Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Combined Phloretin and Idebenone Supplementation Improves Ovine Oocyte Quality and Early Embryonic Development by Attenuating Oxidative Stress and Mitochondrial Dysfunction

li xu

lixushz@163.com

Shihezi University

yinwei zheng

animal husbandy technology center, Aksu

shuhong Tang

animal husbandy technology center, Aksu

Ruiguo Zhao

Shihezi University

feifan xu

Shihezi University

Naerhulan Ayiken

Shihezi University

hu guangdong

Shihezi University

yanping wang

Shihezi University

zeng weibing

Shihezi University

Research Article

Keywords: Sheep, Oocyte, Embryo, Idebenone, Phloretin

Posted Date: March 4th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8864012/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Objective

Phloretin (PHL), a natural dihydrochalcone flavonoid, possesses significant antioxidant, anti-apoptotic, and anti-inflammatory activities. Idebenone (IDE), a structural analog of coenzyme Q10, protects mitochondrial function and enhances antioxidant capacity. This study investigated the combined effects of PHL and IDE on the in vitro maturation (IVM) of ovine oocytes and subsequent embryonic development, aiming to identify their optimal synergistic concentration.

Methods

We used a base medium supplemented with 5 μM PHL as the control to evaluate the effects of different IDE concentrations.

Results

Results demonstrated that the combination of PHL with 10 μM IDE significantly enhanced the rates of oocyte maturation, cleavage, and blastocyst formation compared to the control. This co-treatment also significantly increased intracellular glutathione (GSH) levels and mitochondrial membrane potential (ΔΨm), while reducing reactive oxygen species (ROS) levels in oocytes. Furthermore, it up-regulated the expression of antioxidant genes (CAT, SOD-2, GPX-3) and the follicular development-related gene GDF-9. During early embryo culture, the 10 μM IDE co-treatment similarly elevated GSH levels and ΔΨm in morulae, reduced ROS levels, and up-regulated the antioxidant genes CAT and GPX-3. Concurrently, it up-regulated the embryonic development-related gene SOX-2, suppressed the apoptosis-related gene Caspase-3 and decreased the Bax/Bcl-2 ratio.

Conclusion

In conclusion, the combination of PHL and IDE significantly improves the efficiency of ovine oocyte IVM and early embryonic development by enhancing antioxidant capacity, inhibiting apoptosis, and promoting developmental competence. Collectively, these findings provide a valuable foundation for optimizing in vitro embryo production systems in sheep.

INTRODUCTION

In vitro embryo production (IVP) is a cornerstone technology for propagating elite sheep and preserving genetic resources. However, oocytes are highly vulnerable to oxidative stress during IVP, which impairs maturation rates and subsequent embryonic developmental potential [1]. A common strategy to mitigate oxidative damage is to supplement the in vitro maturation (IVM) medium with bioactive compounds

exhibiting antioxidant, growth-promoting, or anti-apoptotic properties [2]. However, single antioxidants often confer limited protection due to their specific mechanisms and inability to cover all aspects of oocyte maturation, prompting growing interest in combination strategies. Such synergistic strategies aim to enhance oocyte quality and subsequent developmental competence more comprehensively. For example, in porcine oocytes, the combination of resveratrol and melatonin improved oocyte quality and blastocyst rates by synchronizing nuclear and cytoplasmic maturation, enhancing gap junction communication within the cumulus-oocyte complex (COC), and alleviating oxidative stress [3]. In bovine oocytes, co-treatment with ascorbic acid and resveratrol improved the redox status by increasing thiol levels and catalase (CAT) activity. This enhancement preserved extracellular matrix integrity, improved oocyte stability, and helped establish a more physiologically relevant culture microenvironment, thereby increasing overall IVP efficiency [4]. Murine studies further demonstrated that melatonin and L-carnitine act through multi-targeted mechanisms to cooperatively promote nuclear and cytoplasmic maturation, inhibit apoptosis, regulate gene expression, and improve energy metabolism, ultimately enhancing oocyte developmental competence [5]. In summary, leveraging the synergistic effects of multiple antioxidants represents a promising strategy for optimizing IVM systems in livestock and enhancing the efficiency of in vitro embryo production.

Phloretin (PHL), a dihydrochalcone flavonoid abundant in apples, has garnered considerable interest owing to its potent, broad-spectrum antioxidant activity [6]. Its mechanisms of cytoprotection include countering oxidative stress, preserving mitochondrial function, and inhibiting key signaling pathways such as NF-κB and the NLRP3 inflammasome, thereby mitigating oxidative damage and apoptosis [7,8]. Our previous studies demonstrated that supplementing both IVM and early embryo culture media with 5 μM PHL significantly enhances oocyte maturation and early embryonic development [9]. Idebenone (IDE), a synthetic analog of coenzyme Q10, primarily functions by safeguarding mitochondrial integrity [10]. It directly targets and enhances the mitochondrial respiratory chain, stabilizes mitochondrial membrane potential (ΔΨm), and suppresses ROS production at its source, it also enhances cellular resilience to oxidative stress by upregulating intrinsic defense mechanisms and effectively scavenging free radicals [11]. These beneficial mitochondrial-protective and antioxidant effects have been validated in porcine models, including studies on semen and oocytes [12,13]. Moreover, multiple studies consistently demonstrate that idebenone exhibits significant synergistic effects when combined with other compounds such as Quercetin, paroxetine, butylphthalide, or rosuvastatin, showing superior efficacy in alleviating oxidative stress compared to monotherapies. This synergistic activity has been observed in various disease models, including vascular dementia, post-ischemic stroke depression, and atherosclerosis [14–17]. Consequently, combining IDE with other mechanistically distinct agents represents a promising strategy for achieving enhanced outcomes. However, the combined effect of PHL and IDE on in vitro oocyte maturation and early embryonic development in animal models remains unreported.

This study systematically investigated the combined effects of PHL and IDE on the IVM of ovine oocytes and subsequent embryonic development. Building on previous findings establishing the efficacy of 5 μM PHL, we supplemented the culture medium with varying concentrations of IDE to evaluate its impact on

oocyte maturation, embryonic development, and key antioxidant and anti-apoptotic parameters. Our objectives were to identify the optimal synergistic concentration and to provide mechanistic insights into how this combination improves the efficiency of sheep IVP, thereby contributing to refined oocyte and embryo culture protocols.

MATERIALS AND METHODS

Experimental animals

A total of 200 ovaries were collected from Kazakh sheep at a slaughterhouse in Shihezi, Xinjiang, China. All sheep were raised under natural lighting conditions and fed diets formulated to meet their nutritional requirements. Mineral salts were provided ad libitum via salt lick blocks.

Experimental materials

Fetal bovine serum was purchased from Thermo Fisher Scientiffc (Waltham, MA, USA). MEMA-NEAA (100×), GlutaMAX (100×), BME-EAA (50×) and sodium pyruvate (100 mM) were purchased from Gibco (Waltham, MA, USA). Bovine serum albumin (BSA) was purchased from GPCSCI (Beijing, China). TCM199 basal medium was purchased from Pricella (Wuhan, China). Enhanced mitochondrial membrane potential assay kit (JC-1, C2003SC2003S), ROS assay kit (dichloroffuorescein diacetate, S0035S), TRIzol, hyaluronidase and 6-dimethylaminopurine were purchased from Beyotime (Shanghai, China). Monochlorobimane, Idebenone and Phloretin was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Follicle-stimulating hormone (FSH), β-estradiol, gentamicin, and penicillin were purchased from Solarbio (Beijing, China). NaCl, $NaHCO_3$, $CaCl_2$-$2H_2O$, KH_2PO_4, KCl, $MgCl_2$-$6H_2O$, HiFiScript cDNA Synthesis Kit and 2× UltraSYBR Mixture were purchased from CWBIO (Beijing, China). Dimethyl sulfoxide (DMSO)was purchased from Kulaibo (Beijing, China), and ethylene glycol was purchased from Xinbote (Tianjin, China).

Conffguration of Phloretinand Idebenone Concentration

Phloretin and idebenone were first dissolved in DMSO and then diluted in IVM medium to achieve final concentrations of 5 μM for phloretin and 5, 10, 15, or 20 μM for idebenone. The final DMSO concentration was maintained below 0.1% to ensure no cytotoxicity.

Conffguration of Solutions

Oocyte collection solution: TCM199 basal medium with sodium heparin (0.05 g/L), gentamicin (0.05 g/L) and fetal bovine serum (2%). IVM culture medium: TCM199 basal medium with sodium pyruvate (1 mM), FSH (5 μg/mL), β-estradiol (20 ng/mL), gentamicin (200 μg/mL), Glutamax (2 mM) and fetal bovine serum (10%). IVC culture solution: Sterile water with NaCl (107.7 mM), NaHCO3 (25.07 mM), CaCl2-2H2O (1.17 mM), KH2PO4 (1.19 mM), KCl (7.16 mM), MgCl2-6H2O (0.49 mM), GlutaMax (1 mM), sodium

pyruvate (0.4 mM), inositol (2.77 mM), BME-EAA (1%), MEMA-NEAA (1%), BSA (8 mg/mL), gentamicin (25 μg/mL).

Experimental methods

Oocyte Collection and In Vitro Maturation

Ovaries were collected from a slaughterhouse in Shihezi, Xinjiang, China. They were transported to the laboratory within 2 h in sterile saline supplemented with 100 IU/mL penicillin-streptomycin. The ovaries were placed in a collection buffer, and follicles were incised with a scalpel to release cumulus-oocyte complexes (COCs). Only COCs with homogeneous cytoplasm and surrounded by at least three layers of compact cumulus cells were selected for collection. The selected COCs were washed three times in holding medium and then prepared for subsequent experiments. Under a stereomicroscope, oocytes were randomly allocated into five groups and transferred to 4-well plates containing 500 μL of IVM medium supplemented with 5 μM phloretin and different concentrations of idebenone (0, 5, 10, 15, or 20 μmol/L). The total numbers of COCs assessed for cumulus expansion and first polar body extrusion per group were 172, 193, 206, 192, and 189, respectively. The culture wells were covered with mineral oil and incubated for 24 h at 38.5 °C under 5% CO_2 in air. After incubation, oocyte maturation was evaluated based on the degree of cumulus cell expansion and the extrusion of the first polar body. The most effective treatment concentration was identified for subsequent experiments. Cumulus cell expansion rate (%) = (Area change before and after maturation / Total area before maturation) × 100%. Oocyte maturation rate (%) = (Number of oocytes with first polar body extrusion / Total number of oocytes cultured) × 100%.

Parthenogenetic Activation and Embryo Culture

Matured oocytes were transferred into 500 μL of pre-warmed hyaluronidase solution (100 μL in IVM medium). Gentle pipetting was repeated until the cumulus cells were completely removed. The denuded oocytes were subjected to sequential treatments: 1) equilibration in IVC medium for 5 min; 2) exposure to IVC medium supplemented with 7% ethanol for 7 min; 3) treatment in parthenogenetic activation medium containing 6-DMAP for 5 min; and 4) activation in parthenogenetic activation medium for 4 h. After activation, oocytes were washed in IVC medium for 5 min and then randomly allocated into five experimental groups.The parthenogenetically activated oocytes were distributed into separate wells of a 4-well plate, with each well containing 500 μL of IVC medium supplemented with 5 μM PHL and different concentrations of idebenone (0, 5, 10, 15, or 20 μmol/L). The total numbers of oocytes assessed for cleavage and blastocyst rates per group were 131, 142, 153, 129, and 135, respectively. The cultures were overlaid with mineral oil and incubated at 38.5 °C under a humidified atmosphere of 5% CO_2 in air. Cleavage status was assessed at 48 h post-activation , and blastocyst development was evaluated on day 7. The cleavage rate is defined as the proportion of successfully activated oocytes that develop to the cleavage stage. The blastocyst rate was calculated as the proportion of cleaved embryos that developed to the blastocyst stage. These metrics were used to evaluate early embryonic developmental competence and to identify the optimal phloretin concentration for subsequent experiments.Cleavage

rate (%) = (Number of cleaved embryos / Number of activated oocytes) × 100%Blastocyst rate (%) = (Number of blastocysts / Number of cleaved embryos) × 100%

Detection of ROS and GSH Levels

Intracellular reactive oxygen species (ROS) levels were measured using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence assay. The DCFH-DA probe from a commercial ROS detection kit was diluted 1:1000 in TCM-199 medium to obtain a 10 mM working solution. A randomly selected cohort mature oocytes or morula embryos (15-20 per biological replicate) and incubate them in 100 μL DCFH-DA working solution for 25 min at 38.5 °C in the dark. Following incubation, the oocytes and morulae were washed three times with TCM-199 medium and immediately examined under an inverted fluorescence microscope to capture images. Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, USA). A higher relative fluorescence intensity corresponded to a higher intracellular ROS concentration.

Detection of Mitochondrial Membrane Potentials

Mitochondrial membrane potential (ΔΨm) was assessed using a commercial enhanced mitochondrial membrane potential assay kit with the JC-1 fluorescent probe. According to the manufacturer's instructions, a JC-1 working solution was prepared by adding 5 μL of the JC-1 stock solution (200×) to 1 mL of the provided JC-1 staining buffer. Randomly selected matured oocytes or morulae (15-20 per biological replicate) were incubated in 100 μL of the JC-1 working solution for 25 min at 38.5 °C in the dark. After incubation, the oocytes and morulae were washed three times with TCM-199 medium and immediately examined under an inverted fluorescence microscope to capture images. Fluorescence intensity was quantified using ImageJ software. The ratio of red (aggregates, high ΔΨm) to green (monomers, low ΔΨm) fluorescence intensity was calculated to represent the mitochondrial membrane potential.

Real-Time Quantitative RT-PCR

Gene-specific primers (**Table 1**) were designed using Primer Premier 5.0 software based on the sheep (Ovis aries) reference sequences available in the NCBI database. A total of 40-50 matured oocytes or morulae were pooled from both the control and treatment groups for each biological replicate. Total RNA was extracted from the pooled samples using TRIzol reagent. Subsequently, first-strand cDNA was synthesized from the extracted RNA using a commercial reverse transcription kit. The 20 μL reverse transcription reaction mixture contained: 4 μL dNTP Mix (2.5 mM each), 2 μL Oligo$(dT)_{18}$/VN primer mix, 1 μL RNA template, 4 μL 5× RT Buffer, 2 μL dithiotthreitol(DTT) (0.1 M), 1 μL HiFiScript Reverse Transcriptase, and 6 μL RNase-free water. The thermal cycling conditions for reverse transcription were: 42 °C for 15 min (reverse transcription), followed by 85 °C for 5 min (enzyme inactivation). Quantitative real-time PCR (RT-qPCR) was performed using a LightCycler 96 system (Roche Diagnostics, Switzerland). GAPDH was used as the reference gene for normalization. The relative mRNA expression levels were calculated using the 2^(−ΔΔCt) method. The 20 μL RT-qPCR reaction mixture comprised: 10

μL of 2× SYBR Green Master Mix, 0.8 μL of forward and reverse primers (0.4 μL each, 10 μM), 0.4 μL of passive reference dye (ROX Low), 2 μL of cDNA template, and 6.8 μL of nuclease-free H_2O. The RT-qPCR amplification protocol was: initial denaturation at 95 °C for 30 s; followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 32 s.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (version 9.0; GraphPad Software, USA) and SPSS software (version 26.0; IBM Corp., USA). All graphs were generated using GraphPad Prism (version 9.0). Comparisons between two groups were performed using Student's t-test. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Values with the same letter are not significantly different ($P > 0.05$), while those with different letters differ significantly ($P < 0.05$). ns: not significant ($P > 0.05$); *: significant difference ($P < 0.05$); **: highly significant difference ($P < 0.01$).All experiments were independently repeated at least three times (n≥3). Data from continuous variables are presented as mean ± standard deviation.

RESULTS

Effects of Phloretin Combined with Different Idebenone Concentrations on Cumulus Cell Expansion in Sheep Oocytes

Microscopic examination revealed greater cumulus expansion in all treatment groups compared to the control, except for the 20 μM group (Fig. 1A). The 10 μmol/L treatment group (231.47 ± 4.52%) showed a significant increase in the cumulus expansion rate relative to the control (211.54 ± 9.32%; $p < 0.05$). While the 5 μM and 15 μM groups (215.84 ± 5.23% and 214.73 ± 3.31%, respectively) also exhibited increased expansion, these differences were not statistically significant ($p > 0.05$). The 20 μM group (197.43 ± 9.54%) showed the lowest expansion rate among all groups (Fig. 1B).

Effects of Phloretin Combined with Different Idebenone Concentrations on the First Polar Body Extrusion Rate in Sheep Oocytes

Microscopic examination revealed that all treatment groups, except the 20 μM group, exhibited more frequent first polar body extrusion compared to the control (Fig. 2A). Relative to the control group (66.42 ± 2.16%), the 5 μM and 10 μM treatment groups significantly increased the first polar body extrusion rate (72.12 ± 1.12% and 73.83 ± 2.01%, respectively)($p < 0.05$). The 20 μM group (62.11 ± 2.21%) showed a significantly lower rate than all other groups ($p < 0.05$). No significant difference was observed between the 15 μM group (55.44 ± 0.89%) and the control, 5 μM, or 10 μM groups (Fig. 2B; $p > 0.05$). Based on these results, the 10 μM treatment group was selected for subsequent experiments.

Effects of Phloretin and Idebenone Combination on GSH, ROS, and Mitochondrial Membrane Potential in Sheep Oocytes

Compared to the control, the 10 μM treatment significantly reduced reactive oxygen species (ROS) levels (Fig. 3B, $p < 0.05$), increased glutathione (GSH) levels (Fig. 3D; $p < 0.05$), and enhanced mitochondrial membrane potential (Fig. 3F; $p < 0.05$). These quantitative changes were consistent with the corresponding fluorescence intensity observations (Fig. 3A, C, E).

Effects of Phloretin and Idebenone on Antioxidant and Paracrine-Related Gene Expression in Sheep Oocytes

Compared to the control, the 10 μM treatment group showed significantly elevated expression levels of antioxidant genes (SOD-2, CAT, and GPX-3); (Fig 4A; $p < 0.05$). The expression of the paracrine signaling gene GDF-9 was also markedly up-regulated (Fig. 4B; $p < 0.05$), while BMP-15 expression increased to a lesser, non-significant extent (Fig. 4B; $p > 0.05$).

Effects of Phloretin and Idebenone Combination on Early Embryonic Development in Sheep

Microscopic examination revealed a higher number of blastocysts in the 10 μM treatment group compared to all other groups (Fig. 5A). The cleavage rate was significantly higher in the 10 μM group (84.20 ± 3.27%) than in the control group (79.21 ± 2.81%) (Fig. 5B, $p < 0.05$). Similarly, the blastocyst rate was significantly elevated in the 10 μM group (41.31 ± 1.82%) versus the control (35.23 ± 2.08%) (Fig. 5C; $p < 0.01$). Based on these results, the 10 μM phloretin treatment group was selected for further experimentation.

Effects of Phloretin and Idebenone on GSH, ROS, and Mitochondrial Membrane Potential in Early Ovine Embryos

Compared to the control, the combination treatment (10 μM) significantly reduced ROS levels (Fig. 6B; $p < 0.05$), highly significantly increased GSH levels (Fig. 6D; $p < 0.01$), and markedly enhanced mitochondrial membrane potential in morulae (Fig. 6F; $p < 0.01$). These quantitative changes were consistent with the corresponding fluorescence intensity observations (Fig. 6A, C, E).

Effects of Phloretin and Idebenone on Antioxidant, Developmental, and Apoptosis-Related Genes in Early Ovine Embryos

Compared to the control, the 10 μM treatment group showed highly significant upregulation of antioxidant genes GPX-3 and CAT (Fig. 7A; $p < 0.01$). While SOD-2 expression also increased, the change was not statistically significant (Fig. 7A; $p > 0.05$). Among developmental marker genes, SOX-2 expression was significantly elevated, whereas Oct-4 showed a non-significant increase (Fig. 7B; $p > 0.05$). For apoptosis-related genes, the Bax/Bcl-2 ratio was markedly reduced (Fig. 7C; $p < 0.01$), and Caspase-3 expression was significantly downregulated (Fig. 7D; $p < 0.05$).

DISCUSSION

This study demonstrates that co-treatment with phloretin and an optimal concentration of idebenone significantly enhances the maturation rate of ovine oocytes. The most pronounced effects were observed with the combination of 5 μM PHL and 10 μM IDE. This optimal combination improved oocyte quality by reducing ROS levels, elevating GSH concentrations, and enhancing ΔΨm. Furthermore, it significantly up-regulated the expression of key antioxidant genes (SOD-2, CAT, GPX-3) and the paracrine factor GDF-9, thereby establishing a molecular foundation supporting oocyte maturation and subsequent developmental competence. The efficacy of this combinatorial approach is supported by previous studies on the individual compounds. For instance, phloretin effectively scavenges excess ROS in oocytes, thereby maintaining redox homeostasis [18]. Its potent antioxidant capacity, which is mediated through Nrf2 pathway activation via Keap1 dissociation, has been demonstrated in murine endothelial and neuronal cells [19,20]. Similarly, idebenone has demonstrated protective effects in models such as ram semen preservation and various cell lines, attributable to its mitochondrial-targeting and antioxidant properties [21,22]. Furthermore, idebenone alleviates heat stress-induced impairment of porcine oocyte maturation and embryonic development by enhancing antioxidant capacity and improving mitochondrial function [12]. Evidence from sheep oocytes confirms that combinatorial antioxidant strategies confer more comprehensive and sustained protection against oxidative stress than single compounds [23,24], providing a solid rationale for our combined treatment approach. In the present study, the PHL and IDE combination significantly enhanced both cumulus expansion and the rate of first polar body extrusion. This suggests that the treatment promotes nuclear maturation by improving overall cellular health and energy metabolism, which is consistent with findings in bovine oocytes treated with antioxidant combinations [25]. During IVM, oxidative stress leads to ROS accumulation, which depletes GSH, damages mitochondria, and reduces ΔΨm, ultimately impairing oocyte developmental potential [26]. Our combination treatment effectively counteracted these detrimental effects by elevating GSH, reducing ROS, and enhancing ΔΨm. This aligns with results from multi-species studies on combined antioxidants [27,28] and indicates a synergistic protective effect. The up-regulation of SOD-2, CAT, and GPX-3 indicates that the PHL+IDE combination not only provides exogenous antioxidant support but also activates the oocyte's intrinsic defense system—a mechanism crucial for meiotic stability, membrane integrity, and embryonic viability [29-31]. Furthermore, the significant increase in GDF-9 expression suggests improved granulosa cell function, thereby supporting both nuclear and cytoplasmic maturation. This finding aligns with murine and human studies that have established the critical role of oocyte-secreted factors, including BMP-15 and GDF-9, in regulating cumulus expansion, granulosa cell proliferation, and overall folliculogenesis [32,33]. Although BMP-15 expression increased non-significantly in our study, the well-documented synergy between GDF-9 and BMP-15 implies that the combination treatment may influence a broader network of paracrine factors, warranting further investigation.

During the IVC stage, the combination of 5 μM PHL and 10 μM IDE continued to exert beneficial effects. This combination significantly improved the cleavage and blastocyst formation rates. Furthermore, in morulae, it reduced ROS levels while enhancing both GSH content and ΔΨm. Moreover, the treatment significantly up-regulated the expression of antioxidant genes (GPX-3, CAT) and the early embryonic

development-related gene SOX-2. Concurrently, it suppressed the expression of the apoptosis-related gene Caspase-3 and significantly lowered the Bax/Bcl-2 ratio. Cleavage and blastocyst formation rates are key indicators of embryonic developmental potential and quality. The significant improvement in these rates induced by the PHL+IDE combination aligns with studies utilizing single antioxidants or mitochondrial enhancers in sheep oocytes [34,35], confirming its potential for optimizing embryonic development. Embryogenesis is an energy-intensive process accompanied by substantial ROS generation. Although oocytes were protected during IVM, embryos remain vulnerable to oxidative stress during IVC. Elevated ROS levels and mitochondrial dysfunction are major causes of developmental arrest and quality decline in early embryos [36]. The sustained protective effect of the PHL+IDE combination, which extended from IVM into IVC by maintaining redox homeostasis, is consistent with findings from other antioxidant studies [37], highlighting its cross-stage efficacy. The OCT-4/SOX-2 transcription complex plays a central role in maintaining pluripotency and regulating cell fate in murine embryos [38]. The up-regulation of SOX-2 suggests that the combination treatment not only safeguards cellular health but also promotes early differentiation and developmental progression. Additionally, the reduced CASP3 expression and Bax/Bcl-2 ratio indicate suppressed apoptosis. Similar anti-apoptotic outcomes have been observed in sheep oocytes treated with L-carnitine [39], demonstrating that the PHL and IDE combination enhances embryonic quality and survival by inhibiting apoptotic pathways.

This study also revealed that 20 μM IDE significantly inhibited both oocyte maturation and subsequent embryonic development, indicating cytotoxicity at this concentration. Consistent with this, several reports indicate that idebenone, when exceeding a specific threshold, can induce notable cytotoxicity in various cell types—including murine neurons and retinal cells—by inhibiting mitochondrial complex I, inducing oxidative stress, and activating apoptotic pathways [40]. This collective evidence suggests that the detrimental effects of high-dose idebenone on cell function are widespread; however, the specific toxic mechanisms in oocytes warrant further investigation.

CONCLUSION

This study demonstrates that the combination of phloretin and idebenone enhances the in vitro maturation of ovine oocytes and subsequent early embryonic development by synergistically enhancing antioxidant capacity, optimizing mitochondrial function, and regulating key gene expression. The most effective formulation was 5 μmol/L PHL combined with 10 μmol/L IDE. These findings provide a valuable scientific basis for improving elite sheep breeding efficiency and genetic resource preservation.

Declarations

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHORS' CONTRIBUTION

Conceptualization:

Data curation: Li X, Zheng YW, Tang SH, Zhao RG, Zeng WB.

Formal analysis: Li X, Zeng WB.

Methodology: Li X, Naerhulan Ayiken, Hu GD, Wang YP, Zeng WB.

Software: Li X, Xu FF, Zeng WB.

Validation: Li X, Zheng YW, Zeng WB.

Investigation: Li X, Zheng YW, Xu FF, Naerhulan Ayiken, Hu GD, Wang YP, Zeng WB.

Writing - original draft: Li X, Zeng WB.

Writing - review & editing: Li X, Zheng YW, Zeng WB.

FUNDING

This work was funded by grants from the Biological Breeding-Major Projects (2023ZD040510306), Key Scientific and Technological Program in the Priority Areas of the Corps (2021AB014), Aksu Prefecture Agricultural Science and Technology Joint Innovation Project (2025AGTIF06), and Science and Technology Program of XPCC (2024DA006) .

ACKNOWLEDGMENTS

Not applicable.

SUPPLEMENTARY MATERIALS

No supplementary materials are required in this article

DATA AVAILABILITY

Upon reasonable request, the datasets of this study can be available from the corresponding author.

ETHICS APPROVAL

The animal use protocol was approved by the Animal Ethics Committee of Shihezi University (Approval No. A2023-236).

DECLARATION OF GENERATIVE AI

No AI tools were used in this article.

References

1. Soto-Heras S, Paramio MT. Impact of oxidative stress on oocyte competence for in vitro embryo production programs. Res Vet Sci 2020;132:342-50. https://doi.org/10.1016/j.rvsc.2020.07.013
2. Kereilwe O. Advances in supplementation strategies for enhancing bovine in vitro embryo production: a review of growth factors and antioxidants. JARG 2025;42:3167-81. https://doi.org/10.1007/s10815-025-03636-w
3. Lee S, Jin JX, Taweechaipaisankul A., Kim GA., Lee BC. Synergistic effects of resveratrol and melatonin on in vitro maturation of porcine oocytes and subsequent embryo development. Theriogenology 2018;114:191-98. https://doi.org/10.1016/j.theriogenology.2018.03.0404
4. Costa FC, Silva BR, Filho FFC, et al. Ascorbic acid and resveratrol improve the structural integrity of the extracellular matrix and enhance follicular survival in cultured bovine ovarian tissue. Theriogenology 2025;235:231-44. https://doi.org/10.1016/j.theriogenology.2025.01.020
5. Chegini R, Sadeghi M, Shirian S, et al. Effects of combination of melatonin and L-carnitine on in vitro maturation in mouse oocytes: An experimental study. Int J Reprod Biomed 2024; 22:527-38. https://doi.org/10.18502/ijrm.v22i7.16961
6. Chen M, Gowd V, Wang M, Chen F, Cheng KW. The apple dihydrochalcone phloretin suppresses growth and improves chemosensitivity of breast cancer cells via inhibition of cytoprotective autophagy. Food Funct 2021;12: 177-90. https://doi.org/10.1039/d0fo02362k
7. Chen W, Liu M, Li Z, Luo Z, Wu J. Phloretin alleviates sleep deprivation-induced cognitive impairment by reducing inflammation through PPARγ/NF-κB signaling pathway. Ex. Neurol 2024;382:114949. https://doi.org/10.1016/j.expneurol.2024.114949
8. Hao X, Bai Y, Li W, Zhang MX. Phloretin attenuates inflammation induced by subarachnoid hemorrhage through regulation of the TLR2/MyD88/NF-kB pathway. Sci Rep 2024;14:26583. https://doi.org/10.1038/s41598-024-77671-5
9. Li X, Tang S, Zheng Y, et al. Phloretin enhances the efficiency of sheep oocyte IVM and early embryonic development by bolstering antioxidant defenses, inhibiting apoptosis, and promoting development. Reprod Biol 2025;26:101102. https://doi.org/10.1016/j.repbio.2025.101102
10. Brière JJ, Schlemmer D, Chretien D, Rustin P. Quinone analogues regulate mitochondrial substrate competitive oxidation. Res Commun 2004;316:1138-42. https://doi.org/10.1016/j.bbrc.2004.03.002
11. Jaber S, Polster BM. Idebenone and neuroprotection: antioxidant, pro-oxidant, or electron carrier?. Bioenerg Biomembr 2015;47:111-8. https://doi.org/10.1007/s10863-014-9571-y
12. Pantecostoma RR, Jiwakanon J, Sirisathien S. Effect of idebenone supplementation in a semen extender on boar spermatozoa quality during liquid storage. Vet World 2025;18:1479-86. https://doi.org/10.14202/vetworld.2025.1479-1486
13. Shi XY, Jin XH, Lin JY, et al. Idebenone relieves the damage of heat stress on the maturation and developmental competence of porcine oocytes. Reprod Domest Anim 2022;57:418-28. https://doi.org/10.1111/rda.14080
14. Fadda LM, Hagar H, Mohamed AM, Ali HM. Quercetin and Idebenone Ameliorate Oxidative Stress, Inflammation, DNA damage, and Apoptosis Induced by Titanium Dioxide Nanoparticles in Rat Liver.

Dose Response 2018;16:1559325818812188. https://doi.org/10.1177/1559325818812188

15. Zhang H, Wu H, Qi X, Wu F, Zhang D. Effect of butylphthalide combined with idebenone on vascular dementia: A retrospective observational analysis. Medicine (Baltimore) 2024;103:e37495. https://doi.org/10.1097/MD.0000000000037495
16. YYuan LL, Chen TY, Huang ZQ. Effects of paroxetine hydrochloride combined with idebenone on inflammatory factors and antioxidant molecules in treatment of depression after ischemic stroke. Pak J Med Sci 2023;39:17-22 https://doi.org/10.12669/pjms.39.1.5679
17. Yu W, Jiang W, Wu W, et al. Combining idebenone and rosuvastatin prevents atherosclerosis by suppressing oxidative stress and NLRP3 inflammasome activation. Eur J Pharmacol 2023;955:175911. https://doi.org/10.1016/j.ejphar.2023.175911
18. Shahrajabian, Mohamad H, Wenli Sun. "Importance of thymoquinone, sulforaphane, phloretin, and epigallocatechin and their health benefits." Lett Drug Des Discov 2024;21:209-25. https://doi.org/10.2174/1570180819666220902115521
19. Liu Y, Zhang L, Liang J. Activation of the Nrf2 defense pathway contributes to neuroprotective effects of phloretin on oxidative stress injury after cerebral ischemia/reperfusion in rats. J Neurol Sci 2015;351:88-92. https://doi.org/10.1016/j.jns.2015.02.045
20. Wu X, Ding C, Liu X, et al. Liposome of Phlorizin promote the repair of carotid atherosclerosis in rats by regulating inflammation and the Nrf2 signaling pathway. Biochim Biophys Acta Mol Cell Biol Lipids 2025;1870:159613. https://doi.org/10.1016/j.bbalip.2025.159613
21. Sun Z, Wen P, Yang D, et al. Idebenone improves mitochondrial respiratory activity and attenuates oxidative damage via the SIRT3-SOD2 pathway in a prion disease cell model. Life Sci 2025;366-7:123481. https://doi.org/10.1016/j.lfs.2025.123481
22. Lone FA, Naikoo M, Khatun A, et al. Idebenone improves quality of ram sperm by mitigating oxidative stress during cryopreservation. Cryobiology 2019;90:15-20. https://doi.org/10.1016/j.cryobiol.2019.09.004
23. Wang DH, Ren J, Zhou CJ, et al. Supplementation with CTGF, SDF1, NGF, and HGF promotes ovine in vitro oocyte maturation and early embryo development. Domest Anim Endocrinol 2018;65:38-48. https://doi.org/10.1016/j.domaniend.2018.05.003
24. An L, Liu J, Du Y, et al. Synergistic effect of cysteamine, leukemia inhibitory factor, and Y27632 on goat oocyte maturation and embryo development in vitro. Theriogenology 2018 ;108:56-62. https://doi.org/10.1016/j.theriogenology.2017.11.028
25. Maddahi A, Saberivand A, Hamali H, Jafarpour F, Saberivand M. Exploring the impact of heat stress on oocyte maturation and embryo development in dairy cattle using a culture medium supplemented with vitamins E, C, and coenzyme Q10. J Therm Biol 2024;119:103759. https://doi.org/10.1016/j.jtherbio.2023.103759
26. Yao X, Jiang H, Liang S, et al. Laminarin enhances the quality of aged pig oocytes by reducing oxidative stress. J Reprod Dev 2018;64:489-94. https://doi.org/10.1262/jrd.2018-031

27. Chen Z, Zhang C, Yan C, et al. The combination of FLI, ITS, pFF, and citrate improves the developmental potential of gilt oocytes during in vitro maturation. Anim Reprod Sci 2025;279:107945. https://doi.org/10.1016/j.anireprosci.2025.107945
28. Cañón-Beltrán K, García-García RM, Cajas YN, et al. Improvement of oocyte competence and in vitro oocyte maturation with EGF and IGF-I in Guinea pig model. Theriogenology 2024;214:206-14. https://doi.org/10.1016/j.theriogenology.2023.10.029
29. Zou X, Ratti BA, O'Brien JG, et al. Manganese superoxide dismutase (SOD2): is there a center in the universe of mitochondrial redox signaling? J Bioenerg Biomembr 2017;49:325-33. https://doi.org/10.1007/s10863-017-9718-8
30. Baker A, Lin CC, Lett C, et al. Catalase: A critical node in the regulation of cell fate. Free Radic Biol Med 2023;199:56-66. https://doi.org/10.1016/j.freeradbiomed.2023.02.009
31. Zhang N, Liao H, Lin Z, Tang Q. Insights into the Role of Glutathione Peroxidase 3 in Non-Neoplastic Diseases. Biomolecules 2024;14:689. https://doi.org/10.3390/biom14060689
32. Sanfins A, Rodrigues P, Albertini DF. GDF-9 and BMP-15 direct the follicle symphony. J Assist Reprod Genet 2018;35:1741-50. https://doi.org/10.1007/s10815-018-1268-4
33. Peng J, Li Q, Wigglesworth K, et al. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. Proc Natl Acad Sci U S A 2013;110:E776-85. https://doi.org/10.1073/pnas.1218020110
34. Heydarnejad A, Ostadhosseini S, Varnosfaderani SR, et al. Supplementation of maturation medium with CoQ10 enhances developmental competence of ovine oocytes through improvement of mitochondrial function. Mol Reprod Dev 2019;86:812-24. https://doi.org/10.1002/mrd.23159
35. Ren J, Hao Y, Liu Z, et al. Effect of exogenous glutathione supplementation on the in vitro developmental competence of ovine oocytes. Theriogenology 2021;173:144-55. https://doi.org/10.1016/j.theriogenology.2021.07.025
36. Almansa-Ordonez A, Bellido R, Vassena R, Barragan M, Zambelli F. Oxidative Stress in Reproduction: A Mitochondrial Perspective. Biology (Basel) 2020;9:269. https://doi.org/10.3390/biology9090269
37. Wei P, Wang J, Yu H, et al. Effects of Leonurine on oocyte maturation and parthenogenetic embryo development in sheep. Reprod Domest Anim 2024;59:e14546. https://doi.org/10.1111/rda.14546
38. Abulaiti X, Zhang H, Wang A, et al. Phosphorylation of Threonine343 Is Crucial for OCT4 Interaction with SOX2 in the Maintenance of Mouse Embryonic Stem Cell Pluripotency. Stem Cell Reports 2017;9:1630-1641. https://doi.org/10.1016/j.stemcr.2017.09.001
39. Mishra A, Reddy IJ, Gupta P, Mondal S. Developmental regulation and modulation of apoptotic genes expression in sheep oocytes and embryos cultured in vitro with L-carnitine. Reprod Domest Anim 2016;51:1020-9. https://doi.org/10.1111/rda.12789
40. Varricchio C, Beirne K, Heard C, et al. The ying and yang of idebenone: Not too little, not too much - cell death in NQO1 deficient cells and the mouse retina. Free Radic Biol Med 2020;152:551-60. https://doi.org/10.1016/j.freeradbiomed.2019.11.030

Tables

Table 1
Symbol of target genes, number GeneBank, forward (F) and reverse (R) primer sequences, primer length (bp) used to complete quantitative PCR (qPCR).

Symbol of target genes	Number GeneBank	Forward (F) and reverse (R) primer sequences	Primer Length(bp)
CAT	NM _001752.4	F:GTGCGGAGATTCAACACTGC	110
		R:CCGGCAATGTTCTCACACAG	
SOD-2	NM_001024465.3	F:CAGCACTAGCAGCATGTTGAG	144
		R:GCGTTGATGTGAGGTTCCAG	
GPX-3	XM_015096153.3	F:ACATCCTGACCTACATGCGG	149
		R:AGCATGGGAGTGTGGCATAG	
GDF-9	NM_001142888.2	F:CGGACATCGGTATGGCTCTC	81
		R:GGTCTTGGCACTGAGGAGTC	
BMP-15	NM_001114767.2	F:AGCATGATGGGCCTGAAAGT	136
		R:ACCCGAGGACATACTCCCTT	
OCT-4	NM_001285569.1	F:CGCCCTATGACTTGTGTGGA	95
		R:CTCGGGCTGAGGGGTCT	
SOX-2	NM_001318074.1	F:CTACAGCATGATGCAGGACCA	133
		R:CTGCGAGCTGGTCATAGAGT	
BCL-2	XM_012103831.5	F:CCTGTGGATGACCGAGTACC	148
		R:CAGACTGAGCAGTGCCTTCA	
BAX	XM_027978594.3	F:GTGCCCGAGTTGATCAGGAC	123
		R:CAGGCTCTTCAGCGACTCA	
Caspase-3	XM_015104559.3	F:AGGAGTGGAATCCAGCAGAG	141
		R:TGGCCTGGTAGAGTTCTAATT	
GAPHD	XM_060411593.1	F:CGGCACAGTCAAGGCAGAGAAC	114
		R:CACGTACTAGCACCAGCATCAC	

Figures

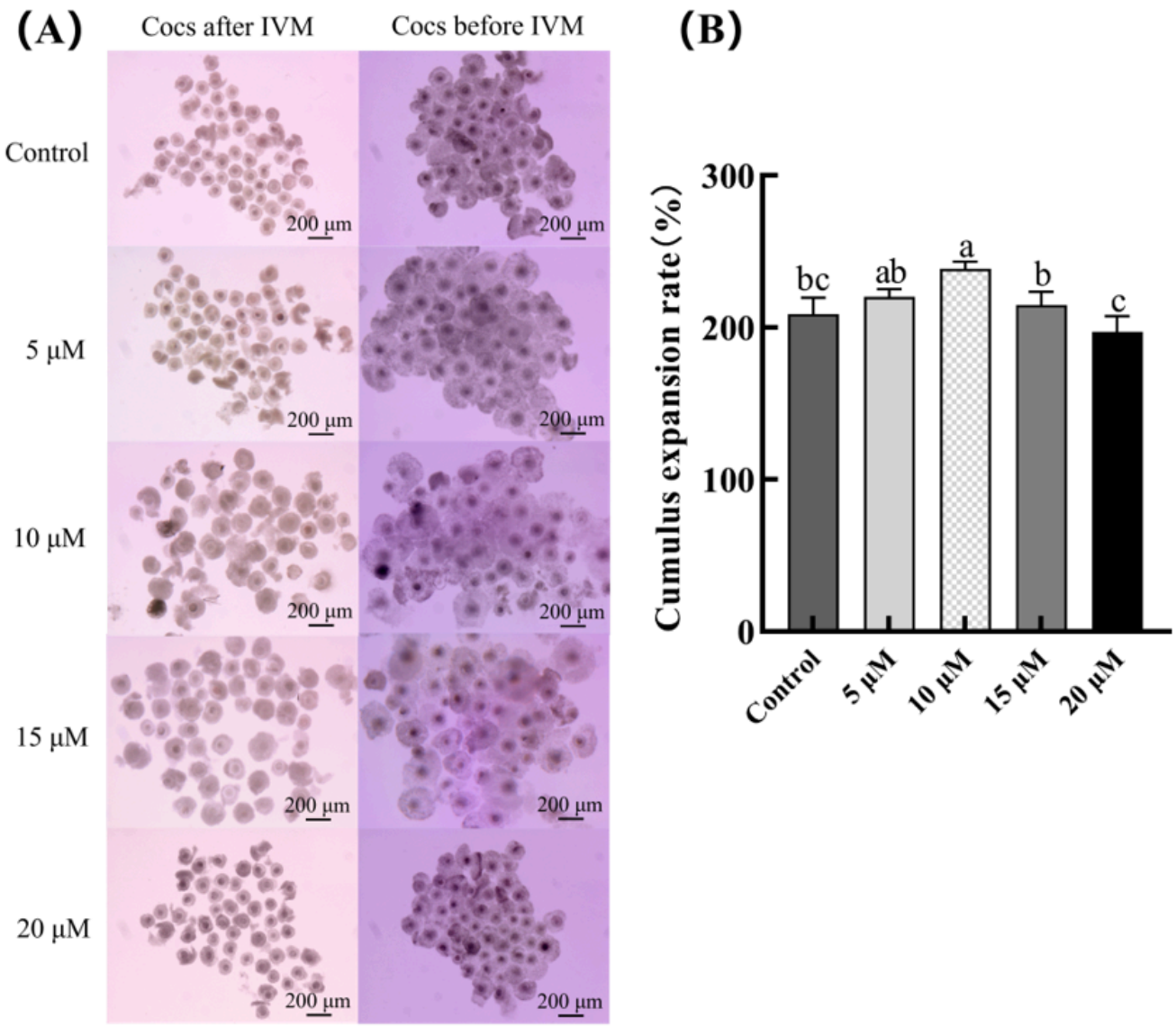


Figure 1

Effects of phloretin combined with different idebenone concentrations on cumulus expansion in sheep oocytes. (A) Representative images of cumulus cell expansion in each treatment group. (B) Quantitative analysis of cumulus expansion rates.Data labeled with different superscript letters (a, b, c…) denote statistically significant differences ($p < 0.05$)

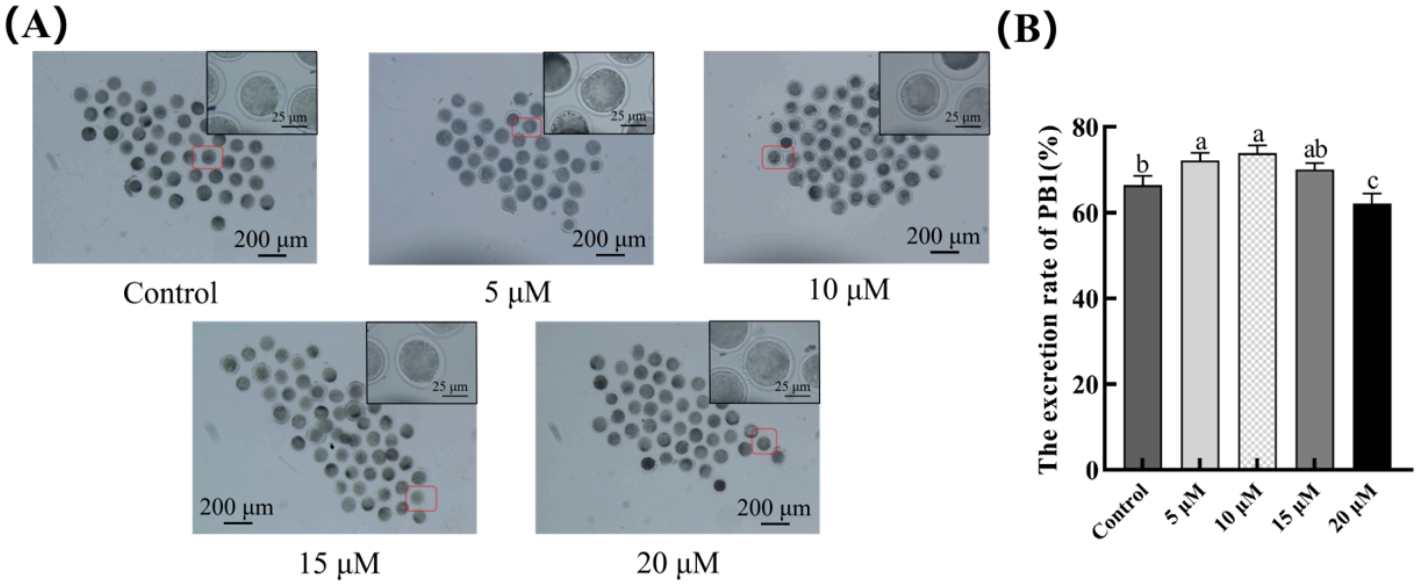


Figure 2

Effects of phloretin combined with different idebenone concentrations on the first polar body extrusion rate in sheep oocytes. (A) Representative images of first polar body extrusion in each treatment group. (B) Quantitative analysis of first polar body extrusion rates. The inset in the upper right corner of (A) shows a magnified view of a single oocyte (indicated by red box).Data labeled with different superscript letters (a, b, c…) denote statistically significant differences ($p < 0.05$)

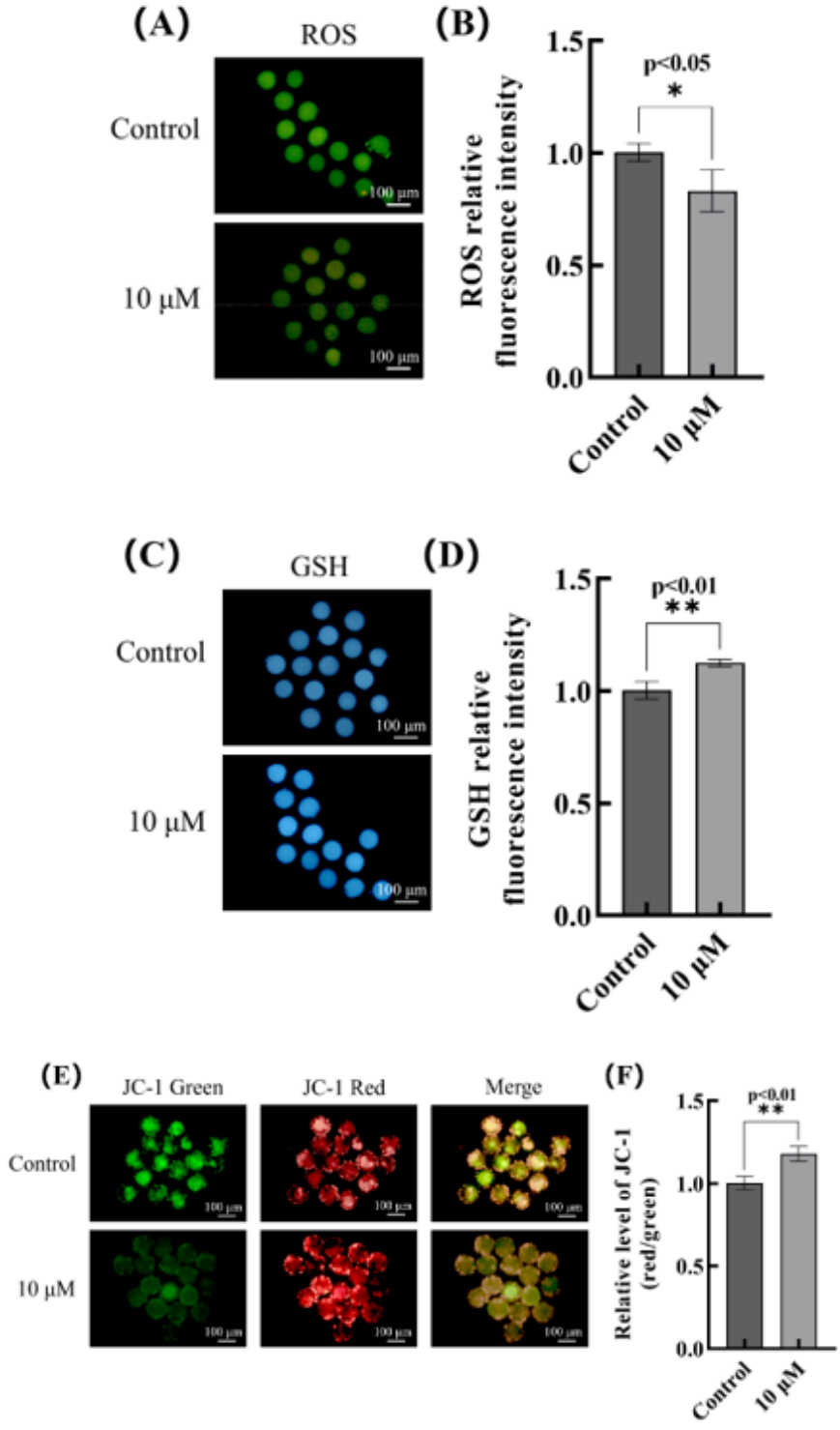


Figure 3

Effects of phloretin and idebenone combination on GSH, ROS, and Mitochondrial Membrane Potential in sheep oocytes. (A) Representative fluorescence images of ROS. (B) Quantitative analysis of ROS levels. (C) Representative fluorescence images of GSH. (D) Quantitative analysis of GSH levels. (E) Representative fluorescence images of mitochondrial membrane potential. (F) Quantitative analysis of

mitochondrial membrane potential levels.(The t test was used for the above analyses comparing two individual samples. * $p<0.05$; ** $p<0.01$).

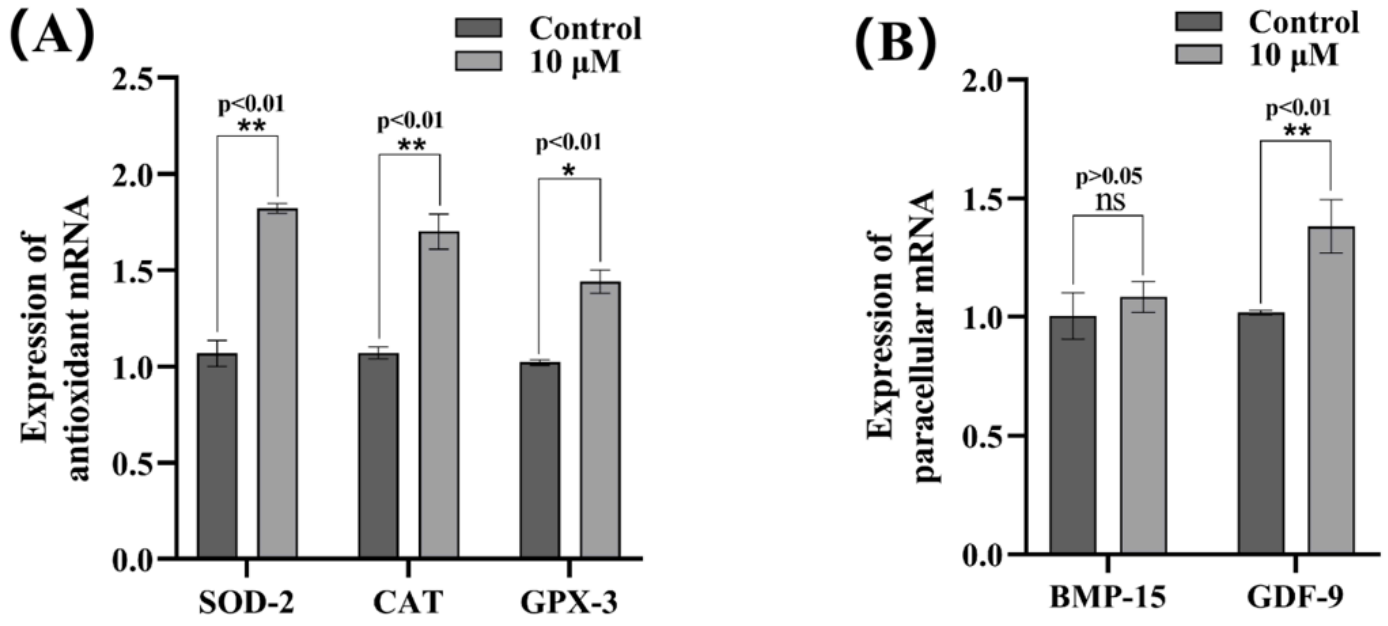


Figure 4

Effects of phloretin and idebenone on antioxidant gene expression and paracrine-related gene expression in sheep oocytes. (A) Expression levels of antioxidant genes in oocytes. (B) Expression levels of paracrine-related genes in oocytes.The t test was used for the above analyses comparing two individual samples. ns $p>0.05$; * $p<0.05$; ** $p<0.01$.

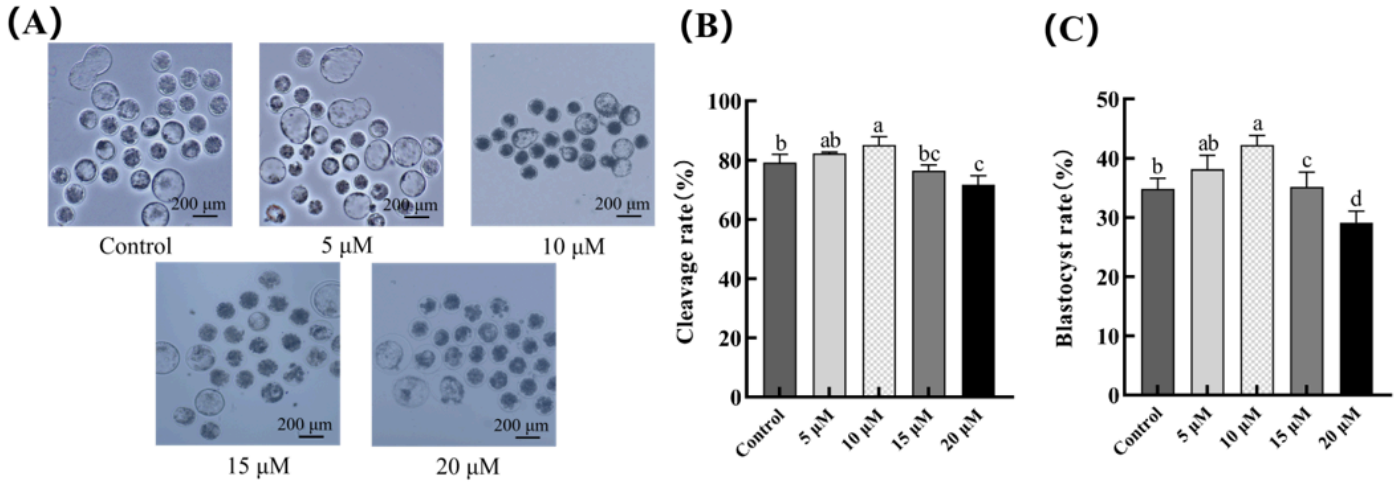


Figure 5

Effects of phloretin combined with different idebenone concentrations on early embryonic development in sheep oocytes. (A) Representative images of embryonic development in each treatment group. (B)

Cleavage rate (48 h). (C) Blastocyst rate (7 d).Data labeled with different superscript letters (a, b, c…) denote statistically significant differences ($p < 0.05$)

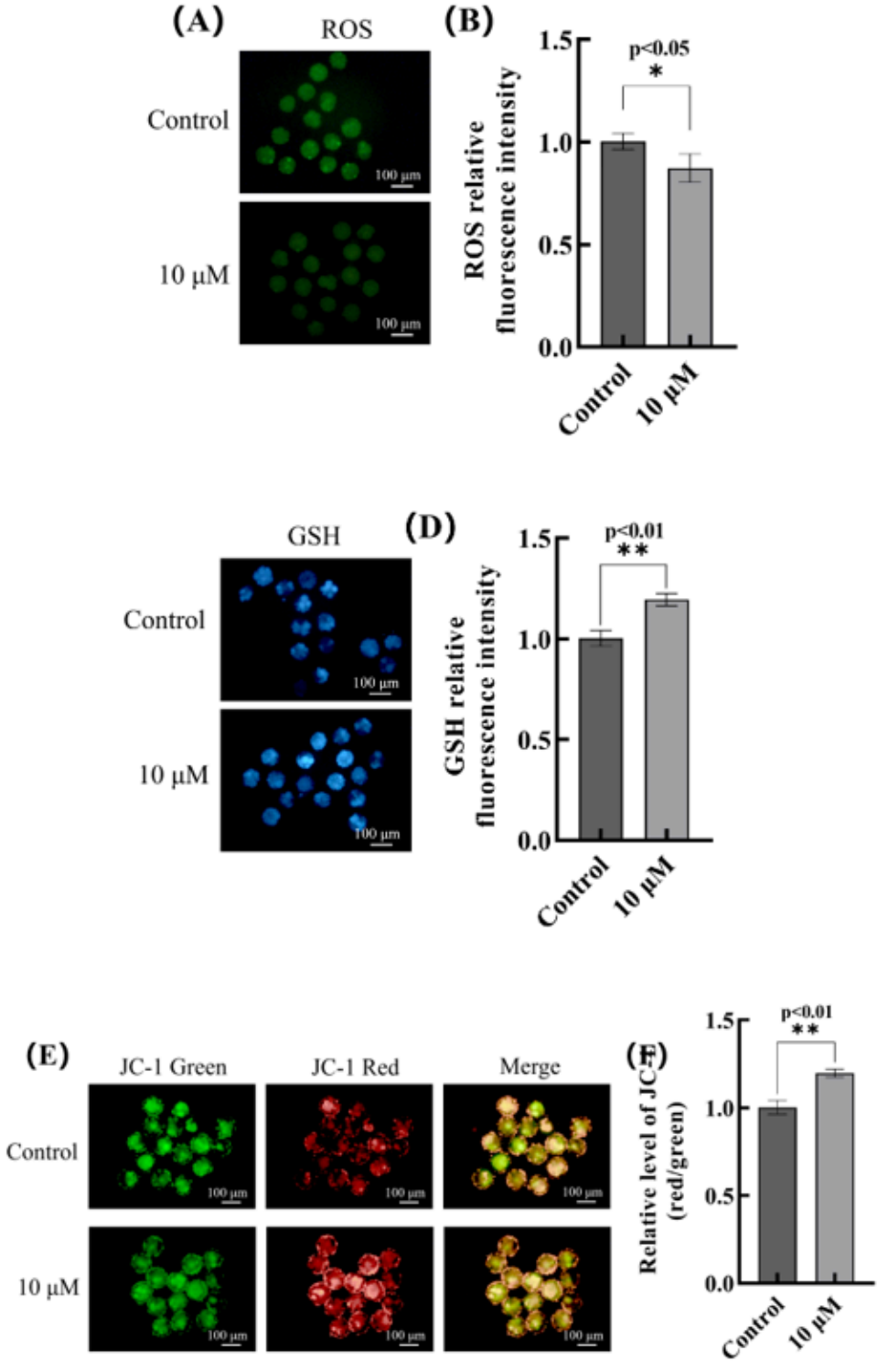


Figure 6

Effects of phloretin and idebenone **on ROS, GSH, and mitochondrial membrane potential in sheep morulae. (A) Representative fluorescence images of ROS. (B) Quantitative analysis of ROS levels. (C)**

Representative fluorescence images of GSH. (D) Quantitative analysis of GSH levels. (E) Representative fluorescence images of mitochondrial membrane potential. (F) Quantitative analysis of mitochondrial membrane potential levels. The t test was used for the above analyses comparing two individual samples. * $p<0.05$; ** $p<0.01$.

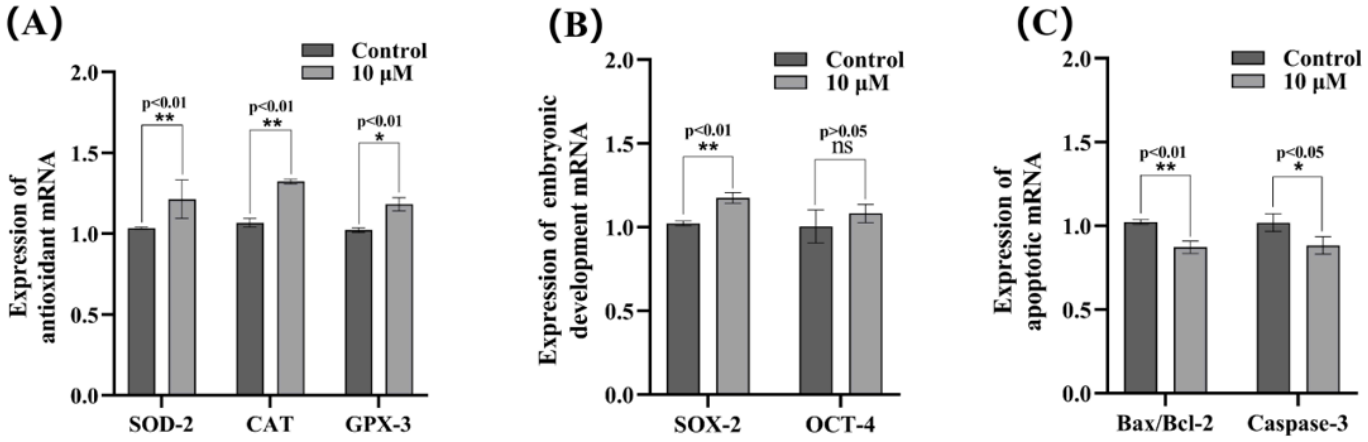


Figure 7

Effects of phloretin and idebenone **on antioxidant genes, early embryonic development-related genes, and apoptosis-related genes in sheep morulae. (A) Expression levels of antioxidant genes (SOD-2, CAT, and GPX-3) in morulae. (B) Expression levels of early embryonic development-related genes (SOX-2 and Oct-4) in morulae. (C) Expression levels of apoptosis-related genes (Caspase-3 and Bax/Bcl-2 ratio) in morulae.**The t test was used for the above analyses comparing two individual samples. ns $p>0.05$; * $p<0.05$; ** $p<0.01$.