrachloro1,1',3,3' tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, USA) on blastocysts. MMP (δΨm) blastocysts (duplicates; a total of 16-20 blastocysts per treatment) was evaluated using the cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-

tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen, Carlsbad, CA, USA), dissolved in H-CDMM for oocytes and HCDM-2 media for blastocysts, as an indicator of mitochondrial activity, as previously described (Pasquariello et al., 2019). Briefly, blastocysts from the respective treatment groups were incubated with 2 μM JC-1 for 30 min in the dark at 38.5 °C under their optimal culture conditions. Following incubation, samples were washed three times with DPBS (1×) supplemented with 0.1% PVP and immediately imaged using an inverted laser scanning confocal microscope. Blastocysts were imaged using the ZEISS LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective. Fluorescence intensity was quantified from .czi images. Imaging J-monomers was conducted using a wavelength of 488 nm for J-monomers and excitation/emission settings at 490/525 nm (FITC filter, green fluorescence), indicative of low MMP. Similarly, images for J-aggregates were carried out using a wavelength of 561 nm and excitation/emission at 596/615 nm (TRITC filter/red fluorescence), indicating high MMP. Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, USA). The ratio of red to green fluorescence was subsequently calculated to determine MMP, expressed as an arbitrary unit.

3.6.2 Oxygen consumption rate and extracellular acidification rate assays

Metabolic analyses of individual day 7 blastocysts (n=10-13) were performed using a microchamber with electrochemical-based oxygen and pH sensors, as described (Y. Obeidat et al., 2019; Y. M. Obeidat et al., 2019). Briefly, the microchamber was filled with 150 μL of CSU chemically defined media (CDM-2). Then individual embryos were placed in the middle of each of the six channels containing CDM-2 media. The three-electrode oxygen sensor was connected to a potentiostat (Quadstat EA 164H, eDAQ Inc., Colorado Springs, CO) that applied a voltage to the sensor and monitored the decrease in oxygen-reduction current over time. The applied potential for all amperometric oxygen assays was set to −0.6 V. The pH sensor was connected to a custom-made

Ina333 instrumentation amplifier circuit that measured the change in voltage. The starting (room air-saturated) oxygen concentration and pH of the media were measured as baseline values for 2 min and calibrated as previously described in detail (Obeidat et al. 2019).

3.7 Embryo Sex Determination

Following completion of the respective assays (ROS, MMP, OCR, ECAR, or PLA), individual blastocysts were recovered and subjected to genomic DNA extraction for sex determination. Single embryos were lysed in 10 µL of lysis buffer (Lucigen, Palo Alto, CA, USA) by incubation at 65°C for 6 min followed by 98°C for 2 min. Embryo sex was subsequently determined by conventional PCR amplification of the sex-determining region Y (SRY) gene and the bovine-specific protein (BSP) gene. Amplification of BSP served as a positive control for DNA recovery and PCR efficiency. Embryos positive for both BSP and SRY amplification were classified as male, whereas embryos positive only for BSP amplification were classified as female.

3.8 Statistical analysis

Data were analyzed in GraphPad Prism version 8.4.2 (GraphPad; San Diego, CA, United States). For sex-specific endpoints, female and male embryos were analyzed independently. Within each sex, data were analyzed using a two-way ANOVA with oxygen tension (5% or 20% O_2) and quercetin supplementation (vehicle or 5 µM QUE) as fixed factors, followed by Tukey's multiple-comparison test. Data are presented as the Mean ± SEM of biological replicates. Statistical significance was identified at $p \leq 0.05$.

4. Results

4.1 Quercetin supplementation improves embryo developmental competence under oxidative stress conditions

Exposure to atmospheric oxygen tension (20% O_2) during in vitro culture significantly impaired

embryo developmental competence compared with embryos cultured under physiological oxygen conditions (5% O_2 control; *p=0.0045*). However, supplementation with 5 μM quercetin (QUE) during culture under 20% O_2 significantly improved blastocyst development compared with untreated embryos exposed to 20% O_2 (*p=0.0116*), restoring developmental competence to levels comparable to those observed under physiological oxygen conditions (Figure 4.2). These findings indicate that oxidative stress induced by atmospheric oxygen negatively affects preimplantation embryonic development and that QUE supplementation during the early post-IVF stages effectively mitigates the detrimental effects observed at the blastocyst stage.

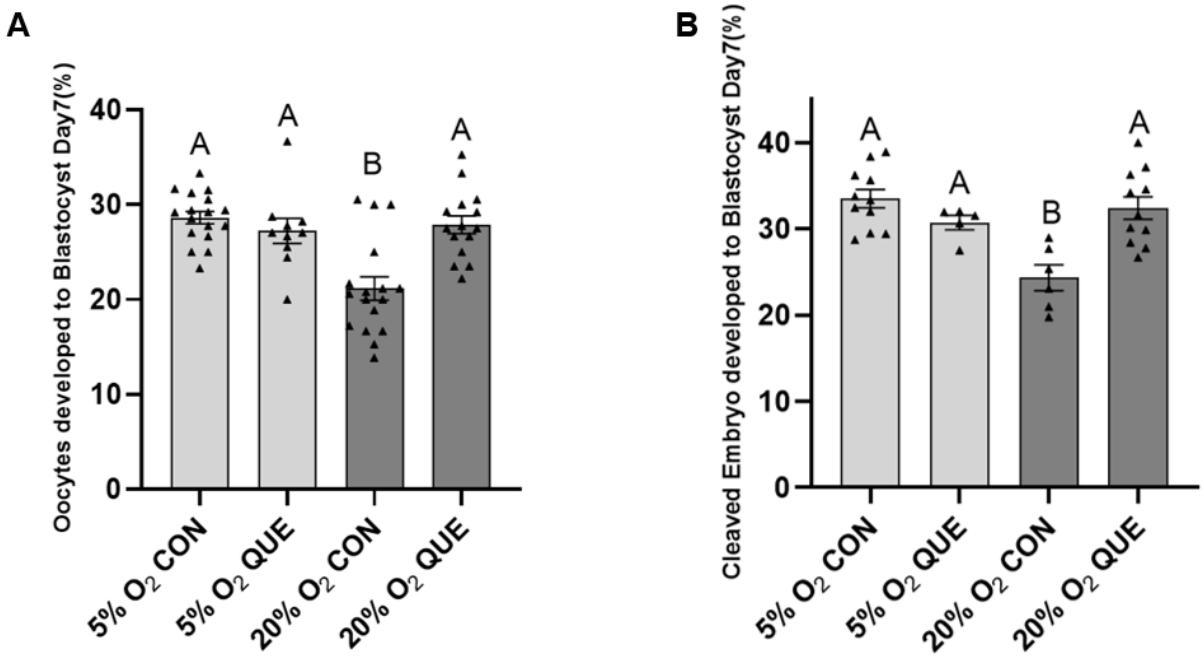

Figure 4.2 Developmental phenotypes of embryos cultured at different oxygen tensions in the presence or absence of QUE supplementation. (**A**) Percentage of oocytes developed into blastocysts for each treatment. (**B**) Percentage of cleaved embryos developed to blastocyst, for each treatment. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters show statistically significant differences between treatment groups ($p < 0.05$).

4.2 Quercetin mitigates mitochondrial dysfunction and reduces oxidative stress in female and male blastocysts under suboptimal culture conditions

To determine whether the improvement in developmental competence was associated with enhanced mitochondrial function and redox homeostasis, MMP and intracellular ROS levels were evaluated in Day-7 blastocysts. Embryos cultured under 20% O_2 exhibited significantly lower MMP compared with embryos cultured under 5% O_2 in both female and male blastocysts ($p=0.0058$ and $p=0.0039$, respectively; Figure 4.3). These results indicate that oxidative stress compromises mitochondrial activity regardless of embryo sex. In contrast, QUE supplementation during culture under 20% O_2 restored MMP to levels comparable to those observed in embryos cultured under physiological 5% oxygen conditions. No sex-specific differences were detected in the response to QUE treatment, suggesting that mitochondrial protection was achieved in both female and male embryos. Consistent with the observed mitochondrial dysfunction, embryos cultured under 20% O_2 accumulated significantly higher levels of ROS compared with embryos cultured under 5% O_2 ($p<0.05$; Figure 4.4). Elevated ROS levels were observed in both female and male embryos, indicating that oxidative stress affected embryos of both sexes. Importantly, QUE supplementation substantially reduced ROS accumulation under 20% O_2 conditions, restoring intracellular ROS levels comparable to those observed in the physiological 5% oxygen control group. Together, these findings demonstrate that QUE mitigates oxidative stress and preserves mitochondrial integrity during embryo culture.

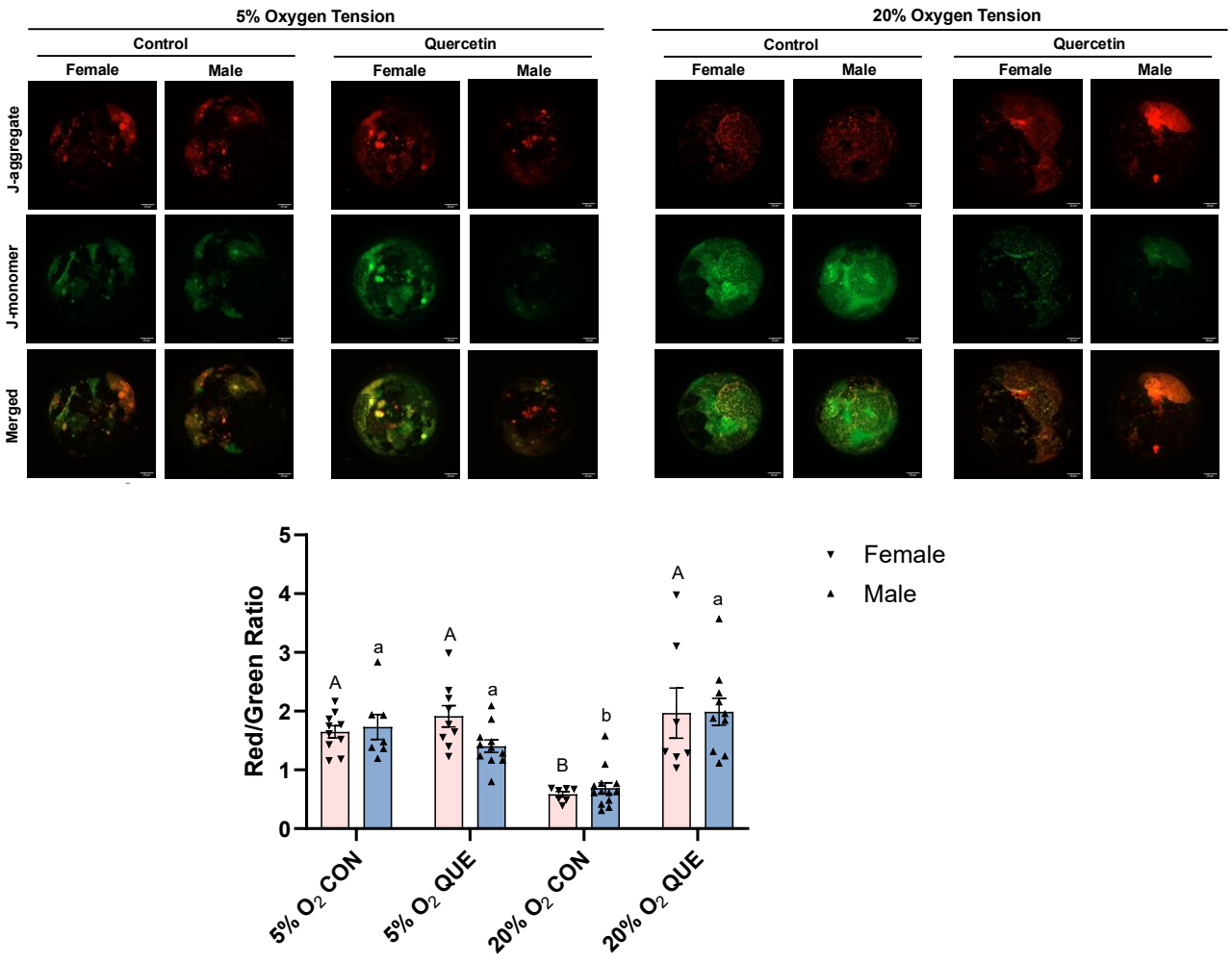

Figure 4.3 Mitochondrial membrane potential (MMP) in preimplantation embryos from QUE-supplemented zygotes exposed to oxidative stress. Quantitative Red/Green fluorescence intensity ratio using JC1 staining in Day 7 Embryos. Different uppercase letters indicate significant differences in female treatment groups, and lowercase letters indicate significant differences in male groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons.

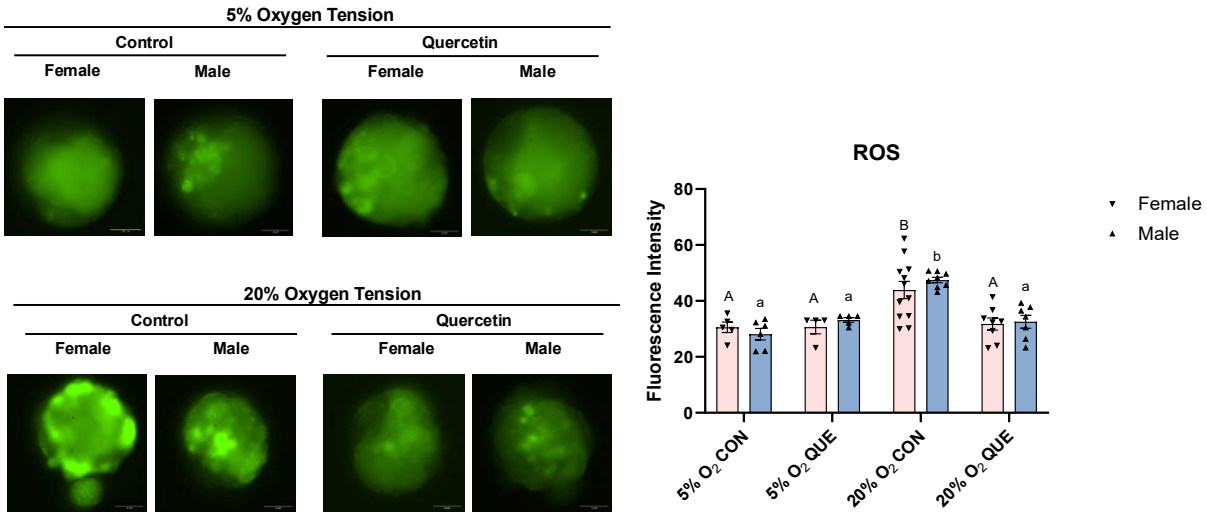

Figure 4.4 ROS accumulation in blastocysts from antioxidant-supplemented zygotes exposed to oxidative stress. ROS accumulation assay 2',7'- DCFH-DA. Quantification of ROS staining on antioxidant-supplemented bovine zygotes under 5 and 20% Oxygen tension. Different uppercase letters indicate significant differences in female treatment groups, and lowercase letters indicate significant differences in male groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons.

4.3 Quercetin reduces KEAP1–NRF2 protein interactions under oxidative stress conditions

To investigate the involvement of NRF2 signaling in the protective effects of QUE, KEAP1–NRF2 interactions were quantified using a proximity ligation assay (PLA). Embryos cultured under 20% O_2 exhibited a marked increase in KEAP1–NRF2 interactions compared with embryos cultured under 5% O_2 ($p<0.0001$; Figure 4.5). Increased interaction between KEAP1 and NRF2 is indicative of enhanced NRF2 sequestration, which may limit activation of antioxidant defense pathways under prolonged oxidative stress conditions. In contrast, supplementation with QUE under 20% O_2 significantly reduced KEAP1–NRF2 interactions, restoring PLA signal intensity to

levels comparable to those observed in embryos cultured under physiological oxygen tension. These results suggest that QUE promotes disruption of the KEAP1–NRF2 complex, thereby facilitating NRF2 activation and enhancing antioxidant defense mechanisms during oxidative stress.

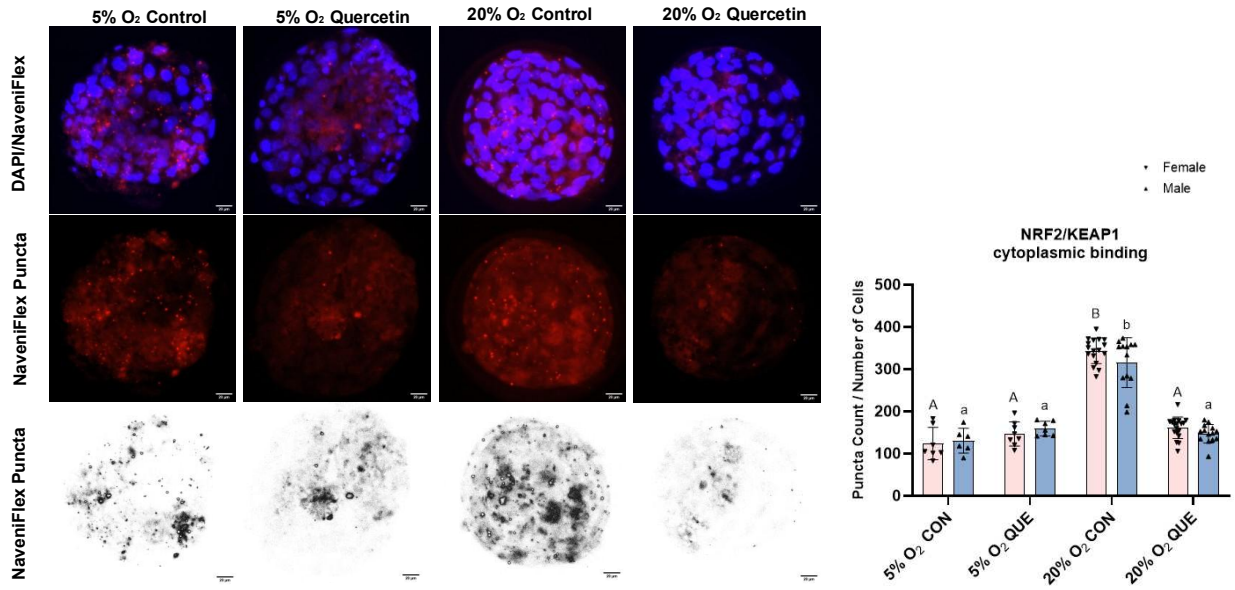

Figure 4.5 NRF2-KEAP1 Protein-Protein Interaction in blastocysts from antioxidant-treated zygotes cultured under oxidative stress. Proximity Ligation Assay (PLA) was used for quantification of cytoplasmic protein binding between NRF2 and KEAP1 on blastocyst from antioxidant-supplemented bovine zygotes under 5 and 20% Oxygen tension. Different uppercase letters indicate significant differences between groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons.

4.4 Quercetin improves the bioenergetics of embryos under oxidative stress conditions

Because oxidative stress can alter embryonic metabolic activity, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed in individual blastocysts. Across all

treatment groups, male embryos exhibited significantly higher OCR values than female embryos (*p<0.05*; Figure 4.6A), indicating inherent sex-specific differences in metabolic activity in preimplantation embryos. Exposure to 20% O_2 significantly increased the embryonic OCR in both female and male embryos compared with their respective 5% O_2 controls (p=*0.0067* and p=*0.0067*, respectively). Elevated OCR is consistent with increased energetic demand and metabolic stress associated with oxidative challenges. Supplementation with QUE during culture under 20% O_2 normalized OCR in both sexes, reducing oxygen consumption to levels comparable with those observed in embryos cultured under physiological oxygen conditions. These findings suggest that QUE alleviates the increased metabolic burden induced by oxidative stress. A similar pattern was observed for ECAR measurements. Embryos cultured under oxidative stress exhibited elevated extracellular acidification rates, whereas QUE supplementation significantly reduced ECAR values in both female and male embryos compared with untreated 20% O_2 controls (*p=0.0174* and *p=0.0174*, respectively; Figure 4.6B). The reduction in ECAR following QUE treatment indicates improved metabolic efficiency and suggests that NRF2 activation contributes to the maintenance of metabolic homeostasis during preimplantation development.

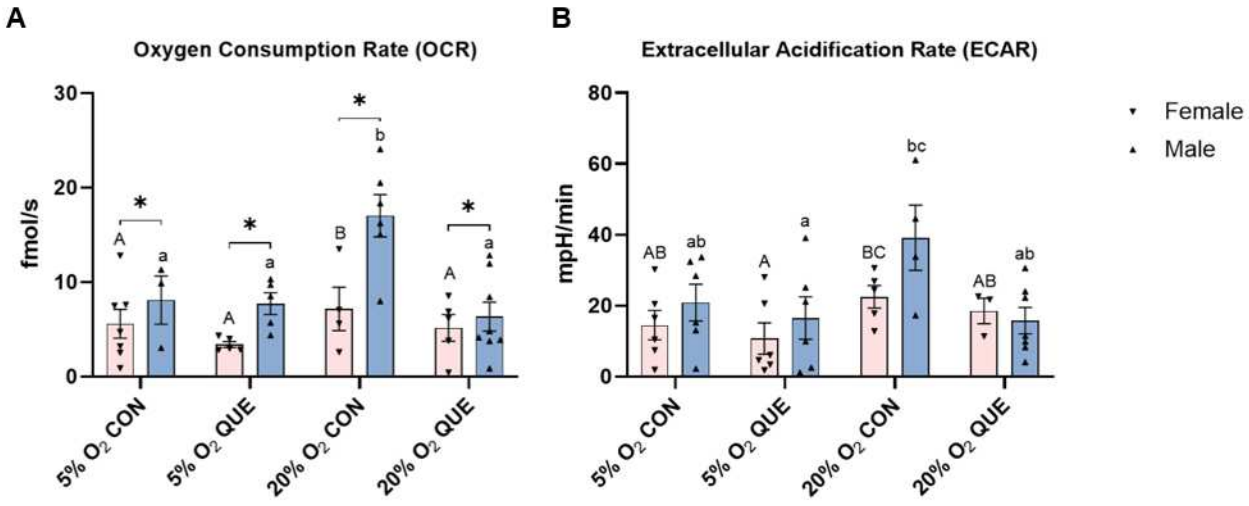

Figure 4.6 Metabolic function of preimplantation embryos from antioxidant-treated zygotes

cultured under oxidative stress. Metabolic Multisensor Platform was used on single pre-implantation embryos to quantify: (A) Oxygen Consumption Rate (OCR). (B) Extracellular Acidification Rate (ECAR). Different uppercase letters indicate significant differences in female treatment groups, and lowercase letters indicate significant differences in male groups. *$*p < 0.05$, **$p < 0.001$, ***$p < 0.001$* within treatment groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons.

5. Discussion

The present study demonstrates that transient QUE supplementation during the early stages of embryo culture effectively protects bovine preimplantation embryos against culture induced oxidative stress. Consistent with previous observations by (Khadrawy et al., 2020), 20% oxygen tension impairs developmental competence, causes ROS accumulation, and mitochondrial dysfunction, while QUE supplementation alleviated these detrimental effects.

Previous studies in bovine granulosa cells subjected to oxidative damage have demonstrated that disruption of NRF2 signaling is associated with increased ROS accumulation, impaired mitochondrial activity, and reduced cellular function, whereas QUE supplementation restores cellular homeostasis by activating NRF2-dependent antioxidant responses (Khadrawy et al., 2019). Consistent with these observations, the present study showed that transient QUE supplementation during early cleavage stages improved redox balance and preserved mitochondrial function under oxidative stress conditions.

Importantly, our findings further extend the current understanding of NRF2 regulation by demonstrating that transient QUE supplementation not only restored redox homeostasis and

mitochondrial function but also normalized embryo bioenergetic activity and disrupted KEAP1-mediated NRF2 sequestration. In contrast to previous reports describing sexually dimorphic NRF2 responses under oxidative stress, the protective effects of QUE were evident in both female and male embryos (Taqi et al., 2019), suggesting that NRF2-mediated adaptation represents a conserved mechanism for maintaining developmental competence despite sex-specific metabolic characteristics.

Beyond its well-established role in antioxidant defense, NRF2 has emerged as an important regulator of mitochondrial bioenergetics and cellular metabolism, coordinating cellular adaptation to oxidative stress by regulating redox homeostasis, mitochondrial function, and energy production (Amin et al., 2014). In the present study, embryos cultured under atmospheric 20% oxygen tension exhibited elevated OCR and ECAR values, indicating increased energetic demands and metabolic adaptation in response to oxidative stress. These findings align with the concept that environmental stressors can induce a metabolically active state characterized by increased substrate utilization and mitochondrial activity, thereby disrupting cellular homeostasis (Ramírez et al., 2026).

The biological significance of these metabolic changes can be interpreted within the framework of the Quiet Embryo Hypothesis, originally proposed by Leese (2002), which suggests that embryos with the highest developmental potential exhibit lower metabolic activity and utilize available resources more efficiently than their less-competent counterparts. However, subsequent refinements of this concept have emphasized that embryo viability is not necessarily associated with the lowest metabolic activity, but rather with the maintenance of metabolism within an optimal physiological range, often referred to as the "Goldilocks zone," where metabolic activity is sufficiently robust to sustain development while avoiding excessive energetic expenditure and cellular stress (Leese et al., 2022). Under this updated model, both excessively high and

excessively low metabolic activity may reflect suboptimal physiological states, whereas developmental competence is associated with metabolic homeostasis and flexibility.

Interestingly, our findings revealed clear sex-specific differences in embryo bioenergetics. Male blastocysts consistently exhibited higher OCR values than their female counterparts across treatment groups, suggesting inherently greater mitochondrial activity and oxygen utilization. These observations are consistent with previous reports demonstrating that male and female embryos differ in their transcriptional profiles, epigenetic regulation, and metabolite consumption from the earliest stages of development (Bermejo-Alvarez et al., 2011). Such developmental dimorphism has long been proposed as a potential explanation for sex-specific responses to environmental stressors, although the direction and biological significance of these differences remain controversial. Some studies suggest that female embryos possess enhanced tolerance to oxidative stress through differential regulation of genes involved in redox balance and glucose metabolism, including the X-linked gene G6PD, which contributes to NADPH production and antioxidant defense (Pérez-Cerezales et al., 2018). Conversely, other studies have reported increased susceptibility of female embryos to apoptosis under specific in vitro culture conditions (Ghys et al., 2016). Collectively, these findings indicate that embryo sex influences the mechanisms by which embryos respond to environmental insults rather than directly determining developmental competence.

Collectively, these findings support the emerging concept that developmental competence depends not only on antioxidant capacity but also on embryos' ability to maintain bioenergetic homeostasis under stress conditions. In this context, NRF2 appears to function as a central integrator of redox regulation and metabolic adaptation, enabling embryos to respond to oxidative stress while maintaining metabolic activity within a range compatible with optimal developmental progression.

The observed normalization of OCR, ECAR, mitochondrial function, and KEAP1–NRF2 interactions following quercetin supplementation further supports the notion that transient NRF2 activation contributes to embryo resilience by restoring both redox and metabolic homeostasis under suboptimal culture conditions.

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CHAPTER V—General summary, conclusions, and future directions

1. Summary and conclusion

Reproductive efficiency is a fundamental determinant of sustainability and productivity in livestock systems. Nevertheless, reproductive cells and embryos are continuously challenged by a variety of stressors that disrupt cellular homeostasis and compromise processes that are vital for successful reproduction. Environmental challenges are not only a phenomenon in the in vivo reproduction but also in the in vitro production of embryos which are normally intended to overcome fertility problems in livestock and human reproduction. These challenges arise not only from environmental conditions, such as heat stress (HS), but also from the intrinsic limitations of in vitro embryo production (IVP) systems, where non-physiological culture conditions can promote oxidative stress (OS) and impair normal developmental processes. In granulosa cells, oocytes, and preimplantation embryos, excessive accumulation of reactive oxygen species (ROS) can impair mitochondrial function, activate cellular stress pathways, induce apoptosis, and reduce developmental potential. While significant advances have been made in understanding the detrimental effects of these stressors, effective strategies to enhance cellular resilience and preserve reproductive competence remain a major goal in reproductive biology. Therefore, investigating endogenous antioxidant defense mechanisms and targeted interventions to mitigate stress-induced damage is essential for improving fertility and embryo production under both environmental and in vitro conditions. Hence, the overall objective of this dissertation was to investigate the mechanisms by which HS and in vitro culture–associated OS impair female reproductive function and early embryonic development, and to evaluate the potential of NRF2 pathway activation through antioxidant interventions to enhance cellular adaptation, mitigate oxidative damage, and improve developmental competence in granulosa cells, oocyte maturation

and preimplantation embryos.

The literature review presented in Chapter I established the biological framework for Chapters II and III of this dissertation by summarizing current knowledge regarding the effects of thermal stress on ovarian follicular dynamics, granulosa cell function, oocyte maturation, and preimplantation embryo development. The evidence reviewed demonstrated that HS disrupts reproductive processes at multiple levels, including altered endocrine function, impaired follicular development, mitochondrial dysfunction, oxidative damage, endoplasmic reticulum stress, apoptosis, and epigenetic dysregulation. Importantly, these effects are not restricted to a single developmental stage but instead propagate throughout the reproductive continuum, ultimately compromising fertility and embryonic competence. The review further highlighted oxidative stress as a central mechanism linking environmental challenges to reproductive dysfunction and identified the NRF2 signaling pathway as a promising therapeutic target.

Building upon this foundation, Chapter II investigated whether enhancing endogenous antioxidant defenses through NRF2 activation could protect bovine granulosa cells against HS-induced oxidative damage. Granulosa cells represent a critical component of the follicular microenvironment, providing metabolic support, hormonal regulation, and signaling cues required for oocyte development. Exposure to elevated temperature increased ROS accumulation, disrupted mitochondrial function, and reduced cellular viability, confirming the high sensitivity of granulosa cells to thermal stress (Zhang et al., 2023). Supplementation with the naturally occurring antioxidants quercetin (QUE), sulforaphane (SFN), and carnosol (CAR) improved cellular resilience; however, QUE and SFN consistently exhibited the strongest protective effects. Both compounds enhanced NRF2 nuclear activation, reduced ROS accumulation, preserved mitochondrial membrane potential, and attenuated apoptosis under HS conditions. These findings

demonstrate that activation of NRF2-mediated antioxidant responses effectively restores redox homeostasis and protects follicular somatic cells from thermal injury. Furthermore, the results emphasize the importance of maintaining granulosa cell function as a prerequisite for preserving the developmental competence of the enclosed oocyte.

The work described in Chapter III extended these observations to the oocyte and its subsequent embryonic development. Oocytes exposed to HS during in vitro maturation exhibited elevated oxidative stress, mitochondrial dysfunction, and altered expression of stress-response genes in the surrounding cumulus cells. These detrimental effects persisted beyond maturation, resulting in reduced embryonic developmental competence and compromised blastocyst quality. Remarkably, supplementation with QUE or SFN during maturation effectively mitigated these adverse outcomes. Oocytes matured in the presence of exogenous antioxidants exhibited reduced ROS accumulation and restored mitochondrial function, resulting in improved blastocyst development rates and increased total blastocyst cell numbers. Moreover, embryos derived from antioxidant-treated oocytes exhibited improved embryo competence indices and more favorable metabolic profiles characterized by reduced oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). These findings support the concept that oxidative damage occurring during oocyte maturation can exert long-lasting carryover effects on embryonic development and demonstrate that targeted NRF2 activation during maturation improves both oocyte quality and embryo competence.

In Chapter IV, the focus shifted from maternal HS to OS occurring directly during embryo culture. In vitro embryo production systems expose embryos to environmental conditions that differ substantially from those encountered within the reproductive tract, particularly with respect to oxygen concentration (Hasler, 2000). Exposure to atmospheric oxygen tension (20% O_2) induced

oxidative stress, increased ROS accumulation, impaired mitochondrial function, altered metabolic activity, and reduced blastocyst developmental competence compared with embryos cultured under physiological oxygen conditions (5% O_2) (Ciray et al., 2009). Supplementation with QUE during early embryo culture significantly improved developmental outcomes, restored mitochondrial membrane potential, reduced ROS accumulation, and normalized metabolic activity. Furthermore, proximity ligation assays revealed reduced KEAP1–NRF2 interactions in QUE-treated embryos, indicating enhanced NRF2 activation. Importantly, these protective effects were observed in both female and male embryos. Interestingly, our findings revealed pronounced sex-specific differences in embryonic bioenergetics. Across treatment groups, male blastocysts consistently exhibited higher OCR values than female blastocysts, indicating greater mitochondrial activity and oxygen consumption. These observations align with previous studies demonstrating that sexual dimorphism emerges during early embryonic development and is reflected in differences in gene expression, epigenetic regulation, metabolic activity, and developmental trajectories (Bermejo-Alvarez et al., 2011). Together, these findings provide mechanistic evidence that transient activation of NRF2 signaling during preimplantation development enhances embryonic resilience to OS and improves developmental competence under suboptimal culture conditions.

Although each experimental chapter focused on a distinct biological system, all studies converged on a common mechanistic theme: disruption of redox homeostasis represents a major driver of reproductive dysfunction, while enhancement of endogenous antioxidant defenses improves cellular survival, mitochondrial function, and developmental competence.

A major strength of this dissertation lies in its integrative approach. Rather than examining isolated cellular events, this work followed the progression of oxidative stress across the reproductive

continuum, from the ovarian follicle to the developing embryo. The results demonstrate that oxidative stress-induced dysfunction is not restricted to a single developmental stage but can originate within the follicular environment, affect oocyte competence, and subsequently influence embryonic development. Conversely, interventions targeting antioxidant defense mechanisms at multiple stages can generate beneficial outcomes throughout the reproductive process.

From a practical perspective, these findings have important implications for livestock production under conditions of increasing environmental stress. HS is projected to become more prevalent as global temperatures continue to rise, posing a growing threat to reproductive efficiency and food security. The identification of NRF2 as a central regulator of cellular resilience provides a mechanistically grounded strategy for improving reproductive performance in cattle. Furthermore, because OS is a common challenge across mammalian species, the findings presented here may also have broader relevance to assisted reproductive technologies in other agricultural species and, potentially, in human reproductive medicine.

Ultimately, this dissertation advances our understanding of how OS compromises reproductive competence and establishes the NRF2-mediated oxidative stress response pathway as a therapeutic target for designing solutions to improve reproductive outcomes under adverse environmental conditions.

2. Major contributions of this dissertation

This dissertation provides several novel contributions to the field of reproductive biology, including:

A. It demonstrates that HS-induced oxidative damage in bovine granulosa cells can be effectively mitigated by activating the NRF2 pathway with naturally occurring

antioxidants.

B. It establishes mechanistic relationships between NRF2 activation, mitochondrial function, redox homeostasis, and cellular survival within the ovarian follicular microenvironment.
C. It demonstrates that antioxidant supplementation during oocyte maturation improves subsequent embryonic development, highlighting the long-term developmental consequences of oocyte quality.
D. It provides evidence that modulating OS responses during oocyte maturation influences embryonic metabolic efficiency and competence.
E. It demonstrates that transient activation of NRF2 during embryo culture improves blastocyst development and mitochondrial function under OS conditions.
F. It identifies sex-specific metabolic responses to oxidative stress and antioxidant supplementation during preimplantation development.
G. It provides a comprehensive framework that links OS among granulosa cells, oocytes, and embryos through a common NRF2-dependent mechanism.

3. Future directions

3.1 Establishing the causal role of NRF2 signaling

Although the findings strongly support a protective role of NRF2 activation, future studies should directly establish causality through functional modulation of the pathway. Approaches such as NRF2 inhibition, KEAP1 overexpression, siRNA-mediated knockdown, or CRISPR/Cas9-based genome editing could be employed to determine whether the beneficial effects of QUE and SFN are entirely dependent on NRF2 signaling. Such studies would strengthen mechanistic understanding and identify potential alternative pathways contributing to cellular protection.

3.2 Transcriptomic and multi-omics characterization of antioxidant responses

The molecular mechanisms underlying NRF2-mediated protection remain incompletely characterized in reproductive cells. Future studies should integrate transcriptomic, proteomic, metabolomic, and epigenomic analyses to identify downstream regulatory networks activated following antioxidant supplementation. In particular, RNA-sequencing approaches could reveal novel NRF2 target genes associated with mitochondrial function, metabolism, stress adaptation, and developmental competence. Multi-omics integration would provide a more comprehensive understanding of how oxidative stress shapes reproductive outcomes at the systems level.

3.3 Sex-specific responses to oxidative stress during embryonic development

The sex-specific differences observed in embryonic metabolic activity suggest that male and female embryos may utilize distinct adaptive strategies in response to OS. Further research should explore whether these differences arise from sex-chromosome dosage effects, mitochondrial regulation, epigenetic modifications, or differential activation of antioxidant pathways. Understanding sexual dimorphism during preimplantation development may provide important insights into embryo selection, developmental programming, and long-term offspring health.

3.4 Long-term developmental consequences of early oxidative stress

The present studies focused primarily on preimplantation development. However, OS experienced during follicular growth, oocyte maturation, or early embryogenesis may influence later developmental stages through persistent epigenetic modifications. Future research should evaluate the effects of antioxidant supplementation on implantation success, fetal development, placental function, pregnancy outcomes, and postnatal health. Such studies would determine whether improvements observed during preimplantation development translate into long-term reproductive and developmental benefits.

3.5 Translation to commercial embryo production systems

The practical implementation of NRF2-targeting strategies requires validation under commercial conditions. Future studies should evaluate antioxidant supplementation protocols in large-scale embryo production systems using multiple sire and donor combinations. Additional investigations should assess cryotolerance, embryo transfer success, pregnancy establishment, and calf performance. Such studies would facilitate the translation of these findings into commercially applicable reproductive technologies.

3.6 Development of precision antioxidant supplementation strategies

The successful identification of effective QUE and SFN supplementation regimens across multiple reproductive models represents an important step toward developing targeted antioxidant interventions for assisted reproductive technologies. Building upon these findings, future studies should focus on refining the temporal use of NRF2-activating antioxidants and evaluating whether sequential or combinatorial antioxidant strategies can further enhance cellular adaptation to stress. In particular, investigating potential synergistic interactions among QUE, SFN, and even CAR, may provide a means of achieving greater cytoprotective effects while minimizing the need for prolonged exposure to individual compounds. These precision supplementation strategies could facilitate the design of next-generation embryo culture systems that maintain redox homeostasis without disrupting the physiological signaling processes essential for normal development.

4. Final perspective

The reproductive challenges associated with climate change, environmental stress, and artificial culture systems demand innovative strategies to enhance cellular resilience without disrupting normal developmental processes. The work presented in this dissertation demonstrates that OS is

a common mechanistic denominator linking reproductive dysfunction across the ovarian follicle, oocyte, and developing embryo. By identifying NRF2-mediated antioxidant signaling as a critical regulator of cellular adaptation and survival, this research provides both mechanistic insights and practical opportunities to improve reproductive efficiency.

As environmental pressures continue to intensify, the development of interventions that strengthen endogenous cellular defense mechanisms will become increasingly important. The findings presented here suggest that targeted activation of NRF2 is a promising approach to improving reproductive performance, enhancing embryo quality, and increasing resilience to environmental stressors. Beyond their relevance to cattle reproduction, these discoveries contribute to a broader understanding of how redox regulation governs reproductive success and developmental competence across mammalian species.

Taken together, this dissertation advances the field of reproductive biology by demonstrating that modulation of OS responses can improve reproductive outcomes from the ovarian follicle to the preimplantation embryo, thereby establishing NRF2 as a key molecular target for future reproductive and developmental research.

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APPENDIX I
- Original Publications

Animal Reproduction

Molecular and developmental consequences of heat stress on oocyte and embryo competence

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Complete List of Authors:	Ramirez, Ghyslaine; Colorado State University College of Veterinary Medicine and Biomedical Sciences, Biomedical Sciences Menjivar, Nico; Stanford Medicine Gebremedhn, Samuel; JR Simplot Co Gad, Ahmed; Colorado State University, Department of Clinical Sciences; Cairo University, Department of Animal Production Held-Hoelker, Eva; University of Bonn; Georg-August-Universitat Gottingen Hoelker, Michael; Georg August University Gottingen Center for Research on Teaching and School Research, Dept of Animal Sciences Zewdie, Genet; Addis Ababa University Tesfaye, Dawit; Colorado State University, Dept of Biomedical Sciences
Keyword:	Heat stress, Reproduction, Ovary, Oocyte, Embryo

1. Title page

Article type: Review article for conference proceedings (42nd Annual Scientific Meeting of the Association of Embryo Technology in Europe (AETE), September 2-4, 2026, in Rennes, France)

Running title: Impact of Thermal Stress on Mammalian Reproduction

Molecular and developmental consequences of heat stress on the bovine oocyte and embryo competence

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2. Abstract

Seasonal heat stress (HS) is a pervasive environmental challenge with profound consequences for female reproductive physiology, affecting ovarian function, oocyte maturation, and early embryonic development. At the ovarian level, HS disrupts follicular growth, impairs steroidogenesis, and compromises granulosa cell function, thereby creating a suboptimal microenvironment that reduces oocyte competence. In oocytes, HS induces oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, spindle abnormalities, chromosomal missegregation, and persistent epigenetic alterations. These disruptions extend into early embryonic development, where redox imbalance, apoptosis, ER stress, and altered lineage allocation reduce cleavage and blastocyst formation, compromise trophectoderm and inner cell mass integrity, and impair implantation potential. Maternal heat exposure further exacerbates embryonic vulnerability by altering the oviductal and uterine environment, reducing embryotrophic factors and antioxidant defenses, and ultimately influencing offspring phenotype and fertility, potentially across generations. Accordingly, this review aims to synthesize current knowledge on the physiological and molecular impacts of heat stress on ovarian function, oocyte maturation, and early embryonic development. To this end, we consider studies conducted under both in vivo and in vitro conditions, highlighting shared and distinct mechanisms of thermal stress at the organ and cellular levels to identify potential targets for intervention.

3. Key words

Heat stress, Reproduction, Ovary, Oocyte and Embryo

4. Introduction

Global warming induced seasonal thermal stress is continuing to rise (Hansen et al., 2006) with an exponential increase in the number of heat days in different regions of the world (Davariashtiyani et al., 2023). Extreme HS during summer disrupts various reproductive processes ranging from ovarian functionality to pronounced depression of conception rate in beef and dairy cows worldwide (Davariashtiyani et al., 2023). HS exerts a significant impact on animal productivity, health, reproduction, and overall welfare. The situation is aggravated for dairy cows, as the sector is under tremendous pressure to increase milk production amid rising demand worldwide for milk and milk products. The environmental HS and endogenous heat production due to high metabolic rate in dairy cow's compromise animal reproductive performance, which is an essential component for farm productivity (Tao et al., 2020).

The impact of seasonal thermal stress on mammalian female reproduction is multifaceted. One of the most affected dynamic organs is the ovary, and its follicular microenvironment. Ovaries are responsible for safeguarding female gametes and secreting ovarian steroids that ultimately regulate reproductive functions. HS disrupts reproductive function through physiological, behavioral, and endocrine alterations that directly affect the hypothalamic–pituitary–ovarian (HPO) axis. Ultrasonographic studies have demonstrated that HS alters ovarian follicular dynamics and impairs follicular growth rate and prolongs follicular wave duration, indicating compromised follicular function (Dovolou et al., 2023). HS increased the number of midsize follicles thereby reducing follicular dominance, which is accompanied by decreased estradiol and inhibin secretions and increased circulating FSH levels (Parker et al., 2003; Wolfenson et al., 1995a). As the functional unit of the ovary, the impact of HS on the follicle has a far-reaching effect on the oocyte and the functionality of the surrounding somatic cells (granulosa and theca cells).

In addition to seasonal measurements on the impact of thermal stress on oocyte maturation, several in vitro experiments using temperatures up to 43°C to induce HS in oocytes indicated a significant impact on oocyte growth (Kawano et al., 2022), reduced mitochondrial membrane potential (MMP), increased accumulation of reactive oxygen species (ROS), oxidative stress, and induced apoptosis (Feng et al., 2024a; Lee et al., 2023; Roth & Hansen, 2005; Wrzecińska et al., 2023). Taken together, the detrimental effects of HS are observed across in vivo and in vitro models, which significantly impair oocyte quality by disrupting both nuclear and cytoplasmic maturation, increasing chromosomal and spindle abnormalities, elevating oxidative stress and apoptosis, and compromising subsequent embryo development.

Several studies have demonstrated the carryover effects of HS exposure during in vitro oocyte maturation on the developmental competence and molecular architecture of the resulting blastocysts. Specifically, altered MMP, reduced ATP levels, and decreased mtDNA copy number have been reported in blastocysts derived from HS-exposed oocytes (Feng et al., 2024b; Miętkiewska et al., 2022). HS is closely associated with reduced reproductive performance in cattle, in part due to its detrimental effects on blastocyst formation, implantation rates, and increased embryonic mortality. The severity of these impairments is largely stage-specific, with early developmental stages, particularly cleavage and blastocyst formation, being most susceptible. In this literature review, we systematically summarize findings from both in vitro and in vivo studies that evaluate the molecular, functional, and developmental impacts of environmental thermal stress on ovarian and follicular function, oocyte maturation, and early embryonic development.

The impact of thermal stress on ovarian function

Impact of heat stress on follicular growth and dominance

Summer HS adversely affects animal performance and health, disrupting follicular growth (Wolfenson et al., 1997), corpus luteum (CL) function, preimplantation embryo development, and the uterine environment and receptivity. A particularly pronounced effect of HS occurs during follicular development. HS impairs the growth and function of small antral follicles, thereby disrupting the formation of the preovulatory dominant follicle and compromising both ovulation and CL formation (Roth et al., 2000; Wolfenson et al., 1995b). The vulnerability of ovarian follicles to heat stress depends on the developmental stage. Preantral, primary, and secondary follicles are susceptible to elevated temperatures under in vitro conditions, with secondary follicles exhibiting the highest sensitivity, followed by early secondary follicles (L. H. de Aguiar et al., 2020b). Notably, dairy cows exposed to elevated temperatures exhibited reduced diameters of dominant follicles during the first and second follicular waves, resulting in disrupted follicular dominance (Reddy & Anitha, 2024; Badinga et al., 1993; Wilson et al., 1998). This disruption is characterized by an increased number of mid-sized dominant follicles and the absence of a single dominant follicle, leading to a significant reduction in plasma inhibin concentration and an increase in follicle-stimulating hormone (FSH) levels (Roth et al., 2000). These alterations may explain the higher incidence of abnormal ovulation patterns and twinning observed during summer (Ryan & Boland, 1991). The impact of HS on follicular development is long-lasting; cows exposed to HS require at least four to six follicular waves to recover and produce developmentally competent oocytes following the HS period (Hulshof et al., 1994; Roth et al., 2001).

Impact of elevated temperature on follicular microenvironment

A study performed in dairy cows to determine the impact of HS on the metabolic characteristics of the follicular fluid revealed that HS significantly changes the abundance of metabolites that are associated with oxidative stress, including SOD, GSH-Px, CAT, MDA, T-AOC, ROS,

inflammatory factors including IL-1, IL-6, TNF-α, heat shock proteins, HSP70, and HSP90, and steroid hormones estradiol and progesterone (Li et al., 2025). The study further reported 1,544 differential metabolites in the follicular fluid of the heat-stressed group, which were mainly enriched in pathways such as steroid hormone biosynthesis, neuroactive ligand-receptor interactions, D-amino acid metabolism, tyrosine metabolism, phenylalanine metabolism, and tryptophan metabolism. Molecular analysis revealed that mural granulosa cells from heat-stressed cows had lower expression of key steroidogenic proteins, including StAR, LHR, aromatase (CYP19A1), and lower follicular estradiol levels, along with increased oxidative stress markers, including SOD1 and SOD2, and the apoptosis effector caspase-3, which were positively correlated with oxidative stress-induced apoptosis (Kim et al., 2026). These results demonstrated that thermal stress induces coordinated disruptions in molecular, hormonal, and oxidative pathways within the ovarian microenvironment, ultimately impairing oocyte maturation and reducing fertility. The molecular consequences of thermal stress on ovarian follicular development, endocrine function, cellular proliferation, EV cargo composition, and subsequent impact on reproductive outcomes are summarized in **Figure 1.**

Impact of heat stress on the function of follicular cells

Granulosa cells are considered the most important follicular somatic cells that support the growth and development of oocytes, provide a suitable microenvironment that ensures the maturation and ovulation of developmentally competent oocytes, and secrete hormones (Edson et al., 2009). Recently, a field experiment on the transcriptomic profile of GCs derived from beef cows obtained during summer and winter seasons revealed distinct transcriptomic profiles and genes involved in pathways including cell adhesion molecules, chemokine signaling, cytokine-cytokine receptor interaction were enriched by genes down regulated in GCs derived from summer season, while

genes upregulated are involved in protein processing and metabolic pathways (Joyce et al., 2024). This signifies the seasonal alteration in the gene expression profile of GCs in beef cows, which can potentially reflect seasonal variability in pregnancy success. Under in vitro conditions, several reports have shown adverse effects of HS on granulosa cell survival and the expression of key genes. Bovine GCs subjected to high temperatures (41°C) showed increased ROS at 24h, activating NRF2 and its antioxidant targets CAT and PRDX1. It also induced strong endoplasmic reticulum stress, marked by elevated GRP78 and GRP94 expression and increased GRP78 protein levels, along with upregulation of apoptotic genes BAX and CASPASE-3 (Alemu et al., 2018) **(Figure 2)**. Concurrently, HS reduced cell proliferation, reflected by decreased PCNA expression. Overall, elevated temperature triggered oxidative stress, ER stress, apoptosis, and reduced proliferation, prompting NRF2-mediated protective responses in bovine GCs (Alemu et al., 2018). Similarly, GCs subjected to in-vitro HS showed increased ROS, oxidized proteins, apoptosis, and elevated expression of heat shock proteins and antioxidants, alongside reduced cell viability (Gebremedhn et al., 2020). Interestingly, in the same study, exposure to higher temperature altered the expression of cellular miRNAs in GCs and the extracellular vesicles-mediated release of miRNAs in response to HS. The study further revealed that supplementing GCs cultures with EVs from heat-stressed cells promoted an adaptive response during subsequent heat exposure.

Molecular impact of heat stress on oocyte maturation

HS during oocyte maturation reduces bovine oocyte developmental competence, cleavage rates, and blastocyst formation (de Barros & Paula-Lopes, 2018; Feng et al., 2024b). It triggers oxidative damage, ER dysfunction, and altered gene expression in oocytes as well as surrounding cumulus cells (Miętkiewska et al., 2022; Naranjo-Gómez et al., 2021). These changes have far-reaching consequences affecting the pre- and post-implantation phases of embryonic development (Feng et

al., 2024b;). Thermal stress adversely affects both nuclear and cytoplasmic maturation processes, frequently leading to aberrant spindle architecture, impaired mitochondrial function, promoting DNA fragmentation, and contributing to diminished embryo developmental potential (Aroyo et al., 2007).

Redox imbalance in oocytes under heat stress

At the cellular level, HS is commonly modeled at 40–41.5°C during in vitro maturation (IVM) and has been shown to disrupt mitochondrial distribution and alter the expression of mitochondrial genes involved in the respiratory chain in metaphase II (MII) oocytes (Gendelman & Roth, 2012). Furthermore, elevated temperatures increase mitochondrial workload and electron transport chain leakage, elevating mitochondrial ROS in germinal vesicle and MII oocytes, overwhelming antioxidant defenses like SOD and GSH-Px (Feng et al., 2024b; Lang et al., 2024). In bovine IVM at 41°C, mitochondrial ROS levels in oocytes reach those observed following direct oxidant exposure, while glutathione buffering capacity becomes compromised (Held-Hoelker et al., 2025; Payton et al., 2018). Several studies have shown that ROS surges during IVM at 41°C, with NRF2 pathway activation as a compensatory response, though insufficient to prevent apoptosis. Moreover, this leads to lipid peroxidation, protein oxidation, and DNA damage, reducing mitochondrial membrane potential, ATP levels, and mtDNA copy number (Payton et al., 2018; Tariq et al., 2025). Redox imbalance has been shown to go along with an increased expression of pro-apoptotic genes, while the expression of anti-apoptotic genes decreases (Held-Hoelker et al., 2025; Itami et al., 2018). If adaptive antioxidant responses like SOD and GSH-Px fail, this pushes oocytes towards apoptosis (Cao & Kaufman, 2014; Latham, 2015; Zhao et al., 2017). Consequently, the accumulation of excessive ROS and chronic ER stress caused by elevated temperatures leads to spindle abnormalities, errors in chromosome segregation, and defects in the

cytoskeleton in oocytes, thereby reducing their developmental competence (Payton et al., 2018; Song et al., 2024).

ER stress response in oocytes exposed to thermal stress

In bovine and other mammalian oocytes, elevated mitochondrial ROS disturbs protein folding in the ER and calcium homeostasis, triggering the UPR and, if unresolved, induce apoptosis (Cao & Kaufman, 2014; Held-Hoelker et al., 2025; Payton et al., 2018; Zhao et al., 2017). The ER and mitochondria physically interact via mitochondria-associated membranes (MAMs), which regulate Ca^{2+} transfer and apoptotic signaling in oocytes (W. Lin et al., 2021; Zhao et al., 2017). In mouse oocytes, increased MAM abundance and higher IP3R1 and PACS-2 expression enhance ER–mitochondria coupling, sensitizing mitochondria to ER Ca^{2+} release and promoting ROS-linked dysfunction (Zhao et al., 2017). These contacts create microdomains in which mitochondrial ROS and Ca^{2+} flux can rapidly affect the ER redox state and vice versa (Cao & Kaufman, 2014; W. Lin et al., 2021). Furthermore, redox imbalance fuels ER stress through mitochondrial-ER contacts, amplifying UPR and epigenetic disruptions. In agreement with this, antioxidant interventions (e.g., HO-1) or chaperone supplementation (HSP70) have been shown to partially rescue maturation rates (Divvela et al., 2025; Wang et al., 2019). Disturbed UPR mechanisms cause misfolded protein buildup, which, in turn, triggers ER stress markers, such as GRP78/BiP sequestration, in bovine oocytes and cumulus cells, compromising Ca^{2+} homeostasis and maternal mRNA storage. Resulting effects include spindle abnormalities and impaired blastocyst formation (Alemu et al., 2018; T. Lin et al., 2019). Elevated temperatures increase mitochondrial ROS, further oxidizing ER luminal and membrane proteins, disrupt Ca^{2+} pumps and channels, and exacerbate ER Ca^{2+} depletion, ultimately leading to the accumulation of misfolded proteins (Cao & Kaufman, 2014; Latham, 2015; Zhao et al., 2017).

Thermal stress-induced epigenetic alterations in oocytes

Beyond the immediate impact of elevated temperatures on the intrinsic quality of oocytes and embryos, heat stress induces epigenetic modifications, including alterations in chromatin remodeling and DNA methylation patterns, in both oocytes and early embryos (de Barros & Paula-Lopes, 2018; Diaz et al., 2021; Zhu et al., 2021). HS has been shown to downregulate histone modifications (H1, H2A, H2B, and H4) and DNA methylation in both bovine oocytes and embryos (Diaz et al., 2021; Feng et al., 2024b). Exposure to HS before fertilization induced the accumulation of H3K9me3 and HP1 in early embryos. Specifically, an increased accumulation of H3K9me3 was observed in the nuclei of four-cell and eight-cell stage embryos derived from heat-shocked oocytes (Diaz et al., 2025). Furthermore, a higher proportion of four-cell-stage embryos derived from heat-shocked oocytes showed increased HP1 fluorescence, suggesting abnormal chromatin compaction (Camargo et al., 2019). Recent reports indicate that both in vivo and in vitro HS altered DNA methylation patterns and reduced developmental competence in bovine embryos, evidencing stage-dependent differences and highlighting susceptibility at early cleavage stages (Diaz et al., 2025). Feng and colleagues showed that HS significantly reduced global DNA methylation (5mC) and hydroxymethylation (5hmC) levels at all stages from oocyte to blastocyst, with a minimum at the 8-cell stage and persistently lower 5hmC in thermal-stressed embryos (Feng et al., 2024b). This might be explained by reduced mRNA levels of DNMT1, DNMT3A, DNMT3B, and histone H2A in blastocysts derived from heat-stressed oocytes, indicating an impaired active and maintenance methylation machinery. Consequently, HS during bovine oocyte maturation might leave a persistent molecular “imprint” that is clearly detectable in early embryos up to the blastocyst stage, particularly in terms of redox homeostasis, ER stress/UPR signaling,

and epigenetic programming (de Barros & Paula-Lopes, 2018; Feng et al., 2024b; Kawano et al., 2025).

Embryonic consequences of heat stress during maturation

It is important to understand that HS effects during maturation affect the vitality of the resulting embryos. Zygotes and cleavage-stage embryos derived from heat-stressed oocytes show increased ROS levels, along with impaired antioxidant capacity and reduced developmental competence (Divvela et al., 2025). In agreement with findings in oocytes, altered mitochondrial membrane potential, reduced ATP, and mtDNA copy number were also detected in the resulting blastocysts (Feng et al., 2024b; Miętkiewska et al., 2022). Furthermore, antioxidant systems (SOD, CAT, GSH-Px, peroxiredoxins) are downregulated or functionally overwhelmed in heat-stressed ovaries, follicles, and embryos, resulting in increased apoptotic index and TUNEL-positive nuclei in morulae and blastocysts derived from heat-stressed oocytes or early embryos (de Barros & Paula-Lopes, 2018; Feng et al., 2024b; Naranjo-Gómez et al., 2021). Reduction of inner cell mass and compromised trophectoderm integrity, as well as altered expression of stress-related and developmentally important genes (e.g., HSPs, BAX/BCL2, antioxidant enzymes, pluripotency markers) in blastocysts, was reported previously (de Barros & Paula-Lopes, 2018; Held-Hoelker et al., 2025; Kawano et al., 2025; Lang et al., 2024). Several studies revealed that blastocysts derived from heat-stressed bovine oocytes exhibit increased expression of ER stress markers (e.g., GRP78/BiP, XBP1, and ATF6 pathway genes), as well as genes associated with apoptosis and mitochondrial dysfunction (Feng et al., 2024b; Held-Hoelker et al., 2025; Lang et al., 2024). Together, heat exposure during maturation disrupts the cytoskeleton, mitochondrial distribution, and calcium homeostasis and promotes apoptosis in the developing embryo (de Barros & Paula-Lopes, 2018; Lang et al., 2024; Naranjo-Gómez et al., 2021).

Molecular consequences of heat stress on early embryonic development

The severity of impediments to embryonic development at the helm of HS is largely stage-specific and primarily occurs during the pre-attachment stage, affecting cleavage and blastocyst formation, although its impact dwindles as the embryo continues in development (Ealy et al., 1993). The greatest detrimental effects to the early developing embryo occur during the pre-genome activation period (1–8 cell stage), whilst the embryo relies heavily on maternally inherited RNAs and proteins, lacking a robust stress response. For instance, early embryonic sensitivity to HS has been shown through the exposure of two-cell embryos and their subsequent rapid transcriptional increase of HSP70 Member 1A (HSPA1A), Heat Shock Protein beta-1 (HSPB1), and the synthesis of HSP70 (AKhan et al., 2020; Rivera & Hansen, 2001). Additionally, embryos exposed to elevated temperatures (e.g., 40–41 °C) during early cleavage frequently exhibit reduced progression through successive mitotic divisions and significantly lowered rates of blastocyst formation, indicating compromised developmental competence and viability relative to embryos maintained at physiological temperatures (~38.5 °C) (Rivera & Hansen, 2001). Short- to moderate HS exposure during the zygote or two-cell stage of development has also been shown to disrupt cytoskeletal integrity, increase ROS, and alter mitochondrial function, all of which hinder the progression of early cleavage stage embryos through blastulation (Abdelnour et al., 2020; Al-Katanani et al., 2002; Roth & Hansen, 2004, 2005). Nevertheless, even embryos exposed to HS amid the early cleavage window are less likely to form morphologically ‘normal’ blastocysts, often exhibiting epigenetic and apoptotic changes that reflect cellular stress and reduced developmental potential, as shown by our group to be partially mitigated via practical abatement strategies such as extracellular vesicles (Menjivar et al., 2026; Menjivar, Gad, Gebremedhn, et al., 2023) and the use of antioxidants (Ramírez et al., 2026) cultured in vitro.

HS alters cell-fate decisions and subsequent implantation potential during early embryonic development via perturbations in lineage allocation, cellular survival pathways, and blastocyst quality, ultimately reducing the embryo's competence for attachment and pregnancy establishment. Exposure of early embryos to elevated temperatures induces cellular damage and oxidative stress that are known to decrease blastocyst total cell number, with particularly pronounced reductions in trophectoderm (TE) cells (Sakatani et al., 2004, 2007, 2008, 2012), the lineage responsible for placental formation and implantation (Sakatani, 2017). Additionally, HS-induced apoptosis via caspase-dependent pathway activation duly alters the balance between surviving blastomeres, undermining lineage allocation in a developmentally regulated manner (Paula-Lopes & Hansen, 2002). In addition, HS exposure during early embryogenesis has also been shown to induce abnormal chromatin remodeling and epigenetic modifications (e.g., altered H3K9me3 accumulation and heterochromatin organization), which have the capacity to reprogram gene expression patterns that govern differentiation of the inner cell mass (ICM) and TE lineages, thereby affecting downstream developmental trajectories and embryonic competence for implantation (Camargo et al., 2019). In early cleavage heat shock, it has also been shown that past a reduced blastocyst yield, further alterations in mitochondrial function and DNA integrity persist, suggesting selective survival of metabolically altered embryos with potential impacts on their post-transfer viability (Carvalheira et al., 2024). Collectively, such efforts to reshape cell-fate decisions, affecting embryonic lineage composition and the molecular programming of the early embryo, are determinants that, in part, compromise pregnancy establishment, increasing early embryonic loss in cattle.

A prime determinant governing the earliest stages of embryonic development is the supraphysiological milieu within the oviduct and uterine environments. Particularly, HS disrupts

the composition of oviductal and uterine secretions (histotroph), reducing concentrations of key embryotrophic factors such as insulin-like growth factor-1 (IGF-1), colony-stimulating factor-2 (CSF2), amino acids, and antioxidants that aid in sustaining embryo metabolism and survival (Sakatani, 2017). Additionally, HS increases ROS production in reproductive tract epithelial cells, leading to oxidative damage, impaired mitochondrial function, and decreased antioxidant capacity in luminal fluids, thereby indirectly exposing developing embryos to cellular stress (Sakatani, 2017; Wolfenson & Roth, 2019). Additionally, thermoregulatory blood flow redistribution reduces uterine and ovarian perfusion, limiting tissue oxygenation and impairing oviductal transport and endometrial preparation for implantation (Wolfenson & Roth, 2019). However, mechanisms to study such undertakings in vitro are largely stunted due to the inaccessibility of physiological organs and inherent drawbacks, including critical restrictions on the formation of vivo tissue-like structures and long-term physiological function when cultured in 2D. Thus, our group specifically has made strides in using organoid models to better understand the molecular undertakings of the bovine oviduct to HS (Menjivar, Gad, Thompson, et al., 2023), mapping the architecture of in vitro organoid-derived EVs with those collected in vivo and from in vitro cells in 2D and elucidating their subsequent impact on embryos developed under conditions of HS (Menjivar et al., 2026).

5. Conclusions:

HS is an escalating environmental challenge with profound consequences for female reproductive performance, particularly through its detrimental effects on ovarian and follicular function, oocyte maturation, and early embryonic development. This review summarizes the current state of the art on the physiological and molecular impacts of HS on ovarian function, oocyte competence, and early embryo development. Moreover, it highlights key mechanisms underlying HS-induced damage at the organ, cellular, and molecular levels, enabling the identification of potential targets

for intervention. In addition to various pharmacological strategies, emerging molecular approaches, including EV-mediated signaling, offer promising avenues to enhance cellular resilience in oocytes and embryos.

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Figure legends

Figure 1. A model illustrating the molecular consequences of environmental heat stress (HS) on ovarian function and its subsequent impact on reproductive outcomes. Reduced fertility reflects the cumulative effects of HS on follicular cells (granulosa and theca cells), including disrupted steroidogenesis, increased oxidative stress, mitochondrial dysfunction, and altered gene expression, which collectively compromise follicular homeostasis. In parallel, HS perturbs extracellular vesicle (EV)-mediated molecular signaling, impairing the transfer of regulatory molecules (e.g., miRNAs, proteins, and lipids) and disrupting intercellular communication within the follicular microenvironment. These alterations, together with impaired oocyte growth, meiotic competence, and cytoplasmic maturation, ultimately result in reduced developmental competence, compromised embryo quality, impaired implantation potential, and decreased pregnancy rates.

Figure 2. Molecular mechanisms underlying the response of antral follicle–enclosed somatic cells and oocytes to thermal stress. Heat stress induces oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction in both somatic (granulosa and theca) cells and the oocyte. The primary line of defense, and among the most extensively studied pathways, include the NRF2-mediated antioxidant response and the unfolded protein response (UPR), which act coordinately

to restore cellular homeostasis by regulating redox balance, protein folding, and stress adaptation. When these protective mechanisms are insufficient or overwhelmed, persistent stress leads to apoptosis, impaired cellular communication, and compromised oocyte competence, ultimately affecting follicular integrity and developmental potential.

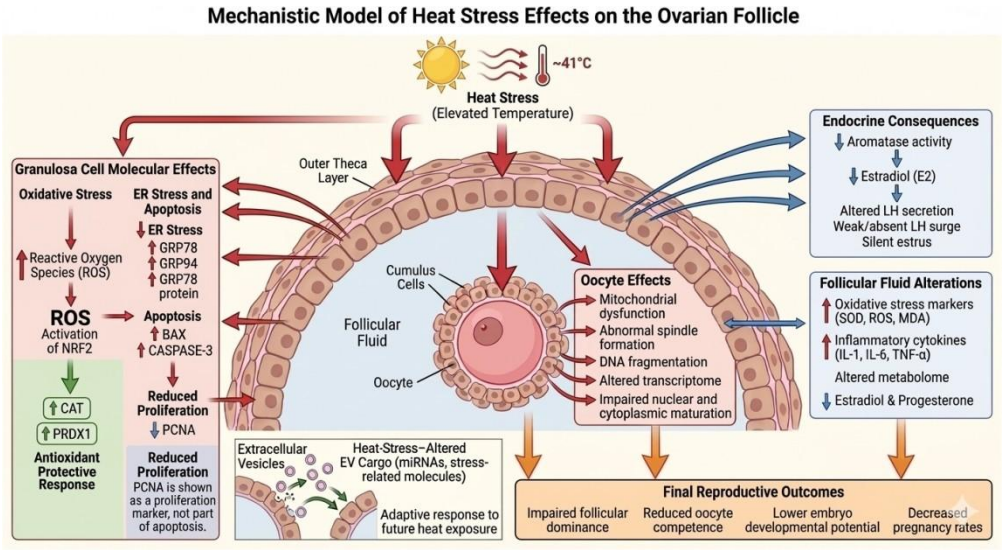

Figure 1

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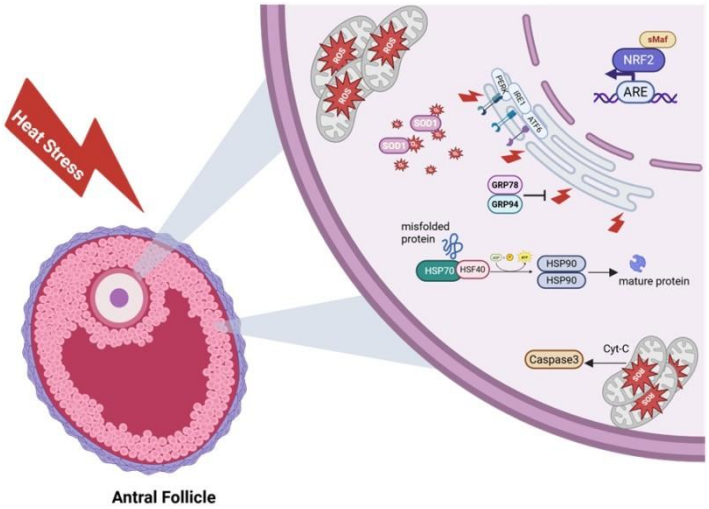

Figure 2

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Enhancing antioxidant capacity via NRF2 pathway activation to mitigate heat stress-induced oxidative damage in bovine granulosa cells, oocytes, and embryos

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Global warming-induced thermal stress is an escalating threat to livestock fertility, perturbing ovarian function, oocyte maturation, and preimplantation embryo development through excessive accumulation of reactive oxygen species (ROS), which drive follicular oxidative damage. Although embryo transfer technologies offer a practical abatement strategy for mitigating such implications caused by HS, *in vitro* systems lack the endogenous antioxidant defenses present *in vivo*, leaving follicular cells, gametes, and embryos particularly vulnerable. Here, we aimed to investigate whether antioxidants, including quercetin (QUE), carnosol (CAR), and sulforaphane (SFN), mitigate HS-induced follicular oxidative damage in bovine granulosa cells (GCs), oocytes, and embryos. For this, antioxidant supplementation, either individually or in combination, was performed during *in vitro* GC culture and oocyte maturation under normothermic (NT) or HS conditions. Across all models, QUE and SFN supplementation activated nuclear NRF2, reduced ROS accumulation, and restored mitochondrial function and apoptosis levels under conditions of HS. In oocytes exposed to thermal stress, QUE and SFN supplementation also led to increased blastocyst rates and total cell numbers. Single-embryo metabolic profiling revealed reduced oxygen consumption (OCR) and extracellular acidification (ECAR) rates in blastocysts derived from antioxidant-treated oocytes, indicative of enhanced metabolic efficiency. Moreover, quantitative analysis of recently defined embryo competence-associated genes demonstrated a restoration of the embryo competence index (ECI) following QUE and SFN supplementation. In conclusion, antioxidant supplementation during GC culture and oocyte maturation

alleviates HS-induced reproductive dysfunction by restoring redox homeostasis, preserving metabolic efficiency, and re-establishing embryo competence, thereby providing a mechanistically grounded strategy to mitigate climate-driven fertility decline in cattle.

KEYWORDS

heat stress, NRF2 pathway, antioxidants, embryonic development, oocyte maturation, granulosa cells, quercetin, sulforaphane

1 Introduction

Heat stress (HS), defined as the inability to adequately dissipate body heat, exerts well-documented detrimental effects on cattle reproductive physiology and productivity (Khan et al., 2023; Lovarelli et al., 2024; Wolfenson et al., 2000; Wolfenson and Roth, 2019). The impact of HS on female fertility is profound and multifaceted. At the molecular level, within the ovarian follicular microenvironment, HS leads to the accumulation of reactive oxygen species (ROS), triggering oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction, and apoptosis (Amaral et al., 2020). Specifically in the ovary, ROS accumulation has been shown to deteriorate oocyte quality, largely by inducing GC apoptosis and accelerating corpus luteum degeneration (Liu et al., 2020; Zhang et al., 2023). As oocyte maturation occurs synchronously with follicular development, and oocyte competence depends on the bidirectional communication with the surrounding GCs, oocytes are therefore highly vulnerable to ROS. Cumulus cells (CCs) deliver mRNA cargo to the oocyte through trans-zonal projections (TZP), which contribute to the oocyte mRNA reserve (Macaulay et al., 2016). These early oogenic events are crucial for the embryo's developmental potential. Moreover, the long-term impact of HS stress during oocyte maturation on embryonic developmental potential and molecular architecture, mediated in part through epigenetic modifications, has been previously demonstrated (Amaral et al., 2020; Bie et al., 2017; Kono et al., 1996).

In response to thermal stress, GCs, oocytes, and embryos rely on the cascade of protective antioxidant systems to maintain cellular homeostasis, including the induction of heat shock proteins (HSPs) and the unfolded protein response (UPR) (Marei and Leroy, 2022). A key component of the antioxidant defense machinery is the transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2), which regulates enzymes that mitigate oxidative damage in GCs, oocytes, and preimplantation embryos (Amin et al., 2014; Feng et al., 2023; Khadrawy et al., 2020). As a master regulator of cellular redox homeostasis, NRF2 controls the expression of more than 100 oxidative stress-associated genes, mitigating cellular damage caused by pro-oxidants (Chen, 2022; Lu et al., 2016; Thiruvengadam et al., 2021). Under physiological conditions, NRF2 is tightly regulated by Kelch-like ECH-associated protein 1 (KEAP1), facilitating NRF2's continuous ubiquitination and degradation. We have previously shown that failure of embryos to activate their NRF2-mediated oxidative stress response can lead to accumulated ROS, reduced mitochondrial function, and a subsequent reduction in developmental competence (Aglan et al., 2020; Amin et al., 2014; Khadrawy et al., 2019; Khadrawy et al., 2020; Sohel et al., 2018; Taqi et al., 2021). Pharmacological activation of NRF2 through natural plant compounds has also emerged as a promising strategy (Khadrawy et al., 2019; Taqi et al., 2021). Although the precise molecular interactions of most plant compounds remain incompletely characterized, the binding interactions of quercetin (QUE), carnosol (CAR), and sulforaphane (SFN) with KEAP1 are well established (Hong et al., 2005; Ji et al., 2023; Luo et al., 2022). QUE, a natural antioxidant polyphenol, binds to KEAP1 at Arg483 through hydrogen bonds and also promotes NRF2 phosphorylation through PKC, thereby enhancing NRF2 activity and reducing oxidative stress (Luo et al., 2022). CAR, another natural polyphenol, is reported to form hydrogen bonds with amino acids Serine602 and Glutamine530 and π-π stacking interaction with Tyr572 of KEAP1, activating NRF2 signaling and ameliorating PCOS-related phenotypes in KGN cells (Ji et al., 2023). SFN, abundant in cruciferous vegetables, modifies KEAP1 cysteine residues through a reversible reaction, mimicking ROS-induced KEAP1 modification (Hong et al., 2005). Functional studies demonstrate that SFN induces NRF2 nuclear translocation, increases antioxidant enzyme expression, and rescues GCs from oxidative damage *in vitro* (Sohel et al., 2017). This highlights the need to unveil whether exogenous antioxidant supplementation effectively enhances the antioxidant capacity of GCs, oocytes, and the resulting blastocysts, thereby mitigating the impact of environmental stress. Here, we investigated whether supplementation with QUE, CAR, and SFN during GCs and oocyte culture under normothermic and HS conditions could enhance NRF2-mediated cellular defense mechanisms and improve metabolic and embryonic competence.

2 Materials and methods

2.1 Ovarian collection

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 1 h. Ovaries were placed in an insulated container filled with physiological saline solution (0.9% NaCl) at 37 °C. Upon arrival, ovarian samples were immediately washed three times with a warmed 0.9% NaCl solution, followed by a rinse with 70% ethanol.

2.2 Granulosa cells (GCs) and cumulus-oocyte-complex (COCs) isolation and culture

Follicular fluid containing GCs and COCs was aspirated from ovarian follicles ranging between 3 and 8 mm in diameter using a vacuum pump (GenX International; Guelph, ON, Canada)

calibrated to 50 mmHg. The pumping system was connected to a standard 18-gauge hypodermic needle (Covidien Monoject™; Mansfield, MA, United States) and paired with a sterile 50 mL collection tube (CELLTREAT® Scientific Products; Pepperell, MA, United States). The aspirated follicular fluid was left to settle undisturbed for 10 min, allowing a visible cellular pellet containing COCs to form at the bottom of the tube. The supernatant containing GCs was then carefully transferred to a sterile 15 mL tube (Thermo Fisher Scientific; Waltham, MA, United States) with 3 mL of warm Dulbecco's Phosphate Buffered Saline (DPBS (1x)) without calcium and magnesium chloride (Sigma-Aldrich; St. Louis, MO, United States).

2.2.1 Antioxidant dilutions and preparation

Quercetin (QUE; Sigma-Aldrich; St Louis, MO, United States), Carnosol (CAR; MedChemExpress; Monmouth Junction, NJ, United States), and Sulforaphane (SFN; Selleck Chemicals; Houston, TX, United States) were prepared at 10 mM stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich; St Louis, MO, United States). To ensure consistency across treatments, the final DMSO concentration was standardized and maintained constant for all experimental groups, including the vehicle controls. Working dilutions were freshly prepared immediately before use.

2.2.2 Granulosa cells (GCs) culture, antioxidant supplementation, and heat stress (HS) exposure

The follicular fluid suspension containing GCs was centrifuged at 500 × g for 7 min, after which the GC pellet was collected and the supernatant discarded. The pellet was resuspended in red blood cell (RBC) lysis buffer (Sigma-Aldrich; St Louis, MO, United States) and incubated for 3 min. The reaction was stopped by washing the cells in Dulbecco's Modified Eagle's Media/Ham's Nutrient Mixture F12 (DMEM/F12) (Sigma-Aldrich; St. Louis, MO, United States) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich; St. Louis, MO, United States), 1% Penicillin-Streptomycin (Sigma-Aldrich; St. Louis, MO, United States), and 1% Amphotericin B solution (Sigma-Aldrich; St. Louis, MO, United States). The cells were then centrifuged again at 500 × g for 5 min. This process was repeated until a pure pellet was obtained. The supernatant was discarded, and the pellet was washed with DPBS (1x) before being resuspended in the DMEM/F12 media described above. Quantity and viability of GCs were assessed using a hemocytometer (Hausser Scientific, Horsham, PA, United States) and the trypan blue exclusion method (Sigma-Aldrich; St. Louis, MO, United States). Depending on the assay, GCs were seeded at 2×10^5 cells per well in 24-well plates or at 1×10^4 cells per well in 96-well tissue-culture-treated plates (United States Scientific, Inc., Ocala, FL) in DMEM/F12 media as described above.

Bovine GCs were initially cultured for 24 h until reaching subconfluency. Antioxidant supplementation was performed following a brief wash a with DPBS (1x) for QUE (5 μM), CAR (10 μM), SFN (1 μM), a combination of three (MIX), vehicle (DMSO), and an untreated control for 24 h. Subsequently, control groups were maintained under normal temperature (NT, 38.5 °C) for 24 h, while the HS-treated groups were transferred for 8 h to a different incubator programmed at 42 °C and then returned to normal temperature (NT) for the remaining 16 h, a method to mimic daylight conditions, and summing in a 72 h total length of culture. The 42 °C temperature is commonly used *in vitro* to induce a robust and reproducible heat-stress response without causing excessive cell death in bovine GCs (Roth and Hansen, 2004). A schematic overview of the experimental design is shown in (Supplementary Figure S1A).

2.2.3 *In vitro* maturation (IVM), antioxidant supplementation, and heat stress (HS) exposure

COCs were collected from abattoir ovaries as described above. Retrieved COCs were grouped (50 per well) and placed into 4-well culture dishes (Thermo Fisher Scientific, Waltham, MA, United States) containing 1 mL of chemically defined media for *in vitro* maturation of oocytes (IVM) pre-equilibrated at 38.5 °C in 5% CO_2 (De La Torre-Sanchez et al., 2006). At the time of equilibration, all IVM media were supplemented with 15 ng/mL NIDDK-oFSH-20, 1 μg/mL USDA-LHB-5, 1 μg/mL estradiol 17β, 50 ng/mL epidermal growth factor, and 0.1 mM cysteamine (De La Torre-Sanchez et al., 2006). Based on their ability to enhance nuclear activation of the NRF2 protein in cultured GCs, only QUE and SFN were selected for further evaluation during the oocyte maturation experiment. Consequently, the six experimental groups used during GC culture were reduced to four groups for the oocyte maturation experiments: 5 μM QUE under HS, 1 μM SFN under HS, and vehicle control (DMSO) under both thermoneutral and HS conditions.

In vitro oocyte maturation, which lasted 23 ± 1 h, was initiated by incubating all treatment groups under normothermic conditions (NT; 38.5 °C) for the first 8 h. Subsequently, the HS QUE, HS SFN, and HS control groups were transferred to a separate incubator set at 41 °C for the remaining 15 h, whereas the NT control group remained under NT conditions throughout the IVM period. Previous studies have shown that exposure of bovine oocytes to 41 °C during IVM is sufficient to impair developmental competence, supporting its use as a physiologically relevant heat stress (HS) model (Paula-Lopes and Hansen, 2002). A schematic overview of the experimental design is shown in (Supplementary Figure S1B).

2.3 *In vitro* fertilization (IVF) and embryo culture

For *in vitro* fertilization (IVF), COCs were transferred (in <20 μL of maturation media) into 4-well culture dishes containing 430 μL of equilibrated CSU chemically defined media for IVF (F-CDM) (De La Torre-Sanchez, Jose Fernando et al., 2006) and maintained at 38.5 °C and 5% CO_2 in humidified air. Frozen-thawed bull sperm from a single ejaculation were processed to separate viable and motile sperm using a multilayer 45%/90% Percoll® (Sigma-Aldrich; St. Louis, MO, United States) gradient and centrifuged at 800 × g for 20 min. Following this, the supernatant was discarded, and the remaining sperm pellet was washed with 2 mL of CSU chemically defined media for the handling of early embryos (HCDM-1) (De La Torre-Sanchez et al., 2006) and centrifuged at 300 × g for 5 min. Sperm concentration was determined using a hemocytometer, and the suspension was adjusted to 5×10^6 sperm/mL using equilibrated F-CDM. Diluted sperm was then added to COCs at a final concentration of 0.5×10^6 sperm/mL and co-incubated for 18 h at 38.5 °C in 5%

CO2 in humidified air. Following fertilization, presumptive zygotes were vortexed for 90 s at maximum speed to remove cumulus cells (CCs). Denuded zygotes were then transferred in groups of 50 to pre-equilibrated 4-well dishes containing 500 µL of CSU chemically defined media for *in vitro* culture of early embryos (CDM-1). Embryos were cultured for 56 h in a mixed gas incubator maintained at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2. Embryos with ≥4 blastomeres were then transferred in groups of 35 to pre-equilibrated 4-well dishes containing 500 µL of CSU chemically defined media for *in vitro* culture of late embryos (CDM-2). Embryos were cultured for 120 h in a mixed gas incubator maintained at 38.5 °C in 5% CO_2, 5% O_2, and 90% N_2 (De La Torre-Sanchez et al., 2006). Blastocyst developmental rates were evaluated on day 7 (168 h) and day 8 (192 h) post-IVF. A schematic overview of the experimental design is shown in (Supplementary Figure S1C).

2.4 Antioxidant dose optimization and cell viability assay for bovine GCs

Bovine GC collection, culture, HS treatment, and antioxidant supplementation were performed as indicated above. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technology, Japan). Doses tested were 1, 5, 10, and 20 µM for QUE, CAR, and SFN under NT and HS conditions. For this, 10 µL of the reagent was added to each well and incubated for 3 h at 38.5 °C in a 5% CO2 incubator. Optical density of the formazan dye was measured at 450 nm using a microplate reader (BioTek Instruments, Germany). Wells containing only media with the antioxidant, but no cells, served as blanks for background correction. All assays were performed in quadruplicate. Based on these results, the highest non-toxic doses were selected: 1, 5, and 10 µM for SFN, QUE, and CAR, and an antioxidant mix was based on these doses (Supplementary Figure S2). Additionally, these doses were used for further experiments.

2.5 Active NRF2 binding assay on nuclear extracts from bovine GCs

Nuclear activation of NRF2 in antioxidant-supplemented bovine GC nuclear extracts under NT and HS was assessed using the commercial Trans-AM® NRF2 kit (Active Motif, Carlsbad, CA, United States). Following culture, the NEPER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific; Waltham, MA, United States) was employed to separate nuclear and cytoplasmic protein fractions from bovine GCs from four biological replicates. Protein concentrations in the resulting nuclear extract suspensions were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, United States) following the manufacturer's instructions. Subsequently, the TransAM® NRF2 ELISA was used to analyze the nuclear protein extracts from individual and combined antioxidant-supplemented groups under NT and HS conditions. Optical density was measured at 450 nm with a reference wavelength of 655 nm using a microplate reader (BioTek Instruments, Germany). Wells containing all reactive agents was used as a blank for background correction.

2.6 Annexin V/propidium iodide (PI) staining on bovine GCs

To assess the apoptotic index (early apoptosis + late apoptosis) in bovine GCs, co-staining of annexin V and propidium iodide (PI) was performed using the Cell Meter APC-Annexin V Binding Apoptosis Assay Kit (Biomol International; Plymouth Meeting, PA, United States), according to the manufacturer's recommendations with minor modifications. GCs were analyzed using the Cytek 4 laser-Aurora™ (Cytek Biosciences; Fremont, CA, United States). Assays were performed in duplicate, and the percentages of different cell populations within the treatment groups (live and healthy, early apoptotic, late apoptotic, necrotic) were recorded for analysis.

2.7 Gene expression analysis of antioxidant and stress-related markers in GCs, denuded oocytes, and CCs, and embryo competence-associated genes in blastocysts

The impact of antioxidants on modulating stress response genes in GCs, denuded oocytes, and CCs was investigated using qRT-PCR. Total RNA from GCs was isolated from three replicates using the miRNeasy® mini kit (Qiagen; Hilden, Germany). Moreover, total RNA was extracted from four pooled replicates of denuded oocytes and CCs (50 COCs) and from blastocysts (10 blastocysts) using the RNeasy® plus micro kit (Qiagen; Hilden, Germany). Total RNA sample concentrations and integrity were assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, United States). RNA samples were stored at −80 °C until further use.

Following this, cDNA was synthesized by reverse transcription using oligo$(dT)_{18}$ primers and the First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific; Waltham, MA, United States). For each sample type, cDNA synthesis was performed using the maximum amount of high-quality RNA available per biological replicate, resulting in different RNA input amounts for granulosa cells (33.2 ng), denuded oocytes (22.2 ng), cumulus cells (29.4 ng), and blastocysts (16.8 ng). These differences reflect biological and technical limitations in RNA yield inherent to each sample type, rather than differences in experimental normalization. Gene expression analyses were conducted for each sample type, and relative expression levels were normalized to the geometric mean of the reference genes β-ACTIN and GAPDH. Gene-specific primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the list of primers is indicated in Supplementary Table S1. Formal qRT-PCR analysis was performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad; Hercules, CA, United States). The following thermocycling conditions were applied for amplification: initial denaturation at 95 °C for 3 min, followed by 40 cycles of amplification at 95 °C for 15 s, and 60 °C for 60 s. The specificity of the amplification was

determined by melting curve analysis generated at the end of each run, and the data were analyzed using the comparative C(T) method (Schmittgen and Livak, 2008).

2.8 Proximity ligation assay (PLA) of KEAP1-NRF2 protein-protein interactions in preimplantation embryos

Following the manufacturer's NaveniFlex PLA protocol (Navinci Diagnostics, Uppsala, Sweden), the embryos were counterstained using two primary antibodies (NRF2 polyclonal antibody, Invitrogen; Carlsbad, CA, United States, unconjugated, rabbit polyclonal antibody, PA138312; KEAP1 (G-2), Santa Cruz Biotechnology; Dallas, TX, United States, mouse monoclonal antibody, sc-365626) and mounted on a poly-L-lysine-coated slide (Newcomer Supply; Middleton, WI, United States) with 5–10 µL of ProLong Diamond Antifade Mountant (Invitrogen; Carlsbad, CA, United States) and a coverslip, allowing them to dry flat (>24 h). PLA/DAPI-labeled embryos were imaged using the LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective with fitted blue (345/455) and red (596/615) fluorescent filters. For the analysis, fluorescent images were converted to grayscale, puncta counts and total cell counts were obtained using ImageJ software (National Institute of Health, Bethesda, MD, United States). The ratio of PLA puncta count normalized to total cell number from individual blastocysts was subsequently calculated. For each treatment group, 6–11 blastocysts were analyzed.

2.9 Mitochondrial function assays on bovine GCs, denuded oocytes, and preimplantation embryos

2.9.1 Intracellular reactive oxygen species (ROS) accumulation assay

The impact of thermal stress on intracellular ROS accumulation was assessed using 10 µM 2′, 7′-Dichlorofluorescein Diacetate (H2DCFDA) (Sigma Aldrich; St. Louis, MO, United States) on GCs, bovine denuded oocytes, and blastocysts. GCs were seeded at 2 × 10^5 cells per well in 24-well plates containing 600 µL of media as described above. Following the culture (washing, antioxidant supplementation, and heat exposure), the media was removed, and cells from both NT and HS treatments were washed twice with DPBS (1x) (Sigma-Aldrich; St. Louis, MO, United States). Cells were then incubated with 10 µM H2DCFDA diluted in 200 µL of non-supplemented F12 culture media for 30 min in a CO2 incubator. After incubation, cells were washed three times using DPBS (1x) (Sigma Aldrich; St. Louis, MO, United States), and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Fluorescence intensity was quantified from. jpg images from different biological replicates, taken from two specific locations per well, across four wells per treatment group per replicate (n = 16 images per group). All images were acquired with identical exposure and light settings, avoiding signal saturation, using a green fluorescent filter (excitation 494 nm, emission 518 nm). Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, United States).

ROS accumulation in denuded oocytes (n = 20 per treatment) and blastocysts (n = 14 per treatment) was assessed as described above. Briefly, denuded oocytes and blastocysts from each treatment group were washed twice in phosphate-buffered saline supplemented with 0.1% polyvinylpyrrolidone (DPBS (1x)-PVP) (Sigma Aldrich; St. Louis, MO, United States). Samples were then incubated for 30 min at 38.5 °C in 50 µL drops of 10 µM H2DCFDA dissolved in H-CDMM (for oocytes) or HCDM-2 (for blastocysts). Following incubation, oocytes and blastocysts were washed three times in DPBS (1x) containing PVP (0.1%) and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Fluorescence intensity was quantified from. jpg images by manually selecting the oocyte or blastocyst area. All images were acquired with identical exposure and light settings, avoiding signal saturation, using a green fluorescent filter (excitation 494 nm, emission 518 nm). Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, United States).

2.9.2 Mitochondrial Membrane Potential (MMP) δΨm assay

The detrimental effect of heat stress on mitochondrial depolarization was analyzed using the cationic dye 5,5′,6,6′-tetrachloro1,1′,3,3′ tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, United States) on GCs, denuded oocytes, and blastocysts. GCs were seeded at 2 × 10^5 cells per well in 24-well plates containing 600 µL of media as described above. Following the culture (washing, antioxidant supplementation, and heat exposure), the media was removed, and cells from both NT and HS treatments were washed twice with DPBS (1x) (Sigma-Aldrich; St. Louis, MO, United States). Cells were then incubated with 2 µM JC-1 staining diluted in 200 µL media culture for 30 min in a CO2 incubator. After incubation, cells were washed three times using DPBS (1x) (Sigma Aldrich; St. Louis, MO, United States) and immediately imaged using an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan). Images were captured at two defined locations per well, across four wells per treatment group per replicate (n = 16 total per treatment).

MMP (δΨm) in oocytes (duplicates; a total of 20 oocytes per treatment) and blastocysts (duplicates; a total of 14–15 blastocysts per treatment) was evaluated using the cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Invitrogen, Carlsbad, CA, United States), dissolved in H-CDMM for oocytes and HCDM-2 media for blastocysts, as an indicator of mitochondrial activity, as previously described (Pasquariello et al., 2019). Briefly, denuded mature oocytes and blastocysts from the respective treatment groups were incubated with 2 µM JC-1 for 30 min in the dark at 38.5 °C under their optimal culture conditions. Following incubation, samples were washed three times with DPBS (1×) supplemented with 0.1% PVP and immediately imaged using an inverted laser scanning confocal microscope. Oocytes were imaged using the ZEISS LSM800 equipped with a 20× air objective, while blastocysts were imaged using the ZEISS LSM980 (Carl Zeiss Microscopy, Jena, Germany) equipped with a 60× oil objective.

Fluorescence intensity was quantified from. jpg (for GCs) and. czi (for oocytes and blastocysts) images obtained from different biological replicates. Imaging for J-monomers was conducted using a wavelength of 488 nm for J-monomers and excitation/emission settings at 490/525 nm (FITC filter, green fluorescence), indicative of low MMP. Similarly, images for J-aggregates were carried out using a wavelength of 561 nm and excitation/emission at 596/615 nm (TRITC filter/red fluorescence), indicating high MMP. Fluorescence intensity was analyzed using ImageJ software (National Institute of Health, Bethesda, MD, United States). The ratio of red to green fluorescence was subsequently calculated to determine MMP, expressed as an arbitrary unit.

2.9.3 Preimplantation embryo oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) assays

Metabolic analyses of individual day 7 blastocysts (n = 4–17) were performed using a microchamber with electrochemical-based oxygen and pH sensors, as described (Obeidat Y. et al., 2019; Obeidat Y. M. et al., 2019). Briefly, the microchamber was filled with 150 µL of CSU chemically defined media (CDM-2). Then individual embryos were placed in the middle of each of the six channels containing CDM-2 media. The three-electrode oxygen sensor was connected to a potentiostat (Quadstat EA 164H, eDAQ Inc., Colorado Springs, CO) that applied a voltage to the sensor and monitored the decrease in oxygen-reduction current over time. The applied potential for all amperometric oxygen assays was set to −0.6 V. The pH sensor was connected to a custom-made Ina333 instrumentation amplifier circuit that measured the change in voltage. The starting (room air-saturated) oxygen concentration and pH of the media were measured as baseline values for 2 min and calibrated as previously described in detail (Obeidat Y. et al., 2019).

2.10 Statistical analysis

Data were analyzed in GraphPad Prism version 8.4.2 (GraphPad; San Diego, CA, United States). For experiments in which all treatments were tested at both temperatures, a two-way Analysis of Variance (ANOVA) was performed with temperature and treatment as fixed factors, followed by Tukey's multiple-comparison test. When treatments were applied at only one temperature (HS), data were analyzed using one-way ANOVA, and Tukey's *post hoc* test was used to compare treatment groups with the corresponding temperature-matched control. Data are presented as the Mean ± SEM of biological replicates. Statistical significance was identified at $p \leq 0.05$.

3 Results

3.1 Dose optimization for antioxidant supplementation in bovine GCs

To determine the optimal antioxidant concentrations, we first evaluated doses of 1, 5, 10, and 20 µM for QUE, CAR, and SFN. As indicated in Supplementary Figure S2, most of the doses tested did not compromise cell viability, confirming their safety and efficacy under conditions of HS. Based on these results, we then selected the highest non-toxic, biologically active concentrations,1 µM SFN, 5 µM QUE, and 10 µM CAR, for all subsequent experiments. In addition, a combinatorial treatment (MIX) was also included to assess potential synergistic effects antioxidants. For all endpoints evaluated, two-way ANOVA revealed a significant temperature × antioxidant treatment interaction ($p < 0.05$), indicating that antioxidant effects were dependent on the presence or absence of thermal stress conditions.

3.2 Impact of QUE, CAR, and SFN on the cellular NRF2-mediated oxidative stress response pathway

Gene expression analysis revealed that *NRF2* transcript levels were upregulated in all antioxidant-treated groups under HS conditions compared to the respective control, with statistical significance for only HS QUE ($p = 0.0260$) and HS SFN ($p = 0.0163$) compared to HS control (Supplementary Figure S3). Moreover, increased expression of downstream antioxidant genes *SOD1, HO1, and PRDX1* ($p < 0.05$) was observed in the SFN-treated group compared with the nontreated control and vehicle groups under HS condition. Quantification of nuclear NRF2 protein in cultured GCs demonstrated an increase in QUE ($p = 0.0002$) and SFN ($p = 0.0007$) treated groups under HS conditions compared to the non-supplemented HS control and vehicle groups (Figure 1A). ROS accumulation was reduced for QUE ($p < 0.0001$), CAR ($p < 0.0001$), SFN ($p < 0.0001$), and MIX ($p < 0.0001$) compared to the control and vehicle under HS conditions (Figure 1B). Additionally, MMP was increased in GCs treated with QUE ($p = 0.0130$), CAR ($p = 0.0048$), SFN ($p = 0.0382$), and MIX ($p < 0.0001$) under conditions of HS compared to the control and vehicle-treated groups (Figure 1C), indicating improved mitochondrial function and maintenance of cellular homeostasis. Reductions in the total rate of apoptosis in GCs were also observed for QUE ($p = 0.0187$), CAR ($p = 0.0234$), SFN ($p = 0.0165$), and MIX ($p = 0.0228$) compared to the control group under HS conditions (Figure 1D; Supplementary Figure S3). Among the three antioxidants evaluated in cultured GCs, QUE and SFN induced a statistically significant increase in nuclear activation of the NRF2 protein, as determined by nuclear extract ELISA assays (Figure 1A). Therefore, given the central role of NRF2 in regulating antioxidant defense mechanisms, subsequent oocyte experiments were conducted using only these two antioxidants, QUE and SFN.

3.3 Antioxidant supplementation effectively mitigates the detrimental effects of HS during oocyte maturation via modulating the physiology of the surrounding CCs

Cumulus cells displayed distinct transcriptional responses to antioxidant supplementation under HS conditions (Figure 2A). Our results showed that *HSP90* expression was reduced in the QUE-treated group compared to the HS control ($p = 0.04$), while *GRP94* expression was also lower in the QUE-treated group ($p = 0.008$). Similarly, *SOD1* expression was upregulated in CCs from both the

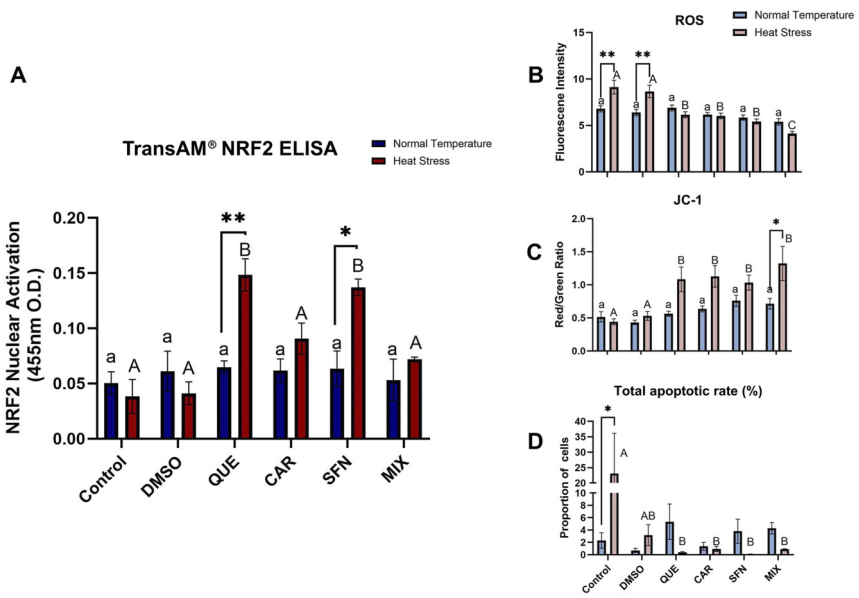

FIGURE 1
The beneficial effect of antioxidant supplementation on NRF2 protein nuclear expression and cellular homeostasis in bovine GCs. **(A)** TransAM® NRF2 ELISAs were used for nuclear protein extracts from antioxidant-supplemented bovine granulosa cells under Normal Temperature and Heat Stress. Gene expression analysis to quantify selected stress-related genes NRF2, SOD1, HO1, PRDX1, CAT, HSP70, HSP90, GRP78, and GRP94 is shown in Supplementary Figure S3A. Blue bars represent Normal Temperature groups, and red bars represent Heat Stress treatments. **(B)** Reactive Oxygen Species (ROS) accumulation assay 2′,7′- DCFH-DA quantification of the relative fluorescence intensity of Reactive Oxygen Species (ROS) in bovine GCs. **(C)** Quantitative red/green fluorescence intensity ratio using 5,5′,6,6′tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for the measurement of Mitochondrial Membrane Potential (ΔΨm), an indicator of mitochondrial activity in bovine GCs. Green fluorescence represents JC-1 monomers, whereas red fluorescence represents JC-1 aggregates. **(D)** Annexin V/PI staining was evaluated using flow cytometry to determine the total apoptotic rate, including the proportion of early and late apoptotic cells. Representative panels shown in Supplementary Figure S3B. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001 between NT and HS conditions within the treatment group. Different lowercase letters indicate significant differences between NT treatment groups, and uppercase letters indicate significant differences between HS treatment groups.

QUE group ($p = 0.003$), and the SFN group ($p = 0.0001$). Also HS SFN showed increased expression of *CAT* ($p = 0.02$) compared to the HS control group. However, transcript levels in denuded oocytes remained unaltered across all treatment groups (Supplementary Figure S4). ROS accumulation assay revealed that both antioxidants successfully reduced ROS accumulation (Figure 2B). Consistently, the MMP assay assessed by JC-1 staining was enhanced in both the QUE ($p = 0.0004$) and SFN-treated ($p < 0.0001$) groups compared to the HS control group (Figure 2C).

3.4 The effect of antioxidant supplementation during oocyte maturation on the developmental capacity of oocytes following IVF

As shown in Figure 3A, compared to those cultured under thermoneutral conditions, exposure of oocytes to HS results in a drastic reduction in the number of oocytes that develop to the blastocyst stage following IVF ($p < 0.0001$). However, priming oocytes with both QUE and SFN resulted in higher blastocyst rates compared to the non-supplemented HS group with $p < 0.0001$ and $p = 0.0002$, respectively, following the same trend in blastocyst rate from cleaved embryos (Figure 3B). Similarly, while HS reduced total cell number per blastocyst compared to the NT group ($p = 0.0166$; Figure 3C), supplementation with QUE and SFN restored the cell number to the level comparable to the NT group.

3.5 Antioxidant supplementation under HS conditions during IVM modulates ROS accumulation and mitochondrial membrane potential of the resulting blastocysts

To elucidate the long-term impact of antioxidant priming and HS exposure during oocyte maturation on the molecular architecture and viability of the resulting blastocysts, various metabolic and molecular parameters were investigated. As shown in Figure 4A, although IVF and IVC were conducted under thermoneutral conditions without antioxidant supplementation, priming oocytes with QUE or SFN effectively restored ROS levels in blastocysts to those observed under thermoneutral conditions, compared with HS groups (HS QUE, $p = 0.0024$; HS SFN, $p = 0.0002$). Furthermore, both antioxidant treatments successfully recovered MMP of blastocysts compared with the non-supplemented HS control (HS QUE, $p = 0.0008$; HS SFN, $p < 0.0001$).

To further investigate the impact of antioxidant supplementation on the activity of the NRF2-KEAP1 signaling pathway in the resulting blastocysts, the highly sensitive

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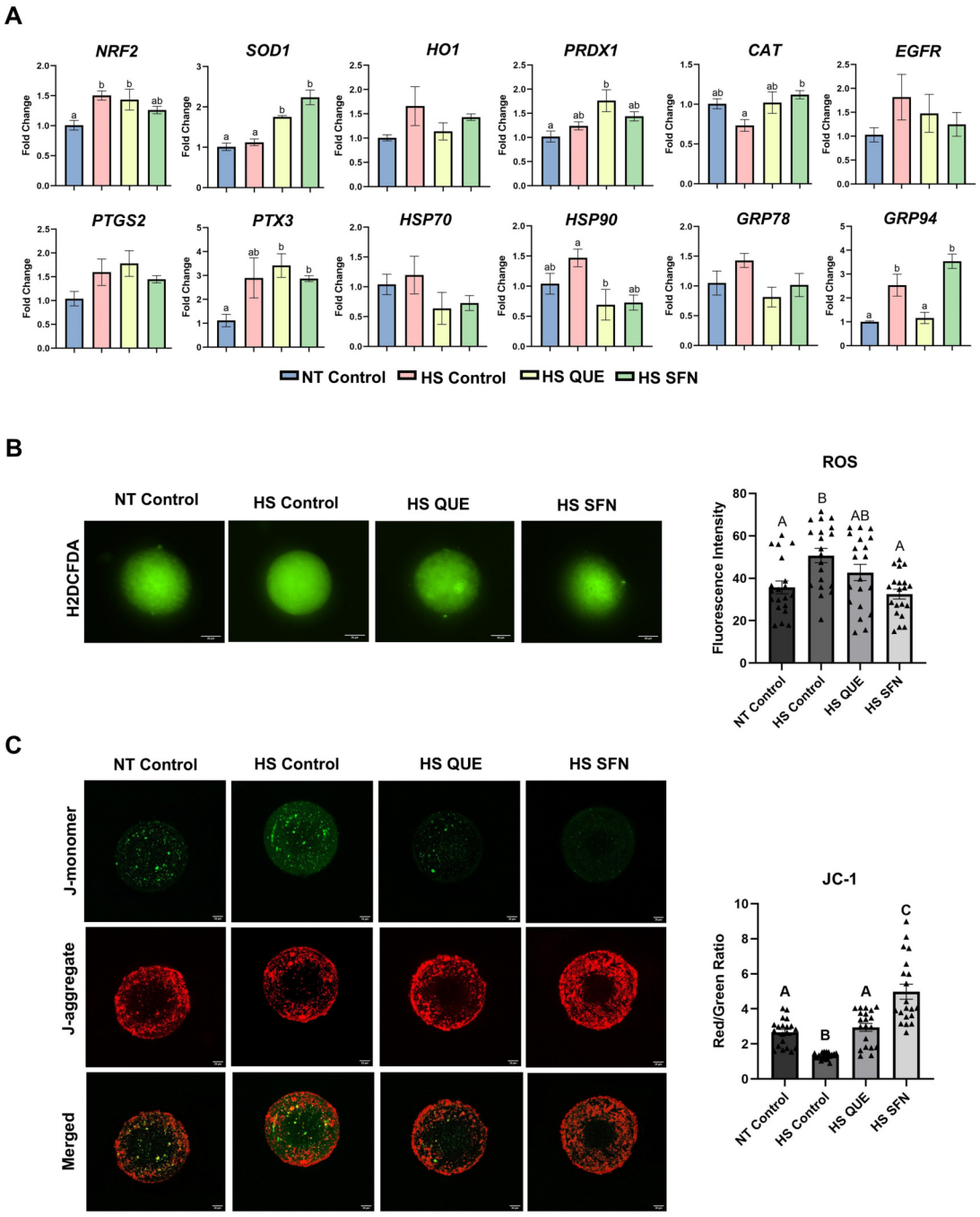

FIGURE 2
The protective effect of antioxidant supplementation mitigating HS effects on cumulus cells transcripts and oocyte function following maturation. **(A)** Stress-related gene expression analysis in cumulus cells, qRT-PCR was performed to quantify selected stress-related genes *NRF2, SOD1, HO1, CAT, PRDX1, HSP70, HSP90, GRP78, GRP94,* as well as cumulus cell expansion genes *PTGS2, EGFR*, and *PTX3.* Gene expression analysis in denuded oocytes did not show variations and is shown in Supplementary Figure S4. Results are from cumulus cells removed from four pools, each containing 50 oocytes, for a total of 200 oocytes per treatment. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Different lowercase letters indicate significant differences between treatment groups. **(B)** Reactive Oxygen Species (ROS) accumulation assay 2′,7′- DCFH-DA quantification of the relative fluorescence intensity of Reactive Oxygen Species (ROS) in single oocytes was carried out in triplicate (total = of 15–20 oocytes per treatment). Fluorescence images of *in vitro* matured bovine oocytes treated with H2DCFDA were captured for the measurement of intracellular ROS levels in single oocytes. Scale bar, 20 μM. **(C)** Fluorescence images of *in vitro* matured bovine oocytes treated with 5,5′,6,6′tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for the measurement of MMP (δΨm), an indicator of mitochondrial activity in single oocytes. Scale bar, 20 μM. Quantification of the relative fluorescence intensity (red/green ratio) in single oocytes was carried out in duplicates (total = of 15–20 oocytes per treatment). Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different upper case letters indicate a statistical difference between treatment groups $p \leq 0.05$).

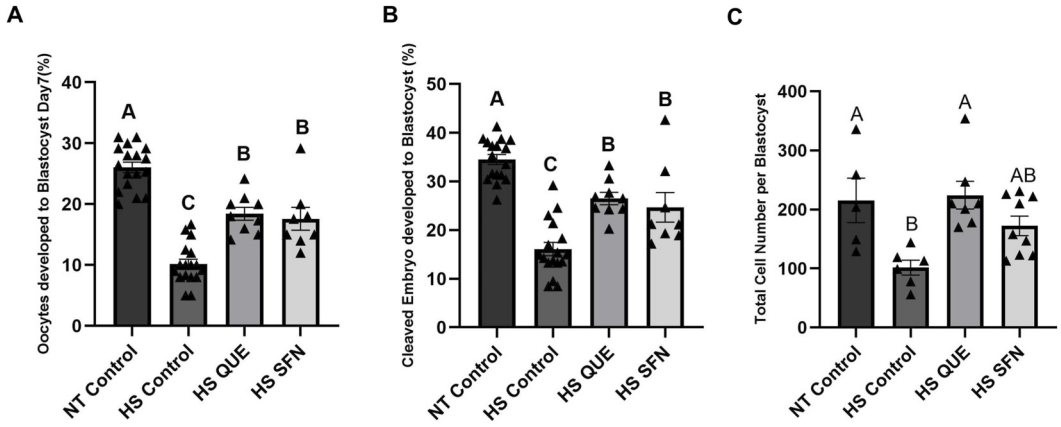

FIGURE 3
The effect of antioxidant supplementation on the developmental capacity of oocytes matured under Heat Stress. Results are from a total of 8-9 replicates (780–900 total oocytes per treatment). **(A)** Percentage of oocytes developed into blastocysts. **(B)** Percentage of cleaved embryos developed to blastocyst. **(C)** Total cell number per single blastocyst, quantification of total cell numbers in single blastocysts was carried out in quadruples (a total of 5–10 blastocysts per treatment). Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters show a statistically significant differences between treatment groups with $p \leq 0.05$.

Proximity Ligation Assay (PLA) was used to assess protein-protein interactions between KEAP1 and NRF2 (Figure 4C). In representative images, Naviflex Puncta (red fluorescence), the cytoplasmic binding between NRF2 and KEAP1 across different treatment groups was presented in puncta. Antioxidant supplementation during IVM promoted dissociation of KEAP1-NRF2 complexes, as evidenced by reduced puncta count per blastomere compared with the HS control group (HS QUE $p = 0.0007$; HS SFN $p < 0.0001$). Puncta counts were normalized to the total cell number of each corresponding embryo.

3.6 Metabolic function of preimplantation embryos derived from antioxidant primed oocytes matured under HS conditions

In the present study, we integrated a metabolic multi-sensor platform capable of monitoring a single embryo oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real-time, by applying amperometric methods described in (Obeidat Y. et al., 2019). The use of this microchamber enables monitoring of single-embryo metabolism during development in a small volume of media. OCR, which is an indicator of aerobic metabolism, was measured in fmol per second per embryo. ECAR, which is an indirect marker of anaerobic metabolism, was measured in mpH per minute per embryo. As shown in Figure 5, supplementation with both QUE and SFN reduced OCR to the thermoneutral level, compared with non-supplemented HS control groups (HS QUE, $p = 0.0078$; HS SFN, $p = 0.0048$). Similarly, ECAR values were reduced to levels comparable to the thermoneutral embryos when compared to the HS control group (HS QUE $p = 0.0009$; HS SFN $p = 0.0067$).

3.7 Antioxidant supplementation during oocyte maturation restored the developmental capacity of blastocysts as determined by embryo competence index

As shown in Figure 6, the ECI values were reduced for blastocysts derived from non-supplemented HS control oocytes compared with the thermoneutral group ($p = 0.0070$). However, supplementation with QUE and SFN during oocyte maturation under thermal stress conditions rescues the ECI of the resulting blastocysts compared with HS controls ($p = 0.0052$ and p = 0.0054, respectively). Among the competence-associated genes (Figure 6A), CHSY1 was significantly reduced in the non-supplemented HS control group compared to the thermoneutral control group ($p = 0.0339$). However, the supplementation of SFN increased CHSY1 expression compared to the HS control group ($p = 0.0008$). Among the non-competence-associated genes (Figure 6B), CCNA2 was upregulated in the HS control group compared to the NT control ($p = 0.0149$), while supplementation of QUE and SFN downregulated CCNA2 expression as compared to the HS control ($p = 0.0464$ and 0.0047, respectively). Moreover, QUE supplementation reduced EIF4A3 expression compared to the HS control group ($p = 0.0180$). Collectively, supplementation with QUE and SFN during oocyte maturation under thermal stress conditions rescues the ECI of the resulting blastocysts compared with their non-treated thermal stressed counterparts (Figure 6C).

4 Discussion

Environmental thermal stress remains one of the most detrimental factors compromising reproductive efficiency in livestock, exerting profound effects on ovarian function, oocyte maturation, and early embryo development through oxidative

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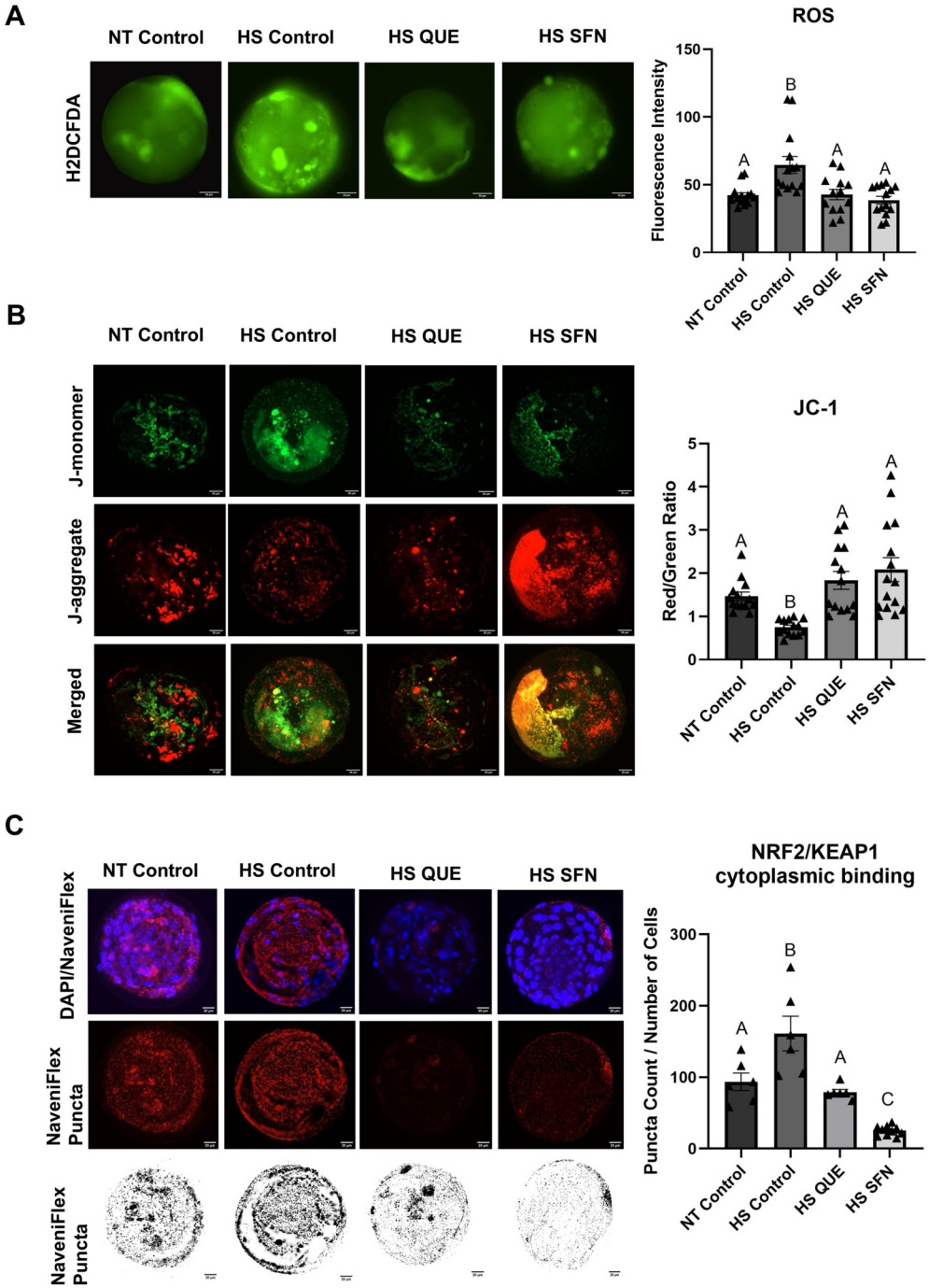

FIGURE 4
Functional alterations of antioxidant supplementation under HS conditions during *in vitro* maturation on blastocyst quality and NRF2-KEAP1 Protein-Protein Interaction. **(A)** Fluorescence images of *in vitro* cultured bovine blastocysts treated with 2′,7′-Dichlorofluorescin Diacetate (H2DCFDA) were captured for the measurement of intracellular ROS levels in single blastocysts. Scale bar, 20 μM. Quantification of the relative fluorescence intensity for reactive oxygen species (ROS) in single blastocysts was carried out in triplicate (a total of 12–15 blastocysts per treatment). **(B)** Fluorescence images of *in vitro* cultured bovine blastocysts treated 5,5′,6,6′tetrachloro-1,1′,3,3′tetraethylbenzimidazolylcarbocyanine iodide (JC-1) for the measurement of MMP ($\delta\Psi m$), an indicator of mitochondrial activity in single blastocysts. Scale bar, 20 μM. Quantification of the relative fluorescence intensity (red/green ratio) in single blastocysts was carried out in duplicates (a total of 12–15 blastocysts per treatment). **(C)** To further investigate the mechanistic role of antioxidant signaling in embryonic development, the colocalization of KEAP1 and NRF2 protein-protein interaction was evaluated using the highly sensitive Proximity Ligation Assay (PLA) and confocal fluorescence microscopy. Representative fluorescence images show Naviflex Pucta (red fluorescence) represents cytoplastic binding between NRF2 and KEAP1 in different treatment groups. Puncta count was optimized based on the total cell number of the corresponding embryo. A total of 6–10 individual embryos were used per group for four different replicates. Fewer puncta were correlated with greater free, active NRF2 availability in the blastocyst cytoplasm. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters show a statistically significant differences between treatment groups with $p \leq 0.05$.

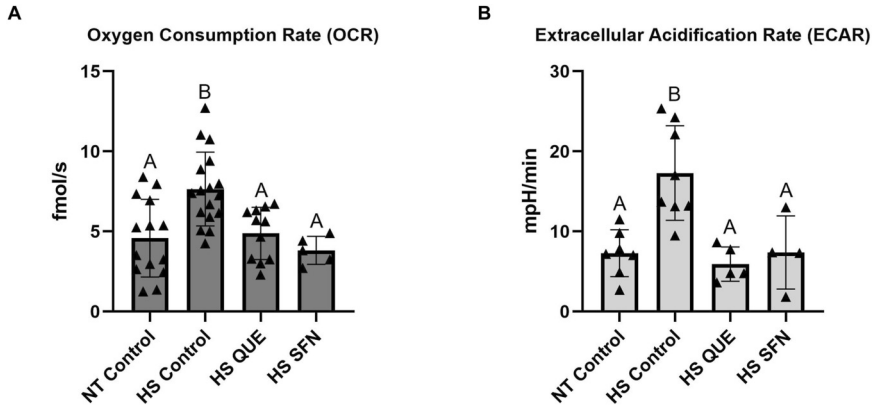

FIGURE 5
Metabolic function of preimplantation embryos from antioxidant-treated oocytes matured under HS conditions. The Metabolic multisensor platform was used on single pre-implantation embryos to quantify: **(A)** Oxygen Consumption Rate (OCR) in quercetin and sulforaphane treatments under HS conditions measured in single embryos (n = 4–17 per group). **(B)** Extracellular Acidification Rate (ECAR) in quercetin and sulforaphane treatments under HS conditions measured in single embryos (n = 4-7 per group). Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Different uppercase letters show a statistically significant differences between treatment groups with $p \leq 0.05$.

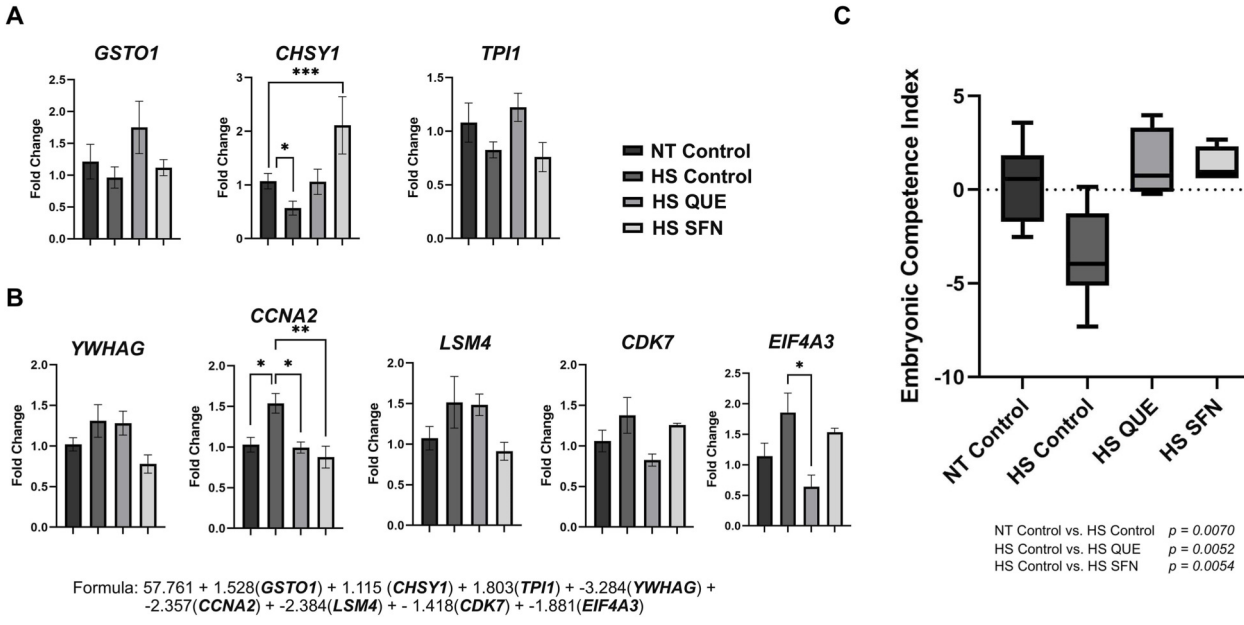

FIGURE 6
The Embryonic Competence Index (ECI) is restored by antioxidant supplementation during IVM under HS. This predictive tool is based on a Bayesian multiple regression formula that identifies transcriptomic biomarker abundance to distinguish in vivo-derived blastocysts that resulted in pregnancy from those that did not, and it validates a predictive formula for assessing embryo vitality. qRT-PCR was performed to quantify: **(A)** Markers corresponding to glutathione S-transferase omega 1 (*GSTO1*), chondroitin sulfate synthase 1 (*CHSY1*), and triosephosphate isomerase 1 (*TPI1*), which are increased in competent embryos. **(B)** Markers: tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*YWHAG*), cyclin A2 (*CCNA2*), LSM4 homolog (U6 small nuclear RNA and mRNA degradation associated, *LSM4*), cyclin-dependent kinase 7 (*CDK7*), and eukaryotic translation initiation factor 4A3 (*EIF4A3*) increased in noncompetent embryos. **(C)** Embryonic Competence Index (ECI). The following formula (R2 = 0.96) was used to determine the ECI of blastocysts based on the expression level of the 8 candidate genes: ECI = 57.761 + 1.528(*GSTO1*) + 1.115 (*CHSY1*) + 1.803(*TPI1*) + −3.284(*YWHAG*) + −2.357(*CCNA2*) + - 2.384(*LSM4*) + −1.418(*CDK7*) + −1.881(*EIF4A3*). Data are presented as mean ± SEM, and the mean differences were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons. Statistical differences for each gene between treatment groups is denoted with *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. P-values for ECI comparisons between treatment groups are shown in the graphic.

stress, mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis (Amaral et al., 2020; Gaskins et al., 2021; Sammad et al., 2020). The objective of this study was therefore to determine whether supplementation with plant-derived antioxidants during *in vitro* culture of bovine follicular cells and oocytes under HS could mitigate oxidative and mitochondrial damage in GCs,

oocytes, and restore the developmental competence of the resulting embryos.

As an initial screening step, we evaluated three antioxidants (QUE, CAR, and SFN), as well as their combination, in GCs exposed to HS, assessing cellular function, maintenance of oxidative homeostasis, and their ability to activate NRF2. Although all antioxidants improved redox homeostasis, only QUE and SFN induced a significant increase in nuclear NRF2 activation (Figure 1A), as confirmed by our nuclear extracts ELISA assays. Because NRF2 activation is a key regulator of antioxidant defense mechanism, these results provided a mechanistic rationale to narrow subsequent IVM experiments using QUE and SFN. This approach ensured that only compounds showing both functional improvement and mechanistic role in activating the antioxidant capacity of the oocytes and embryos via NRF2-KEAP1 pathway were selected.

Oocytes are particularly vulnerable to thermal and oxidative insults, with sensitivity reported as early as 105 days before ovulation and persisting through ovulation and fertilization (Torres-Júnior et al., 2008). Given this prolonged window of susceptibility, impaired developmental outcomes under HS conditions are largely attributable to compromised oocyte quality rather than sperm factors (Rahman et al., 2018; Roth, 2017). Excess ROS in the ovary deteriorates oocyte competence, induces GCs apoptosis, disrupts GCs–oocyte communication, alters cytoskeletal and spindle assembly dynamics, and accelerates ovarian aging through mitochondrial dysfunction, telomere shortening, inflammation, and apoptosis (Prasad et al., 2016; Yaacobi-Artzi et al., 2022; Yang et al., 2021). Consistent with these observations, HS in our model increased ROS and reduced MMP in oocytes (Figures 2B,C), despite minimal transcriptomic changes in the oocyte itself (Supplementary Figure S4). However, transcripts in the CCs exhibited clear antioxidant activation as both QUE and SFN increased NRF2 downstream genes (Figure 2A). ROS accumulation (Figure 2B), markedly elevated under HS, was reduced by both antioxidants to levels comparable to the NT control. The MMP, which is reduced under HS, as previously reported (Menjivar et al., 2023), was restored by QUE and SFN, demonstrating the ability of these antioxidants to preserve mitochondrial function during IVM. These findings parallel previous reports where SFN alleviated chemically induced oxidative stress in mouse oocytes (Li et al., 2023), and protected bovine oocytes against paraquat toxicity through NRF2-mediated mechanisms (Feng et al., 2023). The increased expression of NRF2-downstream genes in cumulus cells (Figure 2A) further supports the role of the cumulus compartment in mediating antioxidant protection during oocyte maturation. Together, our data reinforce that QUE and SFN offer a targeted strategy to counteract HS-induced oxidative stress during IVM via modulating the antioxidant capacity of the surrounding cumulus cells.

Thermal stress exposure during oocyte maturation induced a pronounced carryover effect, leading to reduced blastocyst development (Figures 3A,B), likely due to the cumulative impact of oxidative stress on embryonic developmental capacity. Moreover, the resulting embryos exhibited elevated ROS levels (Figure 4A) and decreased MMP (Figure 4B), consistent with our observation in GCs and oocytes. To evaluate whether antioxidant supplementation during IVM influenced NRF2 signaling in the blastocysts, we employed the highly sensitive protein-protein interaction assay, to assess KEAP1–NRF2 interactions (Figure 4C). In control embryos derived from non-supplemented heat-stressed oocytes, abundant KEAP1–NRF2 puncta indicated cytoplasmic sequestration of NRF2. In contrast, QUE and SFN supplementation during IVM significantly reduced puncta counts per blastomere, reflecting greater availability of free, active NRF2 in the cytoplasm (Figure 4C). These findings mirror our observations in GCs, where both antioxidants increased NRF2 translocation into the nucleus, despite exposure to thermal stress. This concept aligns with our previous studies showing that QUE during bovine embryo culture activates NRF2 and improves mitochondrial activity and developmental outcomes (Khadrawy et al., 2020).

On the other hand, HS exerts profound adverse effects on early embryo development, particularly prior to zygotic genome activation (ZGA), and is a major contributor to early pregnancy loss in cattle (de Barros and Paula-Lopes, 2018). Recent advances in embryo biology have identified molecular markers predictive of embryonic competence, enabling the development of the Embryonic Competence Index (ECI) to quantify the likelihood of embryo survival and pregnancy establishment (Rabaglino et al., 2023; Rabaglino and Hansen, 2024). This index is dependent on the expression of competence-associated genes (*GSTO1, CHSY1*, and *TPI1) involved in* energy metabolism, and non-competence-associated genes (*CCNA2, CDK7, EIF4A3, YWHAG*, and *LSM4*) involved in mRNA processing and cell cycle regulation. In the present study, we examined whether antioxidant supplementation (QUE and SFN) during IVM under HS conditions could restore embryonic competence, using both ECI assessment and single-embryo metabolic measurements (Figures 5A,B). Restoration of ECI using antioxidant supplementation was accompanied by reduced OCR and ECAR compared with HS controls, indicating that embryo competence is linked to the level of embryonic energy expenditure (Leese et al., 2022). Reduction of both OCR and ECAR to levels comparable to non–heat-stressed (NT) controls following antioxidant supplementation indicated stabilization of embryonic energy metabolism, consistent with the "Quiet Embryo Hypothesis" (Leese, 2002; Leese et al., 2022), which proposes that developmentally competent embryos exhibit lower metabolic activity than less viable counterparts. These results suggest that QUE and SFN not only stabilize metabolic activity but also positively modulate gene networks underlying embryo competence, enhancing the likelihood of sustaining pregnancy. By incorporating ECI into our study, we demonstrate that antioxidant supplementation during oocyte maturation can mitigate the long-term impact of thermal stress on embryos' ability to induce pregnancy after transfer.

Collectively, our findings demonstrate that supplementation of QUE and SFN during oocyte maturation under thermal stress conditions attenuates the detrimental impact of HS in oocytes, stabilizes energy metabolism, and restores embryonic competence by improving the antioxidant capacity of the oocytes and surrounding cumulus cells and the resulting blastocysts via activating NRF2 mediated oxidative stress response pathway. To our knowledge, this is the first report demonstrating that the antioxidants QUE and SFN can effectively reverse the long-term consequences of heat stress in oocytes, including impaired metabolic

efficiency and altered embryonic competence in preimplantation embryos. These findings highlight a promising strategy to improve assisted reproductive technology (ART) outcomes under environmental stress conditions and provide a mechanistic foundation for integrating pharmacological approaches to enhance antioxidant capacity in ovarian cells, oocytes and preimplantation embryos in livestock species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because No live animals were involved in the experiments.

Author contributions

GR: Writing – review and editing, Data curation, Investigation, Methodology, Writing – original draft. AG: Writing – review and editing, Validation, Investigation, Data curation. NM: Data curation, Writing – review and editing, Conceptualization, Validation, Supervision. SB: Investigation, Writing – review and editing, Data curation, Visualization, Methodology. M-HC: Visualization, Methodology, Investigation, Formal Analysis, Data curation, Writing – review and editing. TC: Data curation, Writing – review and editing, Supervision, Investigation. SG: Visualization, Methodology, Software, Writing – review and editing, Validation. DT: Conceptualization, Supervision, Project administration, Funding acquisition, Resources, Writing – review and editing, Formal Analysis.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY FIGURE 1
Experimental models for **(A)** Bovine granulosa cell in vitro culture. **(B)** In vitro maturation. **(C)** In vitro embryo production. Antioxidant supplementation and heat stress exposure.

SUPPLEMENTARY FIGURE 2
Dose-dependent impact of antioxidant supplementation on bovine granulosa cell viability. Cell Viability assay CCK8 for each antioxidant under heat stress (HS) and normal temperature (NT). **p < 0.01; ***p < 0.001 between NT and HS conditions within the treatment group; different lowercase letters show significant differences between NT treatment groups and uppercase letters for HS treatment groups. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

SUPPLEMENTARY FIGURE 3
The impact of antioxidant supplementation on stress-related genes and apoptotic rate. **(A)** qRT-PCR was performed to quantify selected stress-related genes NRF2, SOD1, HO1, PRDX1, CAT, HSP70, HSP90, GRP78, and GRP94. Data are presented as mean ± SEM, and the mean differences were analyzed using the two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. *p < 0.05, **p < 0.01, ***p < 0.001 between normal temperature (NT) and heat stress (HS) conditions within the treatment group. Different lowercase letters indicate significant differences between treatment groups under NT, and uppercase letters indicate significant differences between treatment groups under HS condition. **(B)** Representative panels show the distribution of cells stained for both Annexin V and PI across the different quadrants between the different treatment groups. Blue bars represent Normal Temperature groups, and red bars represent Heat Stress treatments.

SUPPLEMENTARY FIGURE 4
The impact of antioxidant supplementation on transcript levels of stress-associated genes in denuded oocytes following maturation. To assess stress-related gene expression in denuded oocytes, qRT-PCR was performed to quantify *NRF2, SOD1, HO1, CAT, PRDX1, HSP70, HSP90, GRP78,* and *GRP94*. Results were obtained from denuded oocytes from four replicates (a total of 200 denuded oocytes per treatment). Stress-related gene expression analysis in cumulus cells, qRT-PCR was performed to quantify selected stress-related genes *NRF2, SOD1, HO1, CAT, PRDX1, HSP70, HSP90, GRP78, GRP94*, as well as cumulus cell expansion genes *PTGS2, EGFR*, and *PTX3*. Results are from cumulus cells removed from a total of four pools, each containing 50 oocytes, for a total of 200 oocytes per treatment. Data are presented as mean ± SEM, and the mean differences were analyzed using the One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Different lowercase letters indicate statistically significant differences between treatment groups.

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