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Phosphatidylinositol restores fertilization and enhances embryonic development in post-ovulatory aging porcine oocytes

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ABSTRACT

Background: Post-ovulatory aging (POA) of oocytes impairs oocyte quality, fertilization potential, and early embryonic development, with lipid metabolism playing a central role. This study explores whether targeting lipid metabolic reprogramming can mitigate POA in porcine oocytes.

Methods: An in vitro POA of porcine oocytes model was established. Targeted lipidomics, organelle function assays, and molecular analyses were performed to assess lipid metabolism and oocyte quality. Data were analyzed using standard statistical methods.

Results: POA oocytes exhibited significant reductions in phosphatidylinositol (PI) and its phosphorylated derivatives (PIP2/PIP3), leading to lipid droplet accumulation, disrupted mitochondria-lipid droplet interactions, impaired fatty acid oxidation, and cellular dysfunction. Supplementation with 30 mM PI partially restored lipid metabolism, improved organelle function, enhanced antioxidant defenses, and enhanced mitochondrial-lipid droplet interactions. Consequently, sperm binding, fertilization rates, and blastocyst formation were significantly increased, while DNA damage and apoptosis were reduced.

Conclusions: PI supplementation effectively restores oocyte quality, fertilization potential, and embryonic developmental competence in POA porcine oocytes. These findings suggest that targeting PI-mediated may represent a promising strategy to improve reproductive outcomes in livestock and assisted reproduction programs.

1. Introduction

POA of oocytes is a major factor affecting oocyte quality, fertilization potential, and embryonic development, with lipid metabolism playing a central role [1]. The POA oocytes is influenced not only by intrinsic biological mechanisms but also by environmental factors and metabolic status. Lipid metabolism is particularly important during oocyte meiotic division and maturation, serving as the primary energy source. Fatty acid oxidation contributes critically to ATP production during oocyte maturation, and inhibiting of this pathway significantly impairs oocyte and early embryo developmental competence. Conversely, enhancing fatty acid oxidation improves oocyte maturation and embryo development [2–4]. In addition to energy provision, dysregulation of lipid

metabolism can accelerate oocyte aging via oxidative stress and inflammatory responses. For example, lipid metabolism abnormalities in oocytes from obese women lead to lipid droplet accumulation and mitochondrial dysfunction. Elevated free fatty acids (FFA) disrupt energy metabolism and impair oocyte signal and material exchange by altering plasma membrane fluidity, thus reducing oocyte maturation and fertilization ability [5,6]. Collectively, these observations underscore the critical role of lipid metabolism in POA and provide a theoretical foundation for targeted interventions.

PI, a major phospholipid component of the cell membrane, and its phosphorylated derivatives (such as PI (4,5) P₂ and PI (3,4,5) P₃) act as second messengers, modulating signaling pathways such as PI3K-AKT and mTOR to regulate cell survival, autophagy, and metabolic

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reprogramming [7–9]. In oocytes, PI metabolism is critical for meiotic resumption and fertilization activation. PI (4,5) P_2 induces calcium oscillations by activating $PLC\zeta$, and inhibition of $PI3K$ activity leads to oocyte arrest at the germinal vesicle stage [7] [10]. Although single-cell transcriptome analyses in mouse oocytes indicate that PI synthase (PIS) and the $PI3K$ regulatory subunit ($PI3KR4$) are downregulated during aging [11], suggesting that dysregulation of PI metabolism may be associated with oocyte aging, direct evidence in large mammalian models such as porcine oocytes is lacking. Consequently, it remains unclear whether exogenous PI supplementation can effectively mitigate aging-related defects in these models.

To address these questions, we established an in vitro POA porcine oocyte model. Exogenous PI supplementation during IVM partially restored cytoplasmic integrity, spindle formation, chromosome alignment, actin cytoskeleton organization, cortical granule distribution, and mitochondrial function in POA oocytes. In addition, PI supplementation enhanced sperm binding, fertilization rates, and early embryo development potential. Mechanistically, PI alleviated POA-induced lipid metabolism disturbances, contributing to improved oocyte quality. Collectively, these findings provide novel insights into the protective role of PI in POA and establish a theoretical basis for optimizing interventions in assisted reproductive technologies (ART).

2. Experimental procedures

2.1. Animals and Ethics

All experimental protocols involving swine oocytes and embryos were approved by the Animal Care and Use Committee of Northwest A&F University.

2.2. PI treatment

Phosphatidylinositol (sigma, 79403) was dissolved in cell culture-grade water to prepare a 30 M stock solution. This stock solution was then diluted with oocyte maturation medium to working concentrations of 10, 20, 30, 40, and 50 mM.

2.3. Collection and In Vitro Maturation (IVM) of porcine oocytes

Porcine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h using phosphate-buffered saline containing antibiotics (100 IU/mL penicillin G and 100 mg/mL streptomycin) at 37°C. Follicular fluid was aspirated using a syringe from follicles with diameters ranging from 3 to 8 mm, and cumulus-oocyte complexes (COCs) were selected under a stereomicroscope. Selected COCs contained at least three layers of compact granulosa cells with uniform cytoplasm. The maturation medium consisted of bicarbonate-buffered TCM-199 (Gibco, BRL, Grand Island, NY, USA) supplemented with 1 mg/mL polyvinyl alcohol (PVA), 1% (v/v) (100×) insulin-transferrin-selenium (ITS), 0.91 mM sodium pyruvate, 3.05 mM glucose, 0.57 mM L-cysteine, 10 IU/mL equine chorionic gonadotropin, 10 IU/mL human chorionic gonadotropin, 2.5 IU/mL follicle-stimulating hormone, 40 ng/mL basic fibroblast growth factor, 20 ng/mL insulin-like growth factor 1, 20 ng/mL leukemia inhibitory factor, and 10 ng/mL epidermal growth factor. The COCs were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 for 44–46 h. After IVM, COCs were treated with 0.1% hyaluronidase and gently pipetted to remove the cumulus cells, and the extrusion of the first polar body was used as a marker of maturation (MII stage). To induce POA, the in vitro matured COCs were maintained intact with their cumulus cells and further cultured in IVM medium for an additional 24 and 48 h. Cumulus expansion was evaluated at each time point using a standardized 0–4 scoring system adapted from recent porcine studies [12]. Scores were based on cumulus layer dispersion and size, with 0 indicating no expansion and 4 indicating fully expanded cumulus cells. Each oocyte

was independently scored by two observers blinded to treatment conditions, and the average score was used for statistical analysis. Samples were collected at the indicated time points for downstream analyses.

2.4. Transmission Electron Microscope (TEM)

Samples were pre-fixed with 2.5% glutaraldehyde, followed by re-fixation with 1% osmium tetroxide. They were then dehydrated through a graded series of acetone concentrations, ranging from 30%, 50%, 70%, 80%, 90%, 95% to 100% (with 100% concentration being changed three times). The samples were infiltrated with a mixture of dehydration agent and Epon-812 embedding medium in 3:1, 1:1, and 1:3 ratios, respectively. Afterward, the samples were embedded in pure Epon-812 embedding medium. Ultrathin sections of 60–90 nm were cut using an ultramicrotome and placed on copper grids. Sections were stained with uranyl acetate for 10–15 min, followed by lead citrate staining for 1–2 min at room temperature. Finally, images were captured using a JEM-1400FLASH transmission electron microscope produced by JEOL (Japan Electron Optics Laboratory Co., Ltd.), with each copper grid first observed under low magnification to select the imaging areas.

2.5. In Vitro Fertilization (IVF) and embryo development

IVF was performed as previously described with some modifications [13,14]. Groups of 30 COCs were transferred to 90 μ L droplets of modified fertilization Tris-buffered medium (mTBM) containing 10 mM Tris, 2 mg/mL bovine serum albumin (BSA, Sigma-Aldrich, USA, A7030) and 2 mM caffeine. Fresh semen from Large White boars (2-to-3-year-old) was washed twice with TL-HEPES (sperm washing solution) containing 2 mg/mL BSA. The sperm suspension was then added to PureSperm 40/80 (Nidacon, Sweden, Ps40-100, Ps80-100), balanced 3 h in advance for centrifugation, and the bottom sperm was washed with the sperm washing solution once. The supernatant was discarded, and the sperm was diluted with mTBM. Sperm activity was assessed on a cell counting plate before use, and 10 μ L of sperm mTBM solution was added to the fertilization droplet. The final sperm concentration was 0.25×10^6 sperm/mL. To quantify sperm binding, oocytes were washed three times with fertilization medium to remove loosely attached sperm, fixed in 4% paraformaldehyde, and stained with Hoechst 33342 (10 μ g/mL) for 10 min at room temperature. The number of sperm attached to the zona pellucida (ZP) was counted under a fluorescence microscope. After IVF, oocytes were denuded using 0.1% hyaluronidase and gently pipetted to remove cumulus cells and loosely attached sperm. Fertilization rate was determined by the presence of male and female pronuclei. Denuded oocytes were fixed and stained with Hoechst 33342, and fertilized egg were identified by the presence of two pronuclei. Fertilization rate (%) was calculated as: Fertilization rate (%) = (Number of oocytes with two pronuclei / Total number of inseminated oocytes) $\times$ 100. The fertilized egg were then cultured in PZM3 medium (6.312 mg/mL NaCl, 0.746 mg/mL KCl, 0.048 mg/mL KH_2PO_4 , 0.048 mg/mL $MgSO_4 \cdot 7H_2O$, 2.106 mg/mL $NaHCO_3$, 0.022 mg/mL sodium pyruvate, 0.617 mg/mL calcium lactate pentahydrate, 0.01 mL/mL (100×) glutamine, 0.546 mg/mL taurine, 0.02 mL/mL β -mercaptoethanol (BME), 0.01 mL/mL minimum essential medium (MEM), and 3 mg/mL BSA), with 5% CO_2 , 38.5°C, and in a humidified incubator. Cleavage rate, cleavage fragmentation rate, and blastocyst formation rate were assessed at defined time points post-fertilization. Cleavage rate (%) was calculated as the number of embryos that reached the 2-cell or higher stage divided by the total number of inseminated oocytes $\times$ 100. Cleavage fragmentation rate (%) was determined as the number of embryos showing cytoplasmic fragmentation at the 2–8 cell stage divided by the total number of cleaved embryos $\times$ 100. Blastocyst formation rate (%) was calculated as the number of embryos that developed to the blastocyst stage by day 7 post-fertilization divided by the total number of inseminated oocytes $\times$ 100. To assess blastocyst quality, total cell number and inner cell mass (ICM) proportion were determined

using immunofluorescence staining: SOX2 for ICM and DAPI for total nuclei. Each blastocyst was manually counted under a fluorescence microscope, and the ICM ratio was calculated as the number of SOX2-positive nuclei divided by the total number of nuclei. Representative images of whole blastocysts and ICM were acquired to ensure consistency between quantified data and visual observation.

2.6. Lipid metabolomics

Biological samples consisted of pooled porcine oocytes (approximately 350–500 MII-stage oocytes per sample) from multiple replicates to obtain sufficient material for metabolite extraction. The pooled samples were vacuum freeze-dried and gently ground into a powder using a ball mill (30 Hz, 1.5 min), and placed into centrifuge tubes with corresponding serial numbers. An equivalent amount of the pooled sample sufficient for extraction was dissolved in 1.2 mL of 70% methanol extraction solution, vortexed every 30 min for 30 s each time for a total of six cycles, and stored overnight at 4°C. After centrifugation (12,000 rpm, 10 min), the supernatant was collected, filtered through a 0.22 µm microporous membrane, and stored in sample vials for analysis by Ultra Performance Liquid Chromatography (UPLC, SHIMADZU Nexera X2, <https://www.shimadzu.com.cn/>) and tandem mass spectrometry (MS/MS, Applied Biosystems 4500 QTRAP, <http://www.appliedbiosystems.com.cn/>). Chromatographic peaks were processed using Analyst 1.6.3 and MultiQuant 3.0.3 software, referencing retention times and peak shapes of standards. Substance identification and qualitative analysis were obtained based on the self-built targeted standard database MWDB (Metware Database). Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were used for differential metabolite analysis. Metabolites with $|\log_2\text{FC}| \geq 1$ and VIP >1 were considered significantly changed metabolites (DEMs). Heatmaps were generated using the Complex Heatmap package in R (v4.2.0). This workflow was specifically adapted to work with small-scale pooled oocyte samples, ensuring sufficient material for accurate and reproducible lipid quantification.

2.7. Microinjection

Germinal vesicle (GV) stage oocytes were used for all microinjections. Prepare injection and holding pipettes using a glass pipette puller and a microforge. Install the holding pipette on the left arm of the micromanipulator and the injection pipette containing 2 µL siRNA (GenePharma) on the right arm (Table S1). The pipettes were aligned horizontally, and the injection tip was gently broken against the holding pipette to form an appropriate opening. Move the injection pipette into paraffin oil and click the "clean" button on the injector to adjust the appropriate pulse pressure. Then, move the injection pipette into the droplet containing the oocytes. The oocyte was secured with the holding pipette, and the zona pellucida and oolemma were pierced while avoiding the polar body. After microinjection, viable oocytes were transferred to maturation medium for further culture, and samples were collected based on experimental requirements.

2.8. Real-time quantitative PCR (RT-qPCR)

The collected oocytes were washed with PBS-PVA-DEPC H₂O (Sigma, USA) and then added to 7 µL of Cells to Signal™ Lysis Buffer (Thermo Fisher, USA) for lysis at 4°C. After lysis, the samples were immediately subjected to reverse transcription. Genomic DNA was digested with 2 µL of 5x gDNA Eraser (1 µL gDNA Eraser to 7 µL total RNA) (Takara, Japan), and the total RNA was directly reverse-transcribed by incubation at 37°C for 15 min. After reverse transcription, the reaction mixes were heated at 85°C for 5 s to inactivate the enzymes and stored at 4°C for subsequent analysis. The RNA was reverse-transcribed into cDNA, and relative gene expression was determined by RT-qPCR using the following reaction system: 5 µL 2x ChamQ

SYBR-Green qPCR Master Mix, 0.2 µL forward primer (F), 0.2 µL reverse primer (R), 1 µL cDNA, 3.6 µL ddH₂O (Vazyme, China). Each RT-qPCR reaction was performed in triplicate. The specific quantitative primers were synthesized by Qingke Co. (Xi'an, China) and are listed in Table S2. The thermocycling protocol was: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s.

2.9. Fluorescence staining and measurement

Collect 500 oocytes for CO-IP. Remove the zona pellucida of the oocytes using Ca²⁺-free DPBS (Sigma-Aldrich, T1788) and lyse the oocytes in Western and IP Lysis Buffer (Beyotime, P0013). Transfer the collected protein samples to a new 1.5 mL centrifuge tube, add the appropriate primary antibodies: VPS15 (17894-1-AP, Proteintech) and YAP1 (Proteintech, 13584-1-AP), and incubate overnight on a rotator at 4°C. After incubation, add 10 µL of Protein A + G Agarose (Beyotime, P2055) to the samples and incubate for 2 h on a rotator at 4°C. After incubation, centrifuge the samples at 1,000xg for 5 min. Carefully pipette off the supernatant, retaining as much Protein A + G Agarose as possible. Resuspend the pellet in 200 µL of Western and IP Lysis Buffer, gently mix by pipetting, and centrifuge again at 1000 xg for 5 min. Repeat the washing of Protein A + G Agarose three times, and after the final centrifugation, resuspend the pellet in 20 µL of RIPA lysis buffer (Beyotime, P0013B). Add an appropriate amount of SDS-PAGE sample loading buffer, mix, and incubate the sample in a 100°C water bath for 10 min. The samples can be used directly for SDS-PAGE protein gel electrophoresis or stored temporarily at –20°C. Prepare the SDS-PAGE gel according to the instructions of the SDS-PAGE Preparation kit (Sagon, C631100-0200). Load the samples into the gel wells slowly using a loading needle and run the gel at a constant voltage of 80 V until the samples reach the separation gel, then adjust the voltage to 120 V for electrophoresis until the bromophenol blue reaches the gel bottom.

2.10. Fluorescence staining and measurement

For immunofluorescent protein detection, samples were fixed in 4% paraformaldehyde (Beyotime, Shanghai, China, P0098) overnight at 4°C, washed with PBS containing 0.2% PVA (PBS/PVA), and permeabilized with 0.1% TritonX-100 (Beyotime, Shanghai, China) at room temperature for 30 min. After washing, the samples were blocked in blocking buffer (Beyotime, Shanghai, China) at room temperature for 3–4 h or overnight at 4°C. The blocked samples were then incubated with anti-VPS15 (17894-1-AP, Proteintech), anti-YAP1 (13584-1-AP, Proteintech), anti-γ-H2AX (Abcam, ab81299, 1:150), anti-SOX2 (Biogenex, USA, 1:200), anti-CDX2 (Biogenex, USA, 1:200), and anti-ac-α-tubulin (ab24610, Abcam, 1:200) for 12–14 h at 4°C. After incubation, embryos were washed three times for 15 min each and incubated with Alexa Fluor 555-labeled Donkey Anti-rabbit IgG (Beyotime, A0453, 1:800), Alexa Fluor 555-labeled Donkey Anti-Mouse IgG (H + L) (Beyotime, A0460, 1:800), Alexa Fluor 488-labeled Goat Anti-Rabbit IgG (H + L) (Beyotime, A0423, 1:800), and Alexa Fluor 488-labeled Goat Anti-Mouse IgG (Beyotime, A0428, 1:800) for 2 h. After washing, the embryos were stained with DAPI and mounted on glass slides for imaging using a laser-scanning confocal microscope (Nikon DS-Ri1). Using the DCFH-DA kit (Beyotime Biotechnology, S0033, China) and Cell-Tracker™ fluorescent probes (Thermo Fisher Scientific, C12881, China), the levels of intracellular ROS and GSH during oocyte maturation were detected. Oocytes were incubated in 0.2%PBS/PVA containing 10 µM 2',7'-DCFH for 15 min. After washing the oocytes three times in 0.2% PBS/PVA, images were captured using a confocal microscope (Nikon, Tokyo, Japan). Oocytes were stained with MitoTracker (Invitrogen, Thermo Fisher Scientific) and Actin-Tracker Red-Rhodamine (Beyotime Biotechnology, C2207S, China) for 30 min at 37°C in the dark. After washing three times in 0.2%PBS/PVA, the oocytes were observed using a confocal microscope (Nikon, Tokyo, Japan), and oocytes with abnormal mitochondrial distribution were recorded. To assess ER

distribution, oocytes were incubated in 1 mL of medium containing 1 μ L ER-Tracker Green (Beyotime Biotech) at 37°C in the dark with 5% CO₂ for 30 min. After incubation, oocytes were washed three times with 0.2%PBS/PVA, mounted on glass slides, and imaged using a confocal laser scanning microscope. Fluorescence intensity was quantified using ImageJ software. For Ca²⁺ imaging, oocytes were incubated in 1 mL of medium containing 100 μ L Fluo-4 AM (Beyotime Biotech) at 37°C in the dark with 5% CO₂ for 50 min. After incubation, oocytes were washed three times with 0.2%PBS/PVA, mounted on glass slides, and imaged using a confocal laser scanning microscope. Fluorescence intensity was quantified using ImageJ software. The Annexin V Staining Kit (Vazyme, Nanjing, China) was used to detect the level of early apoptosis in oocytes. The dye working solution was prepared according to the instructions. Oocytes were washed three times with preheated PBS; then, after washing with binding buffer, the oocytes were incubated in the staining working solution at room temperature in the dark for 15 min. Afterward, the oocytes were washed three times with 0.2%PBS/PVA and could be observed and photographed using a confocal microscope (Nikon, Tokyo, Japan). For the measurement of mitochondrial membrane potential in oocytes, the operation was performed according to the specifications of the assay kit (Beyotime, C1071S). Oocytes were incubated with 1X JC-1 working solution at 37°C for 20 min. The oocytes were then washed three times with warm 0.2%PBS/PVA. To perform lysosomal staining, oocytes were incubated in a medium containing 100 nM cell-permeant LysoTracker Green (Beyotime Biotech) at 37°C in a dark environment with 5% CO₂ in the air for 30 min. After washing three times with 0.2%PBS/PVA, the samples were mounted on glass slides. The oocytes were photographed using a confocal microscope, and the fluorescence intensity was calculated using Image J software. The relative ratio of red to green fluorescence intensity represents the proportion of mitochondrial depolarization.

The DeadEnd TM colorimetric TUNEL system (Promega) was used to detect blastocyst apoptosis. The blastocysts were fixed overnight in 4% paraformaldehyde at 4°C, permeabilized in 0.1% Triton X-100 for 30 min at 25°C, transferred into E buffer micro-droplets at room temperature for 5 min, and incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase at 37°C for 1 h in the dark. 2 $\times$ SSC (SSC: 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate) was added to stop the reaction and samples were allowed to stand for 15 min. Samples were stained with DAPI at room temperature in the dark for 8 min. Staining was observed under a fluorescence microscope (Nikon).

2.11. Statistical analysis

Data processing and graphing were performed using GraphPad Prism (version 8.0; GraphPad Software, USA). Quantitative data, including gene expression, immunofluorescence intensity, and embryo quality parameters, were normalized to internal controls and presented as mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using Student's t-test for comparisons between two groups and one-way ANOVA for comparisons among multiple groups. Tukey's multiple comparison test was applied for all pairwise comparisons, and Dunnett's test was used. All tests were two-sided, with $P < 0.05$ considered statistically significant. Exact P-values, adjusted P-values, effect sizes, and 95% confidence intervals were reported where applicable. Fluorescence signal quantification was performed using Image-Pro-Plus 6.0 and ImageJ software.

3. Result

3.1. Characterization of lipidomic changes and metabolic dysregulation in POA porcine oocytes

To investigate lipid metabolism during POA, we established an in vitro POA model of porcine oocytes by extending MII-stage oocyte culture for 24h (POA 24h) and 48h (POA 48h). As culture time increased,

POA oocytes exhibited progressive deterioration in quality. Specifically, the proportion of oocytes maintaining normal MII morphology was significantly reduced (Control: 59.67 $\pm$ 1.43% vs. POA 24h: 52.56 $\pm$ 0.95%, $P < 0.05$; Control: 59.67 $\pm$ 1.43% vs. POA 48h: 31.98 $\pm$ 1.8%, $P < 0.0001$, Fig. 1A), while cytoplasmic fragmentation increased (Control: 8.04 $\pm$ 0.95% vs. POA 24h: 18.71 $\pm$ 1.55%, $P < 0.001$; Control: 8.04 $\pm$ 0.95% vs. POA 48h: 30.2 $\pm$ 1.58%, $P < 0.0001$, Fig. 1B). The cleavage rate significantly declined (Control: 71.38 $\pm$ 1.75% vs. POA 24h: 59.87 $\pm$ 1.31%, $P < 0.001$; Control: 71.38 $\pm$ 1.75% vs. POA 48h: 33.05 $\pm$ 1.24%, $P < 0.0001$, Fig. 1C), and cleavage fragmentation increased (Control: 7.69 $\pm$ 1.30% vs. POA 24h: 19.61 $\pm$ 1.24%, $P < 0.001$; Control: 7.69 $\pm$ 1.30% vs. POA 48h: 36.37 $\pm$ 1.84%, $P < 0.0001$, Fig. 1D). Additionally, blastocyst formation was significantly reduced in POA oocytes (Control: 40.1 $\pm$ 1.7% vs. POA 24h: 24.17 $\pm$ 1.36%, $P < 0.0001$; Control: 40.1 $\pm$ 1.7% vs. POA 48h: 13.89 $\pm$ 1.13%, $P < 0.0001$, Fig. 1E). Based on these results, POA 24h oocytes were selected for subsequent lipidomics analysis.

To further explore lipid metabolic abnormalities, TEM revealed structural alterations during POA, with enlarged lipid droplets and reduced contact points between lipid droplets and mitochondria. Quantitative analysis confirmed that lipid droplet size was significantly increased in POA 24h and POA 48h oocytes compared to Control (Fig. 1F–G, $P < 0.001$, $P < 0.0001$), indicating a disruption of lipid droplet distribution. These data directly support the observations from TEM images and provide statistical evidence for altered lipid metabolism during POA. Targeted lipidomics (LC-MS/MS) of Control and POA 24h oocytes revealed significant alterations in lipid composition (Fig. 1H). PCA demonstrated clear separation between groups ($R^2X = 0.82$, $Q^2 = 0.75$; Fig. 1I), indicating global reprogramming of lipid metabolism during POA. Using thresholds of $|\log_2FC| > 0.5$ and $VIP > 1$, 82 differential metabolites (DEMs) were identified, including 30 upregulated (e.g., Triglycerides TAG 16:0/18:1/18:1) and 52 downregulated metabolites (e.g., PI 18:0/20:4; Fig. 1J and K). KEGG pathway enrichment indicated that these DEMs were enriched in glycerophospholipid metabolism and general metabolic pathways (Fig. 1L).

Notably, PI and its phosphorylated derivatives (PIP2, PIP3) were strongly negatively correlated with the aging phenotype ($r = -0.93$, $P < 0.001$; Fig. 1M), suggesting that PI metabolic dysregulation is a prominent feature of POA and closely associated with impaired lipid homeostasis, rather than definitively causal. Collectively, these results indicate that changes in PI metabolism are a characteristic of POA and may contribute to the observed oocyte dysfunction. These observations highlight potential targets for interventions to improve oocyte quality, though further functional studies are required to establish direct causal links.

3.2. Exogenous PI improves POA phenotypes in porcine oocytes

Based on lipidomics analysis, PI and its derivatives were found to be significantly downregulated in POA oocytes (Fig. 1M), prompting investigation of exogenous PI supplementation. Various concentrations of PI (10, 20, 30, 40, 50 mM) were added to the maturation medium, and 30 mM PI was identified as the most effective in improving the aging phenotype. POA 24h oocytes exhibited reduced cumulus expansion (61.15 $\pm$ 1.83%) compared to Control oocytes (POA 24h: 61.15 $\pm$ 1.83% vs Control: 85.57 $\pm$ 2.00%, $P < 0.0001$). Treatment with 30 mM PI partially restored cumulus expansion (30 mM PI: 77.7 $\pm$ 1.98% vs POA 24h: 61.15 $\pm$ 1.83%, $P < 0.001$; 30 mM PI: 77.7 $\pm$ 1.98% vs Control: 85.57 $\pm$ 1.60%, $P < 0.001$), whereas other concentrations showed less pronounced effects (Fig. 2A) [12,15,16]. Similarly, the proportion of oocytes maintaining normal MII morphology was significantly increased in the 30 mM PI group compared to the aging group (30 mM PI: 59.93 $\pm$ 0.66% vs POA 24h: 54.68 $\pm$ 1.27%, $P < 0.05$), bringing it closer to control levels (30 mM PI: 59.93 $\pm$ 0.66% vs Control: 62.30 $\pm$ 2.44%, $P < 0.05$) (Fig. 2B). Cytoplasmic fragmentation was significantly increased in POA oocytes compared to Control oocytes

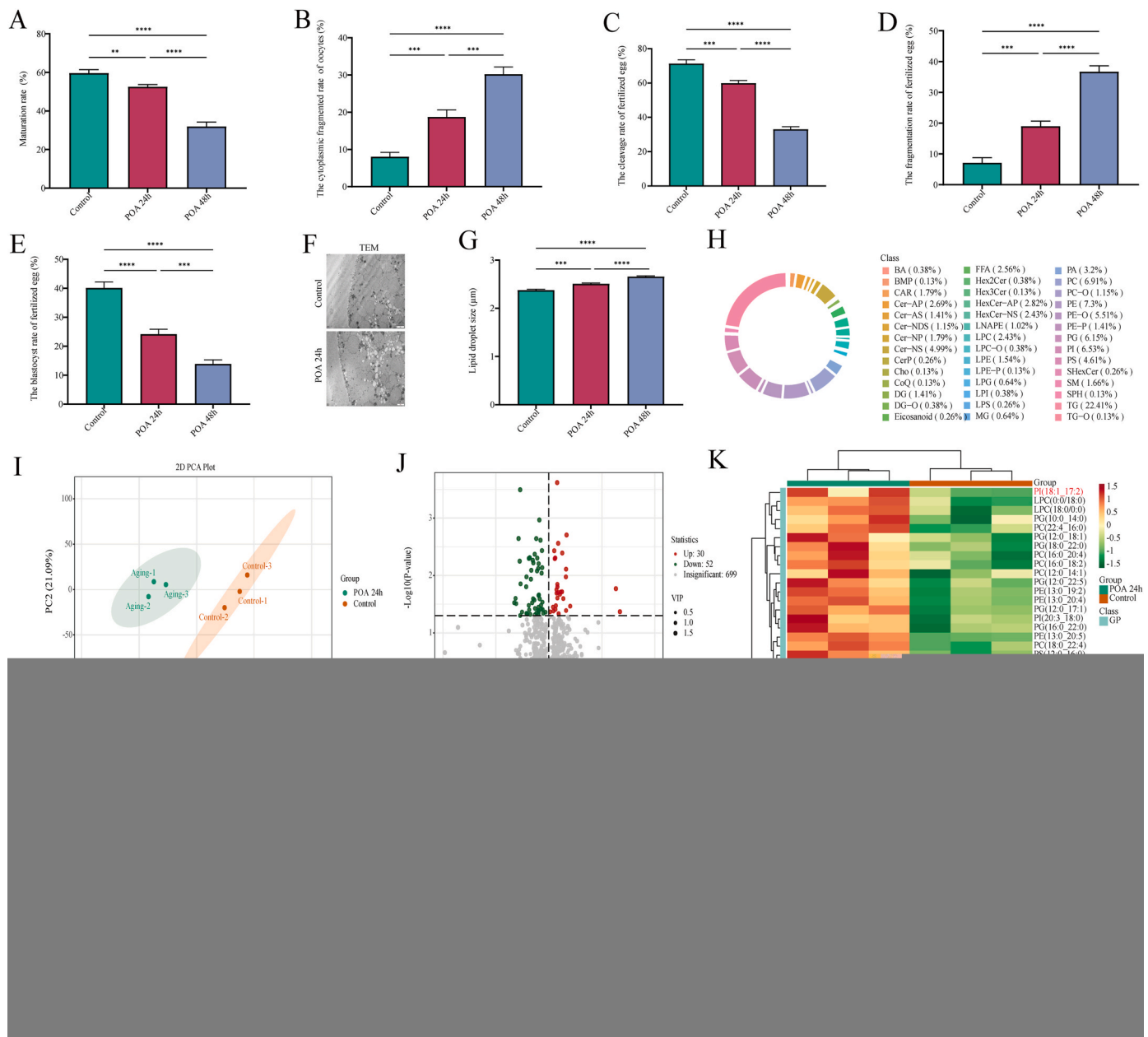


Fig. 1. Targeted lipidomics analysis of POA porcine oocytes. **A.** Proportion of oocytes maintaining normal MII morphology in Control (N = 193), POA 24h (N = 188), and POA 48h groups (N = 168). Normal MII morphology was defined by the presence of an intact spindle, properly aligned chromosomes, and absence of cytoplasmic degeneration or fragmentation. **B.** The cytoplasmic fragmented rate of oocytes (Control: N = 16, POA 24h: N = 34, POA 48h: N = 51). **C.** The cleavage rate of fertilized egg (Control: N = 127, POA 24h: N = 91, POA 48h: N = 39). **D.** The fragmentation rate of fertilized egg (Control: N = 10, POA 24h: N = 18, POA 48h: N = 14). **E.** The blastocyst rate of fertilized egg (Control: N = 48, POA 24h: N = 18; POA 48h: N = 4). **F.** Representative TEM images showing ultrastructural alterations in lipid droplets and mitochondria in Control and POA oocytes, scale bar = 5 μm. **G.** Quantitative analysis of lipid droplet size in different groups (N = 30). **H.** Classification of lipid species detected in control and aged oocytes, illustrating significant alterations in lipid composition. **I.** PCA score plot demonstrating clear separation between control and aged groups ($R^2X = 0.82$, $Q^2 = 0.75$). **J.** Volcano plot showing differential metabolites. Green and red dots represent significantly downregulated and upregulated metabolites, respectively. **K.** Heatmap of differential lipid metabolites in porcine oocytes. **L.** KEGG pathway enrichment analysis showing significantly enriched pathways associated with lipid metabolism and signal transduction. **M.** Relative abundance of phosphatidylinositol (PI 18:0/20:4), which was significantly decreased in POA oocytes compared with Control. Statistical analysis was performed using one-way ANOVA with Tukey's and Dunnett's multiple comparison test. Adjusted *P* values are indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant. Data are presented as mean ± SEM. The experiments were repeated three times. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(POA 24h: $19.26 \pm 1.71\%$ vs. Control: $7.36 \pm 0.61\%$, $P < 0.001$). Treatment with 30 mM PI partially restored cytoplasmic integrity, reducing fragmentation (30 mM PI: $9.71 \pm 0.71\%$ vs POA 24h: $19.26 \pm 1.71\%$, $P < 0.001$; 30 mM PI: $9.71 \pm 0.71\%$ vs Control: $7.36 \pm 0.61\%$, $P < 0.05$), confirming the protective effect of PI on cytoplasmic integrity (Fig. 2C). The cleavage rate was significantly

decreased in POA oocytes compared to Control (POA 24h: $61.44 \pm 2.72\%$ vs. Control: $72.74 \pm 1.62\%$, $P < 0.0001$), and was improved in the 30 mM PI group (30 mM PI: $69.57 \pm 0.51\%$ vs POA 24h: $61.44 \pm 2.72\%$, $P < 0.05$; 30 mM PI: $69.57 \pm 0.51\%$ vs Control: $72.74 \pm 1.62\%$, $P < 0.05$), indicating partial restoration of early developmental potential (Fig. 2D). Similarly, the cleavage fragmentation

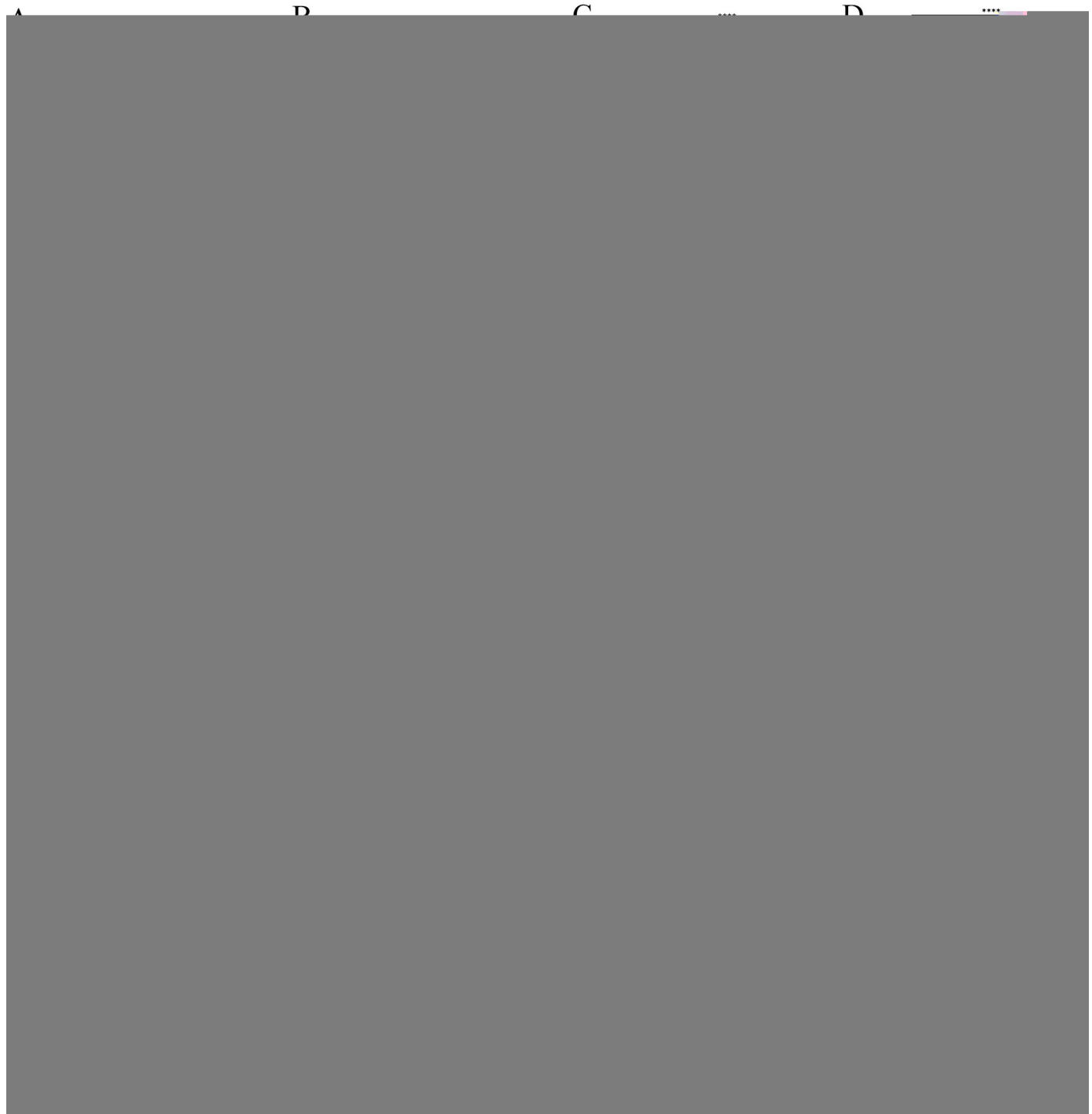


Fig. 2. Effects of exogenous PI Supplementation on POA porcine Oocytes. **A.** Cumulus cell expansion index in Control and POA 24h oocytes after treatment with different PI concentrations (Control: N = 279, 10 mM PI: N = 217, 20 mM PI: N = 247, 30 mM PI: N = 252, 40 mM PI: N = 236, 50 mM PI: N = 182, POA 24h: N = 209). **B.** Proportion of oocytes maintaining normal MII morphology in POA oocytes after PI supplementation (Control: N = 203, 10 mM PI: N = 187, 20 mM PI: N = 189, 30 mM PI: N = 196, 40 mM PI: N = 193, 50 mM PI: N = 181, POA 24h: N = 185). **C.** The cytoplasmic fragmented rate of oocytes (Control: N = 15, 10 mM PI: N = 20, 20 mM PI: N = 22, 30 mM PI: N = 19, 40 mM PI: N = 24, 50 mM PI: N = 28, POA 24h: N = 39). **D.** The cleavage rate of fertilized egg (Control: N = 138, 10 mM PI: N = 107, 20 mM PI: N = 112, 30 mM PI: N = 123, 40 mM PI: N = 108, 50 mM PI: N = 94, POA 24h: N = 88). **E.** The fragmentation rate of fertilized egg (Control: N = 10, 10 mM PI: N = 11, 20 mM PI: N = 13, 30 mM PI: N = 12, 40 mM PI: N = 14, 50 mM PI: N = 14, POA 24h: N = 17). **F.** The blastocyst rate of fertilized egg (Control: N = 53, 10 mM PI: N = 19, 20 mM PI: N = 22, 30 mM PI: N = 38, 40 mM PI: N = 15, 50 mM PI: N = 10, POA 24h: N = 17). **G-H.** Mitochondrial membrane potential measured by JC-1 staining (N = 30), scale bar = 80 μ m. **I-J.** Cortical granule (CGs) subcortical localization abnormalities (N = 30), scale bar = 80 μ m. **K-L.** Annexin-V fluorescence intensity as a marker for apoptosis (N = 30), scale bar = 80 μ m. **M-N.** Meiotic spindle integrity in POA oocytes (N = 30), scale bar = 80 μ m. **O-P.** Actin filament distribution in POA oocytes (N = 30), scale bar = 80 μ m. **Q-R.** Lipid accumulation and fluorescence quantification in POA oocytes after PI supplementation (N = 30), scale bar = 80 μ m. **S.** PI-mediated rescue of lipid metabolism markers. Statistical analysis was performed using one-way ANOVA with Tukey's and Dunnett's multiple comparison test. Adjusted *P* values are indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant. Data are presented as mean $\pm$ SEM. The experiments were repeated three times.

rate was elevated in POA oocytes relative to Control (POA 24h: $19.13 \pm 1.87\%$ vs. Control: $6.89 \pm 1.34\%$, $P < 0.0001$), and reduced by PI treatment (30 mM PI: $9.49 \pm 1.08\%$ vs POA 24h: $19.13 \pm 1.87\%$, $P < 0.0001$; 30 mM PI: $9.49 \pm 1.08\%$ vs Control: $6.89 \pm 1.34\%$, $P < 0.05$), suggesting improved embryo integrity (Fig. 2E). Finally, blastocyst formation was impaired in POA oocytes compared to Control (POA 24h: $24.76 \pm 2.63\%$ vs. Control: $41.39 \pm 2.15\%$, $P < 0.0001$), and partially restored by 30 mM PI treatment (30 mM PI: $33.51 \pm 1.96\%$ vs POA 24h: $24.76 \pm 2.63\%$, $P < 0.01$; 30 mM PI: $33.51 \pm 1.96\%$ vs Control: $41.39 \pm 2.15\%$, $P < 0.001$), demonstrating that PI improves the ability of aging oocytes to form blastocysts (Fig. 2F).

Mitochondrial membrane potential ($\Delta\Psi_m$), as assessed by JC-1 staining, was significantly increased in the PI-treated oocytes (Fig. 2G and H), indicating partial restoration of mitochondrial function in aging oocytes. Moreover, cortical granule (CGs) subcortical localization abnormalities were significantly reduced in the PI-treated group compared to POA oocytes ($P < 0.01$), indicating that PI partially restores CGs distribution. However, the PI-treated group remained significantly lower than Control oocytes ($P < 0.05$), confirming that PI improves CGs integrity and subcortical organization but does not fully restore it to normal levels (Fig. 2I and J). Annexin-V fluorescence intensity, a marker for apoptosis, was significantly reduced in the PI-treated groups ($P < 0.05$), indicating that PI supplementation alleviates POA-induced apoptotic processes (Fig. 2K and L). In addition, PI supplementation significantly improved meiotic spindle integrity in aging oocytes. Compared to normal oocytes, POA oocytes exhibited severe spindle abnormalities, including condensation, elongation, and breakage, as

well as chromosome misalignment. However, PI treatment significantly reduced these abnormalities, and spindle morphology was restored to near control levels (Fig. 2M and N). Confocal microscopy analysis revealed that PI treatment restructured the distribution of actin filaments, with the fluorescence intensity in the cortical region of actin filaments restored to near normal levels (Fig. 2O and P). Most importantly, PI supplementation significantly reversed the lipid content alterations observed in POA oocytes (Fig. 2Q and R), indicating a partial restoration of lipid metabolic homeostasis. In POA oocytes, *ACACA* and *PLIN2* were significantly upregulated, while *FASN*, *FADS1*, *HSL*, and *CPT2* were downregulated. 30 mM PI treatment brought the expression levels of these genes closer to those of the control group, suggesting that PI supplementation helps restore normal lipid metabolic pathways (Fig. 2S).

Taken together, these findings demonstrate that exogenous PI supplementation effectively reverses POA-related defects in oocyte maturation, improves lipid metabolism, and restores the integrity of cellular structures and functions. 30 mM PI was identified as the optimal concentration for intervention, providing critical information for future mechanistic studies.

3.3. Exogenous PI partially restores organelle function and redox balance in POA porcine oocytes

This study systematically evaluated the effects of PI on organelle structure and functional in POA porcine oocytes. Using specific fluorescence probes, we observed typical organelle dysfunction in POA 24h

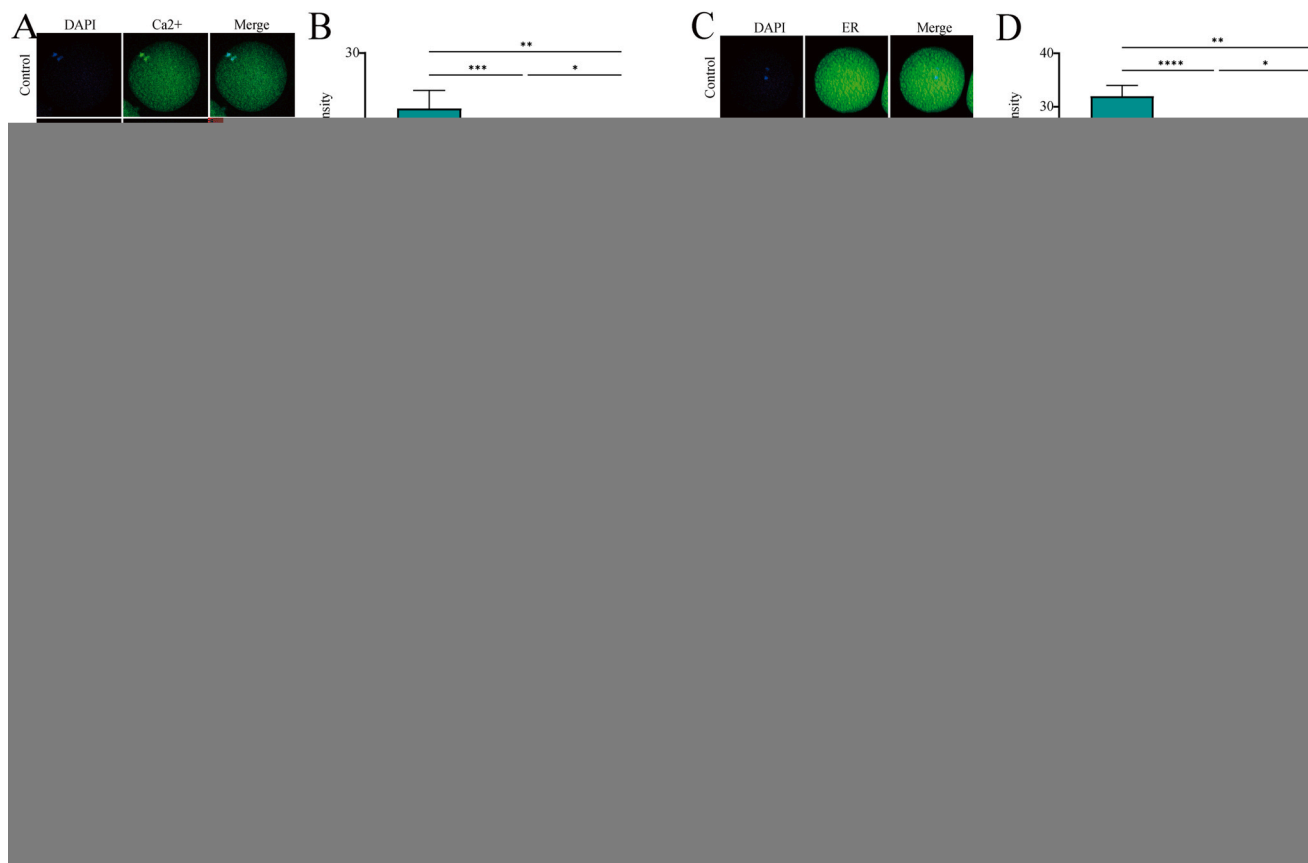


Fig. 3. PI Supplementation partially restores Organelle function and redox balance in POA 24h Oocytes. A-F. Organelle structure assessment, including Ca²⁺ homeostasis (A-B) (N = 30), ER morphology (C-D) (N = 30), and Golgi polarity (E-F) (N = 30), scale bar = 80 μ m. PI treatment partially restored these parameters toward Control levels. G-H. Lysosomal activity in Control, POA 24h, and PI-treated (30 mM) oocytes (N = 30), scale bar = 80 μ m. I. Representative images of ROS, GSH, and mitochondrial staining (N = 30), scale bar = 80 μ m. J-L. Quantification of ROS, GSH, and mitochondrial fluorescence intensity. Statistical analysis was performed using one-way ANOVA with Tukey's and Dunnett's multiple comparison test. Adjusted P values are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant. Data are presented as mean $\pm$ SEM. The experiments were repeated three times.

oocytes compared to control oocytes. POA 24h oocytes exhibited significant Ca^{2+} homeostasis imbalance ($P < 0.01$), ER fragmentation ($P < 0.001$), and loss of Golgi polarity ($P < 0.001$). PI treatment partially restored these parameters toward the levels observed in control oocytes, with statistically significant differences compared to POA oocytes ($P < 0.01$), although values did not fully reach Control levels (Fig. 3A–F). Additionally, lysosomal function was assessed, revealing that POA 24h oocytes had increased lysosomal activity. PI treatment reduced lysosomal activity toward Control levels (Fig. 3G and H), indicating partial correction of abnormal lysosomal accumulation. Mitochondrial distribution and membrane potential, as assessed by Mito-Tracker Red staining, were partially restored toward Control levels in POA 24h oocytes following PI treatment, indicating amelioration of mitochondrial organization and activity. These findings demonstrate that PI can correct mitochondrial dysfunction in POA 24h oocytes. PI supplementation additionally mitigated oxidative stress: ROS levels were reduced and GSH content partially restored toward Control levels (Fig. 3I–L), highlighting improved redox balance. Collectively, these results indicate that PI acts through multifaceted mechanisms, including the partial restoration of Ca^{2+} homeostasis, amelioration of lysosomal and mitochondrial dysfunction, and stabilization of the redox environment, to partially correct organelle dysfunction in POA oocytes.

3.4. *PI3KR4 and YAP1 cooperatively regulate lipid metabolism and oocyte development in POA porcine oocytes*

To investigate potential interactions between PI3KR4 and YAP1 in POA oocytes, we first examined their immunofluorescence distribution. POA 24h oocytes exhibited significantly increased PI3KR4 (Fig. 4A and B) and YAP1 (Fig. 4C and D) signals compared to Control oocytes, suggesting upregulation during POA. Co-IP experiments indicated a potential interaction between PI3KR4 and YAP1 (Fig. 4E), consistent with cooperative involvement in oocyte function. Specific siRNAs were designed to knock down *PI3KR4* and *YAP1*. In the si-*PI3KR4*-994 group, the immunofluorescence signal for *PI3KR4* was significantly reduced, and the quantitative results were consistent with the immunofluorescence data (Fig. 4F–H). In the si-*YAP1* group, the immunofluorescence signal for *YAP1* was also significantly reduced, and the quantitative results matched the immunofluorescence images (Fig. 4I–K). In POA 24h oocytes, knockdown of *YAP1* significantly reduced its immunofluorescence signal, whereas *PI3KR4* signal was modestly increased, likely reflecting compensatory regulation. In the si-*PI3KR4* group, *PI3KR4* immunofluorescence was significantly reduced, while *YAP1* signal was partially retained, indicating that *PI3KR4* downregulation alone did not fully suppress *YAP1* activity, possibly due to other regulatory pathways. Double knockdown of *PI3KR4* and *YAP1* resulted in a significant reduction of both signals (Fig. 4L–O), supporting a cooperative interaction and partial interdependence between these proteins during post-ovulatory aging. Functional assays revealed that knockdown of either *PI3KR4* or *YAP1* disrupted lipid droplet organization, resulting in decreased fluorescence intensity and abnormal distribution compared to POA 24h oocytes (Fig. 4P and Q). In oocyte developmental potential assays, si-*PI3KR4* and si-*YAP1* groups showed a significant decrease in maturation rate (Fig. 4R, $P < 0.05$), a significant increase in cytoplasmic fragmentation (Fig. 4S, $P < 0.05$), and significant changes in cleavage rate (Fig. 4T) and cleavage fragmentation (Fig. 4U) compared to the control group. These changes were more pronounced in the si-*YAP1* + si-*PI3KR4* double knockdown group. Finally, the blastocyst formation rate (Fig. 4V) was significantly reduced in the knockdown groups, further confirming the cooperative role of *PI3KR4* and *YAP1* in oocyte development.

Overall, these findings indicate that *PI3KR4* and *YAP1* likely cooperate to maintain lipid metabolism and oocyte developmental potential in POA oocytes. Knockdown experiments demonstrate that both proteins contribute to lipid droplet organization, meiotic maturation, and embryonic development. While the data support a cooperative role, the

precise downstream signaling pathways mediating these effects remain to be elucidated.

3.5. *Exogenous PI partially restores fertilization and embryonic development in POA porcine oocytes*

This study evaluated the effects of PI supplementation on fertilization capacity and embryonic development in POA porcine oocytes. Since ovastacin release from CGs in oocytes removes the sperm-binding domain from the ZP, we assessed sperm binding. Hoechst staining of sperm heads revealed that POA 24h oocytes exhibited significantly reduced sperm binding compared to Control oocytes ($P < 0.05$). Supplementation with 30 mM PI during IVM partially restored sperm binding toward Control levels (Fig. 5A and B). Moreover, fertilization rates, assessed by the presence of pronuclei after IVF, were significantly decreased in POA 24h oocytes compared to Control ($P < 0.05$). 30 mM PI supplementation partially restored fertilization rates toward control levels, showing a significant improvement compared to POA 24h oocytes ($P < 0.05$), while remaining lower than Control (Fig. 5C). These results indicate that PI treatment enhances the fertilization ability of aging oocytes. Next, we evaluated embryo quality. Total cell number in blastocysts was significantly reduced in POA 24h oocytes compared to Control oocytes ($P < 0.05$) and partially restored with 30 mM PI treatment ($P < 0.05$). Similarly, the number of TE and ICM cells was decreased in POA oocytes relative to Control ($P < 0.05$) and was partially restored by 30 mM PI ($P < 0.05$; Fig. 5D and E) (No additional PI was added during culture in PZM3 medium). Apoptosis and DNA damage were assessed by TUNEL and γH2AX staining. POA 24h oocytes led to elevated apoptosis and DNA damage in blastocysts compared to Control ($P < 0.05$). 30 mM PI treatment during IVM significantly reduced these markers relative to POA 24h oocytes ($P < 0.05$), although levels remained higher than Control ($P < 0.05$) (Fig. 5F–I). Collectively, these results indicate that PI supplementation during IVM partially restores fertilization capacity and embryonic developmental potential in POA 24h oocytes. PI treatment improves sperm binding, fertilization rates, blastocyst formation, TE and ICM cell numbers, and reduces DNA damage and apoptosis, although most parameters do not fully reach Control levels. These findings suggest that PI during maturation may serve as a promising approach to mitigate aging-related declines in oocyte quality and support assisted reproduction outcomes.

3.6. *PI partially restores oocyte function and developmental potential through PI3KR4 and YAP1 in POA porcine oocytes*

Our study demonstrates that exogenous PI supplementation during IVM partially restores oocyte maturation, lipid metabolism, and embryonic developmental potential in POA 24h porcine oocytes. PI treatment improved cumulus expansion, MII morphology, cytoplasmic integrity, sperm binding, and blastocyst formation, while reducing apoptosis and DNA damage. Mechanistic investigations revealed that *PI3KR4* and *YAP1* are upregulated in POA oocytes and likely cooperate to maintain lipid homeostasis, organelle function, and developmental competence (Fig. 6). Functional knockdown of either *PI3KR4* or *YAP1* disrupted lipid droplet organization, reduced oocyte maturation, and impaired embryonic development, with more pronounced effects observed in the double knockdown group. These findings indicate that the protective effects of PI are, at least in part, mediated via *PI3KR4* and *YAP1*, providing novel insights into the molecular mechanisms underlying POA and highlighting potential strategies to preserve oocyte quality.

4. Discussion

Increasing evidence indicates that the POA of oocytes leads to morphological and functional alterations, which impair fertilization potential, reduce developmental competence, and increase the risk of



Fig. 4. *PI3KR4* and *YAP1* interaction and functional effects in POA porcine oocytes. A-B. *PI3KR4* immunofluorescence intensity in control and POA 24h oocytes (N = 30), scale bar = 80 μ m. C-D. *YAP1* immunofluorescence intensity in control and POA 24h oocytes (N = 30), scale bar = 80 μ m. E. Co-IP experiments showing the interaction between *PI3KR4* and *YAP1* (N = 200). F-H. *PI3KR4* expression and knockdown analysis in control and si-*PI3KR4* treated oocytes (N = 30), scale bar = 80 μ m. I-K. *YAP1* expression and knockdown analysis in control and si-*YAP1* treated oocytes (N = 30), scale bar = 80 μ m. L-O. Quantitative analysis of *PI3KR4* and *YAP1* protein and mRNA levels (N = 30). P-Q. Lipid droplet distribution and fluorescence intensity in Control, si-*PI3KR4*, si-*YAP1*, and double knockdown (si-*PI3KR4* + si-*YAP1*) oocytes (N = 30), scale bar = 80 μ m. R. Maturation rate (Control: N = 200, POA 24h: N = 169, si-*PI3KR4*: N = 175, si-*YAP1*: N = 184, si-*PI3KR4*+si-*YAP1*: N = 121). S. The cytoplasmic fragmented rate of oocytes (Control: N = 11, POA 24h: N = 38, si-*PI3KR4*: N = 27, si-*YAP1*: N = 21, si-*PI3KR4*+si-*YAP1*: N = 33). T. The cleavage rate of fertilized egg (Control: N = 141, POA 24h: N = 70, si-*PI3KR4*: N = 91, si-*YAP1*: N = 108, si-*PI3KR4*+si-*YAP1*: N = 43). U. The fragmentation rate of fertilized egg (Control: N = 8, POA 24h: N = 15, si-*PI3KR4*: N = 13, si-*YAP1*: N = 10, si-*PI3KR4*+si-*YAP1*: N = 11). V. The blastocyst rate of fertilized egg (Control: N = 51, POA 24h: N = 12, si-*PI3KR4*: N = 23, si-*YAP1*: N = 33, si-*PI3KR4*+si-*YAP1*: N = 5). Statistical analysis was performed using one-way ANOVA with Tukey's and Dunnett's multiple comparison test. Adjusted *P* values are indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant. Data are presented as mean $\pm$ SEM. The experiments were repeated three times.

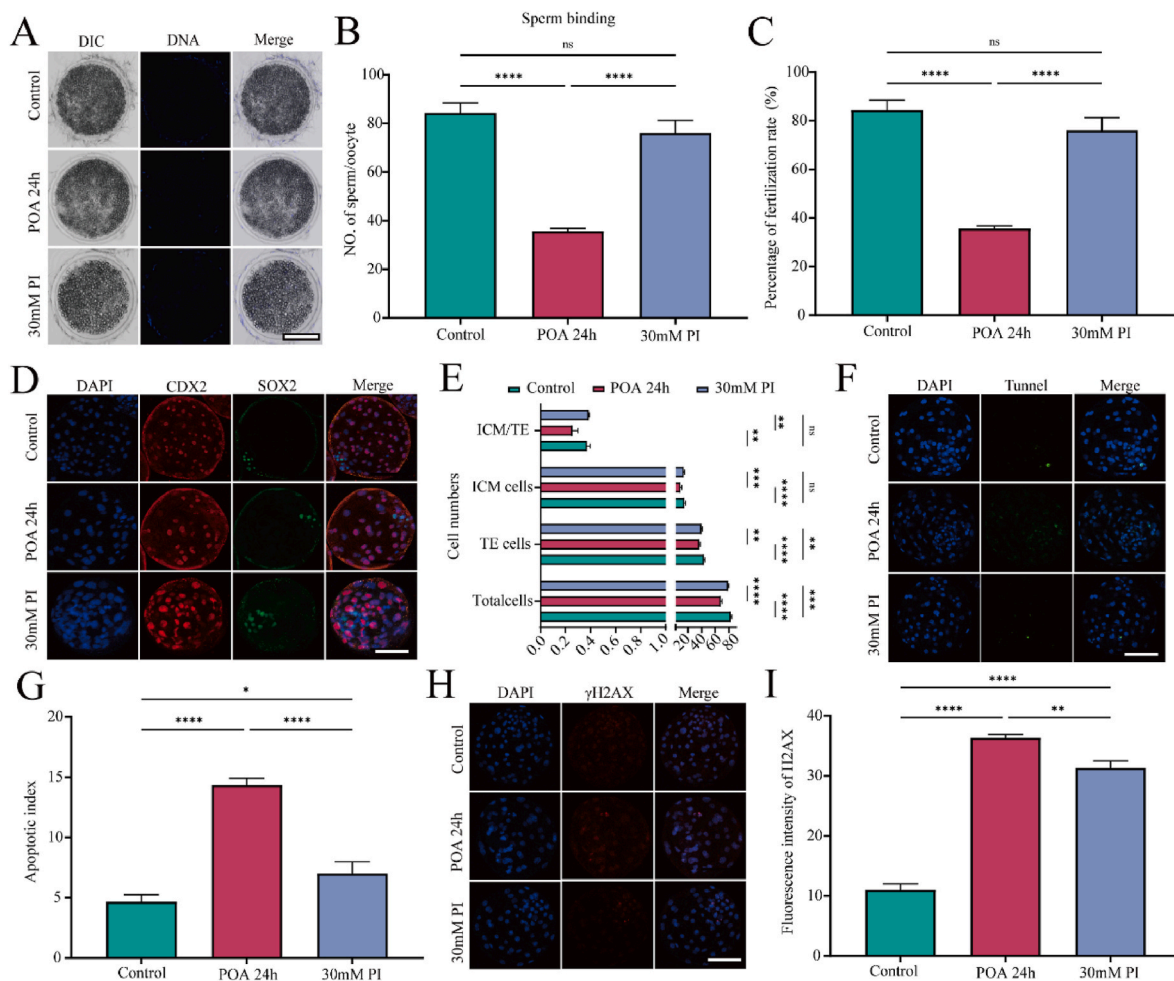


Fig. 5. PI Restores Fertilization Ability and Enhances Embryonic Development in POA porcine oocytes. A-B. Sperm binding to oocytes. Hoechst staining of sperm heads was used to quantify the number of sperm bound to the ZP of control, POA 24h, and POA 24h + PI (30 mM) oocytes (Control: N = 253, POA 24h: N = 107, 30 mM PI: N = 228), scale bar = 60 μ m. C. Fertilization rate in Control, POA 24h, and POA 24h + PI (30 mM) oocytes (Control: N = 222, POA 24h: N = 63, 30 mM PI: N = 165). D-E. Total cell number and TE/ICM composition in blastocysts, assessed by CDX2 and SOX2 staining (N = 30), scale bar = 60 μ m. F-G. Apoptosis in blastocysts, assessed by TUNEL staining and apoptotic index quantification (N = 30), scale bar = 60 μ m. H-I. DNA damage in blastocysts, assessed by γ H2AX staining and fluorescence intensity quantification (N = 30), scale bar = 60 μ m. Statistical analysis was performed using one-way ANOVA with Tukey's and Dunnett's multiple comparison test. Adjusted *P* values are indicated as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, not significant. Data are presented as mean $\pm$ SEM. The experiments were repeated three times.

embryonic loss [17–21]. To further explore the mechanisms underlying POA, we employed pigs as a model due to their physiological similarities to humans, providing a widely accepted preclinical system for studying oocyte aging [22,23]. Targeted lipidomics analysis revealed a marked decline in PI and its phosphorylated derivatives (e.g., PIP2 and PIP3) during POA, which was closely associated with lipid metabolic reprogramming and mitochondrial dysfunction [24]. Disruption of PI metabolism led to abnormal lipid droplet accumulation and weakened interactions between lipid droplets and mitochondria, impairing fatty acid oxidation and exacerbating the energy crisis and cellular dysfunction [5]. These metabolic disturbances not only compromised the energy supply of oocytes but also diminished their developmental potential [25]. Collectively, these observations suggest that PI metabolic alterations are a prominent feature of oocyte aging and may contribute to the functional decline observed during POA, providing a rationale for exploring the protective effects of exogenous PI supplementation in subsequent experiments. The importance of PI in lipid metabolism was further supported by these observations. During POA, the decline of PI and its phosphorylated derivatives was associated with abnormal lipid droplet accumulation and weakened interactions between lipid droplets and mitochondria, which likely impaired fatty acid β -oxidation and

cellular energy production [26]. In normal oocytes, neutral lipids stored in lipid droplets are metabolised to fatty acids, which are transported into mitochondria via the carnitine shuttle and oxidised through β -oxidation to produce ATP, a process essential for oocyte maturation and developmental competence [4,26]. Disruption of this balance due to PI deficiency likely reduced fatty acid oxidation efficiency, limiting cellular energy supply and exacerbating metabolic disturbances during aging [3,11,27]. Together, these observations suggest that changes in PI metabolism are a prominent feature of oocyte aging and may contribute to the observed decline in oocyte quality by influencing lipid metabolism and organelle function. These results are consistent with prior studies demonstrating that modulation of lipid metabolism, including enhancement of β -oxidation via CPT1 activation or L-carnitine supplementation, can improve oocyte developmental outcomes, whereas inhibition of β -oxidation impairs maturation and embryo development across species [26,28]. Additionally, antioxidant interventions such as melatonin and other free-radical scavengers have been reported to mitigate POA-induced oxidative damage, improve mitochondrial function, and enhance fertilisation and blastocyst formation rates in ageing oocyte models [21,29,30]. Compared with these interventions, PI supplementation not only maintains lipid metabolic homeostasis but also

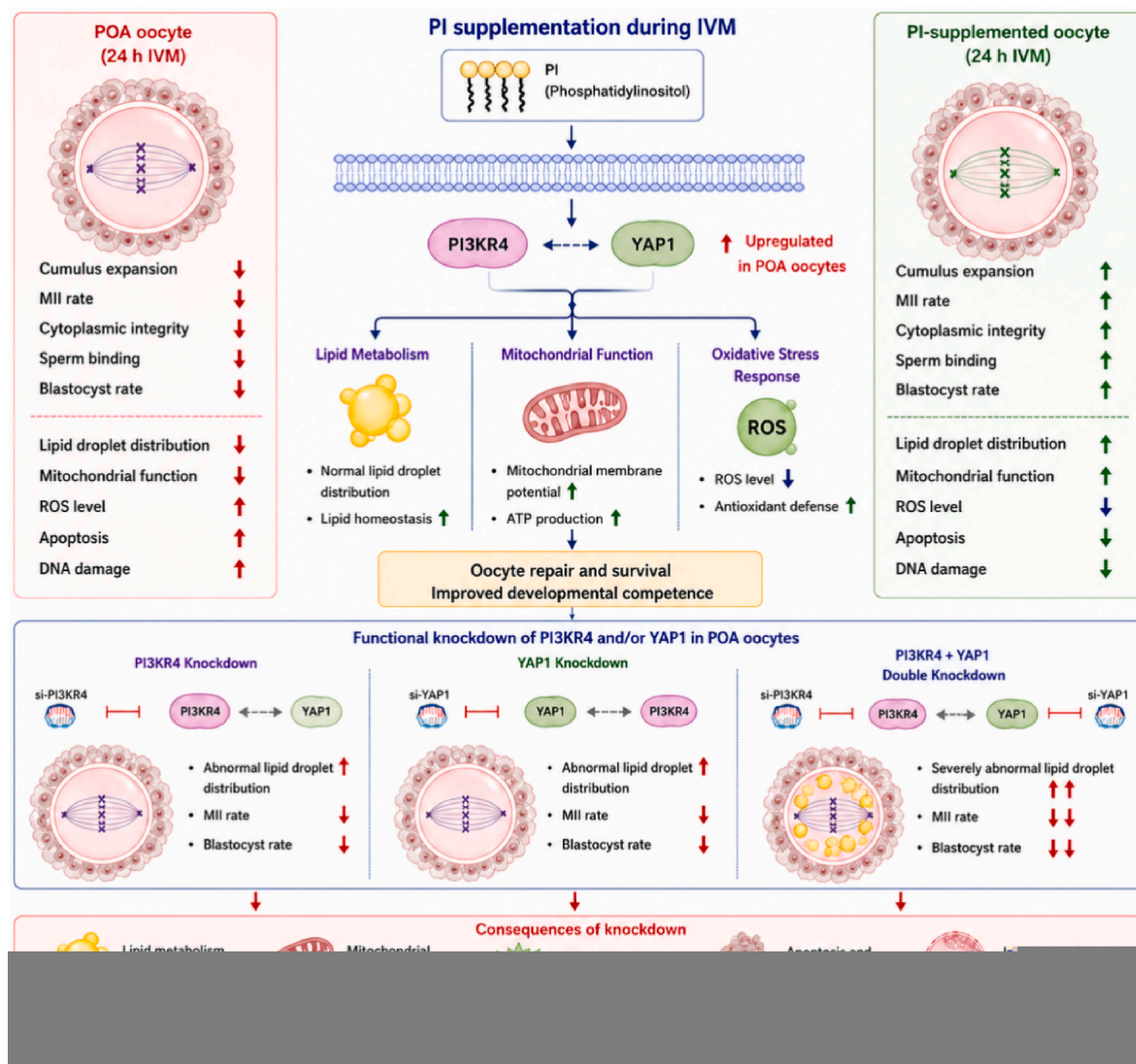


Fig. 6. Mechanistic model illustrating the protective effects of exogenous PI supplementation on POA 24h porcine oocytes. Exogenous PI added during IVM partially restores lipid metabolism, organelle function, and embryonic developmental potential. *PI3KR4* and *YAP1* are upregulated in POA oocytes and likely cooperate to maintain lipid droplet organization, mitochondrial function, and redox balance, thereby improving cumulus expansion, meiotic spindle integrity and chromosome alignment, sperm binding, and blastocyst formation. Knockdown of *PI3KR4* or *YAP1* disrupts these processes, with more pronounced defects observed in the double knockdown group.

stabilises organelle function and reduces apoptosis and DNA damage, highlighting both metabolic and antioxidative protective effects. Taken together, these observations underscore the critical role of lipid metabolism regulation, including phosphoinositide dynamics, in maintaining oocyte function during aging. Moreover, exogenous PI supplementation during IVM significantly improved the fertilization ability and embryonic developmental potential of POA 24h porcine oocytes. PI treatment not only restored the sperm-binding capacity but also significantly increased fertilization rates, with a marked increase in the total cell number of embryos, especially in the TE and ICM cells. These results indicate that PI enhances oocyte developmental potential by improving organelle function and stabilizing lipid metabolism. Additionally, PI also protected the quality of embryo by mitigating DNA damage and apoptosis in POA oocytes. TUNEL staining and γ H2AX analysis showed that PI supplementation significantly reduced DNA damage and apoptosis markers in POA oocytes, further confirming its antioxidative and anti-aging effects. Collectively, these findings provide evidence for the multiple protective roles of PI in POA oocytes. To further investigate the molecular mechanisms underlying the protective effects of PI, we examined the potential roles of *PI3KR4* and *YAP1* in aging oocytes.

In addition to the protective effects of PI, our mechanistic investigations suggest that *PI3KR4* and *YAP1* likely cooperate to maintain lipid metabolism and oocyte developmental potential in POA 24h porcine oocytes. Knockdown of either protein significantly disrupted lipid droplet distribution, reduced oocyte maturation, increased cytoplasmic fragmentation, and decreased cleavage and blastocyst formation rates, with effects being more pronounced in the double knockdown group (Fig. 4P–V, $P < 0.05$). These observations are consistent with previous studies demonstrating that PI3K/AKT signaling plays a critical role in oocyte meiotic progression and developmental competence, where perturbation of this pathway impairs oocyte maturation and embryo development in porcine oocytes [31–34]. Furthermore, *YAP1* has been implicated in regulating lipid metabolism and cellular differentiation in reproductive cells, aligning with the effects observed following *YAP1* knockdown on oocyte quality [35]. Cross-talk between PI3K/AKT and YAP/TAZ pathways has also been described in other cellular contexts, suggesting potential cooperative signaling that warrants further investigation [36]. Collectively, these data indicate that *PI3KR4* and *YAP1* may function in concert to stabilize lipid homeostasis and support embryonic developmental competence, complementing the

protective effects of PI supplementation. While these findings support a cooperative regulatory role, the precise downstream signaling mechanisms linking PI, *PI3KR4*, and *YAP1* remain to be clarified in future studies.

Although our study demonstrates a significant role for PI in the POA process, the precise mechanisms by which PI regulates lipid metabolism and organelle function via specific signaling pathways remain to be elucidated. Evidence from published studies indicates that PI3K/AKT signaling plays important roles in oocyte meiotic progression and developmental competence in mammalian oocytes, including pig oocytes, as perturbation of this pathway impairs oocyte maturation and embryo development [37]. Additionally, modulation of the PI3K/AKT pathway has been shown to affect oocyte maturation and subsequent embryonic development across multiple mammalian species, suggesting functional relevance of this pathway in reproductive processes [33]. However, the specific molecular cascade linking PI supplementation to activation of PI3K/AKT or related pathways, and how such signaling impacts lipid metabolic balance and organelle integrity in POA oocytes, has not been directly demonstrated in our current study. Future research should clarify these mechanistic links, particularly whether PI activates downstream effectors in the PI3K/AKT or *YAP1* axis in aging oocytes. Moreover, exploring the role of PI in other aging models will be important to assess the generality of its effects and evaluate its potential translational applications in reproductive aging and assisted reproductive technologies.

5. Conclusion

In summary, our study demonstrates that exogenous PI supplementation during IVM partially restores organelle function, fertilization potential, and embryonic developmental competence in POA porcine oocytes through multiple mechanisms. These results highlight the role of PI in mitigating age-associated declines in oocyte quality by stabilizing lipid metabolism and supporting organelle integrity. The protective effects of PI, potentially mediated via *PI3KR4* and *YAP1*, provide a mechanistic rationale for its use as a therapeutic intervention. Future studies are warranted to validate the efficacy of PI in other oocyte aging models and to explore its potential translational applications in assisted reproductive technologies.

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CRedit authorship contribution statement

Baobao Zhao: Conceptualization, Investigation, Writing – original draft. **Yang Li:** Conceptualization, Investigation, Writing – original draft. **Jieyu Wang:** Methodology, Software, Visualization. **Zhenzhen Yin:** Methodology, Software, Visualization. **Xiaoli Guo:** Methodology, Software, Visualization. **Zihan Xu:** Methodology, Software, Visualization. **Shiqing Wang:** Formal analysis, Validation. **Meiyun Lv:** Formal analysis, Validation. **Jinwang Liu:** Investigation, Project administration, Validation. **Heqiang Li:** Investigation, Project administration, Validation. **Suzhu Qing:** Conceptualization, Funding acquisition, Methodology. **Yongsheng Wang:** Conceptualization, Funding acquisition, Methodology.

Conflict of interest

All experimental protocols involving swine oocytes and embryos were approved by the Animal Care and Use Committee of Northwest

A&F University.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.theriogenology.2026.118038>.

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