

ORIGINAL ARTICLE

Metformin protects against mouse oocyte apoptosis defects induced by arecoline

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Abstract

Objectives: Arecoline is the main bioactive substance extracted from *Areca catechu* L, which has cell, neural and genetic toxicity. The function of arecoline in reproductive system has not been well explored.

Materials and Methods: To investigate the toxic effects of arecoline on oocyte development, immunofluorescence staining, qPCR, Western blotting, sperm binding assays and in vitro fertilization were performed to evaluate oocyte meiosis competence and embryo development.

Results: Our data revealed that arecoline exposure disrupts actin filament dynamics, spindle assembly and kinetochore-microtubule attachment stability in mouse oocytes, leading to aneuploidy and oocyte meiosis arrest. In addition, arecoline treatment disturbs the distribution of mitochondria, reduces ATP production and increases the level of oxidative stress, which ultimately induces oocyte apoptosis. Supplementation with metformin, a medicine for type 2 diabetes in the clinic, partially alleviates these damages.

Conclusions: Metformin has a protective effect on arecoline-induced mouse oocytes apoptosis.

1 | INTRODUCTION

Areca catechu L. (Arecaceae) is widely distributed in East Asia, South Asia and Southeast Asia such as China, India, Indonesia, Malaysia and the Philippines.¹ It is the fourth most common class of psychoactive substances in the world following tobacco, alcohol and caffeine.² According to statistics, about 600 million people around the world have the habit of chewing betel nut, accounting for about

one-tenth of the world's total population,^{3,4} and at least 400 million people chew betel nuts every day.^{5,6} Based on hundreds of research reports in seven countries, International Agency for Research on Cancer (IARC) officially identified betel nut as a primary carcinogen in 2003.⁷

Arecoline (1,2,5,6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester) is an alkaloid isolated from betel nut and is considered as the main active ingredient of betel nut.⁸ Arecoline has not only cytotoxicity,^{9,10} neurotoxicity,¹¹ genotoxicity¹² and immunotoxicity,¹³ but also reproductive toxicity in male.^{14,15} According to the survey, pregnant women who chew betel nut for a long time will

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experience symptoms such as decreased fertility, premature birth and miscarriage, and most of the babies they give are characterized by underweight and stunting.¹⁶⁻¹⁸ Studies have shown that exposure to arecoline significantly reduced the hatching rate and survival rate of zebrafish embryos, resulting in embryonic development retardation.^{19,20} At the same time, arecoline also reduces the formation of implanted embryos in the early stages of pregnancy and inhibits the growth of blastocyst trophoblast cells.²¹ In addition, arecoline can also cause a decrease in the number of spermatozoa in the epididymis, a decrease in sperm motility and a malformation in sperm morphology.^{14,15} However, the toxic effects of arecoline on oocyte maturation and its specific mechanisms remain unknown.

The mammalian oocytes originate from primordial germ cells, undergo complex processes of mitosis and meiotic maturation and then arrest at the MII stage. Only after fertilization or parthenogenetic activation, the oocytes initiate the early embryonic development.²² During the first meiotic process, with the germinal vesicle breakdown, the chromosome is directly exposed to the cytoplasm of the oocytes, opening a very sensitive time window when chemical substances, such as toxins or drugs, can cause the loss of nuclear and cytoplasmic maturation, affecting the development potential.²² Exposure to chemicals can significantly induce excessive reactive oxygen species (ROS) production²³ causing mitochondrial dysfunction,²⁴ resulting in disruption of cytoskeletal integrity and early apoptosis, which can lead to poor embryo quality and delay or even prevent embryo development.²⁵ It is worth noting that mitochondrial control of apoptosis has been described at several levels, including ATP production,²⁶ mitochondrial membrane potential and mitochondrial membrane permeability to apoptogenic factors, which can be released from the intermembrane space into the cytosol.²⁷

Metformin is recommended by experts from the American diabetes association and the European diabetes research association as a first-line treatment for type 2 diabetes.²⁸ The study found that in addition to hypoglycaemic effects, metformin is also playing an active role in fighting cancer,²⁹⁻³² delaying ageing³³⁻³⁶ and regulating immunity.³⁷⁻³⁹ At the same time, numerous studies have pointed out that metformin plays an important role in the reproductive system.⁴⁰⁻⁴² On the one hand, metformin treatment not only reduces oxidative stress and germ cell loss, thereby limiting spermatogenesis damage, but also increases the number and viability of rat spermatozoa.⁴³ On the other hand, metformin can reverse ovulation dysfunction and improve oocyte quality and embryo development in mice with polycystic ovary syndrome (PCOS) by reducing the level of ROS in oocytes and improving mitochondrial function.⁴⁴ In addition, studies in embryos have shown that metformin can also reduce apoptosis in blastocysts of obese mice.⁴⁵ Although the reproductive safety of metformin is still unclear, in general, metformin helps restore fertility and contributes significantly to the maturity and embryonic development of high-quality female germ cells.

To date, there have been no reports of the effects of arecoline on oocyte maturation. In this study, we explored the toxic effects of arecoline on the quality of cultured mouse oocytes and the rescue function of metformin. The results showed that arecoline leads to

HIGHLIGHTS

- Improvement effects of metformin on arecoline-exposed mouse oocytes.
- Metformin protects the cytoskeletal integrity and euploidy.
- Metformin restores mitochondrial dysfunctions.

disordered progression of oocyte meiosis and increased ROS by destroying cytoskeletal integrity, chromosome euploidy and mitochondrial function, eventually leading to oocyte apoptosis. However, metformin could partially reduce these damages, suggesting that metformin has a protective effect against the apoptosis of oocytes induced by arecoline.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All the experimental procedures in this study strictly implemented the standards of the Ethics Committee of Qingdao Agricultural University. Institute of cancer research (ICR) female mice were fed a regular diet in a temperature-controlled room of 12 hours light/dark cycle. In order to alleviate the suffering as much as possible, the mice were sacrificed humanely by the cervical dislocation during the collection of the oocytes.

2.2 | Oocyte collection and culture

The collection of oocytes was performed as described previously.²² Briefly, after 48 hours of treatment with pregnant mare serum gonadotropin (PMSG), 4- to 6-week old ICR female mice were euthanized and ovaries were collected in M2 medium supplemented with 2.5 $\mu\text{mol/L}$ milrinone. The morphologically intact cumulus-oocyte complexes (COCs) were picked up by a pipette under a stereomicroscope and washed 3-5 times in M2 operating droplets. Each set of 25-30 oocytes in germinal vesicle (GV) stage were placed in approximately 30 μL of M16 medium droplets covered with mineral oil and in vitro matured at 37°C in 5% humidified CO₂ atmosphere.

2.3 | Arecoline and metformin treatment

Arecoline (98%, 252 164; J&K Chemical Ltd., Beijing, China) was dissolved in ultrapure water as stock solution and diluted in the M16 medium as working solution. Metformin (98%, 257 998; J&K Chemical Ltd., Beijing, China) was dissolved in double distilled water (ddH₂O) as stock solution and diluted in the M16

medium as working solution. In order to test effects of arecoline exposure on oocyte meiosis progression, oocytes were matured in M16 medium supplement with different concentration of arecoline with or without metformin, according to experiment design. For detection of maturation rate, the 1st polar bodies were evaluated at 12 hours of in vitro culture. According to the results, we selected 180 µg/mL arecoline and 50 µmol/L metformin for the following experiments because this concentration not only caused meiotic arrest but also allowed proper number of oocytes to develop into the MII maturation stage (~50%) for other investigations.

2.4 | Immunofluorescence and confocal microscopy

The oocytes were first fixed in 4% paraformaldehyde (PFA) for about 30 minutes at room temperature (RT). After several washes, the oocytes were permeabilized for 20 minutes in Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 0.5% Triton X-100 and blocked for 1 hour in D-PBS containing 1% bovine serum albumin (BSA). The oocytes were then incubated in the appropriate primary antibody at 4°C overnight. Next, the oocytes were incubated for 1 hour at RT in the corresponding secondary antibody. The DNA was counterstained with 2-(4-Aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI, C1022; Beyotime Institute of Biotechnology, Shanghai,

China) for about 20 minutes. After washing, they were installed on glass slides with a little fluorescent anti-quenching agent. Representative images were captured by using a laser-scanning confocal microscope (Leica TCS SP5 II, Mannheim, Germany). Among the primary antibodies include anti-centromere CREST antibody (1:200; 15-234-0001; Antibodies Incorporated, CA), anti- α -tubulin antibody (1:500; SC-5286; Santa Cruz Biotechnology, TX) and Phalloidin (1:1000; 23 122; AAT Bioquest, CA).

2.5 | RNA extraction and quantitative real-time PCR (qRT-PCR)

In accordance with the instructions, about 30 oocytes were collected and messenger RNA (mRNA) was extracted using Dynabeads mRNA DIRECT Kit (Thermo Fisher scientific Inc, Waltham, MA, USA). HiScript II Q RT SuperMix (Vazyme Biotech Co. Ltd, Nanjing, China) was used for the generation of first-strand cDNA accord to the instruction. And AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co. Ltd, Nanjing, China) was used for qRT-PCR in an ABI 7500 Sequence Detection System. Ten pairs of primers are listed in the Table 1. Amplification was performed in 20 µL reactions containing 10 µL of SYBR Green Master Mix, 7.2 µL of RNase-free H₂O, 0.4 µL of primers (10 µmol/L), 0.4 µL of ROX Reference Dye2 and 2 µL of cDNA. The amplification conditions of PCR were as follows: reaction was initialled at 95°C for 5 mins, followed by 40 cycles of denaturing at 95°C for 10 s, annealing at 60°C

TABLE 1 Primers and amplification conditions used for qRT-PCR

Genes	Accession (NCBI)	Primer sequences	Size(bp)	Temperature (°C)
<i>Cat</i>	NM_009804.2	F: CAGCGACCAGATGAAGCAGT R: CCTCAAAGTATCCAAAAGCACCC	236	59
<i>Gpx1</i>	NM_001329528.1	F: GGAGAATGGCAAGAATGAAGA R: AGGAAGGTAAAGAGCGGGTG	135	58
<i>Prdx2</i>	NM_011563.6	F: GATGGTGCCTTCAAGGAAATCA R: CCGTGGGGCAAACAAAAGTG	97	60
<i>Prdx6</i>	NM_001303408.1	F: CGCCAGAGTTTGCCAAGAG R: TCCGTGGGTGTTTACCATTG	115	60
<i>Sod1</i>	NM_011434.1	F: GGGTTCCACGTCCATCAGTA R: TTGCCAGGTCTCCAACAT	128	59
<i>Sod2</i>	NM_013671.3	F: CAGACCTGCCTTACGACTATGG R: CTCGGTGGCGTTGAGATTGTT	113	61
<i>Bax</i>	NM_007527.3	F: ATGCGTCCACCAAGAAGCTGAG R: CCCAGTTGAAGTTGCCATCAG	166	62
<i>Bak</i>	NM_007523.3	F: CAACCCCGAGATGGACAACCTT R: CGTAGCGCCGGTTAATATCAT	101	59
<i>Caspase3</i>	NM_001284409.1	F: GACTGGGATGAACCACGACCC R: TCTGACTGAAAGCCGAAAC	205	60
<i>Caspase9</i>	NM_015733.5	F: CTGGGAAGGTGGAGTAGGAC R: GCGGTGGTGAGCAG	189	56
<i>β-actin</i>	NM_007393.5	F: TCGTGGGCCCGCCTAGGCAC R: TGGCCTTAGGGTTTACAGGGGGG	243	59
<i>Gapdh</i>	NM_001289726.1	F: GACAAAATGGTGAAGGTCGGT R: GAGGTCAATGAAGGGGTCG	120	58

for 34 s and extension at 72°C for 20 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression to β -actin or *gapdh*.

2.6 | Western blotting analysis

About 100 oocytes were lysed in the RIPA buffer supplemented with the protease inhibitor cocktail from Beyotime Institute of Biotechnology (P0013B and P1006; Shanghai, China). The target protein was first separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. Next, the membrane was blocked in TBST containing 5% BSA for 1 hour at room temperature, followed by incubation overnight at 4°C with anti-CAT rabbit polyclonal antibody (1:200), anti-BAX rabbit polyclonal antibody (1:500) and anti-ACTB rabbit polyclonal antibody (1:1000). After washing 3-5 times in TBST for 5-10 minutes, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:2000) for 1 hour at 37°C in the dark. All four antibodies were purchased from Sangon Biotech Co., Ltd. (D122036, D120073, D110001 and D110058; Shanghai, China).

2.7 | Parthenogenetic activation

Due to extremely low efficiency of fertilization in vitro using in vitro matured oocytes, the parthenogenetic activation was employed for evaluation of oocytes quality. After extruding the first polar body, 25-30 oocytes were collected and treated in pre-equilibrated Ca^{2+} -free Chatot, Ziomek, Bavister (CZB) medium containing 10 mmol/L $SrCl_2$ (A500908; Sangon Biotech, Shanghai, China) for 4-6 hours at 37°C. The hallmark of successful activation of oocytes is the appearance of pronucleus.

2.8 | Sperm binding assay

Cauda epididymal spermatozoon was isolated from male mice and then placed in human tubal fluid (HTF) medium under mineral oil, which was previously equilibrated to a density of 1×10^6 cells/mL at 37°C, 5% CO_2 atmosphere. After 1 hour of sperm capacitation, 20 μ L of the capacitated sperm solution was added to the HTF medium containing oocytes, and the final sperm concentration was 5×10^5 cells/mL, followed by incubation for 2 hours. The sperm-oocyte combinations were fixed in 4% PFA for 30 minutes and then stained with DAPI.

2.9 | Chromosome spread

The MII stage eggs were first placed in an acidic Tyrode solution (pH = 2.5) for a few seconds. After removing the zona pellucida, the oocytes were transferred to M2 medium as quickly as possible and washed for several times. Then, 25-30 oocytes were transferred to

a glass slide and fixed in an alkaline solution of 1% PFA (pH = 9.2) in distilled water containing 0.15% Triton X-100 and 3 mmol/L dithiothreitol. After the air was naturally dried, the chromosome was counterstained with propidium iodide (PI, E607306; Sangon Biotech, Shanghai, China) and visualized by laser-scanning confocal microscopy.

2.10 | Mitochondrial distribution

Mitochondria were stained by using MitoTracker Deep Red (M22426; Invitrogen, UK) to assess the distribution of mitochondria in oocytes. The oocytes were incubated in a pre-warmed staining solution for 30 minutes at 37°C. After washing, the oocytes were counterstained with DAPI to visualize the nucleus. The image was observed and captured using a laser-scanning confocal microscope. Mitochondria distribution manner were separated into three typical groups include homogenous, perinuclear and clustering, according to Sun et al's description.⁴⁶

2.11 | Mitochondrial membrane potential ($\Delta\psi_m$) assay

To measure mitochondrial membrane potential, oocytes were washed three times with PBS and incubated in culture medium containing 0.5 μ mol/L JC-1 (Invitrogen, Grand Island, NY, USA) at 37°C in 5% CO_2 for 30 minutes. Membrane potential was calculated as the ratio of red fluorescence, which corresponded to activated mitochondria (J-aggregates), to green fluorescence, which corresponded to less-activated mitochondria (J-monomers).⁴⁷ Fluorescence was visualized by laser-scanning confocal microscopy.

2.12 | Adenosine 5'-triphosphate (ATP) content detection

Detection of ATP content was followed the manual of the Enhanced ATP Assay Kit (S0027; Beyotime Institute of Biotechnology, Shanghai, China). Briefly, a same number ($n = 15$) of oocytes obtained from each group were lysed in 4 μ L water by ultrasonic, followed by mixed with 16 μ L of detection solution. After 15 minutes of reaction, the ATP concentration was measured using a luminometer (Berthold, Wildbad, Germany) with a sensitivity of 0.01 pM. The ATP concentration in the control group was arbitrarily set to 1. Three separate experiments were performed with three replicates in each.

2.13 | ROS generation detection

Reactive Oxygen Species Assay Kit (S0033; Beyotime Institute of Biotechnology, Shanghai, China) was performed to detect ROS

content of oocytes. Concisely described as incubation with dichlorofluorescein diacetate (DCFH-DA) probe in the dark at 37°C for 20 minutes, after washing, the oocytes were installed on a glass slide and observed under a laser-scanning confocal microscope with the same scanning parameters for all measurements.

2.14 | Annexin V staining

Apoptosis detection was performed by using the Annexin V-FITC Apoptosis Kit (C1063; Beyotime Institute of Biotechnology, Shanghai, China). Oocytes were stained with 5 μ L of Annexin V-FITC diluted in 195 μ L of binding buffer for 15 minutes at RT in the dark. After washing, the oocytes were mounted on a glass slide to monitor the fluorescence signal and images were captured by using a laser-scanning confocal microscope with the same scanning parameters.

2.15 | Fluorescence intensity analysis

All images were captured using the same scan parameters when performing fluorescence intensity analysis. The average fluorescence intensity per unit area within the region of interest was analysed by using ImageJ software (NIH, Bethesda, MD).

2.16 | Statistical analysis

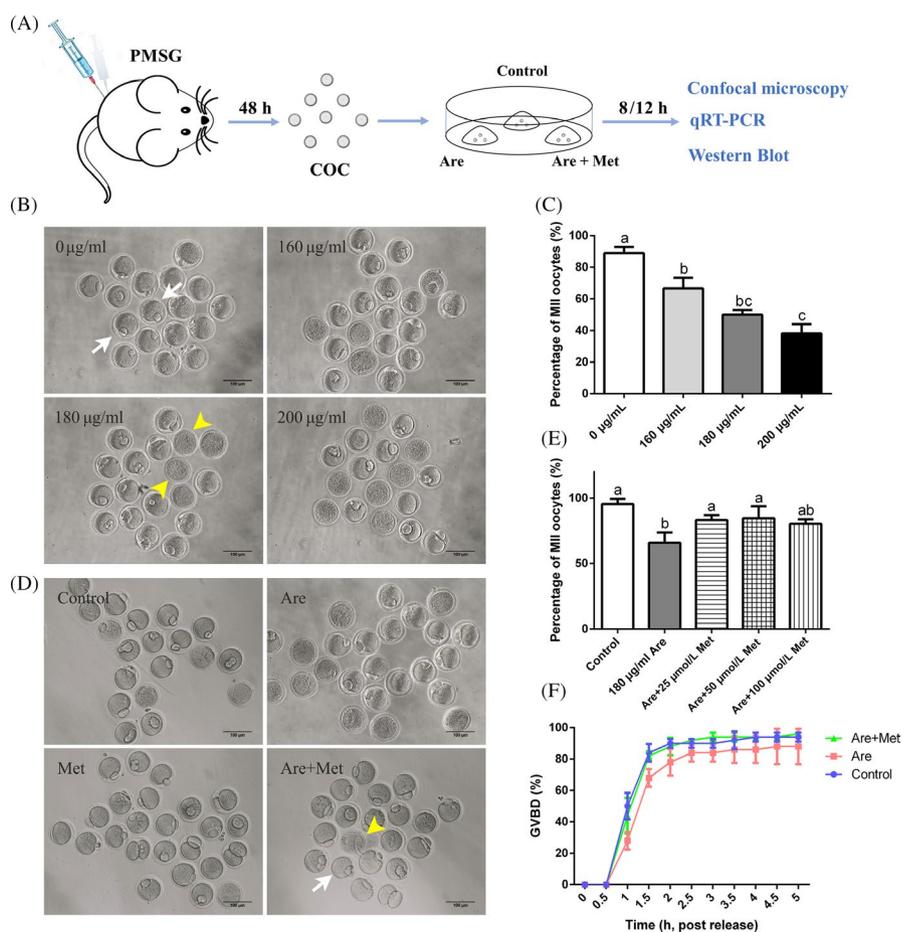
Each experiment was repeated at least 3 times, each group involving 25-30 oocytes. Data were taken as mean \pm standard deviation and analysed by one-way ANOVA using SPSS software. Differences were considered to be statistically significant, $P < .05$.

2.17 | Experiment design

Experiment 1: Metformin improves the meiotic maturation in arecoline-exposed oocytes. To verify whether arecoline toxic to oocytes, as shown in Figure 1A, we obtained COCs from mice 48 hours after PMSG injection, and we counted the first polar body exclusion rate after 12 hours of culture by setting three sets of concentration gradients: 160, 180 and 200 μ g/mL. To verify whether metformin has a certain rescue function for the damage caused by arecoline, oocytes were randomly assigned into three groups: control, arecoline and arecoline-metformin groups. The concentrations of metformin were 25, 50 and 100 μ mol/L, respectively. According to the results, we selected 180 μ g/mL arecoline and 50 μ mol/L metformin for the following experiments.

Experiment 2: Metformin restores the actin dynamics, spindle defects and chromosome misalignment in arecoline-exposed oocytes. The collapsed cytoskeleton was first considered to explain the cause of

FIGURE 1 Effects of metformin (Met) on the meiotic maturation in the arecoline (Are)-exposed oocytes. A, Experimental process overview. B, Representative images of oocytes after 12 h of culture were treated with Are. C, The rates of first polar body extrusion after Are treatment. D, Representative images of oocytes in the control, Are and Are + Met groups. E, The rates of first polar body extrusion in the control, Are and Are + Met groups. F, The rates of germinal vesicle breakdown. Scale bar = 100 μ m. White arrow shows the MII stage oocyte with the first polar body, and yellow arrowhead shows the oocyte that has undergone apoptosis. Data were presented as mean \pm SD. ^{a-c}Means not sharing a common superscript are different ($P < .05$)



the first polar body failed to extrude. Therefore, we next evaluated the two most basic oocyte cytoskeletal proteins in actin filaments and spindle microtubules by fluorescence staining.

Experiment 3: Metformin maintains euploidy by restoring defective kinetochore-microtubule (K-M) attachment in arecoline-exposed oocytes. In general, the failure of the assembly of the spindle tends to impair the stability of the K-M attachment, which in turn causes oocyte aneuploidy. Immediately, we studied the co-localization of spindles, chromosomes and centromeres and the karyotype of oocytes.

Experiment 4: Metformin elevates the rate of homogeneous mitochondrial distribution, ATP content and mitochondrial membrane potential in arecoline-exposed oocytes. To explore the causes of cytoskeletal collapse at a deeper level, we examined the distribution of mitochondria and determined the amount of ATP production, as insufficient energy supply also led to failure of actin filament and spindle assembly. However, the reduction in ATP content is often caused by damage to mitochondria. Therefore, we next analysed the mitochondrial membrane potential of oocytes, a landmark indicator of mitochondrial damage.

Experiment 5: Metformin reduces ROS level and limits early apoptosis in arecoline-exposed oocytes. Mitochondrial dysfunction is often accompanied by the production of large amounts of ROS, which in turn leads to apoptosis in oocytes. To test this hypothesis, we first examined changes in reactive oxygen species and early apoptosis by fluorescence staining. Secondly, in order to further explore the underlying mechanism of apoptosis, we quantified apoptosis-related genes and proteins, and revealed that arecoline has attenuated oocyte apoptosis by activating the caspase signalling pathway. However, metformin could partially reduce these damages, suggesting that metformin has a protective effect against the apoptosis of oocytes induced by arecoline.

3 | RESULTS

3.1 | Metformin improves the meiotic maturation in arecoline-exposed oocytes

Firstly, we investigated the effects of arecoline exposure on the meiotic progression of mouse oocytes. Increasing concentrations of arecoline (160, 180 and 200 $\mu\text{g}/\text{mL}$) were supplemented to M16 culture medium to detect the first polar body extrusion (PBE). As shown in Figure 1B, most oocytes reached the metaphase II (MII) stage with the extrusion of the polar body in the control group ($89.02 \pm 3.75\%$, $n = 305$). However, arecoline exposure resulted in oocyte meiosis arrest with the prominently decreased PBE (160 $\mu\text{g}/\text{mL}$: $66.67 \pm 6.73\%$, $n = 115$, $P < .05$, 180 $\mu\text{g}/\text{mL}$: $50.00 \pm 3.09\%$, $n = 317$, $P < .01$, 200 $\mu\text{g}/\text{mL}$: $38.22 \pm 5.79\%$, $n = 117$, $P < .01$), which indicated that arecoline exposure inhibits meiotic maturation of mouse oocytes in a dose-dependent manner (Figure 1C). Based on the results, we chose 180 $\mu\text{g}/\text{mL}$ arecoline for subsequent experiments because this concentration not only significantly reduced the maturation rate compared to

the control, but also allowed the appropriate number of oocytes to develop into the MII stage for other studies.

To evaluate whether metformin could alleviate the meiotic arrest caused by arecoline, metformin was added into the 180 $\mu\text{g}/\text{mL}$ arecoline-supplemented M16 to yield a final concentration of 25, 50 and 100 $\mu\text{mol}/\text{L}$, respectively. Quantitative analysis revealed that 50 $\mu\text{mol}/\text{L}$ metformin increased significantly the PBE ($84.69 \pm 9.03\%$, $n = 616$, $P < .05$) compared with the exclusive treatment with arecoline (Figure 1D and E; $65.83 \pm 8.04\%$, $n = 247$). Higher concentration of metformin did not visibly promote the PBE rate. According to the results, we selected 50 $\mu\text{mol}/\text{L}$ metformin for the further exploration. In addition, we have also detected the germinal vesicle breakdown (GVBD), another key step in the progression of meiosis. The results indicated that although arecoline caused a slight delay in the occurrence of GVBD, there was no statistical difference compared with the control (Figure 1F).

3.2 | Metformin partially enhances the ability of sperm binding to zona pellucida and parthenogenetic activation in arecoline-exposed oocytes

The study of sperm binding ability is important for the evaluation of the fertilization potential of oocytes. Thus, we counted the number of sperm binding to the zona pellucida surrounding unfertilized eggs by DAPI staining. As shown in Figure S1A and B ($n = 194$), although arecoline did not markedly reduce the number of sperm binding to the zona pellucida compared to the control, the metformin-rescued group significantly elevated the number of bound spermatozoa compared with the arecoline-exposed group.

Parthenogenetic activation is great method for evaluation of embryo development. Then, we calculated the rate of development to blastocysts after parthenogenetic activation of MII stage eggs. The results showed that blastocyst rate dramatically decreased in arecoline-exposed group compared with control. However, metformin did not rescue the embryo development in the presence of arecoline (Figure S1C and D; $77.04 \pm 6.12\%$, $n = 192$ vs $43.18 \pm 3.35\%$, $n = 219$, $P < .01$ vs. $52.88 \pm 1.18\%$, $n = 210$, $P > .05$). Taken together, these observations implied that metformin could improve partially the ability of sperm binding to zona pellucida and parthenogenetic activation.

3.3 | Metformin restores the actin dynamics in arecoline-exposed oocytes

Actin filaments play a key role in cortical polarization and asymmetric spindle localization during oocyte maturation. To determine whether actin dynamics was involved in arecoline-induced meiosis arrest, phalloidin-TRITC was applied to stain the F-actin. As shown in Figure 2A, actin filaments were evenly accumulated on the plasma membrane with robust fluorescent signal in the control oocytes. In contrast, the arecoline-exposed oocytes displayed intermittent distribution of actin filaments with faded fluorescent signals, and

FIGURE 2 Effects of metformin on the actin dynamics in the arecoline-exposed oocytes. A, Representative pictures of actin filaments (Red) in the control, Are and Are + Met groups. Scale bar = 25 μ m. B, The relative fluorescence intensity of F-actin in the control, Are and Are + Met groups. DIC, differential interference contrast. Data were presented as mean \pm SD. ^{a-b}Means not sharing a common superscript are different ($P < .05$)

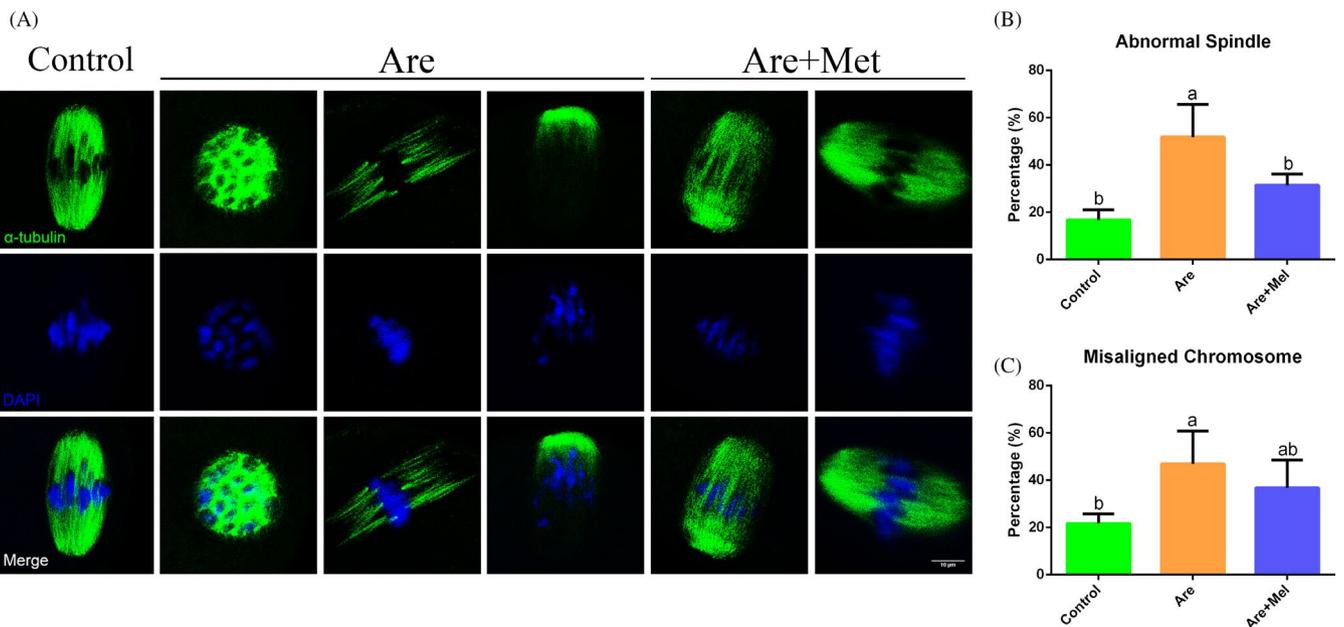
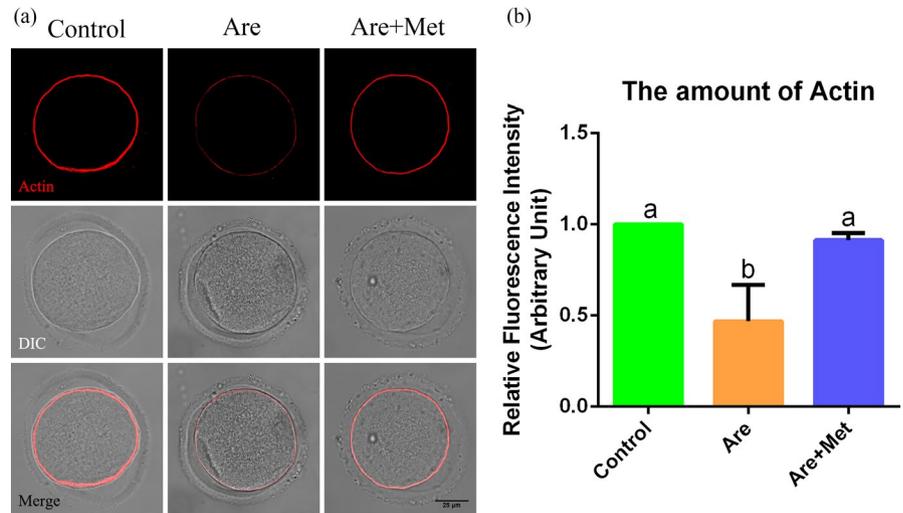


FIGURE 3 Effects of metformin on the spindle assembly and chromosome alignment in the arecoline-exposed oocytes. A, Representative pictures of spindle morphologies and chromosome alignment in the control, Are and Are + Met groups. Spindle (green) and chromosome (blue). Scale bar = 10 μ m. B, The rates of aberrant spindles in the control, Are and Are + Met groups. C, The rates of misaligned chromosomes in the control, Are and Are + Met groups. Data were presented as mean \pm SD. ^{a-b}Means not sharing a common superscript are different ($P < .05$)

these could be rescued to a level indistinguishable from the controls (Figure 2B; $n = 222$), implying that metformin prevented the actin dynamics from damage induced by arecoline.

3.4 | Metformin rescued the spindle defects and chromosome misalignment in arecoline-exposed oocytes

Due to meiotic failure of oocytes is highly correlated with defective spindle assembly and chromosome alignment, we next detected the spindle apparatus formation in metaphase I (MI) stage oocytes. About 8 hours after retrieval, oocytes in MI were immunostained

with anti- α -tubulin-FITC antibody to label the spindle organization and counterstained with DAPI to visualize the chromosome. The results showed that the majority of oocyte in control group exhibited a typical barrel-shaped spindle with a well-aligned chromosome on the equatorial plate (Figure 3A, left). However, a higher frequency of various morphology-aberrant spindles with misaligned chromosomes was present in arecoline-exposed oocytes (Figure 3A, middle). As expected, metformin treatment in arecoline-exposed oocytes reduced partially the abnormal rates (Figure 3A, right and Figure 3B and C; $n = 576$; abnormal spindle: $17.36 \pm 3.72\%$, $n = 172$ vs. $51.86 \pm 13.77\%$, $n = 268$ vs. $31.47 \pm 4.70\%$, $n = 182$, $P < .01$; misaligned chromosome: $22.03 \pm 3.26\%$, $n = 164$ vs. $46.88 \pm 13.90\%$, $n = 176$ vs. $36.77 \pm 11.73\%$, $n = 190$, $P < .05$). All these results

illustrated metformin could restore the dynamic stability of the oocyte cytoskeleton.

3.5 | Metformin maintains euploidy by restoring defective kinetochore-microtubule attachment in arecoline-exposed oocytes

Abnormal spindle formation and chromosome alignment are often associated with the aneuploidy which is usually caused by the defective K-M attachment. Thus, we tested the stability of K-M attachments. As shown in Figure 4A, the kinetochore and microtubule were completely attached in control group. The arecoline-exposed oocytes showed defective K-M attachment. However, the rate of aberrant K-M attachments caused by arecoline in metformin-rescued group reduced to an indistinguishable level compared with the control (Figure 4A and B; $20.21 \pm 6.68\%$, $n = 156$ vs. $37.10 \pm 7.60\%$, $n = 150$ vs. $22.35 \pm 3.67\%$, $n = 164$, $P < .05$).

In addition, we analysed the karyotype of MII eggs by chromosome spread assay. Aneuploidy is defined as less than or more than 20 univalents. The results indicated that aneuploidy rate obviously raised in arecoline-exposed oocytes compared to control, with statistical differences, whereas metformin partially reduced the rate of aneuploidy induced by arecoline (Figure 4C and D; $13.18 \pm 3.83\%$, $n = 134$ vs. $35.68 \pm 5.14\%$, $n = 164$ vs. $25.31 \pm 2.15\%$, $n = 148$, $P < .01$). All these data suggested that arecoline exposure enhanced aneuploidy by inducing cytoskeletal defects, ultimately leading to oocyte meiosis failure, and metformin treatment could break this arrest and restore the meiotic progression of mouse oocytes.

3.6 | Metformin elevates the rate of homogeneous mitochondrial distribution, ATP content and mitochondrial membrane potential in arecoline-exposed oocytes

The functional integrity of mitochondria is critical for oocyte meiosis and considered to be one of the key indicators of oocyte cytoplasmic maturation. We then detected the mitochondrial distribution of MII stage eggs to research whether arecoline exposure would result in mitochondrial dysfunction. As shown in Figure 5A, three typical images from left to right represented homogenous, perinuclear and cluster distribution of mitochondria, respectively. Compared with the control oocytes, arecoline notably reduced the rate of homogenous distribution, which could be restored in metformin-rescued oocyte (Figure 5B; homogenous distribution: $78.33 \pm 4.41\%$, $n = 186$ vs. $45.06 \pm 8.37\%$, $n = 186$ vs. $78.38 \pm 9.14\%$, $n = 192$, $P < .01$).

ATP content is closely related to mitochondrial function. Next, we examined the ATP content of oocytes in different treatment groups. Not surprisingly, arecoline did significantly decreased the ATP content of oocytes compared to the control, while metformin returned ATP to normal levels after supplementation (Figure 5C; $n = 405$). These results showed metformin could increase ATP production by repairing mitochondrial dysfunction in arecoline-exposed oocytes.

To exploring the mechanism of ATP reduction, mitochondrial membrane potential in each group was detected. The results revealed that mitochondrial $\Delta\psi_m$ sharply decreased after arecoline treatment (Figure 5D and E; $n = 375$). In addition, the reduction of $\Delta\psi_m$ could be present in the presence of metformin.

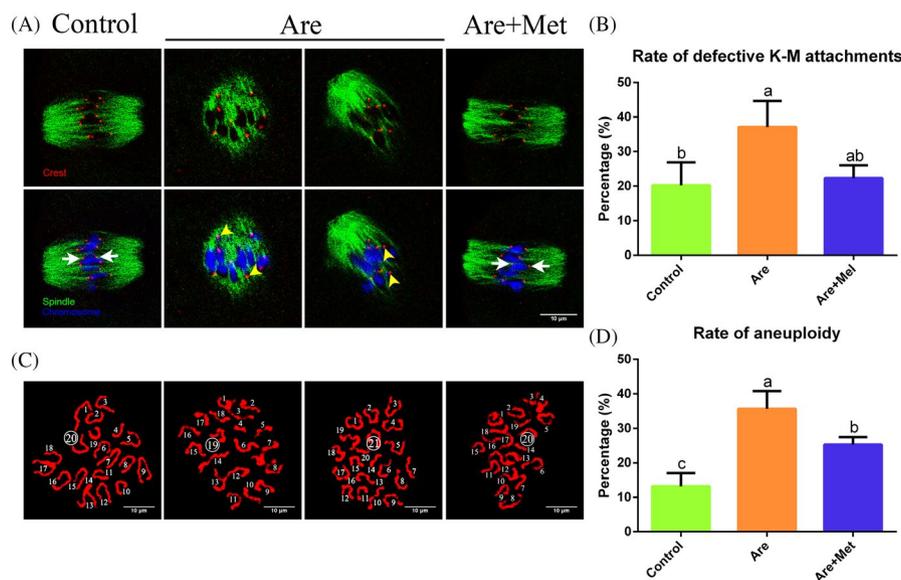


FIGURE 4 Effects of metformin on the kinetochore-microtubule (K-M) attachment and karyotype in the arecoline-exposed oocytes. A, Typical images of K-M attachment in the control, Are and Are + Met groups. Kinetochore (red), microtubule (green) and chromosome (blue). The white arrow represents normal attachments; the yellow arrowhead represents abnormal attachments. B, The rates of defective K-M attachments in the control, Are and Are + Met groups. C, Typical images of euploidy (20 univalents) and aneuploidy (less or more than 20 univalents). D, The rates of aneuploid oocytes in the control, Are and Are + Met groups. Scale bar = 10 μ m. Data were presented as mean \pm SD. ^{a-c}Means not sharing a common superscript are different ($P < .05$)

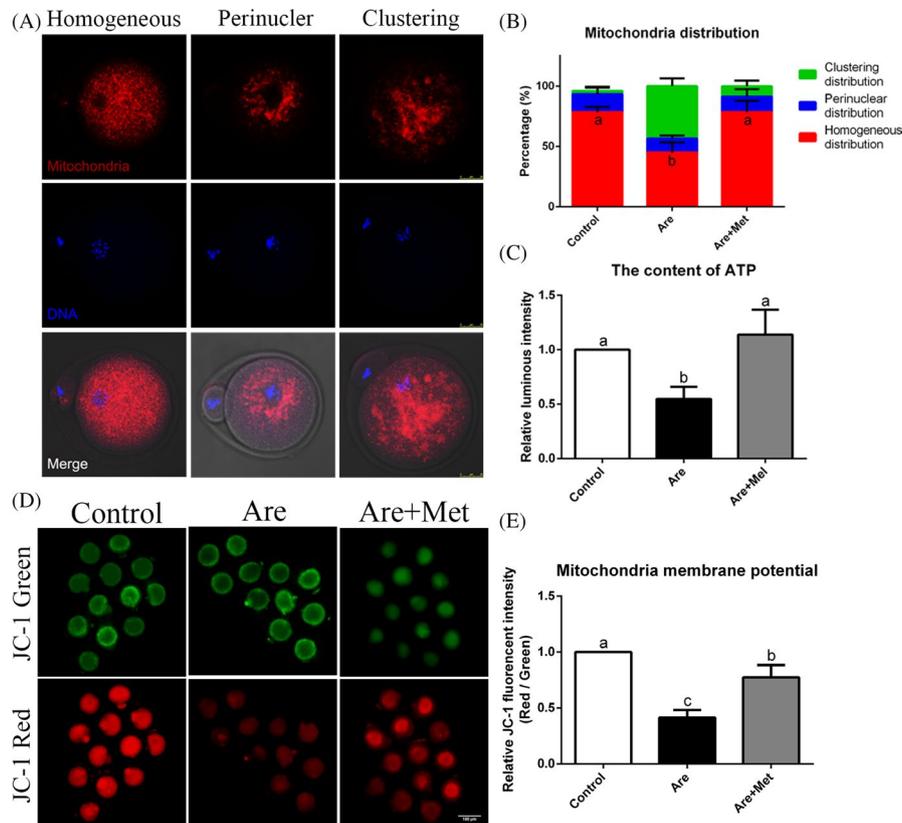


FIGURE 5 Effects of metformin on mitochondrial distribution, ATP content and mitochondrial membrane potential in the arecoline-exposed oocytes. A, Representative photographs of homogenous, perinuclear and clustering distribution of mitochondria in mouse oocytes. Mitochondria (red) and chromosome (blue) were shown. Scale bar = 25 μ m. B, The rates of homogeneous, perinuclear and clustering mitochondrial distribution pattern. C, The content of ATP in the control, Are and Are + Met groups. D, Mitochondrial membrane potential was calculated as the ratio of red fluorescence, which corresponds to activated mitochondria (J-aggregates), to green fluorescence, which corresponds to less-activated mitochondria (J-monomers). Scale bar = 100 μ m. E, Relative fluorescence intensity of the mitochondrial membrane potential in the control, Are and Are + Met groups. Data were presented as mean \pm SD. ^{a-b}Means not sharing a common superscript are different ($P < .05$)

3.7 | Metformin reduces ROS level and limits early apoptosis in arecoline-exposed oocytes

Furthermore, oxidative stress is usually induced by mitochondrial dysfunction, which speeds up the progression of apoptosis. Thus, we investigated the extent of ROS generation in arecoline-exposed and metformin-rescued oocytes. As shown in Figure 6A, the oxidative stress signal of the arecoline-exposed oocytes was significantly increased compared with the control, while obviously reduced in metformin-rescued oocytes. Fluorescence intensity analysis was also consistent with the above observations (Figure 6B; $n = 574$), which was further confirmed by qRT-PCR (Figure 6C; $n = 520$) and Western blot (Figure 6G, upper panel; $n = 453$).

Cumulative studies have shown that excessive ROS could induce oocyte apoptosis, and we next explored the early apoptosis of oocytes. It is worth noting that only oocytes with green fluorescent signals in both the zona pellucida and the cytoplasmic membrane are considered to have experienced early apoptosis.⁴⁸ Our results showed that the number of oocytes undergone early apoptosis in the arecoline-exposed group was raised dramatically, whereas the

metformin-rescued group was reduced to a level indistinguishable from the control group (Figure 6D and E; $24.51 \pm 3.88\%$, $n = 201$ vs $63.28 \pm 6.34\%$, $n = 207$ vs $36.75 \pm 3.84\%$, $n = 186$, $P < .01$). In addition, the results of qPCR (Figure 6F; $n = 330$) and immunoblotting (Figure 6G, lower panel; $n = 369$) showed apoptosis-related genes and protein expression levels, which further verified the above results. The data suggested that arecoline induced a decrease in ATP production and increased ROS synthesis by disrupting mitochondrial integrity, ultimately leading to oocyte apoptosis. Surprisingly, metformin administration partially relieved these damages caused by arecoline exposure.

4 | DISCUSSION

In the present study, we found arecoline displayed extremely toxicity effect on mammalian oocytes maturation via regulating ROS level, further injuring mitochondrial function. Many studies revealed that metformin could reduce ROS content in several kinds of cells, and display positive effects in oocytes maturation and further embryonic development.⁴³⁻⁴⁵ According to the information, we

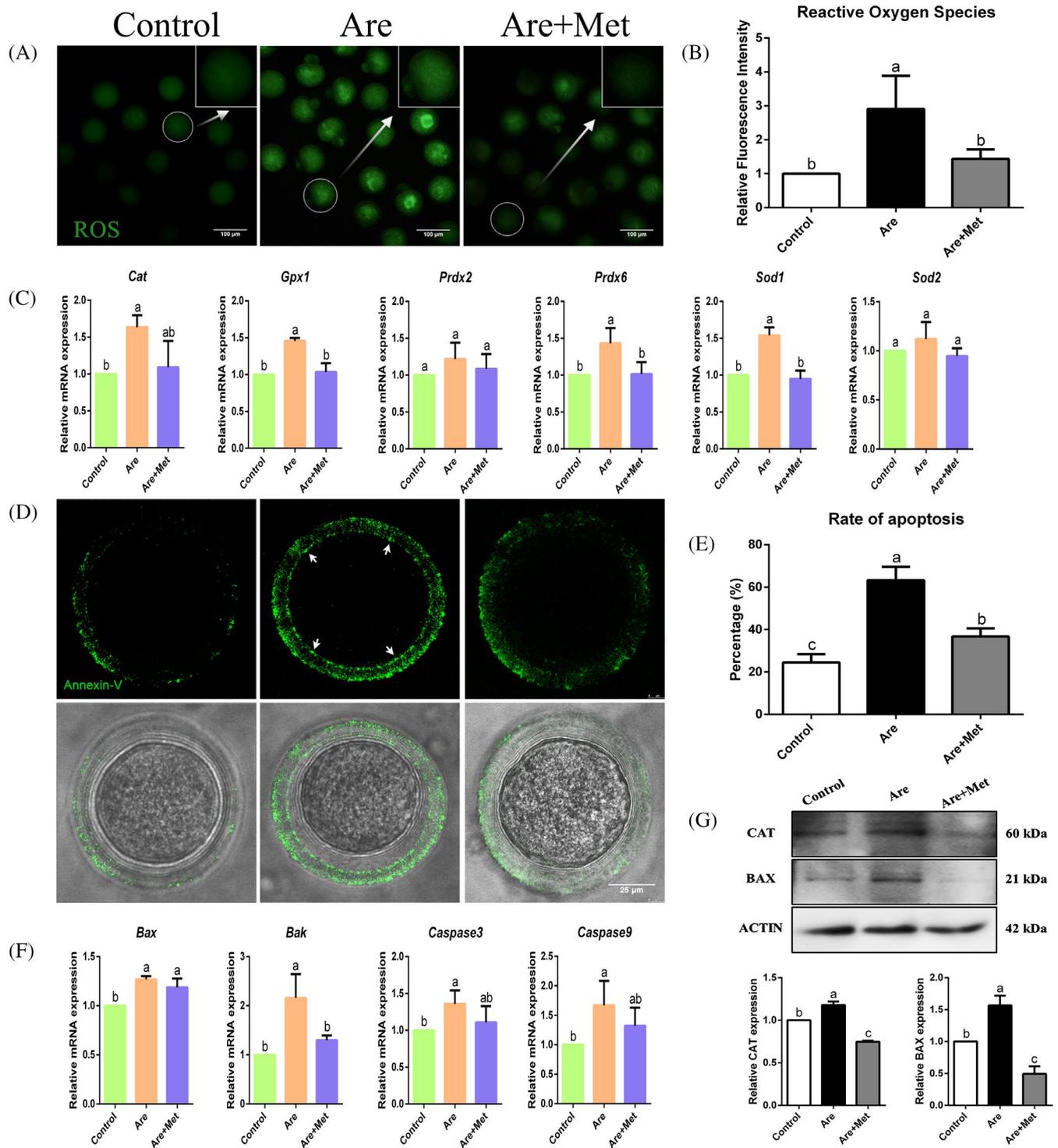


FIGURE 6 Effects of metformin on oxidative stress and early apoptosis in the arecoline-exposed oocytes. A, Representative photographs of ROS levels (green) in the control, Are and Are + Met groups. Scale bar = 100 μ m. B, Relative fluorescence intensity of ROS in the control, Are and Are + Met groups. C, Relative expression of oxidative stress-related genes, including *Cat*, *Gpx1*, *Prdx2*, *Prdx6*, *Sod2* and *Sod1*. D, Representative photographs of early apoptotic oocytes in the control, Are and Are + Met groups. Oocytes with fluorescence signals (green) at both membrane (white arrow) and zona pellucida are regarded as early-stage apoptosis. Scale bar = 25 μ m. E, The rate of early apoptosis oocytes in the control, Are and Are + Met groups. F, Relative expression of apoptosis-related genes, including *Bax*, *Bak*, *Caspase3* and *Caspase9*. (g) Relative level of oxidative stress and apoptosis-related proteins, including CAT and BAX. Data were presented as mean \pm SD. ^{a-c}Means not sharing a common superscript are different ($P < .05$)

hypothesized that metformin could rescue arecoline-induced oocyte cytotoxicity.

To confirm our hypothesis, we first examined the effect of arecoline on GVBD and the PBE of mouse oocytes, which is a sign of meiotic resumption⁴⁹ and nuclear maturation,⁵⁰ respectively. Oocyte maturation is the key event of life initial. Poor quality of matured oocytes directly results in failure of embryo development, lower pregnancy rate and abnormal offspring.⁵¹ Oocyte maturation includes nuclear maturation, which marked as the 1st polar body extrusion, and cytoplasm maturation, which marked as mono-spermic penetration, nuclear reprogramming and embryonic development.⁵²

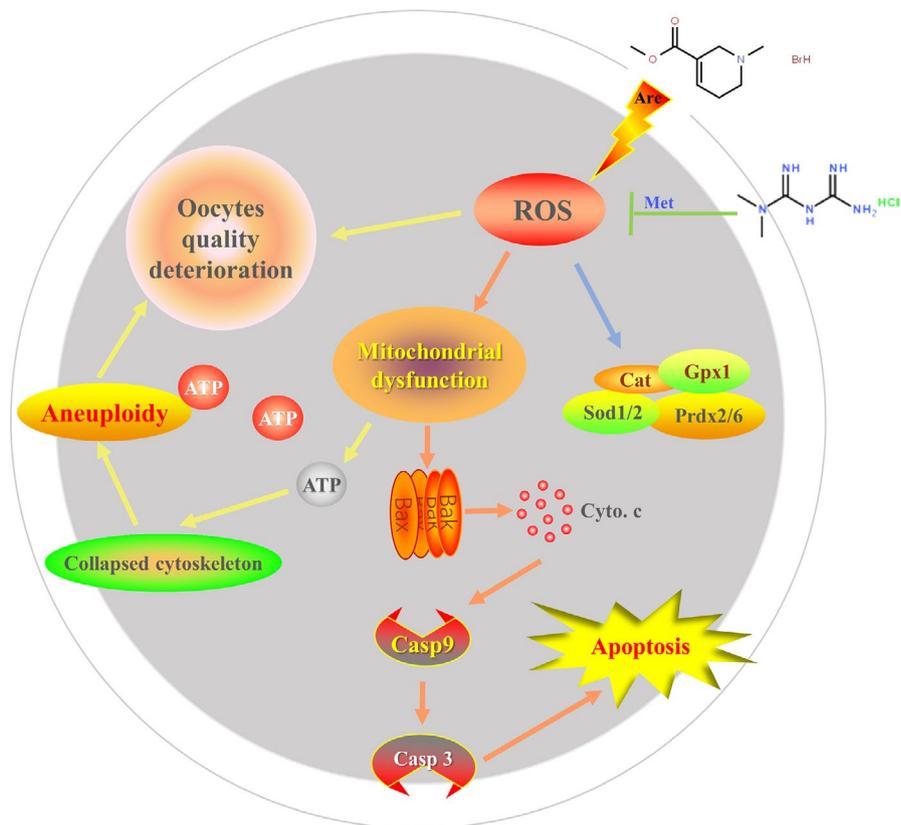
Nuclear maturation is primarily involved in chromosome segregation, which is easily detected by the PBE. Therefore, the PBE failure indicates abnormal chromosome separation, which is usually caused by a damaged cytoskeleton. In oocytes, actin filaments and microtubules are the most important components of the cytoskeleton and play a key role in ensuring proper chromosome segregation during meiosis.⁵³ Studies have shown that abnormal spindle assembly and chromosomal misalignment can impair female fertility in different species.⁵⁴ Our data indicated that arecoline disrupts actin filament dynamics and spindle assembly, suggesting that arecoline destroys cytoskeletal integrity. Compared to the control, metformin partially or completely restored the actin dynamics, spindle defects and chromosomal misalignment in the arecoline-exposed oocytes to indistinguishable levels. Thus, metformin safeguards the cytoskeletal integrity of the arecoline-exposed oocytes and ensures nuclear maturation during oocyte meiosis.

Abnormal cytoskeleton, especially spindle defects and chromosomal misalignment, is always accompanied by errors in K-M attachment, which usually results in oocyte aneuploidy.⁵⁵ Therefore, we examined the K-M attachment stability and euploidy in arecoline-exposed oocytes. The results indicate that arecoline perturbs the stability of K-M attachment and obviously enhances the aneuploidy of oocytes, but these could be rescued by metformin treatment. Therefore, arecoline exposure enhanced aneuploidy by inducing cytoskeletal defects, ultimately leading to oocyte meiosis failure, and metformin treatment could break this arrest and restore the meiotic progression of mouse oocytes.

Arecoline treatment caused widely abnormal on oocyte maturation in the present study, which revealed that arecoline disturbed some basic cell function, further results in the abnormal of nuclear and cytoplasmic dynamics. Almost all cell event associated with energy supply. Metabolic and oxidation respiration are the basic characteristics of life. According to this, we doubt that arecoline inhibit the function of mitochondrial, further induced abnormal of maturation.

Mitochondria are important organelles to produce ATP in oocytes, and their functional integrity is critical for oocyte meiosis and is considered as a key indicators of oocyte cytoplasmic maturation. Mitochondria disperse from the perinuclear area to the cytoplasm during meiotic maturation of oocytes, and their failure to migrate usually marks a failure of cytoplasmic maturation. So next, we examined the distribution of mitochondria in the arecoline-exposed oocytes and then tested the ATP content. The results confirmed that metformin could increase ATP production by repairing mitochondrial

FIGURE 7 Potential mechanism of metformin-mediated improvement of mouse oocyte apoptosis defects induced by arecoline. The arecoline leads to mitochondrial dysfunction by reducing the mitochondrial membrane potential of oocytes. On the one hand, it will cause a decrease in the production of ATP to reduce the energy supply, leading to the collapse of the oocyte cytoskeleton, which in turn causes aneuploidy, resulting in a deterioration in oocyte quality. On the other hand, a decrease in mitochondrial membrane potential causes the release of cytochrome c, which activates the caspase signalling pathway and ultimately leads to oocyte apoptosis. However, metformin could partially reduce these damages, suggesting that metformin has a protective effect against the apoptosis of oocytes induced by arecoline



dysfunction in the arecoline-exposed oocytes. In addition, studies have found that excessive oxidative stress can impede cytoplasmic maturation and embryonic development of oocytes.^{56,57} At the same time, ROS accumulation leads to mitochondrial dysfunction and induces oocyte apoptosis. Our data confirmed that arecoline induces a large amount of ROS, resulting in significant apoptosis of oocytes. And these could be restored after metformin supplementation. These results reveal that arecoline induces a decrease in ATP production and an increase in ROS synthesis by disrupting mitochondrial integrity, ultimately leading to oocyte apoptosis. Fortunately, metformin administration partially alleviates these damages caused by arecoline exposure.

To further explore the specific mechanism of apoptosis caused by arecoline, we examined the mitochondrial membrane potential of oocytes, and the results showed that arecoline significantly reduced the membrane potential of mitochondria. Our previous studies have shown that the decrease of mitochondrial membrane potential in oocytes can cause the release of cytochrome *c*, which in turn activates the up-regulation of intracellular apoptosis-related caspase and leads to apoptosis of oocytes.^{27,58,59} To establish whether this same apoptotic mechanism also occurs in arecoline-exposed oocytes, the expression levels of oxidative stress and apoptosis-related genes and proteins further were validated. Our results clearly revealed that caspase signalling pathway-related genes and proteins *Bax*, *Bak*, *Caspase9* and *Caspase3* were significantly elevated compared with the control group.

Embryonic development which is related with complex biological process includes genome reprogramming⁶⁰ and zygote genomic activation.⁶¹ Although metformin successfully rescued the nuclear maturation of mouse oocytes in the presence of arecoline, the embryo development after parthenote activation is still very poor. The cause for this result may be that the cytoplasmic damage caused by arecoline to oocytes did not been completely recovered by metformin, that is, the cytoplasm did not fully matured. The results indicated that arecoline treatment caused seriously and widely damage on oocyte. However, the deep molecular mechanism should be explored in the future.

Mechanism of metformin on ROS content and oocyte quality regulation has been well explored. Previous studies reported that metformin inhibits the activity of complex I (NADH) of the mitochondrial respiratory chain,⁶²⁻⁶⁵ further reduced production of ROS. This inhibition next led to activation of the AMP-activated protein kinase (AMPK) complex.⁶⁶ AMPK was a key cellular energy sensor to maintain the cellular energy homeostasis. Following AMPK activation, the catabolism pathway for energy production was initiated, including stimulating cellular glucose uptake and mitochondrial biosynthesis, and facilitating glycolysis, β -oxidation of fatty acids and oxidative phosphorylation.⁶⁷ This compensation mechanism was designed to restore sufficient energy to maintain cellular homeostasis.⁶⁸⁻⁷⁰

During maturation, oocytes obtain most of their energy from cumulus cells, which metabolize glucose through glycolysis to produce lactate and pyruvate, the preferred substrates for oocytes.⁷¹ Metformin has been reported to stimulate human granulosa cells to

produce lactic acid,⁷² which can improve oocyte development and increase metabolic substrates for ATP production. Moreover, lipid was also an important energy substance for oocytes. Metformin can increase the usage of lipids and β -oxidation of fatty acids, which was essential for mouse oocyte maturation and early embryo development.^{34,73}

In conclusion, as shown in Figure 7, our study demonstrates that arecoline leads to mitochondrial dysfunction by reducing the mitochondrial membrane potential of oocytes. Furthermore, the dysfunction of mitochondria decreases the production of ATP, leading to the collapse of the oocyte cytoskeleton, which in turn causes aneuploidy, resulting in a deterioration of oocyte quality. On the other hand, damage of mitochondria membrane causes the release of cytochrome *c*, which activates the caspase signalling pathway and ultimately leads to oocyte apoptosis.⁷⁴ Fortunately, metformin could partially reduce these damages, suggesting that metformin has a protective effect against the apoptosis of oocytes induced by arecoline.

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CONFLICT OF INTEREST

There is no conflict of interest in the declaration.

AUTHORS' CONTRIBUTIONS

WL, MZ and QS provided key intellectual input in the conception and design of these studies. WL and MZ wrote the manuscript. WL and CZ performed all experiments. SY and WS contributed to the writing of the manuscript. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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