

Article

Pyrroloquinoline Quinone Alleviates Tris(1,3-Dichloro-2-Propyl) Phosphate-Induced Damage During Mouse Oocyte Maturation

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Simple Summary

Tris(1,3-dichloro-2-propyl) phosphate is a common chemical used to prevent fires that is frequently found in our homes and environment. Recent concerns suggest it may harm reproductive health. Our study aimed to investigate how this fire-retardant chemical affects the development of mouse oocytes and whether a natural protective substance called pyrroloquinoline quinone can reduce this damage. We discovered that the fire retardant significantly hinders the ability of oocytes to develop and prepare for fertilization. It does this by increasing harmful oxygen molecules, damaging the tiny powerhouses that provide energy to the cell, and triggering a process of cell death. Importantly, our results showed that adding the natural protective substance successfully prevented these problems, allowing the oocytes to develop normally and stay healthy. In conclusion, this research demonstrates that while environmental pollutants like fire retardants can damage fertility, natural substances can offer a potential shield. These findings are important for society as they help us understand the risks posed by everyday chemicals and suggest new ways to protect the reproductive health of both humans and animals from environmental threats.

Abstract

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is a ubiquitous organophosphate flame retardant posing potential threats to reproductive health. Given that TDCIPP toxicity is often linked to oxidative stress, pyrroloquinoline quinone (PQQ), a potent natural antioxidant and mitochondrial nutrient, was hypothesized to mitigate these adverse effects. This study investigated the impact of TDCIPP exposure on the in vitro maturation of mouse oocytes and evaluated the protective role of PQQ. Using an in vitro maturation model, we assessed the toxic effects of TDCIPP by examining the first polar body extrusion (PBE) rate and cumulus expansion, followed by analyses of oxidative stress (ROS and GSH), mitochondrial integrity (ATP content and distribution), and apoptosis-related markers through transcriptome sequencing (Smart RNA-seq), quantitative real-time PCR, and immunofluorescence. The results demonstrated that TDCIPP significantly suppressed cumulus expansion and reduced the PBE rate. Mechanistically, TDCIPP induced severe oxidative stress, disrupted mitochondrial function, and activated the apoptotic pathway. Furthermore, TDCIPP triggered early apoptotic signaling by downregulating *Bcl-2* and upregulating *Bax*. Notably, supplementation with PQQ effectively reversed these detrimental effects by reducing intracellular ROS levels, maintaining GSH content, preserving mitochondrial density and ATP



Academic Editor: Raffaele Boni

Received: 29 December 2025

Revised: 12 February 2026

Accepted: 17 February 2026

Published: 21 February 2026

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production, and inhibiting apoptosis. In conclusion, our findings provide new insights into the gamete toxicity of TDCIPP and suggest that PQQ may serve as a potential therapeutic agent to protect oocyte quality against environmental pollutant-induced damage.

Keywords: tris(1,3-dichloro-2-propyl) phosphate (TDCIPP); pyrroloquinoline quinone (PQQ); reactive oxygen species (ROS); mitochondrion; oocyte

1. Introduction

Organophosphate flame retardants (OPFRs) are a class of synthetic chemicals widely used in different processes, such as foam, plastic, textile, and furniture production [1]. In recent years, OPFRs have been frequently detected in environmental samples and biological matrices across the globe [2], which underscores their extensive environmental distribution. Increasing evidence indicates that OPFRs exhibit considerable biological toxicity, thereby raising concerns regarding the potential health risks associated with long-term human exposure [3].

Tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) is one of the most widely used OPFRs and has been detected in diverse environmental media and biological samples worldwide [4]. Its ubiquitous presence in water, air, soil, and dust implies that TDCIPP has the potential to bioaccumulate in aquatic organisms, with subsequent transfer to humans and other animals via the food chain [5]. Multiple studies have confirmed the widespread occurrence of TDCIPP in humans, as evidenced by its consistent detection in breast milk [6,7], placenta [8], urine [9], and hair [10]—findings that indicate long-term human exposure to this compound. Existing evidence demonstrates that TDCIPP exhibits considerable biological toxicity, exerting adverse effects on multiple facets of animal health. Specifically, research has shown that TDCIPP can disrupt normal developmental and reproductive processes, impair nervous system function, interfere with respiratory system homeostasis, compromise skin integrity, and accumulate in various organs [11–14]. Recent studies have further highlighted that gestational exposure to TDCIPP can disrupt embryonic development via specific signaling pathways [15]. In fish toxicology research, TDCIPP has been demonstrated to disrupt thyroid hormone (TH) homeostasis [16], induce developmental toxicity [17], cause neurodevelopmental toxicity [18], and trigger reproductive impairments [6]. To date, extensive studies have confirmed the overt toxicity of TDCIPP in zebrafish embryos and larvae and PC12 cells [19]; however, its specific effects on mammalian oocytes, such as those from mice, remain insufficiently investigated.

With respect to gamete toxicity, studies focusing on in vitro fertilization (IVF) have yielded critical insights. Specifically, these studies suggest that urinary concentrations of OPFR metabolites are negatively correlated with multiple reproductive outcomes. For instance, elevated levels of OPFR metabolites are strongly associated with reduced oocyte fertilization rates, embryo implantation rates, clinical pregnancy rates, and live birth rates [9]. Moreover, previous studies have shown that elevated maternal urinary concentrations of bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), the primary metabolite of TDCIPP, are significantly associated with an increased risk of fetal chromosomal abnormalities [20]. Further investigations have revealed that adult zebrafish exposed to TDCIPP can transfer its metabolite BDCIPP to their offspring, which leads to reduced thyroid hormone levels and impaired growth [6]. At the population level, an epidemiological study reported a significant association between TDCIPP exposure and reduced sperm motility and viability [21]. Given the widespread environmental presence of TDCIPP and its associated

potential hazards, elucidating the molecular mechanisms underlying TDCIPP-induced cellular damage is of considerable importance.

Pyrroloquinoline quinone (PQQ) is a water-soluble, vitamin-like factor that has garnered attention for its role as a redox cofactor and is naturally present in various dietary sources [22,23]. In recent years, PQQ has been shown to exert diverse biological effects, including antioxidant activity, mitochondrial activation, anti-inflammatory and neuroprotective functions, as well as the promotion of animal growth, development, and reproduction. Specifically, PQQ has been demonstrated to mitigate oxidative damage and enhance mitochondrial biogenesis in various models, such as promoting adenosine triphosphate (ATP) synthesis in mouse granulosa cells [24] and restoring ovarian function in premature ovarian insufficiency (POI) mice [25], and enhancing porcine oocyte maturation [26]. Given that TDCIPP exposure often disrupts cellular homeostasis by inducing severe oxidative stress and mitochondrial dysfunction, we hypothesized that PQQ could serve as a targeted rescue agent. Although previous studies have shown PQQ can alleviate defects caused by other toxicants like diisobutyl phthalate (DiBP) [27], its potential efficacy in counteracting TDCIPP-induced oocyte toxicity remains unknown.

Therefore, this study aimed to bridge this knowledge gap by elucidating the toxic effects of TDCIPP on the meiotic maturation of mouse oocytes and systematically evaluating whether PQQ can protect against these effects. By analyzing oxidative stress markers, mitochondrial function, and apoptotic pathways, we sought to verify our hypothesis and provide experimental evidence for PQQ as a potential therapeutic strategy to safeguard female fertility against environmental pollutants.

2. Materials and Methods

2.1. Experimental Materials

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Female ICR mice (8–10 weeks old) were obtained from SPF Biotechnology Co., Ltd. (Beijing, China). The mice were housed in well-ventilated cages under strictly controlled environmental conditions: temperature was maintained at 22 ± 2 °C, and a 12-h light/dark cycle was applied. Throughout the experiment, the mice had free access to standard laboratory food and water ad libitum.

2.2. Oocyte Collection, Treatment, and Maturation

Mice were intraperitoneally injected with 10 international units (IUs) of pregnant mare serum gonadotropin (PMSG), and oocytes were collected 48 h later, following the detailed protocol previously established in our laboratory [28]. Mice were humanely euthanized via cervical dislocation, followed by surgical excision of the ovaries, which were immediately transferred to M2 operating medium. Follicles were punctured using a 1 mL syringe needle to release the cumulus–oocyte complexes (COCs). Subsequently, COCs were examined under a stereomicroscope. Only those exhibiting a uniform ooplasm and surrounded by at least three complete layers of compact cumulus cells were selected for subsequent treatment and in vitro maturation (IVM) procedures.

To determine the optimal toxic concentration of TDCIPP, a dose–response experiment was conducted. COCs were cultured in an IVM medium (MEM α ; Life Technologies Corporation, USA) supplemented with TDCIPP at concentrations of 0, 100, 500, 1000, or 1500 ng/mL for 16 h. These concentrations were selected based on previous toxicological reports [4,21] and environmental exposure levels [19,29]. Similarly, to evaluate the protective effect of PQQ, COCs were treated with PQQ at concentrations of 0, 50, 100, or 150 μ M for 16 h, determined according to previous studies demonstrating its antioxidant efficacy in reproductive cells [24,27]. Subsequently, we performed dose–response experiments within

these ranges to identify the optimal concentrations for our specific experimental model. Additionally, a combination of the optimal concentrations of TDCIPP and PQQ was applied for 16 h. Four experimental groups were established: (1) control group: untreated COCs; (2) TDCIPP group: COCs exposed to the optimal concentration of TDCIPP; (3) PQQ group: COCs treated with the optimal concentration of PQQ; and (4) TDCIPP + PQQ group: COCs treated with the optimal concentrations of both TDCIPP and PQQ.

After 16 h of maturation culture, cumulus cells were gently removed by pipetting COCs in M2 medium containing 0.1% hyaluronidase. Subsequently, oocytes with a visible first polar body were counted to calculate the oocyte maturation rate. Only mature oocytes (MII stage), characterized by the presence of the first polar body, were collected for the subsequent experiments (including ROS/GSH measurement, ATP assay, mitochondrial staining, and immunofluorescence). All experiments were repeated at least three times independently.

2.3. Measurement of Intracellular ROS and GSH Levels

Intracellular ROS and glutathione (GSH) levels were determined using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) and Thiol TrackerTM Violet staining solution (Beyotime, Shanghai, China), respectively, according to previously validated protocols [28,30]. Specifically, denuded oocytes were incubated in a medium supplemented with either 10 μ M DCFH-DA (for ROS detection) or 20 μ M Thiol TrackerTM Violet staining solution (for GSH quantification). Incubations were performed at 37 °C in a 5% CO₂ atmosphere for 30 min. After incubation, oocytes were thoroughly washed three times with Dulbecco's phosphate-buffered saline (DPBS). Fluorescence intensity of the oocytes was then measured using a fluorescence microscope (Olympus CH 30, RF-200, Tokyo, Japan): a 488 nm filter was used for ROS detection, while a 370 nm filter was applied for GSH analysis. The obtained fluorescence data were processed and analyzed using ImageJ software (v2.16) to ensure accurate and reliable results.

2.4. ATP Content Assays

The ATP levels in individual oocytes were determined using the Enhanced ATP Assay Kit (Beyotime, Shanghai, China), following the manufacturer's instructions and modifications for oocyte samples described previously [28]. Briefly, a series of ATP standard solutions with concentrations ranging from 0 to 40 picomoles (pmol) was prepared first. Subsequently, oocytes were lysed in 20 μ L lysis buffer. After lysis, the samples were centrifuged at 4 °C for 5 min to remove cellular debris. Next, the ATP standards, together with suitable diluents and assay buffer, were added to the wells of a 96-well microplate. Immediately after sample loading, luminescence intensity was measured using a Tecan Infinite F200 luminometer (Tecan, Männedorf, Zürich, Switzerland). The ATP content in each sample was calculated via the standard curve method. To determine the average ATP content per oocyte, the total ATP amount of each sample was divided by the number of oocytes in each respective sample, and the results are expressed in picomoles (pmol) per oocyte.

2.5. Mitochondrial Membrane Potential Assessment

Mitochondrial membrane potential was evaluated by selectively labeling active mitochondria in target cells using the Mito-Tracker Red CMXRos mitochondrial fluorescent probe (Beyotime, Shanghai, China), according to previously described protocols [28]. Briefly, the Mito-Tracker Red CMXRos stock solution was diluted to prepare a 20 nM working solution. Next, staining droplets were dispensed onto a culture dish, and mineral oil was immediately overlaid onto the droplets to prevent evaporation. These droplets were then pre-warmed by incubation at 37 °C in a humidified atmosphere with 5% CO₂ until ready

for use. Prior to staining, granulosa cells were removed from the oocytes, and the denuded oocytes were rinsed three times with PBS supplemented with 0.1% polyvinyl alcohol (PBS-PVA) to eliminate residual granulosa cell debris and culture medium. Subsequently, the oocytes were incubated at 37 °C in the dark for 20 min. Following the incubation period, the oocytes were washed three times with PBS-PVA to thoroughly remove any residual staining solution. Fluorescence analysis was conducted using a fluorescence microscope (Olympus CH 30, RF-200, Tokyo, Japan), with the excitation filter set at 594 nm. The fluorescence intensity of the oocytes was then quantified and analyzed using ImageJ software, providing valuable data on mitochondrial function and activity.

2.6. Immunofluorescence Staining of Oocytes

For immunofluorescence staining of CASPASE3, BCL-2, and BAX, oocytes were processed according to established immunocytochemical protocols [31]. Briefly, oocytes were first fixed in 4% paraformaldehyde dissolved in PBS (pH 7.4), followed by permeabilization with 0.5% Triton X-100 at 37 °C for 45 min. After three washes with 0.1% PVA/PBS each at 37 °C for 5 min, non-specific binding sites were blocked with 1% bovine serum albumin (BSA) for 1 h. The oocytes were then incubated overnight at 4 °C with primary antibodies. The specific primary antibodies used were rabbit anti-CASPASE3 (1:500; Cat AC033, Beyotime, Shanghai, China), rabbit anti-BCL2 (1:500; Cat AB112, Beyotime), and mouse anti-BAX (1:500; Cat AB026, Beyotime). Subsequently, the oocytes underwent four washing steps using 0.1% PVA/PBS (20 min per wash at 37 °C) to remove unbound primary antibodies. They were then incubated in the dark at room temperature for 1 h with secondary antibodies: Alexa Fluor 594 Goat Anti-Rabbit (1:1000; Cat# A0516, Beyotime) or Alexa Fluor 488 Goat Anti-Mouse (1:1000; Cat# A0428, Beyotime). Following another series of four washes in 0.1% PVA/PBS at 37 °C for 5 min each, 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used for costaining the oocytes. Finally, the stained oocytes were mounted on glass slides and visualized using a fluorescence microscope (Olympus CH 30, RF-200, Tokyo, Japan).

2.7. Real-Time Fluorescence Quantitative PCR (q-PCR)

Total RNA was isolated from the oocytes and cumulus granulosa cells (CGCs) across different treatment groups using TRNzol reagent (Tiangen, Beijing, China) following the manufacturer's standard protocol. Next, complementary DNA (cDNA) synthesis was performed via the FastKing one-step reverse transcription system (Tiangen, China), a premixed reagent that concurrently eliminates genomic DNA contamination while catalyzing the reverse transcription of RNA into cDNA. All steps were executed strictly in accordance with the manufacturer's instructions. Subsequently, quantitative PCR (qPCR) reactions were carried out using the SuperReal Fluorescence Quantitative Premix (SYBR Green, Enhanced Edition; Tiangen, China) on a CFX Connect real-time PCR system (Bio-Rad, Hercules, CA, USA). To ensure the reproducibility of the results, each reaction was run in triplicate, and cycle threshold (CT) values were recorded for all target genes. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [32]. Detailed sequences of the specific primers employed for amplification are listed in Table 1.

Table 1. Primer pairs for real-time quantitative PCR.

Gene	Primer Sequence (5'-3')	Accession Numbers	Product Length (bp)
<i>Gapdh</i>	Forward: ACAGTCAAGGCAGAGAACGG	NM_008085	235 bp
	Reverse: GGTTCACGCCCATCACAAC		
<i>Bax</i>	Forward: GGAGCCTCAACCCTTCTTCC	NM_007527	159 bp
	Reverse: AGAGGAGTAGGCTGGAGACC		

Table 1. Cont.

Gene	Primer Sequence (5'-3')	Accession Numbers	Product Length (bp)
<i>Bcl-2</i>	Forward: GACTTCTCTCGTCGCTACCG Reverse: CTCTCCACACACATGACCCC	NM_009741	350 bp
<i>Caspase3</i>	Forward: GTCATCTCGCTCTGGTACGG Reverse: CACACACACAAAGCTGCTCC	XM_030243266	169 bp
<i>Has2</i>	Forward: TGTGAGAGGTTTCTATGTGTCCT Reverse: ACCGTACAGTCCAAATGAGAAGT	NM_008216	144 bp
<i>Ptx3</i>	Forward: TTTGGAAGCGTGCATCCTGT Reverse: TCCATCCTTGAAAAGGCGCA	NM_008987	200 bp
<i>Ptgs2</i>	Forward: TTCAACACACTCTATCACTGGC Reverse: AGAAGCGTTTGCGGTACTCAT	NM_011198	271 bp
<i>Tnfaip6</i>	Forward: GCTCAACAGGAGTGAGCGAT Reverse: CTGACCGTACTTGAGCCGAA	NM_009398	148 bp

2.8. Smart RNA Library Construction and Sequencing

MII-stage oocytes were harvested from both the control and TDCIPP-treated groups. The zona pellucida of these oocytes was then removed by exposure to an acidic solution, after which the oocytes were thoroughly rinsed three times in 0.1% PBS-BSA. Cytoplasmic fractions containing RNA were transferred into 45 µL of reverse transcription mixture, which was composed of 200 U/µL SuperScript II Reverse Transcriptase (18064071, Invitrogen, Carlsbad, CA, USA), 10 µL template switch oligo primer, 40 U/µL RNase inhibitor, 10 µM 6-base pair (bp) barcode primer, 10 mM deoxynucleotide triphosphates (4030, Takara Bio Inc., Kusatsu, Japan), 5 M betaine, and 1 M MgCl₂, following the Smart-seq2 protocol [33]. Subsequently, polymerase chain reaction (PCR) was performed on this mixture using a thermocycler with the following parameters: 25 °C for 5 min, 42 °C for 60 min, 50 °C for 30 min, and 70 °C for 10 min. Following the reaction, the resulting cDNA was purified and then subjected to biotin-based PCR amplification and enrichment. Finally, RNA libraries were constructed using the KAPA HyperPrep Kit (Illumina-compatible; KK8504, KAPA Biosystems Inc., Wilmington, MA, USA) and sequenced on an Illumina HiSeq X Ten platform (Illumina Inc., San Diego, CA, USA) with 150 bp paired-end reads.

2.9. Transcriptomic Data Analysis

Raw sequencing reads were first accurately demultiplexed using cell-specific barcode information appended to the 3' ends of R2 reads during library preparation. The sequence data were then further processed, including alignment with read1 data and integration of unique molecular identifier (UMI) information. Quality filtering was subsequently performed to remove reads containing a high proportion of low-quality bases. The resulting clean reads were mapped to the mouse reference genome, and HTSeq software (v0.6.1) was used to quantify uniquely aligned reads at the gene level [34]. After quantification, the raw gene expression count matrix was normalized to transcripts per million (TPM).

Downstream bioinformatics analyses were conducted using the Seurat package (v4.0.0) [35]. Strict cell and gene filtering was applied based on the following criteria: each cell must express at least 1000 genes, mitochondrial gene reads must constitute <40% of total reads per cell, and the overall alignment rate must exceed 20%. Post-filtering, approximately 2000 highly variable genes (HVGs) with substantial intercellular variation were selected for subsequent dimensionality reduction analyses.

Differentially expressed genes (DEGs) between experimental groups (control, TDCIPP) were identified using Wilcoxon rank-sum tests. *p*-values were adjusted using the Benjamini–Hochberg (BH) method to control the false discovery rate. The DAVID bioinformatics resource was employed to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses on the identified DEGs [36].

2.10. Data Statistical Analysis

All experimental data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Duncan’s post hoc test for multiple comparisons with SPSS software (v29.0). Results are presented as the mean $\pm$ standard error of the mean (SEM). Fluorescence intensity and other image-based quantitative data were analyzed using ImageJ software, while graphical representations of data were generated using GraphPad Prism 5.0. A *p*-value < 0.05 indicated a statistically significant difference.

3. Results

3.1. TDCIPP Exposure Impairs the Maturation of Mouse Oocytes

To investigate the effect of TDCIPP on the nuclear maturation of mouse oocytes, an IVM model was established. Statistical analysis of the experimental data showed that compared with the control group, the rate of first PBE extrusion was significantly decreased in oocytes treated with 1000 ng/mL and 1500 ng/mL TDCIPP (Table 2). Although 1500 ng/mL TDCIPP also induced a notable reduction in PBE rate, 1000 ng/mL TDCIPP was ultimately selected as the optimal treatment concentration for subsequent experiments. This selection was based on two key considerations: first, this concentration still allowed a considerable proportion of oocytes to complete maturation, ensuring an adequate number of samples for subsequent molecular and cellular level analyses; second, it avoided the potential non-specific cytotoxic effects that might be caused by excessively high concentrations (e.g., 1500 ng/mL), which could complicate the interpretation of results related to oocyte maturation.

Table 2. Impact of TDCIPP on the first polar body extrusion rate of mouse oocytes matured in vitro.

TDCIPP Concentrations (ng/mL)	Oocytes/N	PBE/N	PBE/Oocytes (Mean $\pm$ SEM, %)
0	108	78	72.37 $\pm$ 6.9 ^a
100	107	76	71.16 $\pm$ 5.9 ^{ab}
500	107	75	70.57 $\pm$ 7.8 ^{ab}
1000	107	68	63.21 $\pm$ 3.4 ^{bc}
1500	106	65	61.68 $\pm$ 2.9 ^c

Note: Data are presented as mean $\pm$ SEM. Different superscript letters (a, b, c) within the same column indicate statistically significant differences between groups (*p* < 0.05). The same as below. Assessments were performed after 16 h of culture.

Gap junctions facilitate bidirectional communication between CGCs and oocytes, a process indispensable for the coordinated nuclear and cytoplasmic maturation of oocytes. Given that the functional integrity of CGCs exerts a direct regulatory effect on oocyte developmental potential, the current study aimed to investigate the influence of TDCIPP supplementation in culture medium on CGC expansion during the in vitro culture of COCs. RT-PCR was used to detect the mRNA expression levels of cumulus expansion-related key genes *Has2*, *Ptx3*, *Ptgs2*, and *Tnfaip6*. As illustrated in Figure 1, compared with the control group, the TDCIPP treatment group exhibited a significant downregulation in the mRNA expression of these CGC expansion-related genes (*p* < 0.05).

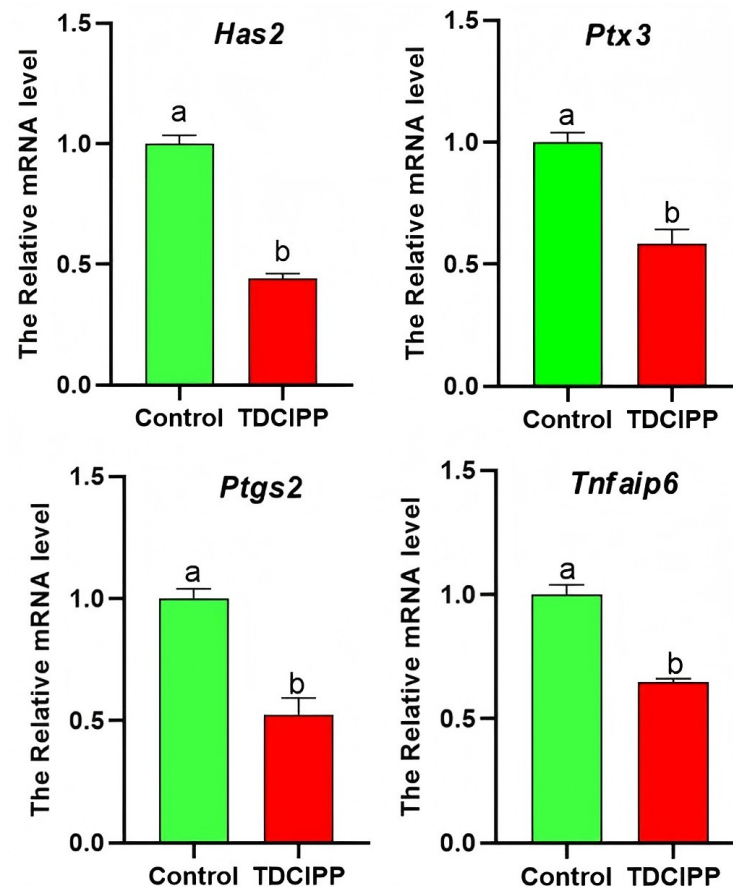


Figure 1. TDCIPP downregulated the mRNA expression levels of genes associated with CGC expansion in mice. Bars marked with different letters (a, b) differ significantly ($p < 0.05$). Data are presented as mean $\pm$ SEM.

3.2. Smart RNA-seq Reveals the Putative Mechanism Underlying Aberrant Meiotic Progression in TDCIPP-Exposed Oocytes

To clarify the potential mechanisms by which TDCIPP-induced oocyte damage, we performed RNA-seq to analyze the transcriptome profiles of oocytes from the control and TDCIPP-exposed groups. The heatmap analysis identified 643 differentially expressed genes (DEGs) in TDCIPP-exposed oocytes compared to the control group (based on $p\text{-adjust} < 0.05$), including 303 upregulated genes and 340 downregulated genes (Figure 2A). Furthermore, using a stricter threshold of $|\log_2FC| \geq 0.5$ and $p\text{-adjust} \leq 0.05$, the volcano plot highlighted 501 significant DEGs, consisting of 285 upregulated and 216 downregulated genes (Figure 2B). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of these DEGs revealed that genes enriched in pathways such as oxidative phosphorylation, chemical carcinogenesis involving reactive oxygen species, progesterone-mediated oocyte maturation, apoptosis, and animal autophagy were abnormally expressed in TDCIPP-exposed oocytes (Figure 2C). Moreover, Gene Ontology (GO) analysis of the DEGs—covering biological processes (Figure 2D), molecular functions (Figure 2E), and cellular components (Figure 2F)—all focused on mitochondrial function, oxidative metabolism, and meiotic development. Specifically, the downregulation of genes enriched in “oxidative phosphorylation” and “mitochondrial respirasome assembly” suggests that TDCIPP exposure compromises mitochondrial energy metabolism, which aligns with the observed ATP deficiency. Furthermore, the significant enrichment of the “apoptosis” and “response to oxidative stress” pathways provides transcriptional evidence that TDCIPP triggers a cell death program likely mediated by oxidative damage. These results indicate that TDCIPP

may induce oxidative stress in oocytes by damaging mitochondrial function, ultimately leading to abnormal meiotic progression of oocytes.

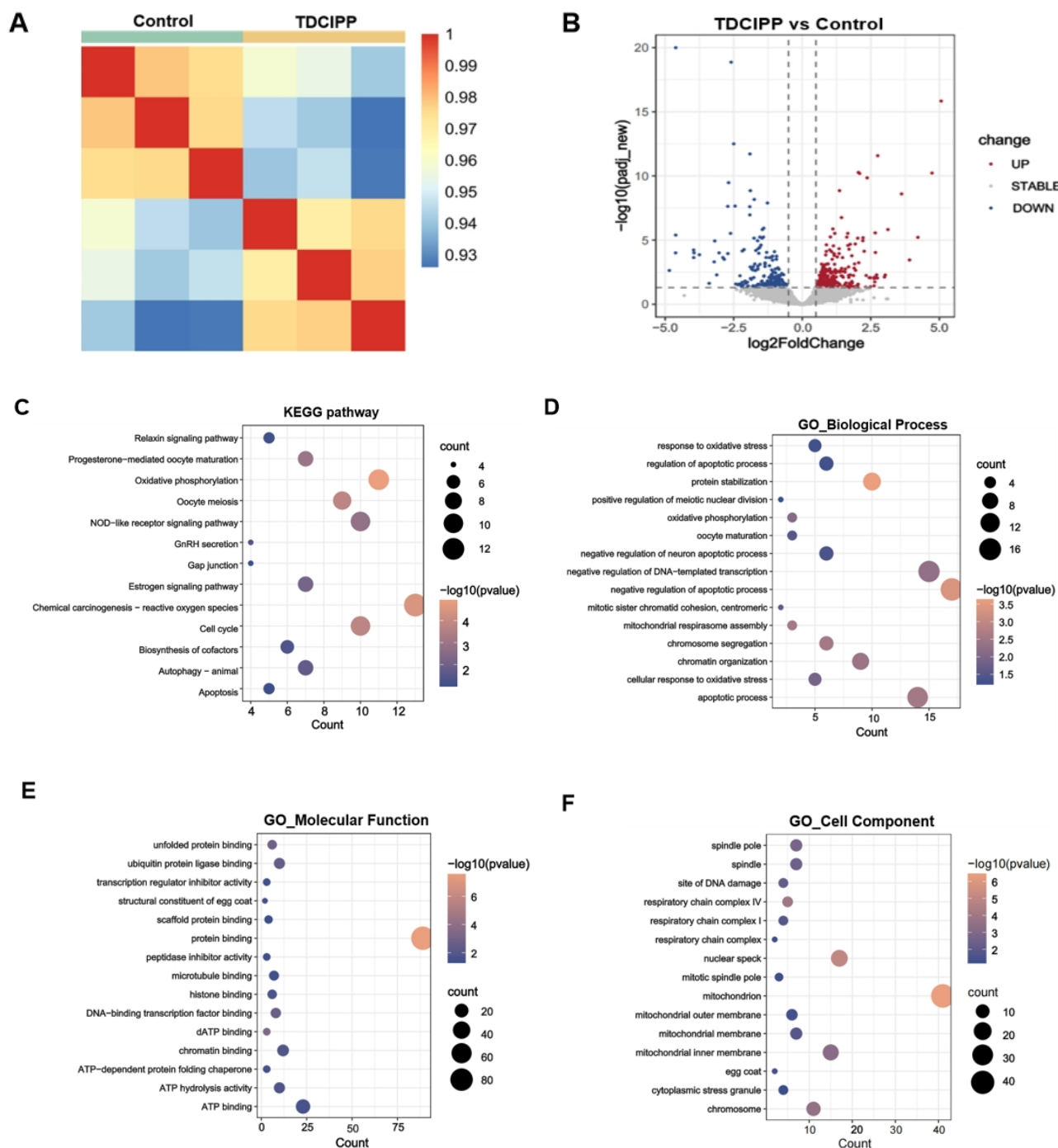


Figure 2. Smart RNA-seq uncovers the potential mechanism underlying abnormal meiotic progression in TDCIPP-exposed oocytes: (A) Heatmap showing transcriptomic differences between oocytes from the control and TDCIPP-exposed groups. (B) Volcano plot illustrating differentially expressed genes (DEGs) in TDCIPP-exposed oocytes compared with control oocytes. (C) KEGG pathway enrichment analysis of DEGs between TDCIPP-exposed oocytes and control oocytes. (D–F) GO enrichment analysis of DEGs between TDCIPP-exposed oocytes and control oocytes, categorized by biological processes (D), molecular functions (E), and cellular components (F).

3.3. PQQ Supplementation Mitigates TDCIPP-Induced Developmental Impairment in Mouse Oocytes

As a highly potent antioxidant, PQQ exerts a prominent role in relieving oxidative stress-triggered damage and regulating other associated physiological processes. To explore whether PQQ could counteract the adverse effects of TDCIPP on oocyte development, we first tested the PBE rates after treatment in the IVM medium with PQQ at different concentrations (0, 50, 100, and 150 μM). The results showed that treatment with 100 μM PQQ resulted in a significant increase in this rate ($p < 0.05$) (Table 3), as detailed in Table 3.

Table 3. Effect of PQQ on the first polar body extrusion rate of mouse oocytes during in vitro maturation.

PQQ Concentrations (μM)	Oocytes/N	PBE/N	PBE/Oocytes (Mean $\pm$ SEM, %)
0	123	91	73.85 $\pm$ 5.8 ^b
50	124	92	74.39 $\pm$ 6.8 ^b
100	125	103	82.42 $\pm$ 3.4 ^a
150	123	93	75.49 $\pm$ 3.9 ^{ab}

Note: Data are presented as mean $\pm$ SEM. Different superscript letters (a, b) within the same column indicate statistically significant differences between groups ($p < 0.05$). Assessments were performed after 16 h of culture.

To evaluate whether PQQ could alleviate the adverse effects of TDCIPP on oocyte development, three experimental groups were established: control, TDCIPP (1000 ng/mL), and TDCIPP + PQQ (1000 ng/mL TDCIPP + 100 μM PQQ) co-treatment groups. The PBE rates of each group were then determined. Representative brightfield images of oocytes with the first PBE are presented in Figure 3, and the quantitative data for PBE rates across groups are summarized in Table 4. Compared with the control group, exposure to TDCIPP alone resulted in a significant reduction in the oocyte PBE rate ($p < 0.05$). Notably, co-treatment with PQQ effectively mitigated the inhibitory effect of TDCIPP: the PBE rate in the TDCIPP + PQQ group was restored to a level that was not statistically different from that of the control group. These results demonstrate that TDCIPP exposure impairs the normal process of first PBE in mouse oocytes, while supplementation with PQQ exerts a protective effect against TDCIPP-induced suppression of PBE. To further characterize the quality of oocytes following treatment, we examined oocyte morphology during data collection. We did not observe overt signs of oocyte degeneration or fragmentation in the TDCIPP-treated group compared to the control, suggesting that the tested concentrations of TDCIPP did not induce immediate cell death. Consequently, the significant reduction in the first PBE rate at 16 h in the TDCIPP group primarily indicates a delay or arrest in meiotic progression from the GV to MII stage. Supplementation with PQQ effectively rescued this meiotic defect, restoring the PBE rate to levels comparable to the control.

Table 4. The first polar body extrusion rate of mouse oocytes treated with TDCIPP + PQQ.

Groups	Oocytes/N	PBE/N	PBE/Oocytes (Mean $\pm$ SEM, %)
Control	305	236	75.84 $\pm$ 6.5 ^{ab}
TDCIPP	308	198	63.47 $\pm$ 9.3 ^c
TDCIPP + PQQ	310	228	71.90 $\pm$ 9.5 ^{bc}

Note: Data are presented as mean $\pm$ SEM. Different superscript letters (a, b, c) within the same column indicate statistically significant differences between groups ($p < 0.05$). Assessments were performed after 16 h of culture.

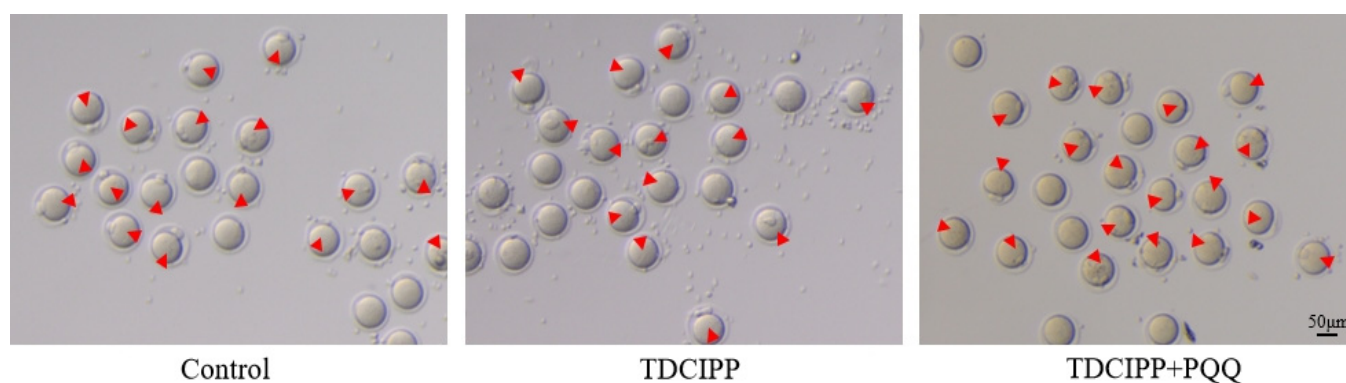


Figure 3. PBE rate of mouse oocytes following treatment with TDCIPP and PQQ. The micrographs display MII-stage oocytes derived from three experimental groups: the control group, the TDCIPP-treated group, and the TDCIPP + PQQ co-treated group. The tip of each red triangle indicates the position of the first polar body extruded by the oocyte. The scale bar in the images corresponds to a length of 50 μm .

3.4. PQQ Supplementation Reduces ROS Levels and Maintains GSH Content in TDCIPP-Exposed Mouse Oocytes

The redox balance between ROS and GSH plays a pivotal role in modulating the cytoplasmic maturation of mammalian oocytes. Specifically, excessive ROS accumulation can elicit oxidative stress responses that impair oocyte integrity, whereas GSH functions as a primary intracellular antioxidant to mitigate oxidative damage and support the acquisition of oocyte developmental competence. To investigate whether PQQ influences TDCIPP-induced redox perturbations in oocytes, we quantified ROS production and GSH content in in vitro-matured mouse oocytes; representative results are illustrated in Figure 4. Relative to the control group, oocytes exposed to TDCIPP alone exhibited a significant elevation in ROS levels, accompanied by a marked reduction in GSH content ($p < 0.05$). In contrast, co-treatment with PQQ in the TDCIPP + PQQ group reversed these TDCIPP-induced changes: ROS levels were significantly lower, and GSH content was significantly higher compared to the TDCIPP-only group ($p < 0.05$). Collectively, these data demonstrate that PQQ effectively mitigates TDCIPP-triggered oxidative stress by restoring the redox equilibrium of oocytes.

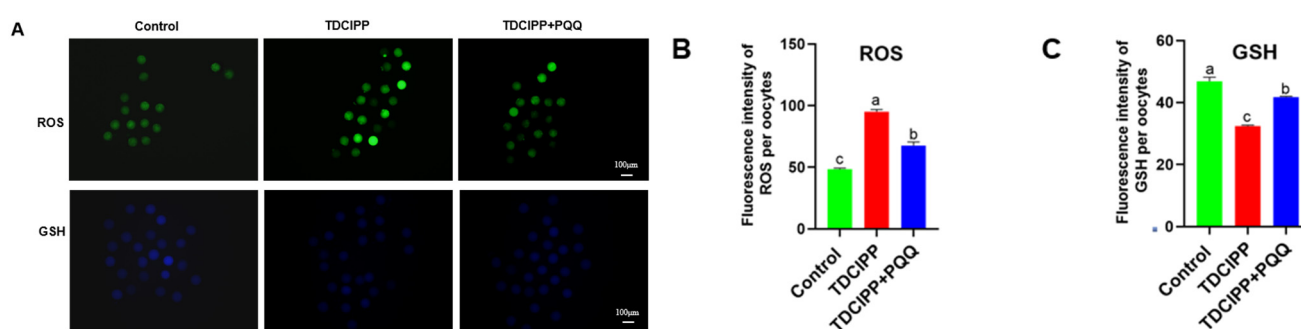


Figure 4. PQQ supplementation reduces the ROS levels and sustains the GSH content in TDCIPP-exposed mouse oocytes: (A) Fluorescence micrographs showing ROS and GSH signals in mouse oocytes from three experimental groups: control, TDCIPP-exposed, and TDCIPP + PQQ-supplemented oocytes. Scale bar = 100 μm . (B) Quantitative analysis of ROS fluorescence intensity in oocytes from the control, TDCIPP-exposed, and TDCIPP + PQQ co-supplemented groups. (C) Quantitative determination of GSH content in oocytes from the control, TDCIPP-exposed, and TDCIPP + PQQ co-supplemented groups. Bars marked with different letters (a, b, c) differ significantly ($p < 0.05$). Data are presented as mean $\pm$ SEM.

3.5. PQQ Supplementation Preserves the Mitochondrial Membrane Potential and ATP Content in TDCIPP-Exposed Mouse Oocytes

Mitochondrial membrane potential, an indicator closely associated with oxidative stress status, plays a critical role in ATP biosynthesis, a process essential for supporting oocyte metabolic function and maturation. To assess whether PQQ modulates TDCIPP-induced mitochondrial dysfunction in oocytes, we quantified mitochondrial intensity and intracellular ATP levels in vitro-matured mouse oocytes, and representative results are presented in Figure 5. Relative to the control group, oocytes treated with TDCIPP alone exhibited a significant reduction in both mitochondrial intensity and ATP content ($p < 0.05$). In contrast, co-supplementation with PQQ in the TDCIPP + PQQ group restored these parameters: no statistically significant differences in mitochondrial intensity or ATP levels were observed between the TDCIPP + PQQ group and the control group ($p > 0.05$). These data demonstrate that PQQ effectively mitigates TDCIPP-induced impairment of mitochondrial function and ATP production in mouse oocytes.

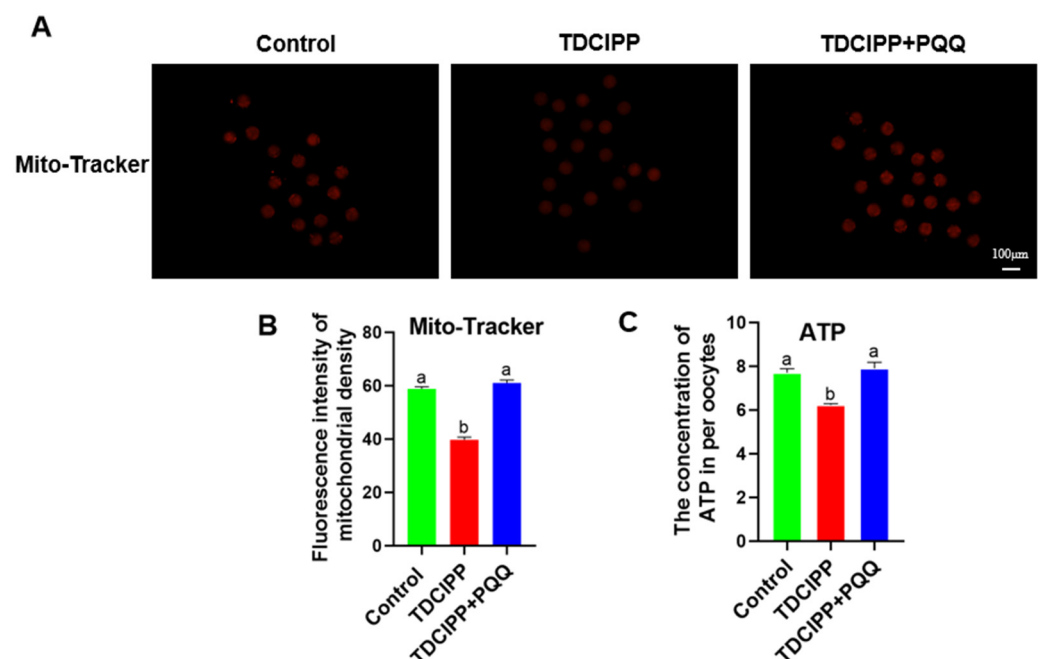


Figure 5. PQQ supplementation preserves mitochondrial intensity and ATP content in TDCIPP-exposed mouse oocytes: (A) Fluorescence micrographs of mouse oocytes stained with the Mito-Tracker Red CMXRos probe, representing three experimental groups: the control group, the TDCIPP-exposed group, and the TDCIPP + PQQ co-supplemented group. Scale bar = 100 μ m. (B) Quantitative analysis of mitochondrial fluorescence intensity in oocytes from the Control, TDCIPP-exposed, and TDCIPP + PQQ co-supplemented groups. (C) Quantitative determination of intracellular ATP content in oocytes from the control, TDCIPP-exposed, and TDCIPP + PQQ co-supplemented groups. Bars marked with different letters (a, b) differ significantly ($p < 0.05$). Data are presented as mean $\pm$ SEM.

3.6. PQQ Supplementation Alleviates Apoptosis in TDCIPP-Exposed Mouse Oocytes

Our RNA-seq analysis identified the “apoptosis” pathway as significantly enriched, with specific alterations observed in Bcl-2 family member transcripts. To validate these transcriptomic findings and confirm the execution of the apoptotic program at the protein level, we quantified the expression levels of three critical apoptotic regulators—*Bcl-2*, *Bax*, and *Caspase3*—using RT-PCR and immunofluorescence staining. As shown in Figure 6A, TDCIPP exposure significantly downregulated *Bcl-2* mRNA expression while upregulating *Bax* mRNA levels compared to the control group ($p < 0.05$). Co-treatment with PQQ reversed these trends: *Bcl-2* mRNA expression was restored to levels comparable to the control group, and accompanied by marked reductions in pro-apoptotic *Caspase3* mRNA

expression ($p < 0.05$). The intracellular distribution patterns of BCL-2, BAX, and CASPASE3 proteins were further visualized via immunofluorescence (Figure 6B,C), with quantitative analyses presented in Figure 6D. TDCIPP exposure significantly decreased the protein expression of the anti-apoptotic protein BCL-2 and increased pro-apoptotic BAX expression relative to the control group ($p < 0.05$). In contrast, TDCIPP + PQQ co-treatment significantly upregulated BCL-2 expression and downregulated BAX expression ($p < 0.05$). Notably, Caspase3 protein expression in the TDCIPP group did not differ significantly from the control group ($p > 0.05$), suggesting that TDCIPP exposure primarily initiated mitochondrial-mediated early apoptotic signaling without leading to the full execution of apoptosis at this stage; meanwhile, the TDCIPP + PQQ co-treatment resulted in a significant reduction in CASPASE3 protein levels ($p < 0.05$).

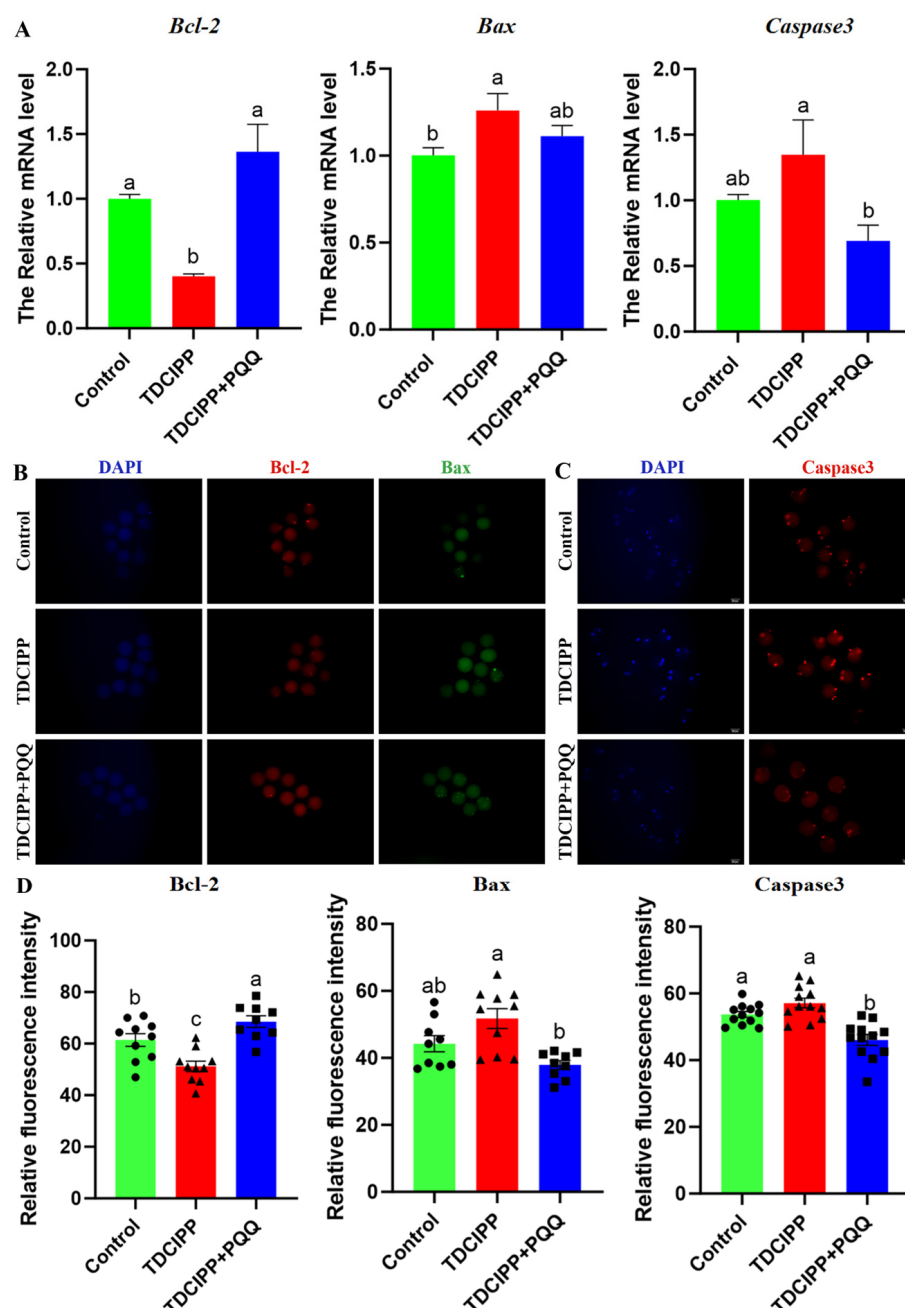


Figure 6. PQQ supplementation alleviates apoptosis in TDCIPP-exposed mouse oocytes: (A) Relative mRNA expression levels of apoptosis-related genes (*Caspase3*, *Bcl-2*, *Bax*) in oocytes from the control group, TDCIPP-exposed group, and TDCIPP + PQQ co-supplemented group. Data are presented as

mean $\pm$ standard error of the mean (SEM). (B) Immunofluorescence micrographs showing the protein distribution of apoptosis-related regulators (CASPASE3, BCL-2, and BAX) in oocytes from the control group, TDCIPP-exposed group, and TDCIPP + PQQ co-supplemented group. Scale bar = 50 μ m. (C) Relative protein expression levels of apoptosis-related regulators (CASPASE3, BCL-2, and BAX) in oocytes from the control group, TDCIPP-exposed group, and TDCIPP + PQQ co-supplemented group. Bars marked with different letters (a, b, c) differ significantly ($p < 0.05$). Data are presented as mean $\pm$ SEM. (D) Relative fluorescence intensity of BCL-2, BAX, and CASPASE3 in oocytes from the control group, TDCIPP-exposed group, and TDCIPP + PQQ co-supplemented group. Bars marked with different letters (a, b, c) differ significantly ($p < 0.05$). Data are presented as mean $\pm$ SEM.

4. Discussion

Numerous investigations have documented that endocrine-disrupting chemicals (EDCs) possess the capacity to compromise female reproductive function [37]. TDCIPP, a widely utilized OHFR, has increasingly been recognized as a prominent environmental pollutant due to its bioaccumulation and potential to induce neurotoxicity, gamete toxicity, and cytotoxicity [38]. TDCIPP is extensively incorporated into consumer goods such as upholstered furniture, textile products, and automotive interior materials [39]. Despite its widespread detection in human biological specimens [21] and the correlation between its metabolites and decreased fertilization rates in IVF clinics [9], the specific effects of TDCIPP on mammalian oocyte quality remain inadequately explored. To address this gap, the present study employed an IVM model to systematically evaluate the risk of TDCIPP to female reproductive health. Our study establishes that TDCIPP exposure exerts multifaceted detrimental effects on mouse oocytes, most notably compromising developmental competence, as evidenced by the marked reduction in the first PBE rate. Beyond oocyte-intrinsic factors, bidirectional communication between GCs and oocytes, facilitated by gap junctions, is indispensable for coordinating both nuclear and cytoplasmic maturation of oocytes [40]. Our observation that TDCIPP significantly downregulated genes essential for cumulus expansion (*Has2*, *Ptx3*, *Ptgs2*, and *Tnfrsf6*) suggests that TDCIPP-induced maturation failure may stem partially from a disrupted follicular microenvironment. This impairment of somatic cell support likely exacerbates the direct toxicity to the oocyte, a mechanism similar to that observed with other EDCs, which disrupts the metabolic coupling within the cumulus–oocyte complex.

To elucidate the molecular mechanisms underlying this meiotic arrest, we performed transcriptomic profiling. The RNA-seq analysis revealed that TDCIPP-induced damage is closely linked to mitochondrial dysfunction and the activation of apoptotic signaling pathways. Unlike bisphenol A (BPA), which primarily disrupts estrogen signaling [41], our transcriptomic data indicate that TDCIPP exerts its toxicity mainly through oxidative stress and metabolic disruption. Mitochondria, commonly denoted as the “powerhouses” of eukaryotic cells, not only play a pivotal role in cellular energy metabolism but also serve as key regulators of intracellular homeostasis [42]. Within the oocyte cytoplasm, mitochondria represent the most functionally crucial organelles, as their integrity and activity directly modulate oocyte maturation, fertilization success, and early embryonic development [43]. The downregulation of genes related to oxidative phosphorylation in our dataset implies that TDCIPP directly targets the oocyte’s energy production machinery, creating a metabolic bottleneck that prevents successful maturation [44].

Validating our transcriptional insights, functional analyses confirmed that TDCIPP disrupts the core biological processes underpinning cytoplasmic maturation. Mechanistically, this disruptive effect appears to be driven by a collapse in redox homeostasis. We observed that TDCIPP exposure triggered excessive intracellular ROS production accompanied by a significant depletion of GSH. This redox imbalance likely serves as the upstream trigger for the observed mitochondrial fragmentation and ATP depletion. Excessive generation

of ROS induces oxidative stress, which has been shown to impair mitochondrial function in bovine oocytes [45,46]. For TDCIPP, previous studies have established that cytotoxic concentrations of this compound trigger the upregulation of heme oxygenase-1 (HO-1), a widely recognized molecular marker of oxidative stress [47]. Previous studies in *C. elegans* [29,48] and zebrafish [19] have also identified oxidative stress as a primary toxicity pathway for TDCIPP. Furthermore, consistent with findings in mouse spermatocytes where TDCIPP induced endoplasmic reticulum stress and mitochondrial apoptosis [49], our data in oocytes revealed an upregulation of *Bax* and downregulation of *Bcl-2*. Collectively, these changes suggest that TDCIPP initiates a mitochondrial-dependent apoptotic cascade in oocytes, driven by unmitigated oxidative stress and energy failure.

Meiotic maturation, particularly the assembly of the meiotic spindle and chromosomal segregation, is an energy-demanding process that relies heavily on ATP derived from mitochondria [35,50]. In the present study, although we did not directly visualize spindle morphology, the significant decline in ATP levels and mitochondrial membrane potential observed in the TDCIPP-treated group provides a plausible mechanistic explanation for the reduced polar body extrusion rate. The energy deficit resulting from TDCIPP-induced mitochondrial dysfunction likely compromises the stability of the cytoskeletal machinery required for cytokinesis [51]. Importantly, PQQ supplementation restored ATP production, which aligns with the observed recovery in oocyte maturation rates, further supporting the hypothesis that PQQ rescues oocyte quality primarily by preserving mitochondrial bioenergetics.

To protect oocytes from TDCIPP-induced mitochondrial damage, we conducted a screening of antioxidants to identify potential intervention agents. PQQ emerged as a promising candidate, as it acts as a cofactor for lactate dehydrogenase and other dehydrogenase enzymes involved in the oxidation of nicotinamide adenine dinucleotide (NADH) to its oxidized form (NAD⁺) [23]. Notably, PQQ has garnered substantial research attention due to its diverse biological activities, which include robust antioxidant capacity, mitochondrial protective effects, anti-apoptotic properties, neuroprotective functions, and anti-inflammatory actions [52]. Prior studies have validated that PQQ's protective efficacy in cellular models: pretreatment with PQQ can significantly attenuate cytotoxicity in cultured cells, preserve DNA integrity in PC12 cells, maintain mitochondrial membrane potential, upregulate *Bcl-2* expression, inhibit Caspase-3 activation, and reverse mitochondrial dysfunction [53]. Furthermore, PQQ has been shown to promote the expression of mitochondrial Na⁺/Ca²⁺ exchanger proteins, thereby reducing ROS production, protecting mitochondrial membrane potential, preventing Ca²⁺ overload, and enhancing mitochondrial biogenesis via upregulation of PGC-1 α and TFAM [54]. Our results showed that PQQ supplementation significantly reduced ROS levels and restored mitochondrial membrane potential and ATP content. This suggests that PQQ protects oocytes not merely by scavenging free radicals, but by preserving mitochondrial bioenergetics and preventing the initiation of the apoptotic cascade. These findings align with previous reports on PQQ's ability to alleviate defects caused by other toxicants like diisobutyl phthalate in porcine oocytes [27], supporting its potential as a broad-spectrum protective agent against environmental reproductive toxins.

Despite these promising findings, our study has certain limitations. First, as an *in vitro* study, the results obtained from isolated oocytes may not fully replicate the complex physiological environment *in vivo*, where metabolism and systemic interactions play critical roles. Therefore, further animal studies are warranted to validate the protective efficacy of PQQ in living organisms. Second, the TDCIPP concentrations used in this study were selected to elucidate acute toxicological mechanisms and may exceed typical environmental exposure levels found in human biofluids. Future investigations should focus on the

long-term effects of chronic, low-dose TDCIPP exposure on reproductive health to better assess real-world risks.

5. Conclusions

TDCIPP exposure elicits gamete toxicity in mouse oocytes during their in vitro maturation process. PQQ effectively alleviates the detrimental impacts of TDCIPP, and this protective action is mediated through two core mechanisms: the maintenance of intracellular redox homeostasis and the regulation of mitochondrial functional integrity. Collectively, these findings demonstrate that PQQ exerts a protective role in mitigating TDCIPP-induced damage to mouse oocytes. From a translational perspective, our study highlights the potential of PQQ as a nutritional supplement to safeguard female fertility against environmental pollutants. Future investigations should focus on validating these protective effects in in vivo models and elucidating the long-term impacts of chronic, low-dose TDCIPP exposure on offspring development.

Author Contributions: L.S. and Z.C.: conceptualization, methodology, formal analysis, writing—original draft, writing—review and editing. L.X. and J.B.: investigation, data curation, and editing. K.L. and Y.Q.: investigation, supervision, and validation. Y.L. and X.X.: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by grants from the National Key Research and Development Program, China (grant number 2021YFD1200402) and the Scientific & Technological Innovation Ability Construction Project of the Beijing Academy of Agriculture and Forestry Sciences (grant numbers KJCX20230218 & KJCX20230204).

Institutional Review Board Statement: This study was reviewed and approved by the Beijing Academy of Agriculture and Forestry Sciences. All animal experiments were carried out according to the International Guiding Principles for Biomedical Research Involving Animals, and the respective permit was issued by the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences (IHVM11-2301-1, approved on 1 January 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: None of the data were deposited in an official repository. All data generated during the study are available from the corresponding authors upon request.

Conflicts of Interest: The authors have no conflicts of interest to declare.

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