

Article

BDNF Promotes In Vitro Maturation of Sheep Oocytes by Alleviating Oxidative Stress and Endoplasmic Reticulum Stress

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Abstract

In vitro maturation (IVM) is highly susceptible to influences of the culture environment, which can lead to increased intracellular reactive oxygen species (ROS) levels and thereby induce a stress response in oocytes, ultimately reducing the developmental potential of early embryos. Brain-derived neurotrophic factor (BDNF) is an ovarian endocrine factor that can enhance the function of follicular granulosa cells and promote oocyte maturation, but the specific pathways remain unclear. We supplemented IVM cultures of sheep oocytes with BDNF and examined aspects of oocyte nuclear and cytoplasmic maturation. The addition of 50 ng/mL BDNF promoted the expansion of cumulus cells and increased the rates of first polar body extrusion, cleavage, and blastocyst formation. Compared with untreated controls, BDNF-treated oocytes had improved Ca^{2+} homeostasis, enhanced expression of antioxidant genes, decreased ROS levels and expression of endoplasmic reticulum stress genes, and increased mitochondrial membrane potential, mitochondrial biogenesis, and numbers of cells with proper distributions of mitochondria and endoplasmic reticulum. Further analysis indicated that BDNF affected oocyte maturation by increasing the numbers of transzonal projections and gap junctions during the IVM process. In summary, the addition of BDNF during the IVM process improved sheep oocyte maturation and embryo development by reducing oxidative stress and endoplasmic reticulum stress. These findings deepen our understanding of the regulatory mechanisms of BDNF during IVM and provide experimental data to improve in vitro embryo production from sheep oocytes.

Keywords: BDNF; sheep oocytes; in vitro maturation; reactive oxygen species; stress response



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1. Introduction

In vitro maturation (IVM) of oocytes is the first and most critical step in the process of in vitro embryo production [1]. The quality of oocytes directly affects the fertilization rate, early embryonic development, pregnancy maintenance, and fetal development in mammals [2]. Reactive oxygen species (ROS) directly impair oocyte quality through the induction of oxidative stress (OS) [3]. In vivo, the stable physiological environment and follicular fluid provide abundant antioxidant factors to neutralize ROS generation. However, during IVM culture of oocytes, the IVM medium typically contains comparatively lower levels of antioxidant enzymes compared with the maternal environment, leading to ROS accumulation. This disruption of redox homeostasis ultimately triggers OS [4,5]. Previous

studies have shown that an imbalance of ROS in oocytes leads to apoptosis, mitochondrial dysfunction, DNA damage, lipid peroxidation, and other damages that hinder oocyte maturation [6,7]. In addition, the endoplasmic reticulum (ER) serves as a central site for intracellular calcium storage and protein folding [8], and ROS accumulation disrupts ER function, impairing protein folding and processing, leading to the misfolded proteins and triggering ER stress (ERS), which ultimately compromises oocyte maturation [9,10].

Brain-derived neurotrophic factor (BDNF) is a widely expressed neurotrophic factor that plays a critical role in supporting neuronal survival and differentiation [11]. BDNF exhibits anti-inflammatory and antioxidant properties by suppressing the expression of pro-inflammatory factors and upregulating anti-inflammatory factors, thereby improving the inflammatory microenvironment [12,13]. BDNF also acts as an ovarian endocrine factor. BDNF is upregulated during the luteinizing hormone (LH) surge, and there is substantial evidence demonstrating its critical role in ovarian follicular development. By binding to its specific receptor, tyrosine kinase receptor B (TrkB), BDNF activates downstream signaling pathways and participates extensively in key physiological processes including steroidogenesis, granulosa cell proliferation, folliculogenesis, oocyte maturation, and ovulation. These mechanisms underscore its essential regulatory functions in ovarian physiology. Dysregulated BDNF expression is closely associated with ovarian pathologies such as premature ovarian insufficiency and polycystic ovary syndrome [14–16]. In mice, BDNF secreted by granulosa cells and cumulus cells promotes extrusion of the first polar body (PBI) of oocytes in a paracrine manner, and it also promotes the development of fertilized eggs into pre-implantation embryos. These two effects mediated by BDNF are specifically blocked by K252a, an inhibitor of the tropomyosin receptor kinase 2 (NTRK2) receptor [17]. Studies using immunofluorescence and confocal laser microscopy showed that BDNF can promote the developmental ability of mouse oocytes during IVM by improving the structure and position of the meiotic spindle and the distribution of cortical granules at meiosis [18]. BDNF is also a regulator of human oocyte maturation and early embryonic development [19]. In a previous study, BDNF increased the proportion of blastocysts formed after parthenogenetic activation of matured bovine oocytes [20]. Furthermore, gonadotropins were shown to promote BDNF expression in human granulosa cells. Treatment of immature oocytes with BDNF increased the number of oocytes reaching the metaphase II stage [21]. As a potential candidate for improving the efficiency of in vitro embryo production from pigs, the addition of BDNF to IVM medium increased PBI extrusion and enhanced development, increasing the number of oocytes achieving the blastocyst stage after in vitro fertilization (IVF) and somatic cell nuclear transfer [22]. Notably, during human oocyte maturation, cAMP acts as a key messenger that stimulates cumulus cells to increase their output of BDNF in a dose-dependent manner [19].

Despite extensive evidence from animal studies that BDNF can promote IVM of oocytes, the precise regulatory mechanisms underlying its role in IVM of sheep oocytes remain unclear. In this study, we investigated the effects and potential mechanisms of BDNF supplementation to the IVM medium during IVM of sheep oocytes. We examined the extent of cumulus expansion and the nuclear maturation rate of cumulus-oocyte complexes (COCs) treated with BDNF, as well as the levels of ROS, ERS, and mitochondrial function in sheep oocytes treated with BDNF. We also measured the expression of genes related to OS and ERS. Finally, we evaluated the developmental ability of embryos based on the cleavage rate, blastocyst formation rate, and total cell count during the in vitro culture stage.

2. Materials and Methods

2.1. *In Vitro* Maturation (IVM)

Sheep ovaries were collected from a slaughterhouse, placed in normal saline at 37 °C, and transported to the laboratory within 2 h. A sterile 10 mL syringe was used to aspirate COCs from antral follicles with a diameter of 3–5 mm. The COCs were washed, and a stereomicroscope was used to select COCs with three or more layers of cumulus cells for subsequent experiments. Collected COCs were cultured in TCM-199 maturation medium (Cat# 12340030, Gibco, Grand Island, NY, USA) consisting of 10% fetal bovine serum (FBS; Cat# FSP500, ExCell Bio, Shanghai, China), 1% penicillin and streptomycin mixture (PS; Cat# 15140122, Gibco), 0.02 IU/mL follicle stimulating hormone (FSH; Cat# B2506171, NSHF, Ningbo, China), 0.02 IU/mL LH (Cat# B2307061, NSHF), 1 µg/mL 17β-estradiol (Cat# E8875, Sigma-Aldrich, St. Louis, MO, USA), and 0.1 mmol/L cysteine (Cat# C7352, Sigma-Aldrich) under mineral oil at 38.6 °C in a humidified atmosphere of 5% CO₂ for 24 h. Three experimental groups were cultured in the same IVM medium as the control group but with the addition of 10 ng/mL, 50 ng/mL, or 100 ng/mL BDNF (Cat# 450-02-10UG, PeproTech, Cranbury, NJ, USA).

2.2. *Sperm Preparation, In Vitro Fertilization (IVF), and In Vitro Culture (IVC)*

After 24 h of culture, cumulus cells were removed by hyaluronidase. Frozen thawed spermatozoa from the same ejaculate of one ram was used across all experimental procedures. Prior to fertilization, sperm preparation was performed using the “swim-up” method [23]. Briefly, the thawed semen was gently layered at the bottom of a centrifuge tube containing IVF medium (synthetic oviductal fluid supplemented with 2% essential amino acids, 1% non-essential amino acids, 3 mg/mL bovine serum albumin (BSA) (Cat# A6003, Sigma-Aldrich), 2% estrous sheep serum, and 6 IU/mL heparin (Cat# H3149, Sigma-Aldrich)). The tube was then incubated at 38.5 °C in a humidified atmosphere of 5% CO₂ for 30 min. After the swim-up process, the upper layer containing motile sperm was carefully aspirated and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the sperm pellet was resuspended in IVF medium to achieve a final concentration of 1×10^7 sperm/mL. The sperm suspension was then added to the COCs, and fertilization was carried out at 38.6 °C in a humidified atmosphere of 5% CO₂ for 18–20 h. Following fertilization, cumulus cells and externally attached sperm were completely removed by gentle pipetting. The fertilized zygotes were placed in embryo development medium (mSOFaa + 3 mg·mL⁻¹ BSA) and cultured at 38.6 °C in an environment with 5% CO₂, 5% O₂, 90% N₂, and saturated humidity. The developmental stage was defined as Day 0 starting at IVF (co-incubation of gametes). Cleavage rates and blastocyst formation rates were recorded at Day 2 and Day 7, respectively.

2.3. *Cumulus Expansion Index (CEI)*

After 24 h of IVM, each oocyte was graded as follows: grade 0, no cumulus expansion; grade 1, expansion of 1–2 layers; grade 2, radial expansion of outer cumulus granulosa cells; grade 3, full expansion except for corona radiata; grade 4, complete expansion. The CEI was then calculated according to the previously reported method [24]: $CEI = [(number\ of\ grade\ 0\ oocytes \times 0) + (number\ of\ grade\ 1\ oocytes \times 1) + (number\ of\ grade\ 2\ oocytes \times 2) + (number\ of\ grade\ 3\ oocytes \times 3) + (number\ of\ grade\ 4\ oocytes \times 4)] \div total\ number\ of\ oocytes$.

2.4. *First Polar Body Rate*

After 24 h of IVM, cumulus cells around the oocytes were removed with hyaluronidase. The oocytes were then washed with oocyte washing solution, and a stereomicroscope was used to count the number of oocytes with PBI as mature nuclear oocytes.

2.5. Total Number of Cells in Blastocysts

Blastocysts collected on the 7th day of development were fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton X-100 (Cat# P0096, Beyotime, Biotechnology, Shanghai, China) for 30 min. Subsequently, the blastocysts were stained with 10 µg/mL Hoechst 33342 (Cat# C1022, Beyotime) and incubated at 37 °C in the dark for 15 min. The stained blastocysts were placed on a glass slide and examined and photographed using an Eclipse Ti2-LAPP inverted fluorescence microscope (Nikon, Tokyo, Japan). The total cell counts within the blastocysts were determined using ImageJ 1.54f software (NIH, New York, NY, USA).

2.6. Detection of ROS and Glutathione (GSH) Levels in Oocytes

After 24 h of IVM, ROS and GSH levels in oocytes were measured using the ROS Assay Kit with DCFH-DA (Cat# S0033S, Beyotime) and CellTracker Blue CMAC (Cat# C2110, Thermo Fisher Scientific, Waltham, MA, USA). The oocytes were incubated in DCFH-DA (1:1000) and CellTracker Blue CMAC (1:1000) droplets at 37 °C under light-protected conditions for 30 min. Subsequently, the cells were washed three times with 0.1% PVA (Cat# P-8136, Sigma-Aldrich) in PBS (Cat# 20012027, Gibco) and then immediately observed and imaged using a confocal laser scanning microscope (FV10i-DOC, Olympus, Tokyo, Japan). The fluorescence signal intensity of each oocyte group was analyzed using ImageJ software.

2.7. Detection of Mitochondrial and ER Distributions in Oocytes

Mitochondrial and ER distribution patterns in oocytes were detected using MitoTracker™ (Cat# M7512, Thermo Fisher) and ER-Tracker™ Green (Cat# E34251, Thermo Fisher) according to the manufacturer's instructions. The collected oocytes were washed three times with 0.1% PVA in PBS, permeabilized with membrane-breaking solution for 2 min, and then incubated with MitoTracker™ (1:1000) and ER-Tracker™ Green (1:1000) under light-protected conditions at 37 °C for 30 min. Fluorescence distribution patterns were observed using a confocal laser scanning microscope.

2.8. Detection of Mitochondrial Membrane Potential (MMP) in Oocytes

MMP in oocytes was evaluated using JC-1 staining (Cat# C2006, Beyotime) according to the manufacturer's protocol. Collected oocytes were washed three times with 0.1% PVA in PBS, incubated with JC-1 working solution under light-protected conditions at 37 °C for 20 min, and then washed three times with JC-1 staining buffer. Fluorescence intensity was detected using a confocal laser scanning microscope. The red/green fluorescence ratio was employed to analyze MMP levels.

2.9. Detection of Calcium Ion Levels in Oocytes

To measure $[Ca^{2+}]_i$ levels, oocytes were incubated with 5 µM Fluo-3 AM (Cat# S1056, Beyotime) at 38.5 °C for 30 min, followed by washing three times with 0.1% PVA in PBS. To measure $[Ca^{2+}]_{ER}$ levels, oocytes were incubated with 10 µM Fluo-4 AM (Cat# S1061M, Beyotime) at 37 °C in the dark for 20 min and then washed three times with 0.1% PVA in PBS. To measure $[Ca^{2+}]_m$ levels, oocytes were incubated with 10 µM Rhod-2 AM (Cat# R1244, Thermo Fisher) at 37 °C in the dark for 20 min, followed by washing with 0.1% PVA in PBS. The fluorescence intensity was detected and analyzed using a confocal microscope.

2.10. Analysis of Transzonal Projections (TZPs) in Oocytes

TZPs play a role in the structural integrity of the cumulus-oocyte complex, which is essential for regulating gene expression in the oocyte. To visualize TZPs in oocytes, they were freed of the granulosa or cumulus cells by physical disruption [25]. The number of

TZPs in oocytes was detected using the Rhodamine Phalloidin Kit (Cat# ab235138, Abcam, Cambridge, UK). Briefly, after IVM (0 h, 8 h, 12 h, or 24 h), cumulus cells were removed. Oocytes were fixed in 4% paraformaldehyde for 30 min. Oocytes were then transferred to a membrane permeabilization solution (1% Triton X-100 in PBS) and blocking (PBS containing 1% BSA) for 2 h. After several washes, oocytes were then incubated with 1' Working Solution (1:1000) at 37 °C in the dark for 2 h to label the F-actin cytoskeleton, washed three times with 0.1% PVA- in PBS, and immediately observed under a laser confocal microscope. Analysis of TZP was performed as previously described [26]. To quantify the number of total TZPs per oocyte, images were analyzed using ImageJ in a confocal optical section obtained at the equatorial plane of the oocyte. Using four arcs set 90° apart around the oocyte circumference, the number of TZPs in the zona pellucida was manually counted within one quarter of the arc regions from maximum-intensity projections (10-µm thick z-stack) of equatorial sections (of consistent depth) obtained from rhodamine phalloidin-stained oocytes. In detail, images were acquired as z-stacks with optical sections taken every 1 µm using 546/575 nm excitation/emission under a 40× objective (exposure time 300–400 ms, laser power 10–15%).

2.11. Real-Time Fluorescence Quantitative PCR (qRT-PCR)

Cumulus cells and oocytes were collected 0 h, 8 h, 12 h, and 24 h after IVM. The cell samples were processed using the Single Cell Sequence Specific Amplification Kit (Cat# P621, Vazyme Biotech Co. Ltd., Nanjing, China). Briefly, 4 µL amplification reagent (2.5 µL 2× Reaction Mix, 0.5 µL 0.1 M primer mixture, 0.1 µL RT/Taq enzyme, and 0.9 µL ddH₂O) was added to the sample. The sample was immediately placed in a −80 °C refrigerator for 2 min, centrifuged for 2 min, and placed in a PCR machine. The sample was incubated at 50 °C for 60 min and then at 95 °C for 3 min, followed by 18 cycles of 95 °C for 15 s and 60 °C for 15 min. After the reaction was completed, the sample was diluted 1:10 and either placed at 4 °C for quantitative tests or stored at −20 °C.

Processed samples were subjected to qRT-PCR detection using the TB Green[®] Ex TaqTMII Kit (Cat# RR830B, TaKaRa Bio, San Jose, CA, USA). The reaction system included 5 µL TB Green[®] Ex TaqTMII, 3 µL ddH₂O, 1 µL upstream and downstream primer mixture, and 1 µL sample. The following parameters were used for qRT-PCR: pre-denaturation at 95 °C for 30 s, PCR with 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and melting curve analysis with 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The qPCR primer sequences are shown in Table 1 (Sangon Biotech, Shanghai, China). *GAPDH* and *β-actin* were used as internal reference genes, and the $2^{-\Delta\Delta C_t}$ method was used to analyze quantitative mRNA data.

Table 1. Primer sequences for qRT-PCR.

Gene	Primer Sequences	Product Length (bp)	Accession Numbers
<i>β-actin</i>	F: GTCATCACCATCGGCAATGA R: CGTGAATGCCGAGGATT	71	XM_060405599.1
<i>GAPDH</i>	F: AGTGTCGCTGTTGAAGTCG R: GAAACCTGCCAAGTATGATG	121	NM_001190390.1
<i>BAX</i>	F: GACAGGGGCCCTTTTGCTT R: TCAGACACTCGCTCAGCTTC	128	XM_027978592.3
<i>BCL2</i>	F: ATGCCTTTGTGGAGCTGTATGG R: ACTGAGCAGTGCCTTCAGAGACA	80	XM_012103831.5
<i>CASP3</i>	F: TTCAGAGGGGACTGTTGCAG R: CAGTCCAGTTCTGTGCCTCG	83	XM_060406953.1
<i>CASP9</i>	F: CCAGAGGAGCTGAGAGTGATG R: TTCCCTTGGCTCTGTCTGAG	196	XM_060396596.1

Table 1. Cont.

Gene	Primer Sequences	Product Length (bp)	Accession Numbers
<i>FAS</i>	F: GCGACGGAGATATGCCAGAATG R: AGGATGAGGAGGAGGAGGATCA	334	NM_001123003.1
<i>P53</i>	F: GGGTGGGAAGGGAATTTACGC R: CCACGAGAGTCTTCCAGTGT	199	XM_042255225.2
<i>ND5</i>	F: TAGCCCAAAATCTCCCACTCTC R: CATCATTCCTTGCGGTACTA	182	NC_0091941.1
<i>HAS2</i>	F: GGGTTCTTCCCTTTCTTTCT R: CCACCCAGCTTTGTTTATTG	240	XM_004011666.5
<i>DUSP1</i>	F: GCCACCATCTGCCTCGCTTAC R: GCTTCTCCTCTGCTTCACGAACTC	84	XM_004016868.5
<i>PTGES</i>	F: ACGACATGGAGACCATCTACC R: TAGGCCACGGTGTGTATCAT	130	XM_027966307.2
<i>PTX3</i>	F: CTCTCTGGTCTGCAGTGTCTG R: TGTCCCACTCGGAGTTCTCA	144	XM_004003220.5
<i>TNFAIP6</i>	F: CAAGCAGTTAGAGGCAGCCAG R: CTCCTTTGCATGTGGGTTGTAG	199	XM_004004704.4
<i>GJA1</i>	F: ATGAGCAGTCTGCCTTTTCGT R: TCTGCTTCAAGTGCATGTCC	249	XM_004011159.5
<i>GJA4</i>	F: CGACGAGCAGTCGGATTT R: AGATGACATGGCCCAGGTAG	155	XM_027967582.2
<i>PRDX1</i>	F: CAGATGGTCAGTTCAAGGAT R: CAGGTGACAGAAGTGAGAAT	191	XM_004001918.4
<i>CAT</i>	F: CCATCTGAAAGACGCACAGC R: ATGCGGGAGCCATACTCAG	84	XM_060400055.1
<i>GPX1</i>	F: GCAACCAGTTTGGGCATCAG R: GCCATTACCTCGCACTTTT	123	XM_004018462.5
<i>SOD2</i>	F: TCACAGCATCTTCTGGACAA R: TGCTCCTTATTGAAGCCAAG	170	NM_001280703.1
<i>CHOP10</i>	F: AGGACCACCAGAGGTCACAC R: TGCCACTTTCCTTTTCGTTTT	193	NM_001078163.1
<i>GRP78</i>	F: TGAAACTGTGGGAGGTGTCA R: TCGAAAGTTCCCAGAAGGTG	171	XM_024998380.2
<i>ATF4</i>	F: CGAGGTGTTTGTGGGGGACT R: AGGAGCCTGCCTTAGCCTTG	149	XM_012158819.3

2.12. Statistical Analysis

Each experiment was performed with at least three replicates. Data are represented as the mean $\pm$ standard error (SEM). Differences between two or multiple groups were analyzed by using independent Student's *t*-test and one-way ANOVA with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA), respectively. ANOVA followed by Tukey's Honestly Significant Difference (HSD) was used to determine statistical differences between groups. The chi-square test was used to evaluate the differences in cleavage rate and blastocyst rate between the two groups. All statistical analyses were conducted using SPSS statistical software. Results were plotted using GraphPad Prism 9 software (GraphPad, La Jolla, CA, USA). The threshold for statistical significance in all tests was $p < 0.05$.

3. Results

3.1. BDNF Treatment Affects the In Vitro Maturation of Sheep Oocytes

COCs were incubated for 24 h in IVM medium alone (control) or supplemented with 10 ng/mL BDNF, 50 ng/mL BDNF, or 100 ng/mL BDNF. After 24 h of IVM, the degree of cumulus expansion was observed (Figure 1A). The results showed that the cumulus cells of all the COCs had diffused. There was no difference in CEI between the control

(2.74 ± 0.16) COCs and the COCs treated with 10 ng/mL BDNF (3.01 ± 0.17); however, the COCs treated with 50 ng/mL BDNF (3.35 ± 0.23) or 100 ng/mL BDNF (3.74 ± 0.12) had a significantly higher CEI than the control ($p < 0.05$ for each comparison; Figure 1B). The PBI extrusion rate was determined using a microscope (Figure 1A). The results are shown in Figure 1C. The PBI extrusion rates in the oocytes treated with 50 ng/mL BDNF ($79.51 \pm 2.09\%$) or 100 ng/mL BDNF ($80.62 \pm 3.16\%$) were both significantly higher than that of the control ($68.93 \pm 1.97\%$; $p < 0.05$ for each comparison), but there was no significant difference between these two groups. Therefore, 50 ng/mL BDNF was selected to treat the oocytes in subsequent experiments.

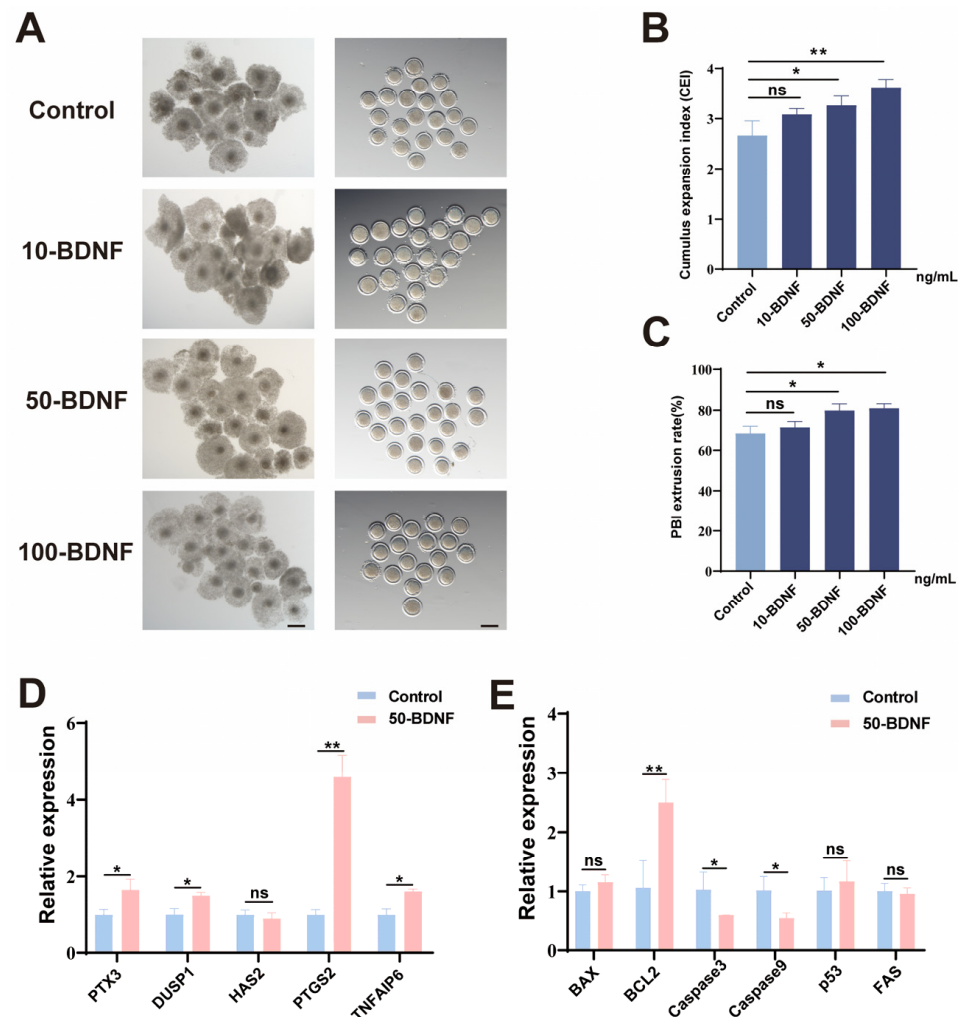


Figure 1. Effects of BDNF on Sheep Oocyte Maturation, Cumulus Expansion, and Apoptosis-Related Gene Expression. (A) In vitro maturation status of the control and the BDNF-treated groups. Cumulus expansion in cumulus-oocyte complexes and first polar body (PBI) extrusion in denuded oocytes was observed under a stereomicroscope (Control, $n = 103$; 10 ng/mL BDNF, $n = 119$; 50 ng/mL BDNF, $n = 122$; 100 ng/mL BDNF, $n = 129$; three biological replicates in each group). Scale bars: 250 μm (left), 100 μm (right). (B) Cumulus expansion index (CEI). (C) PBI extrusion rate. (D) mRNA levels of cumulus expansion-related genes (five biological replicates in each group). (E) mRNA levels of apoptosis-related genes (five biological replicates in each group). * $p < 0.05$, ** $p < 0.01$. ns = no significance.

We examined the expression levels of genes related to cumulus expansion and apoptosis in COCs. Compared with the control, the BDNF-treated COCs significantly upregulated mRNA levels of cumulus expansion-related genes (*PTX3*, *DUSP1*, *PTGS2*, *TNFAIP6*; $p < 0.05$) (Figure 1D), reduced mRNA levels of apoptosis-related genes (*Caspase3*,

Caspase9; $p < 0.05$), and an increased mRNA level of the anti-apoptosis gene *BCL2* ($p < 0.05$) (Figure 1E). In summary, treatment with 50 ng/mL BDNF significantly promoted oocyte maturation and inhibited apoptosis.

3.2. BDNF Reduces Oxidative Stress in Oocytes

After 24 h of IVM, the levels of ROS and GSH in oocytes were detected using DCFH-DA and Cell Tracker Blue CMAC. As shown in Figure 2, compared with the control, oocytes treated with 50 ng/mL BDNF had lower intracellular ROS levels ($p < 0.05$; Figure 2A,B) and higher GSH levels ($p < 0.05$; Figure 2C,D). These results indicate that BDNF can reduce the ROS produced by sheep oocytes during IVM, thereby alleviating OS. Thus, the above demonstrated that BDNF can alleviate OS in sheep oocytes during IVM.

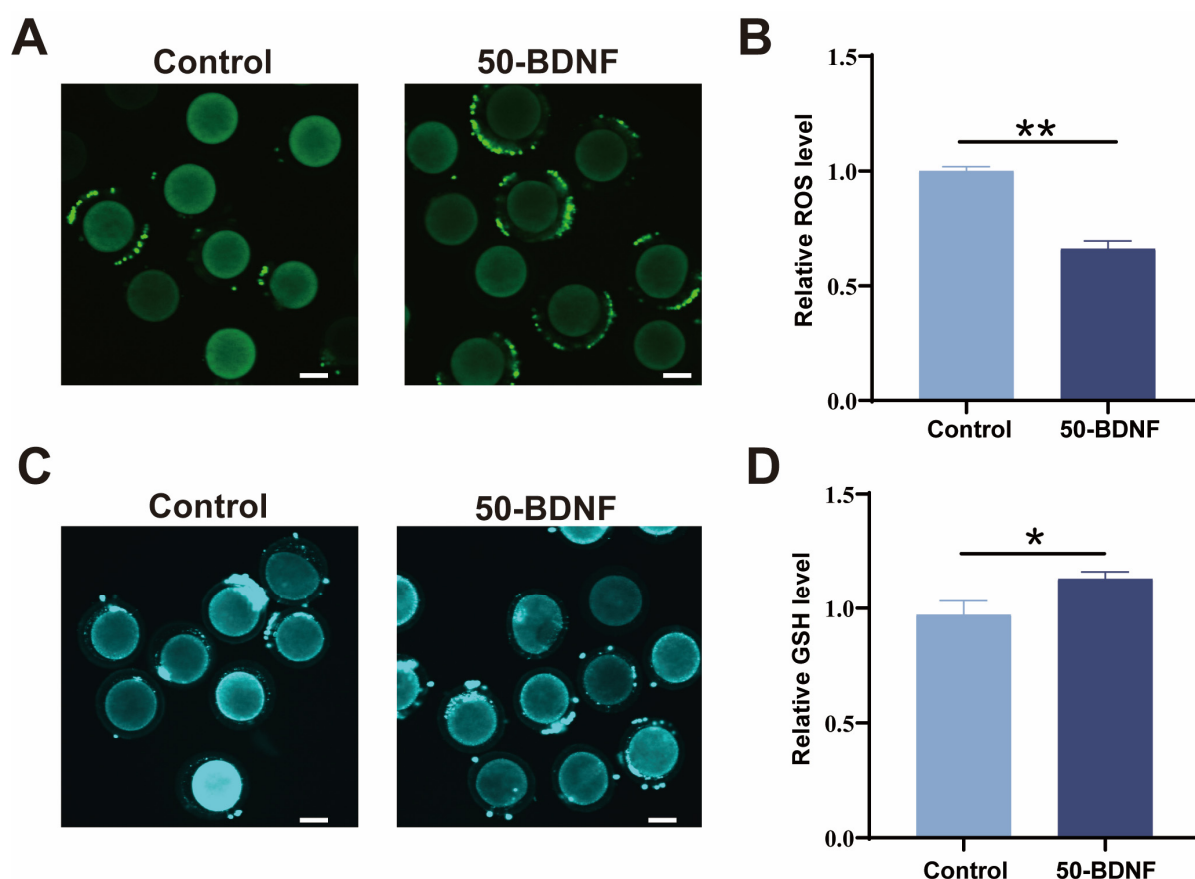


Figure 2. ROS and GSH levels in mature sheep oocytes treated with BDNF. (A) Representative images of ROS in oocytes. Scale bar, 50 μ m. (B) Fluorescence intensity of ROS signals (Control, $n = 45$; 50 ng/mL BDNF, $n = 52$; three biological replicates in each group). (C) Representative images of GSH in oocytes. Scale bar, 50 μ m. (D) Fluorescence intensity of GSH signals (Control, $n = 52$; 50 ng/mL BDNF, $n = 56$; three biological replicates in each group). * $p < 0.05$, ** $p < 0.01$.

3.3. BDNF Enhances the Antioxidant Capacity of Oocytes

Oocyte OS inevitably occurs during IVM, and mitochondria serve as one of the primary sites generating ROS. Mitochondrial distribution patterns in oocytes were detected using MitoTracker staining. Based on previous reports [27], the mitochondrial distribution in oocytes was categorized as proper, characterized by uniform dispersion throughout the entire cytoplasm, or abnormal, manifested as heterogeneous aggregation within specific cytoplasmic regions. The experimental results showed that there was no significant difference in the percentage of cells with a proper distribution of mitochondria between the control and oocytes treated with 50 ng/mL BDNF (Figure 3A,B). The expression of antioxi-

dant genes in oocytes was detected by qRT-PCR, and the results are shown in Figure 3C. The mRNA levels of antioxidant genes, including *SOD2*, *CAT*, *GPX1*, and *PRDX1*, were increased in oocytes treated with 50 ng/mL BDNF compared with those in control oocytes (Figure 2C; $p < 0.05$). Thus, these results suggest that BDNF primarily enhances the oocyte's antioxidant capacity.

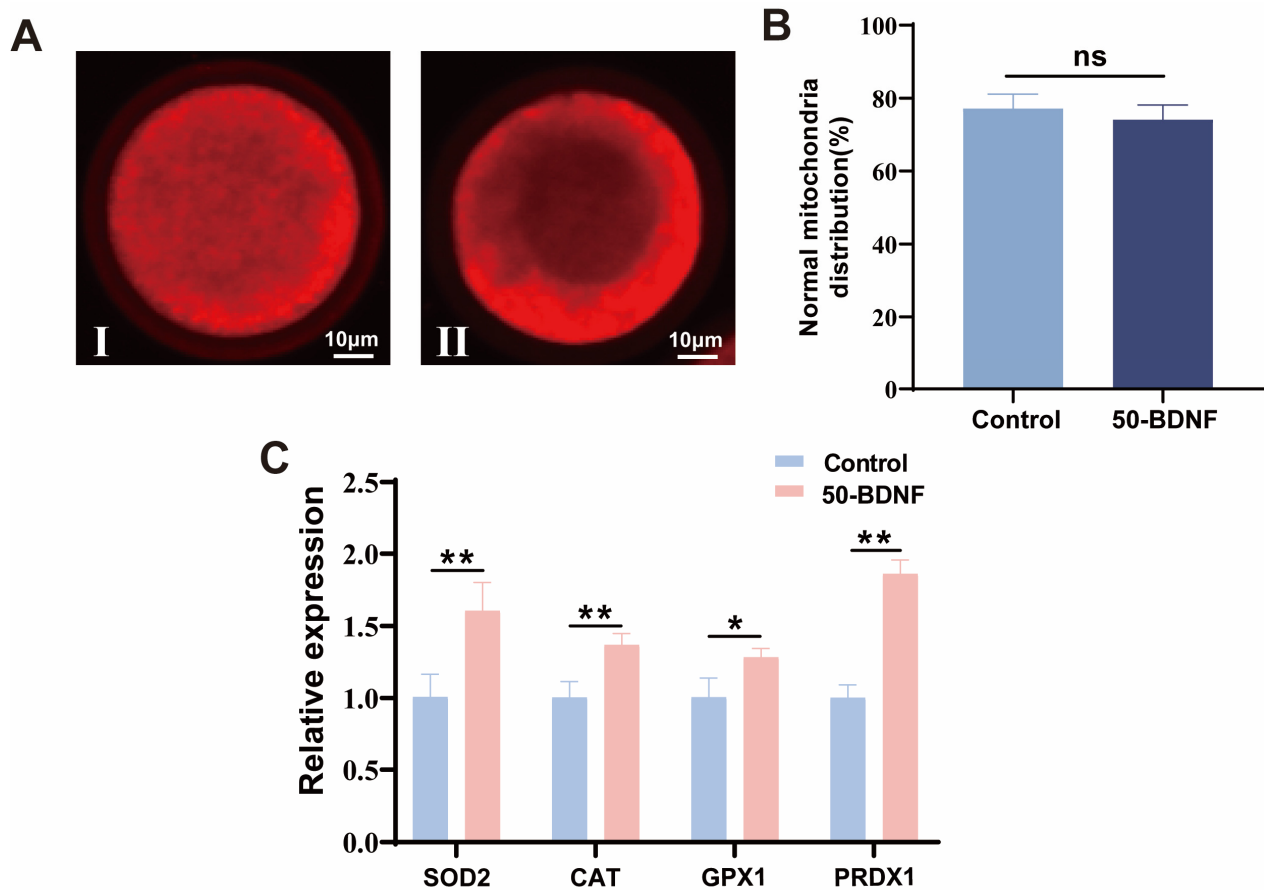


Figure 3. BDNF enhances the antioxidant capacity of oocytes. (A) Representative images of the mitochondrial distribution in oocytes. Type I represents the proper distribution; Type II represents the abnormal distribution. Scale bar, 10 μ m. (B) Mitochondrial distribution patterns (Control, $n = 38$; 50 ng/mL BDNF, $n = 42$; three biological replicates in each group). (C) Relative mRNA expression levels of antioxidant genes in oocytes analyzed by quantitative real-time PCR (five biological replicates in each group). * $p < 0.05$, ** $p < 0.01$. ns = no significance.

3.4. BDNF Regulates Mitochondrial Function in Oocytes

Mitochondria are important organelles in oocytes and are crucial for oocyte maturation. We evaluated the function of mitochondria by measuring the MMP in oocytes. Compared with the untreated control, oocytes treated with 50 ng/mL BDNF had a significantly elevated MMP ($p < 0.05$; Figure 4A,B). Concurrently, we analyzed the mitochondrial biogenesis in oocytes by qRT-PCR. NADH dehydrogenase subunit 5 (ND5) is frequently used as a reliable molecular marker for assessing mitochondrial biogenesis [28]. As shown in Figure 4C, BDNF treatment markedly increased the mRNA levels of ND5, indicating enhanced mitochondrial biogenesis during IVM. These results indicate that BDNF promotes mitochondrial functional activation during oocyte maturation.

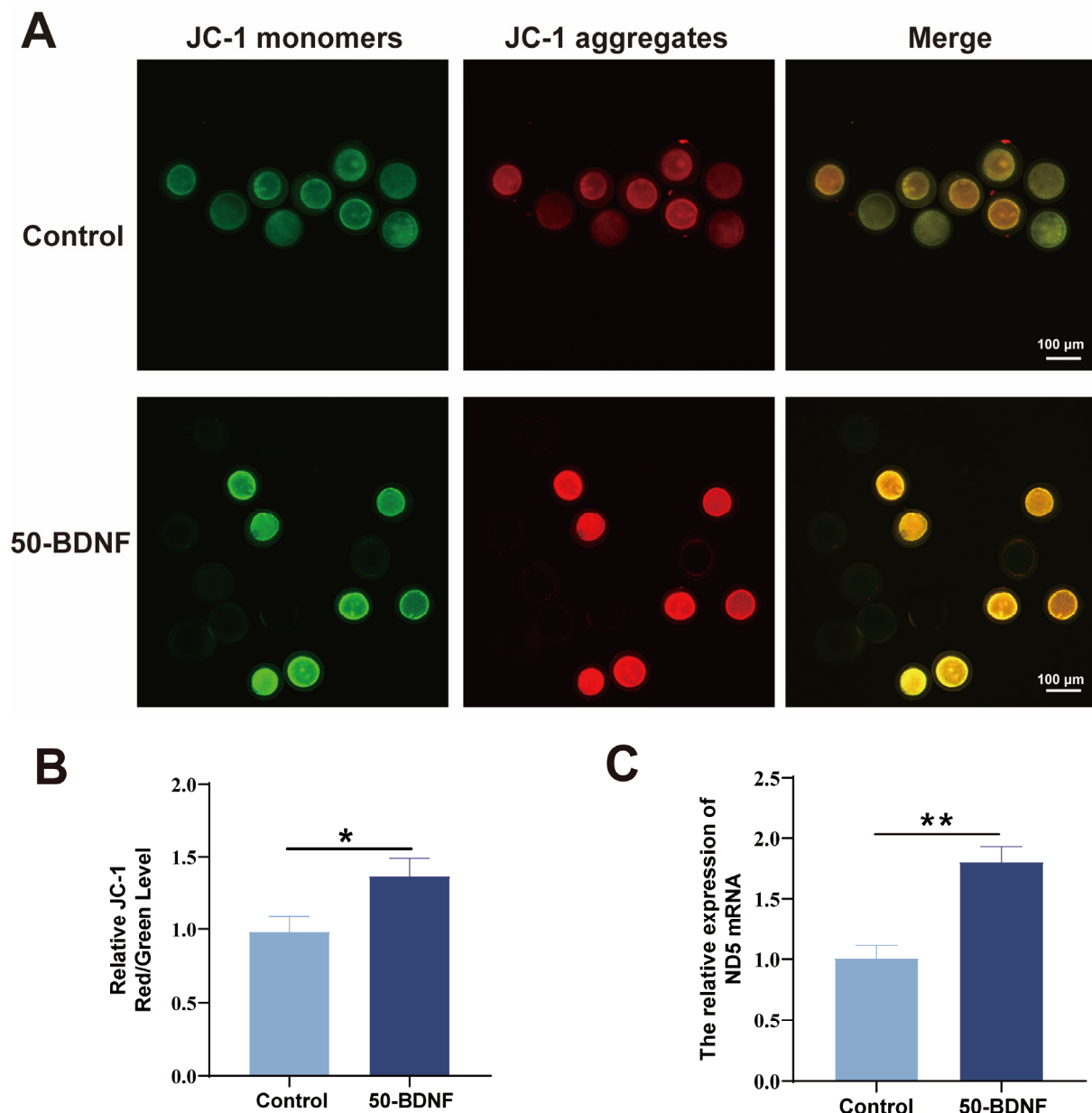


Figure 4. BDNF enhances the mitochondrial function of matured sheep oocytes. (A) Representative images of the mitochondrial membrane potential of oocytes. The red fluorescence represents J aggregates, and the green fluorescence represents JC-1 monomers. The mitochondrial membrane potential is expressed as the ratio of red/green fluorescence intensity. Scale bar, 100 μ m. (B) Quantitative analysis of the mitochondrial membrane potential (Control, $n = 67$; 50 ng/mL BDNF, $n = 74$; three biological replicates in each group). (C) Relative quantitative analysis of the ND5 mRNA level in oocytes (five biological replicates in each group). * $p < 0.05$, ** $p < 0.01$.

3.5. BDNF Alleviates Endoplasmic Reticulum Stress in Oocytes

The ER plays an important role in oocyte maturation. Conditions such as hypoxia, viral infection, calcium depletion, and other stresses can disrupt intracellular homeostasis, triggering ERS. We measured the distribution of the ER in oocytes using ER-Tracker Green dye. Based on previous reports [29], the distribution of the ER in oocytes can be categorized into two situations: proper, characterized by an even distribution throughout the cytoplasm of the oocyte, and abnormal, characterized by an uneven distribution in the peripheral region. As shown in Figure 5A,B, oocytes treated with 50 ng/mL BDNF had a significantly higher rate of proper ER distribution during IVM compared with the control. In addition,

oocytes treated with 50 ng/mL BDNF had significantly lower mRNA levels of ERS-related genes, including *CHOP10*, *GRP78*, and *ATF4*, compared with control oocytes (Figure 5C; $p < 0.05$). These concurrent improvements in ER architecture and reduction in ERS markers indicate that BDNF alleviates ER stress during oocyte maturation.

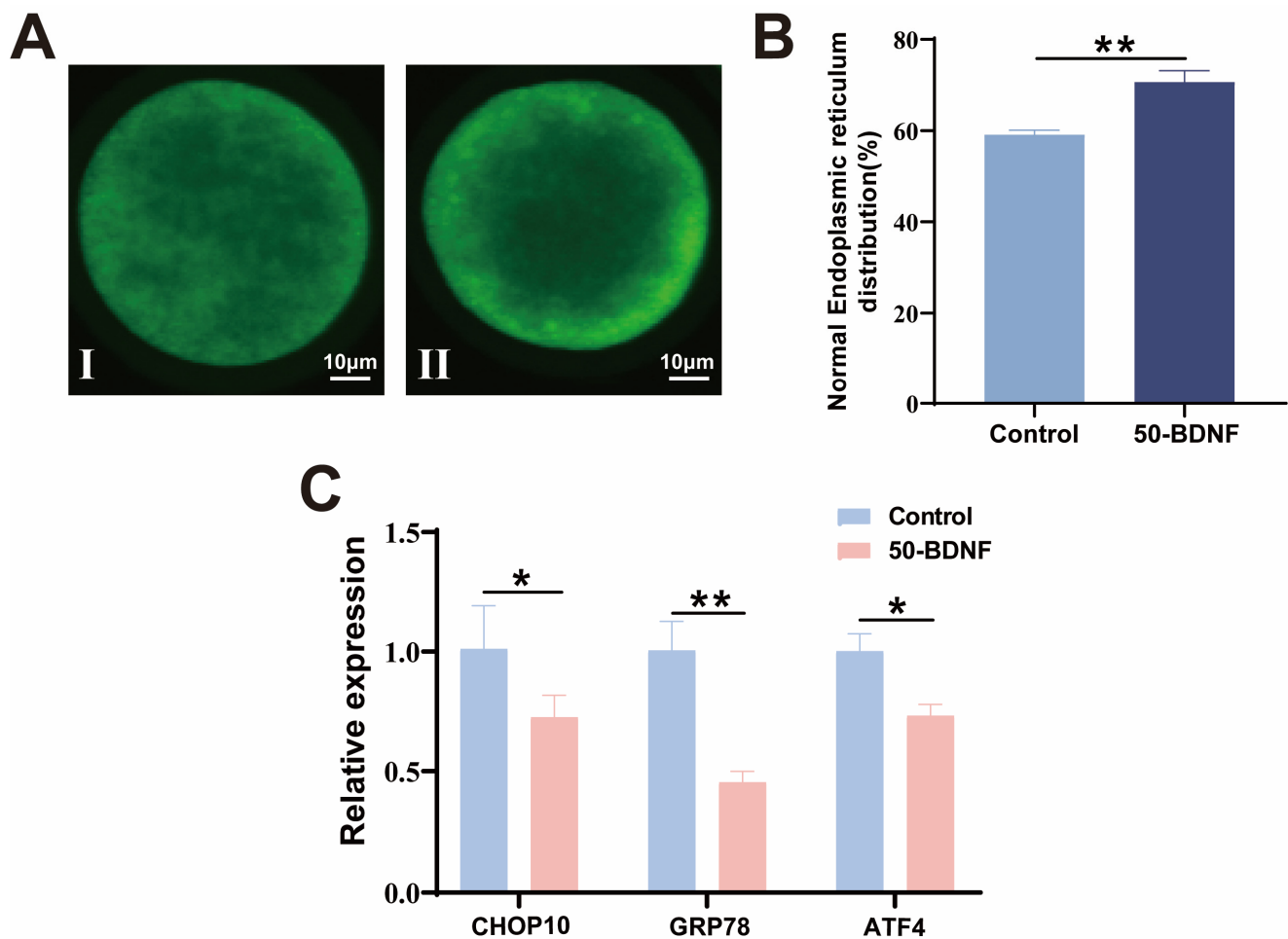


Figure 5. BDNF alleviates endoplasmic reticulum stress (ERS) in mature sheep oocytes. (A) Representative images of the endoplasmic reticulum (ER) distribution in oocytes. Type I represents the proper distribution, and Type II represents the abnormal distribution. Scale bar, 10 μ m. (B) Analysis of the ER distribution (Control, $n = 32$; 50 ng/mL BDNF, $n = 38$; three biological replicates in each group). (C) Relative levels of mRNA expression of ERS genes in oocytes detected by qRT-PCR (five biological replicates in each group). * $p < 0.05$, ** $p < 0.01$.

3.6. BDNF Regulates Ca^{2+} Levels in Oocytes

The ER and mitochondria are recognized as critical organelles for maintaining Ca^{2+} homeostasis. Intracellular Ca^{2+} levels serve as a fundamental regulator of diverse cellular processes, including metabolism, proliferation, differentiation, and apoptosis. During IVM of oocytes, the culture environment induces elevated ROS production, triggering OS. Excessive ROS accumulation disrupts Ca^{2+} equilibrium in oocytes, thereby exacerbating both OS and ERS. To elucidate the mechanistic impact of BDNF on oocyte maturation, we conducted quantitative assessments of Ca^{2+} levels in the mitochondrial, ER, and cytoplasmic compartments. Compared with the control, oocytes treated with 50 ng/mL BDNF exhibited a significant reduction in mitochondrial Ca^{2+} [Ca^{2+}]_m (Figure 6A,B; $p < 0.05$), and increased levels of [Ca^{2+}]_{ER} (Figure 6A,D; $p < 0.05$). In contrast, BDNF treatment did not significantly alter the cytosolic Ca^{2+} level ([Ca^{2+}]_i) compared to the control (Figure 6A,C; $p > 0.05$). Thus,

BDNF treatment triggered a redistribution of Ca^{2+} , decreasing mitochondrial levels while increasing ER stores during oocyte maturation.

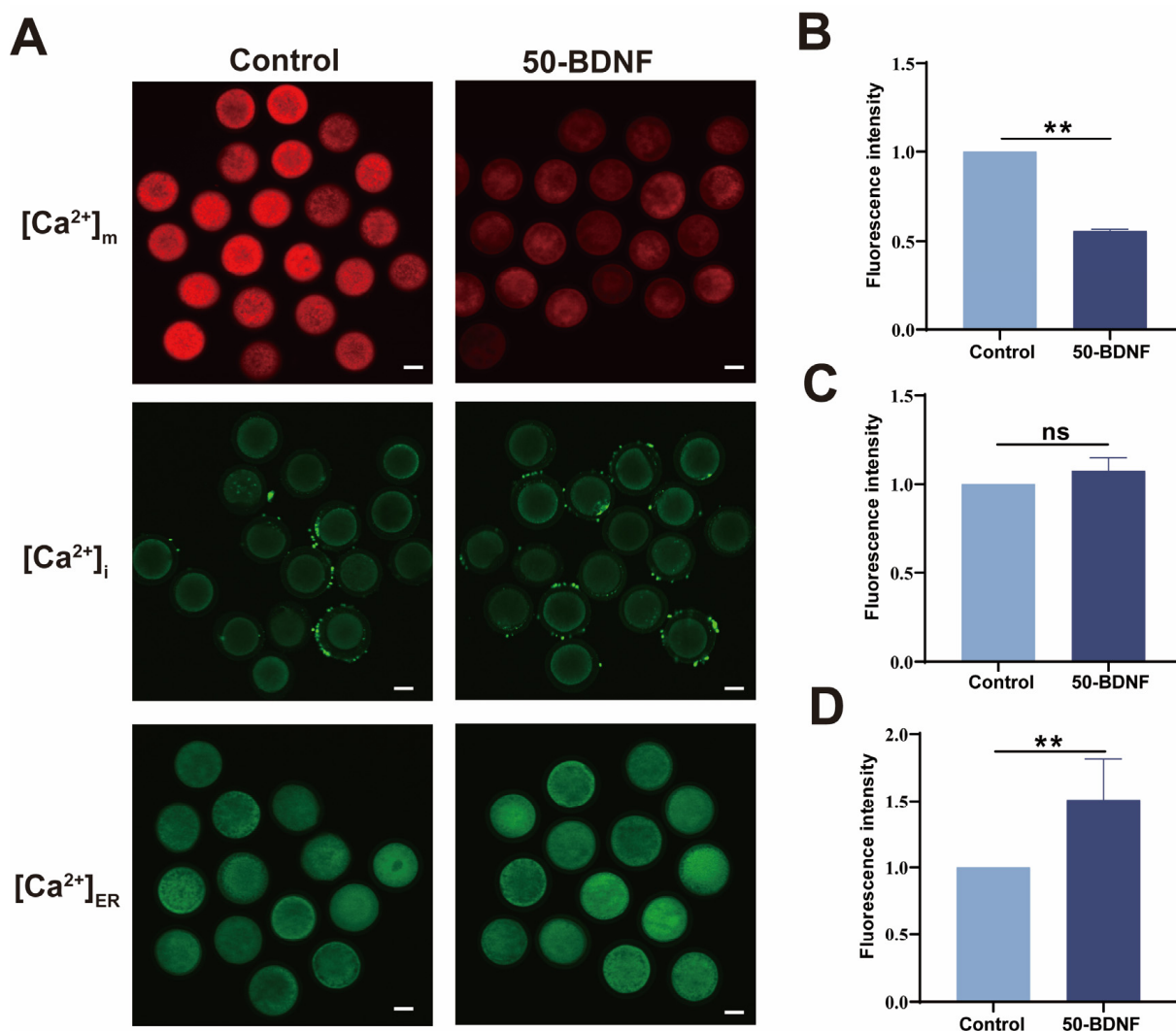


Figure 6. The effect of BDNF on Ca^{2+} in sheep oocytes. (A) Representative images of fluorescent staining of $[\text{Ca}^{2+}]_m$, $[\text{Ca}^{2+}]_i$, and $[\text{Ca}^{2+}]_{ER}$. Scale bar, 50 μm . (B) Fluorescent intensity of $[\text{Ca}^{2+}]_m$ (Control, $n = 30$; 50 ng/mL BDNF, $n = 32$). (C) Fluorescent intensity of $[\text{Ca}^{2+}]_i$ (Control, $n = 28$; 50 ng/mL BDNF, $n = 32$; three biological replicates in each group). (D) Fluorescent intensity of $[\text{Ca}^{2+}]_{ER}$ (Control, $n = 34$; 50 ng/mL BDNF, $n = 33$; three biological replicates in each group). ** $p < 0.01$. ns = no significance.

3.7. BDNF Regulates TZP Structure

During the IVM process, approximately 6 h to 8 h after the oocytes are separated from the follicles, the oocytes resume meiosis, and TZPs begin to be gradually lost. We employed rhodamine phalloidin staining to examine structural and quantitative changes in TZPs during IVM of sheep oocytes. As shown in Figure 7A,B, TZP numbers progressively decreased from 8 h onward. BDNF treatment increased the TZP quantity at 8 h ($p < 0.05$); however, the BDNF-treated oocytes exhibited fewer TZPs than control oocytes at 12 h ($p < 0.05$). After 24 h of IVM, both groups showed reduced TZP numbers compared with earlier time points, with no significant difference between control oocytes and BDNF-treated oocytes.

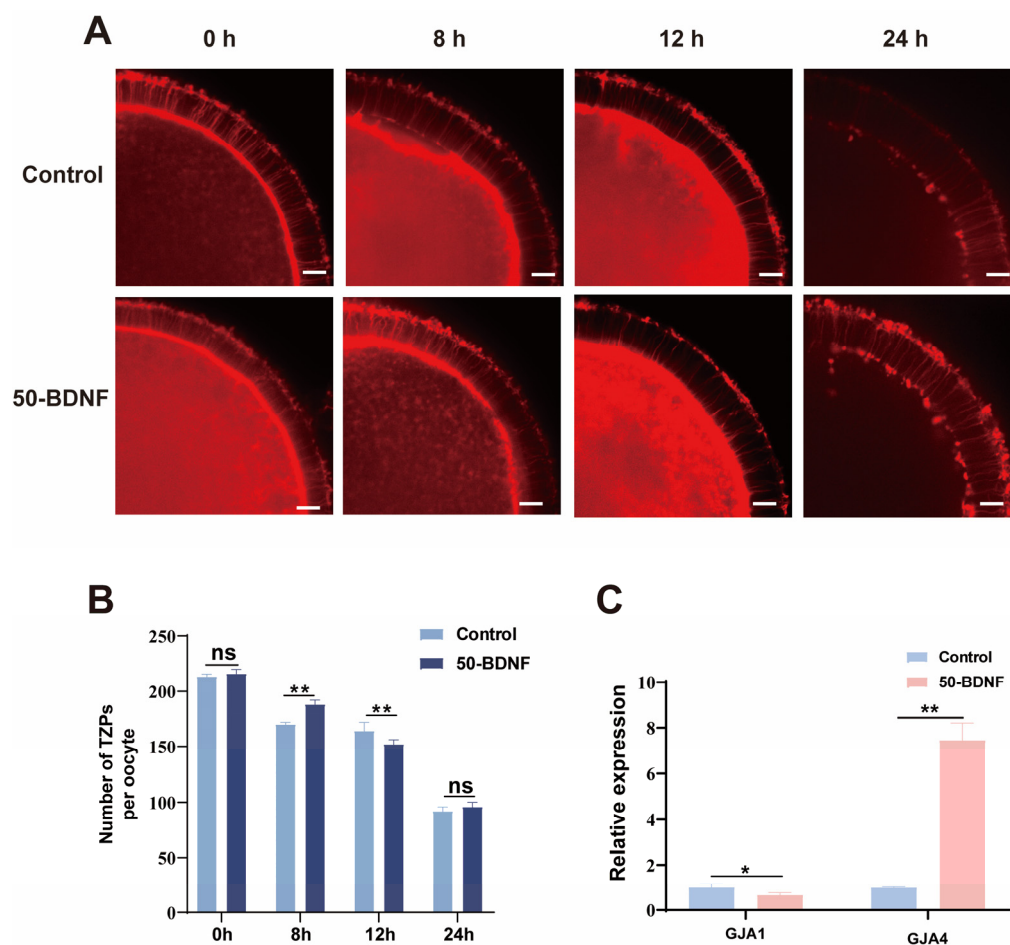


Figure 7. BDNF affects TZP structure. (A) Representative images of transzonal projection (TZP) staining during the in vitro maturation (IVM) process. Scale bar, 5 μ m. (B) Quantitative analysis of TZPs at each time point during IVM. The number of intact TZPs at each time point was compared (Control, $n = 25$; 50 ng/mL BDNF, $n = 25$; three biological replicates in each group). (C) Relative quantitative analysis of the mRNA levels of gap junction genes in cumulus cells (five biological replicates in each group). * $p < 0.05$; ** $p < 0.01$. ns = no significance.

Gap junctions, a ubiquitous form of intercellular communication between adjacent cells, are composed of transmembrane proteins called connexins. *GJA1* and *GJA4* are the two most critical connexin isoforms expressed in mammalian ovarian follicles. We quantified the mRNA expression levels of *GJA1* and *GJA4*. Compared with the control, BDNF treatment significantly downregulated *GJA1* expression, while markedly upregulating *GJA4* expression (Figure 7C; $p < 0.05$ for each comparison). These results suggest that BDNF can regulate oocyte-cumulus cell communication during maturation.

3.8. BDNF Treatment Affects Early Embryonic Development in Sheep

After IVM, oocytes were subjected to IVF and embryonic culture. As shown in Table 2, oocytes that were treated with 50 ng/mL BDNF during IVM exhibited significantly higher rates of cleavage ($88.64 \pm 3.90\%$) and blastocyst formation ($37.27 \pm 1.60\%$) compared with control oocytes ($79.80 \pm 4.72\%$; $23.74 \pm 0.70\%$; $p < 0.05$ for each comparison). Furthermore, quantification of total blastocyst cell numbers revealed a significant increase in the BDNF-treated group relative to the control (Figure 8A,B; $p < 0.05$). Therefore, supplementing 50 ng/mL BDNF during in vitro maturation can significantly improve the blastocyst formation and quality, leading to enhanced in vitro embryo production efficiency.

Table 2. The effect of BDNF on early embryonic development of sheep oocytes.

Groups	No. of Oocytes	No. of Cleavages (Mean $\pm$ SEM, %)	No. of Blastocysts (Mean $\pm$ SEM, %)
Control	198	158 (79.80 $\pm$ 4.72) ^b	47 (23.74 $\pm$ 0.70) ^b
50 ng/mL BDNF	220	195 (88.64 $\pm$ 3.90) ^a	82 (37.27 $\pm$ 1.60) ^a

Note: Percentage of cleavages = No. of cleavages/No. of oocytes; Percentage of blastocysts = No. of blastocysts/No. of oocytes. Different superscript values of a and b represent significant differences between groups ($p < 0.05$). Each experiment was independently repeated five times.

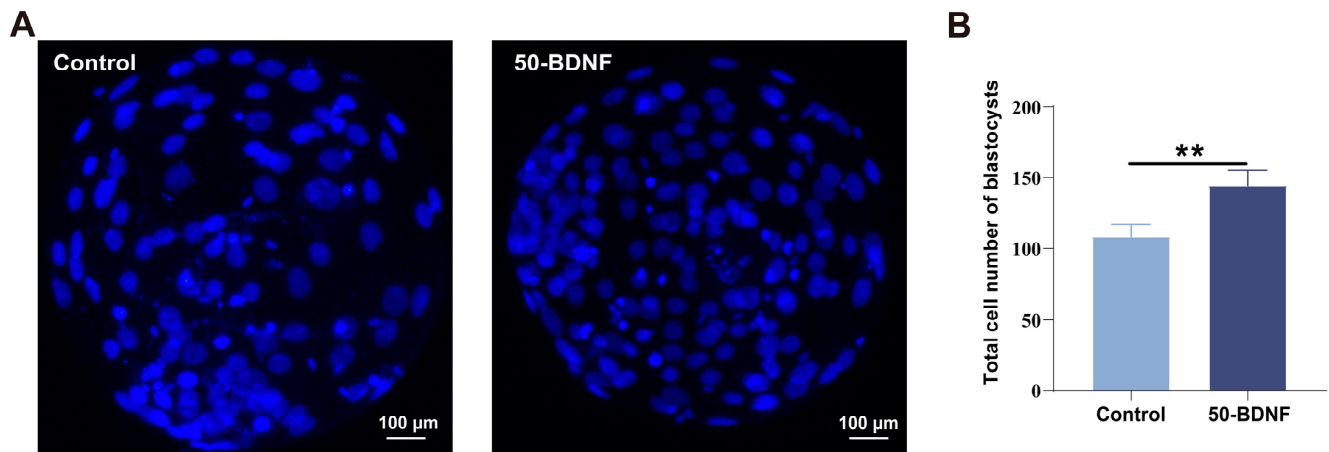


Figure 8. The effect of BDNF treatment during in vitro maturation on the total number of blastocyst cells. (A) Representative images of the total number of blastocyst cells. Scale bar, 100 μ m. (B) Bar chart of the total number of blastocyst cells (Control, $n = 28$; 50 ng/mL BDNF, $n = 34$; three biological replicates in each group). ** $p < 0.01$.

4. Discussion

BDNF has been shown to exert beneficial effects on in vitro-matured oocytes; however, the underlying molecular mechanisms are insufficiently characterized. We first determined the impacts of different concentrations of BDNF on the IVM of sheep oocytes, and we then investigated the specific regulatory pathways that were affected by the BDNF treatment. We found that 50 ng/mL BDNF promoted cumulus expansion and PBI extrusion of sheep oocytes during IVM. Previous studies showed that adding BDNF to the IVM culture medium can increase the PBI extrusion rate in mice, cattle, and wild boars [20,22]. Zhao et al. [30] also found that 10 ng/mL BDNF could bind the receptor TrkB and increase the maturation rate of buffalo oocytes. Cumulus cells protect oocytes from OS and reduce apoptosis. Complete cumulus expansion is crucial for the cytoplasmic and nuclear maturation of oocytes [31–37]. The degree of cumulus expansion is determined by the expression of specific genes, such as HAS2, TNFAIP6, VCAN, and PTX3 [38]. We found that mRNA levels of the cumulus expansion-related genes *PTX3*, *DUSP1*, *PTGS2*, and *TNFAIP6* were increased in BDNF-treated oocytes compared with those in control oocytes, which is largely consistent with the results of a previous study of buffalo oocytes [30]. In addition, BDNF treatment increased the expression of the anti-apoptotic gene *BCL2* and decreased the mRNA levels of the apoptotic genes *Caspase3* and *Caspase9* in oocytes. Based on these results, we hypothesize that treatment with 50 ng/mL BDNF promotes oocyte maturation and improves oocyte quality through cumulus expansion.

Unlike the in vivo environment, standard in vitro oocyte culture employs a gas mixture of 5% CO₂ in air (20% O₂), creating a significantly higher oxygen partial pressure [39]. Therefore, oocytes that mature in vitro are highly susceptible to high ROS levels, which can lead to OS, poor oocyte quality, and abnormal embryonic development [40,41]. GSH is one of the main antioxidants in cells. Its main function is to protect against the damage of OS

and maintain the redox balance within cells [42]. We found that BDNF treatment lowered ROS levels but elevated GSH levels relative to the control group. Mitochondria serve as the primary sites of ATP synthesis during oocyte maturation. As meiosis resumes, the mitochondria undergo spatial reorganization within the oocyte, progressively migrating from cortical regions toward the cytoplasmic center. Uniform intracellular distribution of mitochondria is a critical indicator of cytoplasmic maturation in oocytes [43–46]. We found that BDNF had no effect on the uniform distribution of oocytes, but it significantly increased the MMP and the mitochondrial biogenesis. It is well established that *SOD*, *CAT*, and *GPX* constitute the primary antioxidant defense system, whereas *PRDX* belongs to secondary defense mechanisms. During cellular OS, these enzymes orchestrate the elimination of ROS to maintain intracellular redox homeostasis, thereby safeguarding cellular integrity against oxidative damage [47]. We found that the mRNA levels of *SOD2*, *CAT*, *GPX1*, and *PRDX1* in BDNF-treated oocytes were increased compared with those in control oocytes. This further demonstrates that BDNF can reduce ROS levels in sheep oocytes and resist OS-induced damage during IVM by enhancing mitochondrial function and increasing the activity of antioxidant enzymes.

The ER is the main site for protein synthesis and is also one of the sites where ROS are produced. When cells experience conditions such as hypoxia, viral infection, or OS, misfolded proteins accumulate in the lumen of the ER, thereby inducing ERS [48,49]. In response to ERS, the unfolded protein response (UPR) is activated as a signaling cascade to restore homeostasis within the ER [50,51]. The UPR is mediated by three key ER transmembrane sensors: IRE1 α , PERK, and ATF6 [52]. Normally, the ER chaperone GRP78 is a master regulator, and its expression increases with the severity of ER stress [53]. Substantial experimental evidence indicates that maintaining ER homeostasis is a key mechanism for folliculogenesis and oocyte maturation [54,55]. We found that BDNF restored the spatial organization of ER in oocytes, which was concomitant with transcriptional downregulation of the ERS-related genes *CHOP10*, *GRP78*, and *ATF4*. Collectively, these findings suggest a molecular mechanism whereby BDNF ameliorates the ERS burden in oocytes through targeted suppression of the *GRP78-ATF4-CHOP10* signaling axis.

Mitochondria and the ER mediate the transport of intracellular calcium ions, which function as key second messengers to participate in stress responses [56]. Following its release from the ER via channels such as IP3R or RyR during ERS, calcium fluxes into mitochondria, where elevated levels contribute to increased ROS production [57]. We found that treatment of oocytes with BDNF decreased the level of $[Ca^{2+}]_m$ and increased the level of $[Ca^{2+}]_{ER}$, which is consistent with previous findings that OS damage and ERS can disrupt calcium ion levels in oocytes.

Cumulus cells and oocytes communicate through multiple pathways to meet the nutrient demands of oocyte maturation [58]. One key structure is the TZP, which extends from cumulus cells, traverses the zona pellucida, and contacts the oolemma to establish direct communication [59]. These TZPs are crucial for regulating the growth, development, and meiosis of oocytes [60]. TZPs start to retract within a few hours after the start of IVM, which promotes the resumption of oocyte meiosis [61]. BDNF enhances the sensitivity of cumulus cells to gonadotropins. On one hand, cAMP promotes BDNF secretion in cumulus cells. On the other hand, the combined action of BDNF and FSH activates the downstream cAMP/PKA/CREB signaling pathway, while cAMP is transported from cumulus cells to oocytes [62]. We found that after 8 h of IVM, the number of TZPs began to decrease significantly. Compared with the control, BDNF-treated cells maintained a higher level of TZPs at this stage, which prolonged the meiotic resumption time and promoted cytoplasmic maturation. The reduction of TZPs diminished cAMP transport to oocytes, facilitating meiotic resumption. These findings indicate that the timing of TZP retraction plays a

crucial role in oocyte maturation. Additionally, we examined gap junction genes and found that BDNF upregulated their mRNA expression levels. This enhancement promoted material exchange between cumulus cells and oocytes, thereby improving early embryonic developmental competence.

5. Conclusions

This study reveals a potential molecular mechanism by which BDNF promotes successful IVM of sheep oocytes. BDNF increases the PBI ejection rate and the cumulus expansion of oocytes, reduces OS and ERS, improves mitochondrial function, enhances material exchange between cumulus cells and oocytes, and promotes the maturation of oocytes and the development of early embryos.

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