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SCM-198 ameliorates the quality of postovulatory and maternally aged oocytes by reducing oxidative stress

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Abstract

Oocyte aging is a key constraint on oocyte quality, leading to fertilization failure and abnormal embryonic development. In addition, it is likely to generate unfavorable assisted reproductive technology (ART) outcomes. SCM-198, a synthetic form of leonurine, was found to rescue the rate of oocyte fragmentation caused by postovulatory aging. Therefore, the aim of this study was to conduct a more in-depth investigation of SCM-198 by exploring its relationship with aged oocytes after ovulation or maternal aging and clarifying whether it affects cell quality. The results indicate that, compared to the postovulatory aged group, the 50 μM SCM-198 group significantly improved sperm-egg binding and increased fertilization of aged oocytes, restoring the spindle apparatus/chromosome structure, cortical granule distribution, and ovastacin and Juno protein distribution. The 50 μM SCM-198 group showed significantly normal mitochondrial distribution, low levels of reactive oxygen species (ROS), and a small quantity of early oocyte apoptosis compared to the postovulatory aged group. Above all, in vivo supplementation with SCM-198 effectively eliminated excess ROS and reduced the spindle/chromosome structural defects in aged mouse oocytes. In summary, these findings indicate that SCM-198 inhibits excessive oxidative stress in oocytes and alters oocyte quality both in vitro and in vivo.

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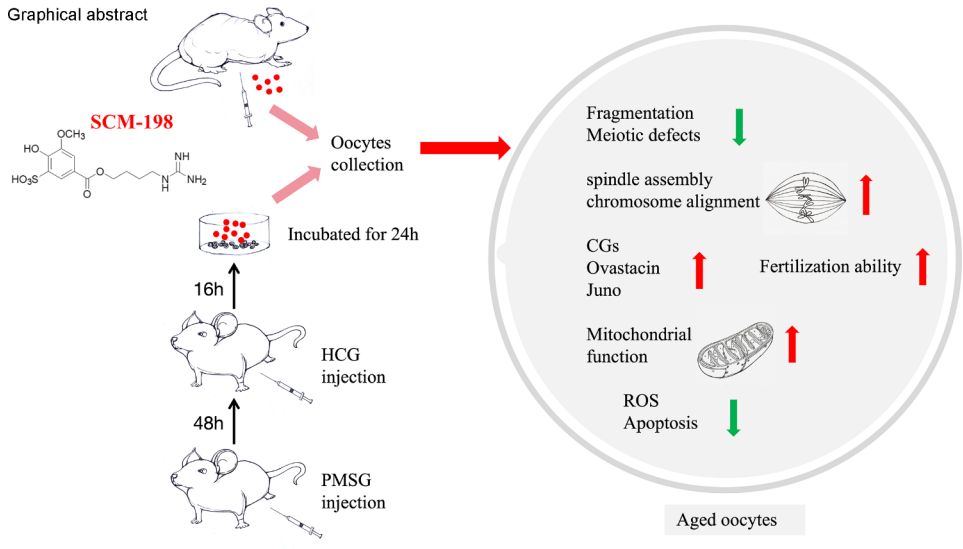
Graphical Abstract



Keywords Apoptosis, Oxidative stress, Oocyte quality, Postovulatory aging, SCM-198

Introduction

Postovulatory aging is defined as oocytes undergoing time-dependent degradation [1]. Postovulatory aging of oocytes leads to inadequate oocyte quality, reduces fertilization rates, reduces embryo viability, and abnormalities in offspring [2, 3]. Postovulatory aging leads to decreased oocyte quality, including increased oocyte fragmentation and apoptosis, sclerosis of the oocyte zona pellucida; and impaired function of oocyte organelles, such as spindles and mitochondria. It also leads to increased sensitivity of oocytes to external stimuli and decreased fertilization and subsequent embryonic development [4]. Postovulatory aging is divided into in vivo and in vitro aging according to the environment in which it occurs. In vitro, aging occurs mainly during oocyte culture. In addition, assisted reproductive technology (ART) is closely linked to the in vitro culture of oocytes. Most poor oocyte quality due to aging can be explained by the initiation of oxidative stress [5]. Therefore, during the in vitro culture of oocytes, antioxidants are added to significantly prevent the apoptotic process and aging of oocytes. For example, melatonin can regulate ovarian function through anti-oxidative stress, and improve the quantity and quality of oocytes [6]. Exogenous Coenzyme Q10 supplementation has been shown to improve ovarian function and delay aging in mice [7]. Coenzyme Q10 also can reduce oxidative stress-induced mitochondrial DNA mutations, restore mitochondrial function, combat oocyte aging, and improve ovarian reserve function [8].

SCM-198 is a chemically synthetic form of leonurine (a bioactive alkaloid extracted from the traditional Chinese medicine Herba Leonuri). SCM-198 exerts various biological effects, including reduction of oxidative stress [9], inhibition of the inflammatory response [10], and anti-apoptosis [11], as well as improvement of microcirculation [12]. Activation of the NRF2 antioxidant pathway [13] decreases the levels of reactive oxygen species (ROS) and increases the antioxidant capacity of cells [14]. SCM-198 reduces oxidative stress in several ways. It can protect endometrial stromal cells from oxidative damage by reducing the production of intracellular ROS and the expression of pro-inflammatory cytokines, inhibiting oxidative stress induced by H_2O_2 [15]. Moreover, SCM-198 can exert effects on some cells, causing a decrease in oxidative stress levels by upregulating the anti-apoptotic gene Bcl-2 and downregulating the apoptotic gene Bax [15]. SCM-198 also affects mitochondria by improving their function and reducing their oxidative stress levels, thereby improving bovine oocytes and effectively promoting subsequent embryonic development [16].

Over the last few decades, studies have found that SCM-198 has preventive and therapeutic effects against many systemic diseases. In cardiovascular diseases, SCM-198 improves the antioxidant capacity of the myocardium and promotes angiogenesis in the ischemic myocardium [14, 17]. It also protects damaged brain tissue after cerebral hemorrhage as well as decreases neuroinflammation and blood-brain barrier disruption [18, 19]. In rheumatoid immune system diseases, SCM-198 inhibits the activation of inflammatory pathways in fibroblast-like synoviocytes in rheumatoid arthritis (RA) and has the potential to treat RA [20]. In movement

system-related diseases, SCM-198 activates autophagy via the PI3K / Akt / mTOR pathway to slow the progression of osteoporosis and may be a potential drug for its treatment [21]. In obstetrics and gynecology, SCM-198 offers a novel prevention strategy for conditions, such as incomplete abortion and endometritis [15, 22]. However, an in-depth research and exploration are still needed on the relationship between SCM-198 and oocytes, and whether it can delay the aging of oocytes and improve their fertilization and subsequent embryo development abilities. These results lay the foundation for preventing oocyte senescence, thereby improving the success of ART in humans.

In this study, an in vitro aging model was used to investigate the effects of SCM-198 on aged mouse oocytes after ovulation, and 10-month-old mice were used as the in vivo model for older mice. SCM-198 supplementation restored the aging-induced decrease in oocyte quality, including abnormalities in spindle assembly and chromosome alignment, cortical granules (CGs), ovastacin, and Juno mislocalization. In addition, SCM-198 alleviated oocyte aging by scavenging excessive ROS, mitigating high levels of oxidative damage inhibiting early apoptosis, and protecting mitochondrial function to improve oocyte quality and enhance oocyte fertilization ability.

Materials and methods

Animals

In this study, the use of animals in experiments, approved by the ethics committee of Nantong University in China, ensuring that the relevant rules and standards of laboratory animal care were followed. Male ICR mice (12 weeks old) and female mice (6 weeks and 10 months old) were used in the experiments. The mice were reared at 20–23 °C and kept under a 12 h dark/light cycle. The mice were provided with appropriate food (Cat# 11010082; Nanjing Xietong Biotechnology Co. Ltd., China).

SCM-198 supplementation

The modified SCM-198 used in this study was provided by Professor Yizhun Zhu from Macau University of Science and Technology, China. He replaced one methoxy group on the benzene ring of SCM-198 with sulfonic acid group to improve its solubility in water and thus improve its efficacy and he applied for patent protection, which does not affect the mechanism of the compound. DMSO was prepared to dissolve SCM-198, and an appropriate amount of culture medium was added to form a concentration of 25, 50, or 100 μM. The DMSO content was less than 0.1%.

Antibodies

Tubulin was immunostained by the Sigma–Aldrich anti-α-tubulin fluorescein isothiocyanate (FITC) antibody (Cat# F2168; St. Louis, Missouri, USA). Fluorescein-labeled mouse monoclonal LCA-FITC was acquired from Vector Laboratories (Cat# FL-1041). The ovastacin / ASTL (D-8) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA; Cat# sc-514054). BioLegend supplied the FITC anti-mouse folate receptor 4 (FR4) antibody (Cat# 125005, USA). GAPDH mouse McAb was acquired from Proteintech (USA; Cat# 60004-1-Ig). In addition, anti-mouse IgG (H+L) was obtained from Cell Signaling Technology (Cat# 5257P, USA), and anti-rabbit IgG (H+L) was purchased from Invitrogen (USA; Cat# A11011).

Oocyte collection and culture

Female mice were administered 5 IU of Pregnant Mare Serum Gonadotropin (PMSG) followed by 5 IU of Human Chorionic Gonadotropin (HCG) to induce superovulation after a time interval of 48 h. Fertilization was conducted within a timeframe of 14 to 16 h, preferably not exceeding 15 h, after HCG injection. Subsequently, oocytes were obtained by euthanizing the mice between 9 and 11 a.m. on the following day. M2 culture medium containing streptomycin sulfate, sodium lactate, HEPES, KCl, and NaCl was prepared. These materials were purchased from Sigma–Aldrich. Ovulated oocytes arrested at metaphase II were collected from the ampulla of female mice was placed in the M2 medium. Hyaluronidase was added to the oocytes at a concentration of 1 mg / mL to remove the cumulus cells. M16 culture medium, which included BSA, glucose, sodium pyruvate, KCl, and NaCl, was prepared. These materials were purchased from Sigma–Aldrich. Oocytes were observed and cultured in M16 medium with 0, 25, 50 or 100 μM SCM-198 for 24 h. The oocytes were placed in a 5% CO_2 incubator at a temperature of 37 °C for 24 h, which was an in vitro aging step.

Immunofluorescence staining and analysis

Oocytes and 4% paraformaldehyde solution were added and fixed for 30 min. The oocytes were maintained at room temperature and 0.5% Triton X-100 was added for 20 min. Next, 1% BSA in PBS was used for blocking for 60 min. Afterward, the mixture was incubated overnight at 4 °C with one of four antibodies: anti-α-tubulin FITC (1:200), rabbit polyclonal anti-ovastacin (1:100), LCA-FITC (1:100), or FITC anti-mouse FR4 antibodies (1:100). The samples were then washed with PBS containing 0.01% Triton X-100. The samples were then washed four times for 5 min each time. An appropriate amount of secondary antibody was added, mixed with the oocytes, and incubated at room temperature for 60 min. The oocytes were washed four times and re-stained for 10 min with 10 μg / mL Hoechst 33,342, a blue dye used to detect DNA. A fluorescence laser-scanning microscope (Axio

Imager M2, Zeiss, Germany) was used in this study. To measure the fluorescence intensity, signals from control and treated oocytes were simultaneously determined using the same procedures and immunolabeling parameters as those used for confocal microscopy. ImageJ software (National Institutes of Health, Maryland, USA) was used to define the regions of interest and detect the average fluorescence intensity per unit area of interest. The average of the measurement results was calculated and the average intensities of the treatment and control groups were evaluated.

Western blot analysis

First, 4 × LDS sample buffer was prepared and 10 × reduction reagent was added (Thermo Fisher Scientific, Waltham, MA, USA). Next, approximately 80 mouse oocytes were added, and these were fully dissolved and heated at 100 °C for 5 min. Bis-Tris gels (10%) were used to separate proteins, prepare PVDF membranes, and transfer the proteins. Quick Block Western blocking buffer was prepared (Beyotime Institute of Biotechnology, Hangzhou, China), and the blots were kept at 20–25 °C for 60 min. These were then mixed with rabbit polyclonal anti-mouse ovastacin protein antibody (1:1000), mouse monoclonal anti-mouse Juno antibody (1:1000), and GAPDH antibody (1:3000) in Solution 1 for Primary Antibodies (Cat# KP31812, Calbiochem, San Diego, CA, USA) at 4 °C overnight. The blots were washed three times with TBST solution for 10 min each time. Subsequently, a goat anti-rabbit IgG secondary antibody was added, which was diluted 3,000 times in Solution 2 for Secondary Antibodies (Cat# KP31855, Calbiochem, San Diego, CA, USA). The sample was then kept at 20–25 °C for 60 min. The blots were visualized using the Amersham Typhoon Odyssey infrared imaging system.

In vitro fertilization

First, PMSG was prepared at a concentration of 5 IU and injected into the abdomen of female mice. Mice were fed normally for 46–48 h, and HCG was prepared at a concentration of 5 IU and injected into the abdomen of the female mice. After 18 h, the male mice were sacrificed by neck amputation and the cauda epididymidis was removed and pierced. Then, the sperm were placed in 200 μL of c-TYH (mouse sperm culture medium) (Cat# 72021; Sudgen Biotechnology Co. Ltd, Nanjing, China) under mineral oil. This was followed by incubation in a 5% CO_2 incubator at 37 °C for 1 h. Meanwhile, the female mice were sacrificed by neck amputation, the abdominal cavity was opened with scissors and the fallopian tubes ampoules on both sides were retrieved. The ampulla was then torn to obtain oocytes containing cumulus cells. Oocytes were collected, the cumulus was removed with hyaluronidase, 100 μL of human oviduct fluid (HTF) culture medium was prepared (Cat# 72002), and the oocytes were added. Sperm was then added to the oocytes at a concentration of 4×10^5 / mL, and the sample was placed in a 5% CO_2 incubator at 37 °C for 24 h. Fertilization was considered successful if there were two pronuclei.

Sperm binding test

Sperm were separated from the cauda epididymides of male mice in HTF medium and placed in a 5% CO_2 incubator at 37 °C for 60 min. After completion of this operation, the sperm was removed and used as a negative control for two-cell embryos. PFA was prepared at a concentration of 4% for two-cell embryos, sperm, and ovulated oocytes, with a sperm concentration of 4×10^5 / mL, and fixed for 0.5 h. Except for two to six sperm, the sperm was removed from typical two-cell embryos using a wide-mouthed pipette. The number of sperm stained with Hoechst 33,342 was calculated using a fluorescence microscope to capture the Z-projection.

Determination of mitochondrial distribution and levels

For staining of active mitochondria, the oocytes were incubated with 500 nM MitoTracker Red (Thermo Fisher, Waltham, MA, USA) for 30 min at 37 °C in a dark environment and an incubator with 5% CO_2. The oocytes were washed thrice with fresh maturing medium for 20 min each and re-stained with 10 μg/mL Hoechst 33,342 for 10 min. The fluorescence signal was measured using a fluorescence microscope with the same parameters. The entire image was analyzed, and the average fluorescence was calculated using ImageJ software.

ROS level measurement

The samples were incubated with dichlorofluorescein diacetate (DCFHDA) in M2 medium in a 5% CO_2 incubator at 37 °C for 30 min. DCFHDA was used at a concentration of 10 μM. A glass slide was prepared and the oocytes were placed on it and observed. The fluorescence signal was measured using a fluorescence microscope with the same parameters. The intensity of ROS in each oocyte from the different groups was measured using ImageJ software.

Annexin-V staining

Oocytes were stained using an Annexin V staining kit (Beyotime Institute of Biotechnology). The manufacturer's instructions were strictly followed during the operation to detect oocyte apoptosis levels. The sample was washed with PBS three times, live oocytes were stained, and 10 μL of Annexin-V-FITC with a volume of 90 μL binding buffer was used. The cells were then placed in a 5% CO_2 incubator at 37 °C for 0.5 h. The oocytes were placed on a glass slide and examined using a fluorescence

Table 1 Primer sequences for qRT-PCR

Gene	Forwards (5′-3′)	Backwards (5′-3′)
Juno	GTGGTTTGAGCCCACCCTTA	AACAGGCACAGAGAGAAGGC
Astl	TAGAGGAGTGCGTGAGTAGGA	ACGGCTAATGGGCTTCACTAT
Gapdh	AATGGATTTGGACGCATTGGT	TTTGCACTGGTACGTGTTGAT

laser-scanning microscope (Axio Imager M2, Zeiss, Germany). Control and treated oocytes were examined using the same method. The entire image was analyzed and the average fluorescence was calculated.

In vivo administration of SCM-198

Six-week-old mice were used as the young control group, and 10-month-old female mice were used as the natural aging model. Aged mice in the treatment group were administered SCM-198 daily by gavage. SCM-198 was administered at a dose of 20 mg/kg. The treatment was continued for 15 or 30 days.

RNA extraction and quantitative real-time PCR

Total RNA from 100 mouse oocytes of each group was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit-BOX 2 (Cat# RC101-01, Vazyme Biotech Co). The quality and quantity of the RNA were determined using a NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific). Then the RNA was reverse-transcribed into first-strand complementary DNA (cDNA) by using PrimeScript™ RT Master Mix (Cat# RR036A, TaKaRa, Japan). The synthesized cDNA was amplified with specific primers and TB Green (Takara, Japan) using the LightCycler® 96 instrument (Roche, Basel, Switzerland). Triplicate samples were examined for each condition. A comparative threshold cycle value was normalized for each sample using the $2^{-\Delta\Delta Ct}$ method. The primers were obtained from AZENTA Life Sciences, and the sequences are listed in Table 1.

Statistical analysis

To ensure the representativeness of results, three parallel experiments were conducted for each group of samples. Spss16.0 software was used to analyze the data, and an analysis of variance was used to compare the means. Statistical software was used to analyze the data and calculate the mean ± standard deviation. Statistical significance was set at $P<0.05$.

Results

SCM-198 effectively decreased the fragmentation rate of postovulatory aged oocytes

To clarify the relationship between postovulatory aged oocytes and SCM-198, oocyte status was first examined in vitro after 24 h of aging in an incubator and supplementation with SCM-198. Oocyte integrity and morphology were analyzed and fragment frequencies calculated. The results serve as indicators to evaluate postovulation oocyte quality. Figure 1A indicates that the control group

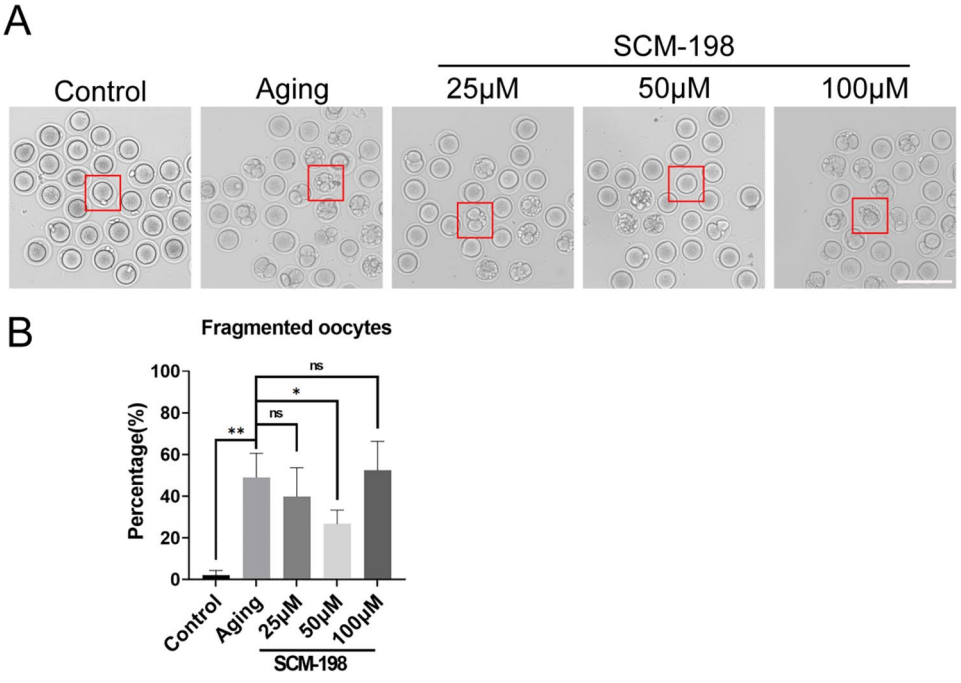


Fig. 1 Effects of SCM-198 on the integrity of postovulatory aged oocytes. ****(A)** Representative images of the relevant content of different groups of samples after 24 h of cultivation. Scale bar = 200 μm. **(B)** The proportion of oocyte fragmentation in different groups. Different concentrations of SCM-198 were added to the culture medium at a concentration of 25, 50, or 100 μM, respectively. Three parallel experiments were conducted, using the average ± SEM to represent the data of **(B)**. *$P<0.05$, **$P<0.01$

(fresh oocytes) had relatively normal oocyte morphology, while the aging group had more oocyte fragments like the one in red box. Quantitative analysis points to a significant age-related increase in oocyte fragmentation compared to the control group (control group, 2.09±2.30%, $n=118$; aging group, 49.01±11.60%, $n=125$, $P<0.01$; Fig. 1B). In order to investigate the relationship between postovulatory aged oocytes and SCM-198 supplementation and analyze whether the latter affects the stability of the former, SCM-198 (25, 50, and 100 μM) was incubated with oocytes at increased doses for 1 day. Specifically, when the concentration of SCM-198 was 50 μM, the fragmentation rate was significantly reduced (50 μM SCM-198 group, 26.67±6.67%, $n=119$, $P<0.05$; Fig. 1B). Therefore, 50 μM SCM-198 was used in subsequent tests.

SCM-198 restored spindle assembly and chromosome alignment in postovulatory aged oocytes

In terms of oocyte quality, similar studies have mainly evaluated whether chromosomes have a normal spindle apparatus morphology. To examine the relationship between oocyte maturation and aging, postovulatory aged oocytes were analyzed and spindle apparatus formation was detected. For testing the shape of the spindle apparatus, the anti-α-tubulin-FITC antibody was used for staining, and the chromosome arrangement analyzed using Hoechst 33,342. The results showed that there was a normal barrel-shaped spindle apparatus on the equatorial plate of control MII oocytes, indicating that the oocytes in this group had well-arranged chromosomes (Fig. 2A). Through observation, it was found that the postovulatory aged oocytes had obvious chromosome dislocation and spindle apparatus morphological disorder, which could be improved after adding an appropriate amount of SCM-198 (control group, 31.58±7.04%, $n=50$; aging group, 79.43±12.50%, $n=53$, $P<0.01$; 50 μM SCM-198 group, 40.03±8.91%, $n=52$, $P<0.01$; Fig. 2B and C). In summary, the addition of SCM-198 improved the chromosomal abnormalities and spindle apparatus defects induced by postovulatory aging.

SCM-198 advanced the fertilization and sperm binding abilities of postovulatory aged oocytes

Abnormal spindles and chromosomes in oocytes cause aneuploidy, and aneuploid eggs lead to fertilization failure. Samples from the aging group were analyzed. It was found that they had a low zygote development ratio after fertilization, and the fertilization ability could be significantly improved after adding an appropriate amount of SCM-198 (control group, 59.90±7.11%, $n=150$; aging group, 24.50±9.41%, $n=147$, $P<0.01$; 50 μM SCM-198 group, 43.06±10.03%, $n=137$, $P<0.05$; Fig. 3A and B).

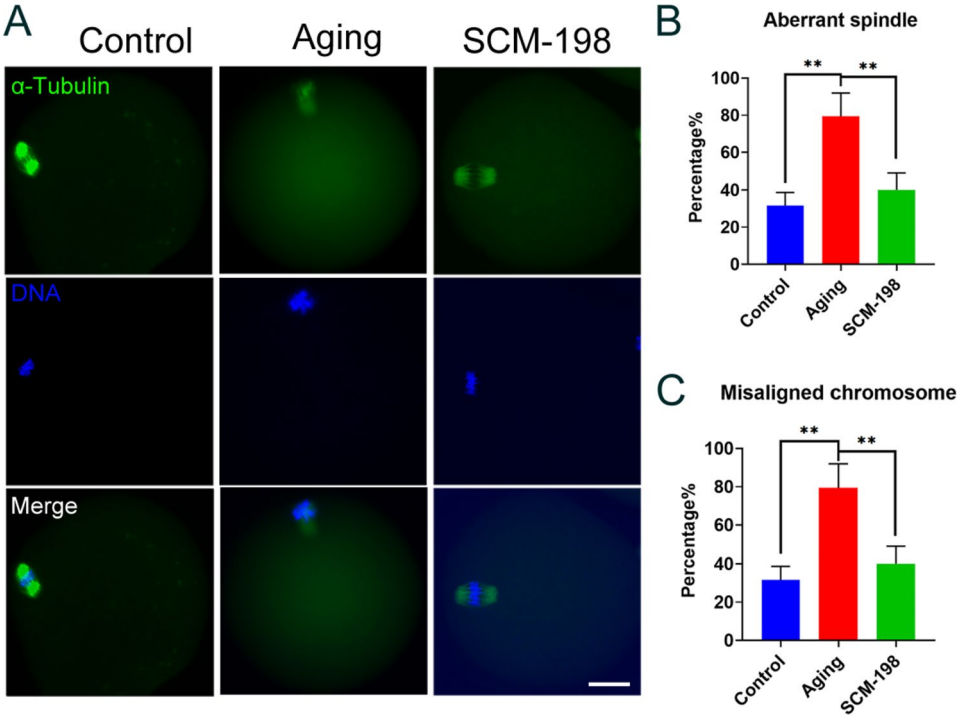


Fig. 2 Effects of SCM-198 on spindle assembly and chromosome alignment in postovulatory aged oocytes. **(A)** Chromosome arrangement and spindle apparatus morphology of oocytes from different groups are shown. Oocytes were immunostained with α-tubulin-FITC antibody to visualize the spindles, and were counterstained with Hoechst to visualize the chromosomes. Scale bar = 20 μm. **(B)** Proportions of abnormal spindle assembly in the control, aged, and SCM-198-supplemented oocytes. **(C)** Proportions of misaligned chromosomes in the control, aged, and SCM-198-supplemented oocytes. Three parallel experiments were conducted, using the average ± SEM to represent the data of **(B)** and **(C)**. $^{**}P < 0.01$

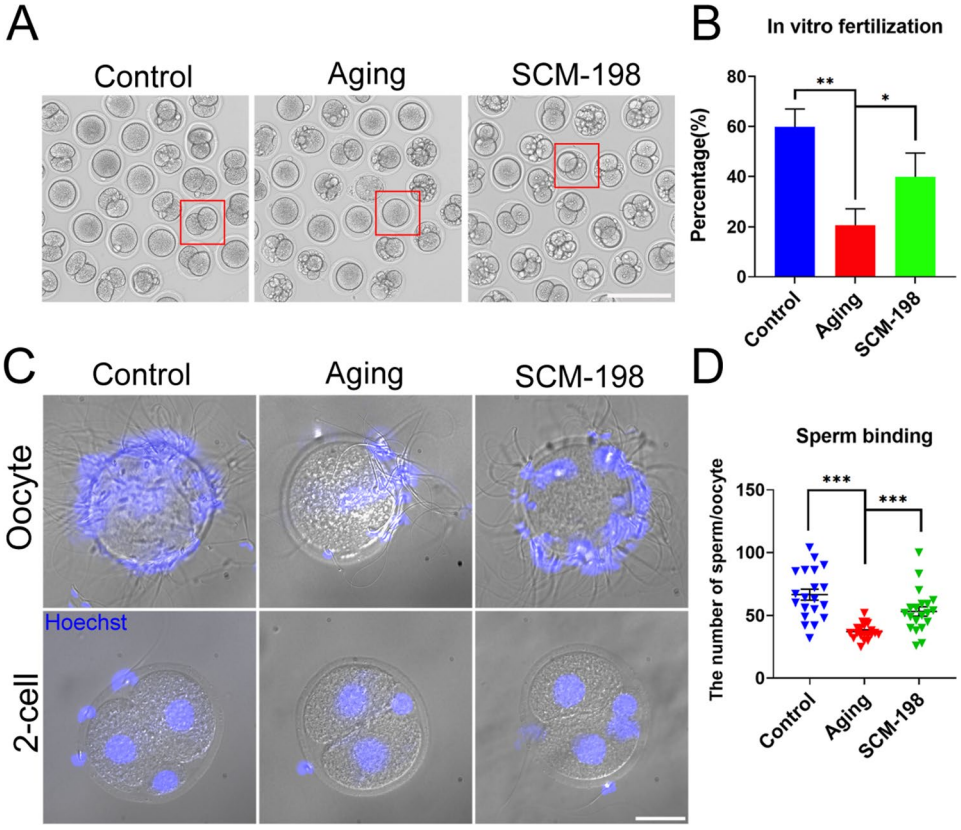


Fig. 3 Effects of SCM-198 on the fertilization and sperm-egg binding ability of postovulatory aged oocytes. **(A)** Pictures of two-cell embryos obtained from different groups. Scale bar = 200 μm. **(B)** Proportions of in vitro fertilization rates in different groups. **(C)** Representative images of sperm binding to matured oocytes or two-cell embryos from different groups. Scale bar = 20 μm. **(D)** Schematic diagram of the results of counting the number of sperm adhered to the surface of ZP. Three parallel experiments were conducted, using the average ± SEM to represent the data of **(B)** and **(D)**. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

The reduction in fertilization rate may indicate that sperm-binding sites may be lost and sperm-binding capacity may be damaged. To validate this hypothesis, sperm-egg binding was measured. The heads of the counterstained sperm were selected and Hoechst 33,342 was used to calculate the number of sperm combined with the zona pellucida. In the unfertilized control eggs, the zona pellucida supported a large amount of sperm binding. To remove unbonded sperm, the two-cell embryos lost sperm-binding sites resulting in the inability of sperm to bind to the egg as a control (Fig. 3C). By comparing different groups of samples, it was found that the number of sperm that could bind to the zona pellucida in the aging group was significantly reduced, but the sperm-binding ability was meaningfully increased in the SCM-198-supplemented group (control group, 66.35 ± 12.59%, $n = 20$; aging group, 37.20 ± 6.26%, $n = 20$, $P < 0.001$; 50 μM SCM-198 group, 53.25 ± 11.34%, $n = 20$, $P < 0.001$; Fig. 3C and D). Overall, SCM-198 improved the fertilization ability of postovulatory aged oocytes and their ability to bind sperm.

SCM-198 rescued CGs and ovastacin dislocation in postovulatory aged oocytes

CGs are small vesicular organelles exclusively available in oocytes that have a signaling function in sperm binding and prevent multiple sperm fertilization. LCA-FITC staining was used to investigate whether postovulatory aging affects CGs dynamics. Immunoassays showed that CGs were present in the subcortical area of oocytes in the control group, but not in the area outside the CG-free domain (CGFD) near the chromosomes (Fig. 4A). In contrast, postovulatory aging revealed that CGs signals were disrupted or completely lost, affecting their normal distribution (Fig. 4A). By comparing different groups of samples, it was found that postovulatory aged oocytes had significantly weaker CG fluorescence signals than the control group (control group, 22.93 ± 6.97 A.U, $n = 20$; aging group, 16.72 ± 4.49 A.U, $n = 25$, $P < 0.001$; Fig. 4B). SCM-198 enhanced the CG signal fluorescence intensity from 16.72 to 20.50% (50 μM SCM-198 group, 20.50 ± 6.49 A.U, $n = 25$, $P < 0.05$; Fig. 4B).

Ovastacin in CGs can play a crucial role in promoting binding between sperm and eggs, thereby increasing

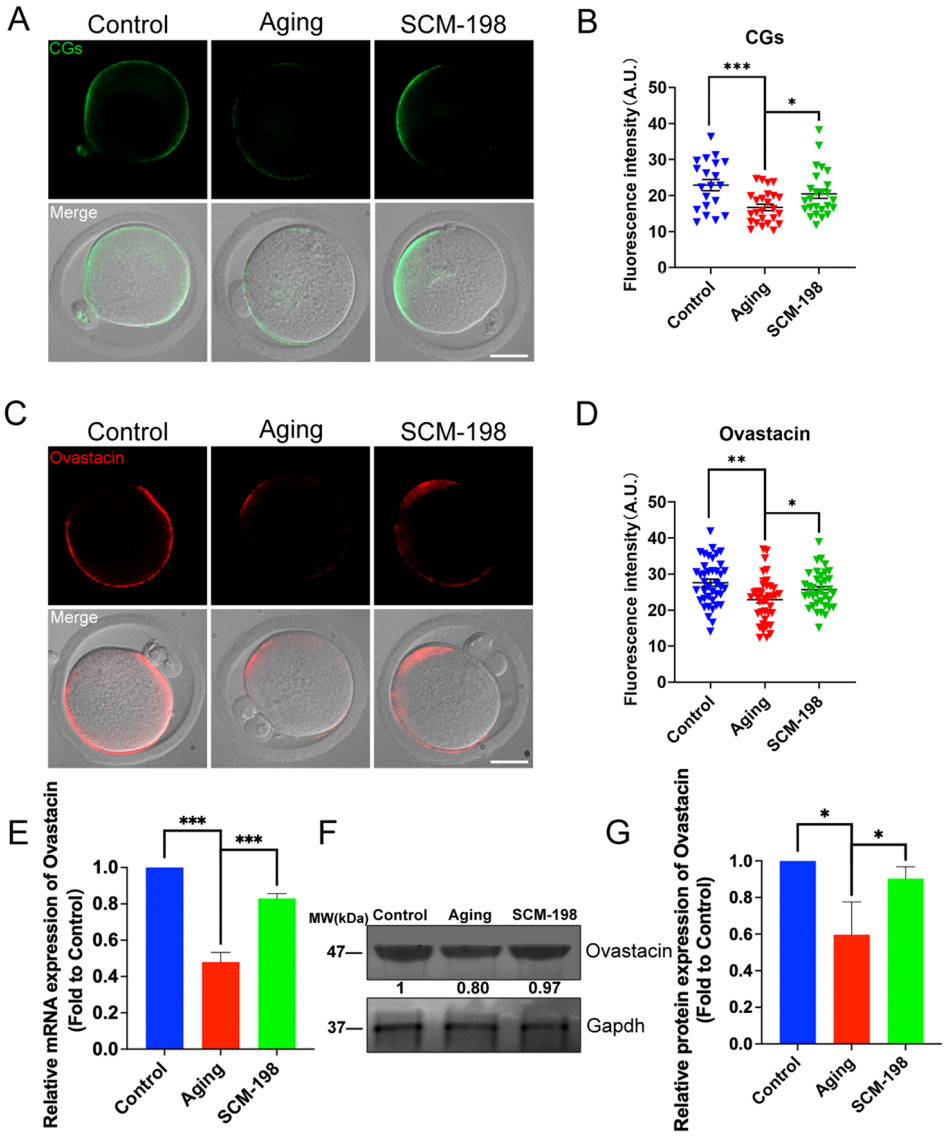


Fig. 4 Effects of SCM-198 on the localization of cortical granules and ovastacin in postovulatory aged oocytes. **(A)** Images of abnormal localization of cortical granules in oocytes from different groups of samples. Scale bar = 20 μm. **(B)** Schematic representation of the fluorescence intensity levels of cortical granule signals in different groups of samples. **(C)** Ovastacin localization images for different groups. Scale bar = 20 μm. **(D)** Results of oocyte ovastacin signal intensity in different groups. **(E)** Relative mRNA expression of Ovastacin from different groups. **(F)** Images of western blotting of Ovastacin from different groups. **(G)** Quantitative analysis of relative protein expression. Three parallel experiments were conducted using the average ± SEM to represent the data of **(B)**, **(D)**, **(E)** and **(G)**. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$

the chances of fertilization. Immunostaining revealed that the localization pattern of ovastacin was similar to that of CGs in control oocytes. Intermittent signals were observed in the CGFD of postovulatory aged oocytes, which could be restored by the appropriate addition of SCM-198 (Fig. 4C). Compared with the control group, postovulatory aged oocytes showed significantly lower ovastacin protein signal intensity compared to different groups of samples (control group, 27.61 ± 6.27 A.U, $n = 40$; aging group, 22.89 ± 6.15 A.U, $n = 40$, $P < 0.01$; Fig. 4D). However, the result was markedly improved in the SCM-198-supplemented group (50 μM SCM-198 group, 25.67 ± 5.11 A.U, $n = 36$, $P < 0.05$; Fig. 4D). According to the results of qRT-PCR and western blotting in this study, there was a significantly lower level of related RNA and proteins in the aging group (Fig. 4E and F). The protein may be prematurely cleaved by zona pellucida before fertilization. After adding an appropriate amount of SCM-198, premature exocytosis of ovastacin was effectively restored, resulting in effective recovery of protein levels (Fig. 4G). Therefore, SCM-198 can alter the movement of ovastacin proteins and CGs in oocytes and exert protective effects. In addition, it avoids premature

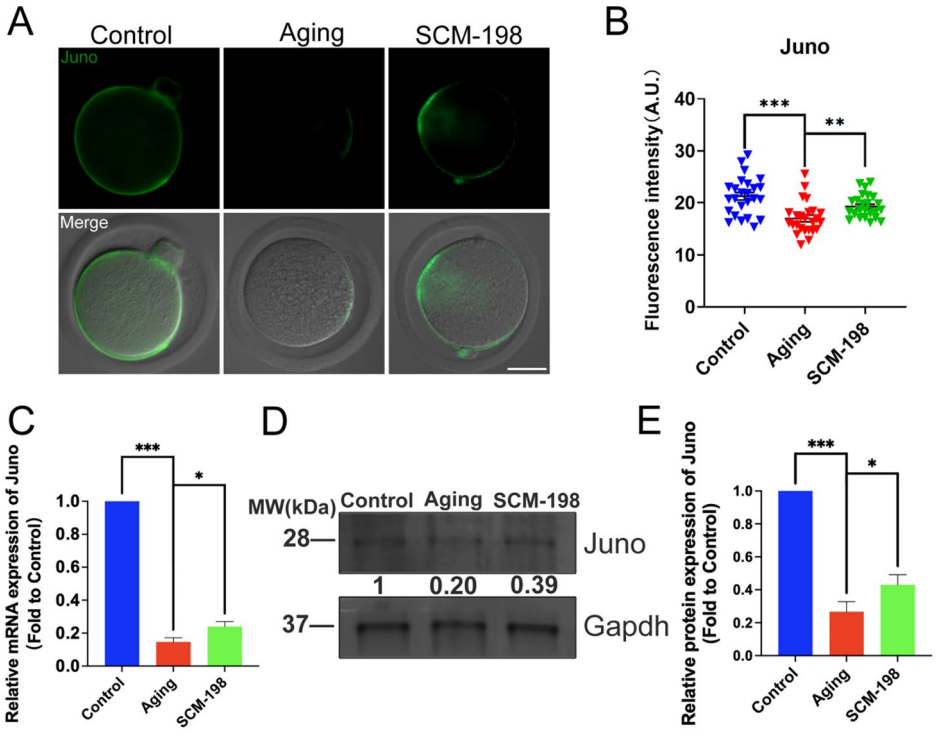


Fig. 5 Effects of SCM-198 on the localization of Juno in postovulatory aged oocytes. **(A)** Representative images of Juno positioning images in different groups. Scale bar = 20 μm. **(B)** Schematic representation of Juno signal fluorescence intensity of control, aged, and SCM-198-supplemented oocytes. **(C)** Relative mRNA expression of Juno in different groups. **(D)** Western blotting was used to detect Juno protein expression levels in different groups. **(E)** Quantitative analysis of relative protein expression. Three parallel experiments were conducted using the average ± SEM to represent the data. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

exocytosis and incorrect protein localization and effectively promotes the fertilization process.

SCM-198 salvages the localization of Juno on the membrane in postovulatory aged oocytes

Juno is a recently discovered sperm receptor that interacts with the sperm plasma membrane protein Izumol at the egg plasma membrane [23, 24]. Female mice eggs lacking this gene cannot bind to sperm and experience infertility [25]. Juno is an essential regulatory protein involved in mammalian oocyte fertilization. Therefore, this study aimed to examine whether SCM-198 could reverse age-related defects in the distribution of Juno. Analysis of the control group revealed evenly distributed Juno on the oocyte membrane, as shown in Fig. 5A. Analysis of the postovulatory aged oocyte group revealed that there was almost no intact Juno on the membrane, and its distribution pattern was relatively abnormal, which may have resulted in the loss of Juno signals on the plasma membrane. SCM-198 group analysis shows that it had a significantly lower localization abnormality rate compared to that of the aging group (control group, 21.25±3.64 A.U, $n=25$; aging group, 16.92±3.23 A.U, $n=25$, $P<0.001$; 50 μM SCM-198 group, 19.29±2.22 A.U, $n=25$, $P<0.01$; Fig. 5A and B). The RNA and protein expression level of Juno in postovulatory aged oocytes was relatively low, and the addition of 50 μM SCM-198 improved this (Fig. 5C, D and E). Therefore, decreased Juno in aged oocytes results in the inability of binding to sperm. After adding an appropriate amount of SCM-198, this abnormality can be restored and the level of fertilization capability damage can be decreased.

SCM-198 exposure recovers the mitochondrial distribution and downgrades ROS levels to inhibit oocyte apoptosis

Mitochondrial dynamics have been shown to produce viable oocytes, and mitochondria are considered one of the most important indicators of cytosolic maturation in oocytes. We examined mitochondrial distribution in postovulatory aged oocytes. Oocytes from the two groups were stained with MitoTracker and evaluated using a fluorescence microscope. In control oocytes, most mitochondria showed a polarized distribution that accumulated peripherally around the chromosome and a homogeneous distribution in the cytoplasm (Fig. 6A). After aging for 24 h in vitro, mitochondria completely or partially lost their normal distribution around the chromosomes and emerged as mitochondria-distributed clusters in the cytoplasm. However, SCM-198 supplementation improved the vulnerable morphology of

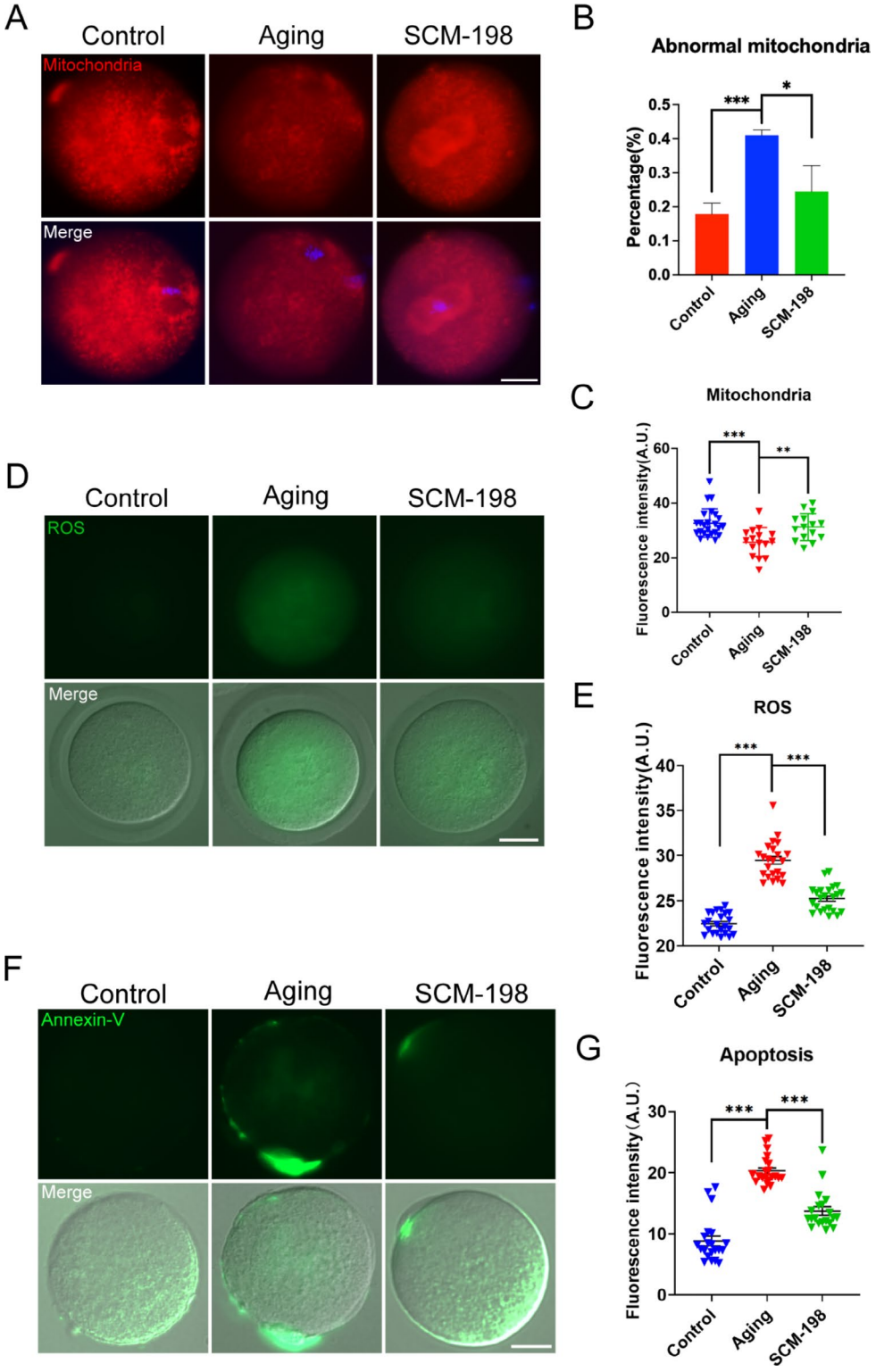


Fig. 6 Effects of SCM-198 on mitochondrial distribution, ROS accumulation, and apoptosis following aging for 24 h of treatment in vitro. **(A)** Representative images of mitochondrial distribution in control, aged, and SCM-198-supplemented oocytes. Scale bar = 20 μm. **(B)** The proportion of abnormal mitochondrial morphology in the control, aged, and SCM-198 groups. **(C)** Mitochondrial fluorescence intensity in the control, aged, and SCM-198-supplemented groups. **(D)** ROS staining results of different groups of sample oocytes. Scale bar = 20 μm. **(E)** ROS fluorescence signal detection results for different groups of sample oocytes. **(F)** Photographs of early apoptosis in Annexin V-stained oocytes from control, aged, and SCM-198-supplemented oocytes. Scale bar = 20 μm. **(G)** The detection results of Annexin V fluorescent signals intensity in different groups of sample oocytes. Three parallel experiments were conducted using the average ± SEM to represent the data of **(B)**, **(C)**, **(E)**, and **(G)**. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

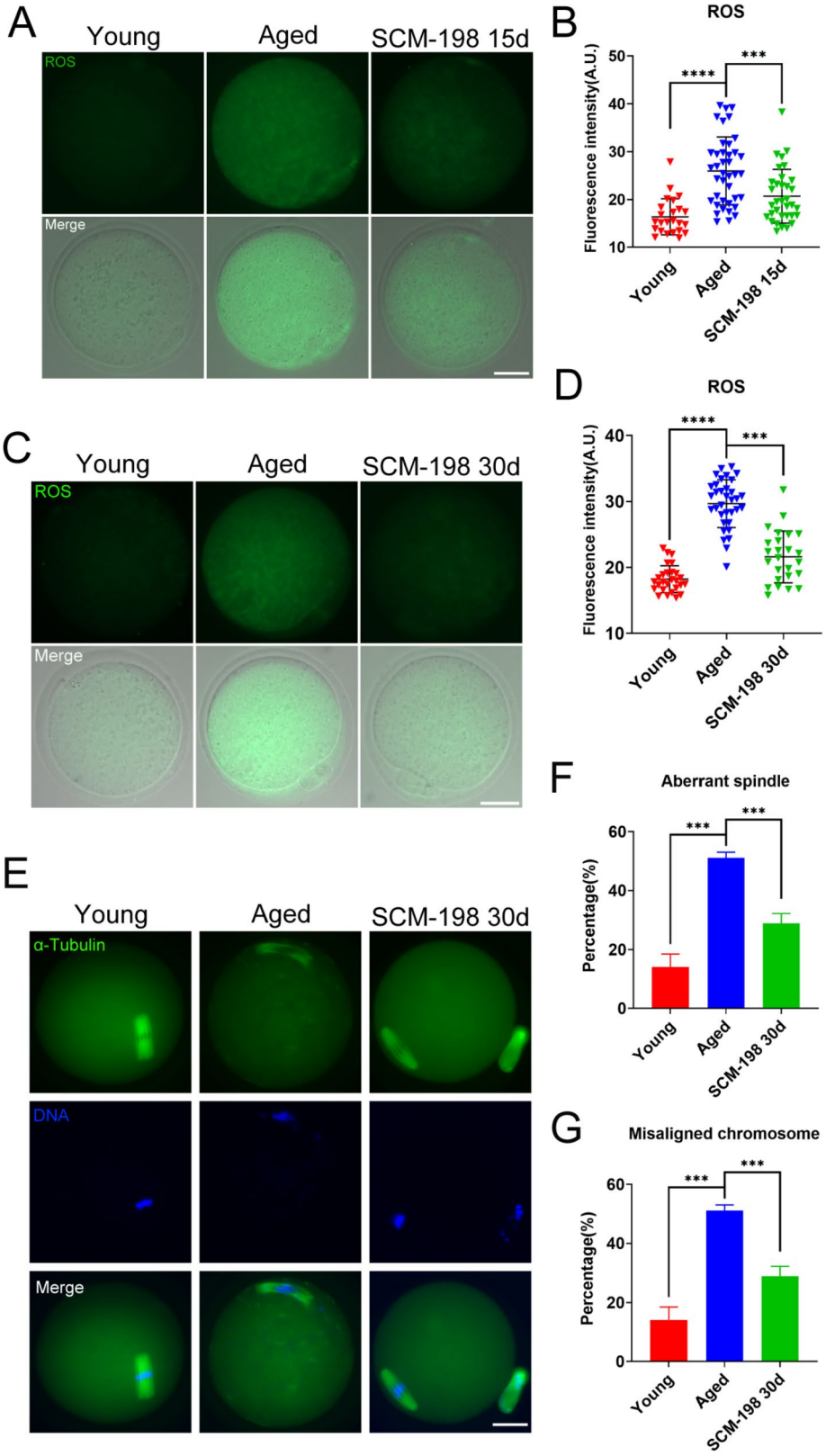


Fig. 7 (See legend on next page.)

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Fig. 7 Effect of SCM-198 supplementation on ROS levels, spindle assembly, and chromosome alignment in vivo. **(A)** Representative images of ROS levels in young, aged, and 15-day SCM-198 treatment groups of sample oocytes. Scale bar = 20 μm. **(B)** The fluorescence intensity of ROS signals was recorded in different groups of sample oocytes. **(C)** Representative images of ROS accumulation in young, aged, and 30-day SCM-198 treatment groups of sample oocytes. Scale bar = 20 μm. **(D)** ROS fluorescence signal detection results for different groups of sample oocytes. **(E)** Representative photos of spindle assembly and chromosome alignment in young and aged oocytes, as well as those supplemented with SCM-198 for 30 days. Oocytes were subjected to immunostaining using anti-α-tubulin-FITC antibody for visualizing spindles; counterstaining with Hoechst was performed to visualize chromosomes. Scale bar = 20 μm. **(F)** Ratios of abnormal spindle assembly in young and aged oocytes, as well as those supplemented with SCM-198 for 30 days. **(G)** Proportions of misaligned chromosomes in the young and aged oocytes, as well as those supplemented with SCM-198 for 30 days. Three parallel experiments were conducted using the average ± SEM to represent the data of **(B)**, **(D)**, **(F)**, and **(G)**. ***$P < 0.001$

mitochondria caused by oocyte aging (control group, 17.83±3.18%, $n=39$; aging group, 40.95±1.65%, $n=17$, $P<0.001$; 50 μM SCM-198 group, 24.44±7.69%, $n=16$, $P<0.05$; Fig. 6B). Statistically, the immunofluorescence signal intensity was also significantly rescued in the SCM-198 group (control group, 32.53±5.25 A.U, $n=24$; aging group, 25.67±5.2 A.U, $n=16$, $P<0.001$; 50 μM SCM-198 group, 31.16±4.92 A.U, $n=15$, $P<0.01$; Fig. 6C), suggesting that SCM-198 supplementation ameliorates impaired mitochondrial dynamics resulting from postovulatory aged oocytes.

ROS is closely linked to mitochondrial dynamics. Mitochondrial dysfunction induces oxidative stress and accelerates apoptosis. To investigate the effects of SCM-198 on oxidative stress in postovulatory aged oocytes, ROS production was measured in control, aged, and SCM-198-treated oocytes. As expected, postovulatory aged oocytes exhibited significantly higher oxidative stress signals than the control group. After adding 50 μM SCM-198, the sample exhibited significantly lower oxidative stress signals than the aging group (control group, 22.47±1.13 A.U, $n=23$; aging group, 29.47±2.06 A.U, $n=23$, $P<0.001$; 50 μM SCM-198 group, 25.25±1.42 A.U, $n=23$, $P<0.001$; Fig. 6D and E). The levels of early apoptosis in oocytes were measured using Annexin V staining. The results of this study indicated that there was no green fluorescence signal present in the control group. Nonetheless, the green fluorescence signal was observed on the plasma membrane in aged oocytes. These results showed that oxidative stress could be induced and oocyte apoptosis promoted in postovulatory aged oocytes. Adding 50 μM SCM-198 significantly restrained the level of oocyte apoptosis (control group, 8.83±3.60 A.U, $n=21$; aging group, 21.12±4.22 A.U, $n=29$, $P<0.001$; 50 μM SCM-198 group, 13.74±3.17 A.U, $n=20$, $P<0.001$; Fig. 6F and G). SCM-198 reduced ROS production, alleviated oxidative stress, and ultimately inhibited apoptosis in the oocytes.

In vivo supplementation of SCM-198 effectively eliminated the excess ROS and reduced the spindle/chromosome structural defects in aged mouse oocytes

Using the established SCM-198 recovery model in aged mice, we initially assessed the regulation of ROS levels in aged oocytes. Oxidative damage resulting from oxidative stress and impaired antioxidant defense systems are major contributors to the decreased quality of aged oocytes [26]. Therefore, to validate the potential for rejuvenating aged oocyte quality with SCM-198 in vivo, we examined the ROS levels in oocytes obtained from young and aged mice. As depicted in Fig. 7A, live staining and quantitative analysis of fluorescence intensity using the oxidation-sensitive fluorescent probe DCFH-DA revealed varying degrees of increased fluorescence signals corresponding to ROS levels in oocytes from aged mice compared with those from young mice, with a significantly higher average fluorescence intensity observed in the former group. These findings indicated that high levels of oxidative stress were induced in aged oocytes. Following 15 days of in vivo treatment with SCM-198, samples exhibited significantly lower ROS levels than the aged group (young group, 16.39±3.77 A.U, $n=24$; aged group, 25.98±7.10 A.U, $n=40$, $P<0.001$; SCM-198 group, 20.69±5.61 A.U, $n=34$, $P<0.001$; Fig. 7A and B). Furthermore, consistent with these results, ROS levels were significantly reduced in oocytes obtained from aged mice treated continuously with SCM-198 for a period of 30 days compared to those from the aging group (young group, 18.22±2.04 A.U, $n=27$; aged group, 29.68±3.96 A.U, $n=34$, $P<0.001$; SCM-198 group, 21.61±3.93 A.U, $n=25$, $P<0.001$; Fig. 7C and D). These outcomes suggest that SCM-198 directly influences ROS levels in aged mouse oocytes and the effect is better with the extension of processing time.

Meanwhile, the decrease in ROS levels in oocytes derived from aged mice following SCM-198 treatment in vivo suggests a potential reduction in defects associated with spindle assembly, and chromosome arrangement may be reduced. Therefore, we visualized the spindle morphology and chromosome arrangement in oocytes using Tubulin and DNA staining. The results of immunofluorescence and data statistics showed that most of the aged oocytes with SCM-198 supplementation in vivo showed typical spindle-shaped spindles, neatly arranged chromosomes on the equator, and restoration of the spindle/chromosome structure (Fig. 7E), and the abnormal rate was significantly different from that of aged oocytes without SCM-198 supplementation (young group, 14.14±4.38%, $n=35$; aged group, 51.11±1.92%, $n=45$, $P<0.01$; SCM-198 group, 28.85±3.33%, $n=38$,

$P<0.01$; Fig. 7F and G). In summary, these results suggest that the abnormal spindle/chromosome structure in aged oocytes can be ameliorated by supplementation with SCM-198 in vivo.

Discussion

In the progression of human ART, postovulatory aging in vitro is an important disadvantage that causes ART failure. The results of this study suggest that the decreased quality of aged oocytes after ovulation can be improved by adding an appropriate amount of SCM-198, which also increases the fertilization rate of oocytes. To validate this hypothesis and explore the relevant mechanisms, in vitro postovulatory aged oocyte and in vivo maternally aged oocyte models were constructed. This provides a reference for the use of SCM-198 for protecting aged oocytes during human ART.

First, the effects of SCM-198 on oocyte morphology and integrity of postovulatory aging in mice were assessed. Previous studies have shown that the fragmentation rate is higher in aged oocytes than in normal oocytes [27]. The addition of 50μM SCM-198 reduced oocyte fragmentation and death, suggesting that SCM-198 may improve oocyte quality. However, it can be seen that a jump difference between 50 and 100μM SCM-198 data. The same thing happened to us that several antioxidants have been found to have toxic effects on oocytes at too high concentrations. For example, High doses of NMN, melatonin, vitamin E and vitamin C may have negative effects on tissues and cells [28–32]. In addition, chromosomal arrangement and spindle apparatus assembly are often used to evaluate the quality of aged oocytes after postovulatory aging. If the spindle apparatus is abnormal during fertilization, the subsequent aneuploid development is affected [33]. Immunofluorescence staining of spindles and chromosomes demonstrated that supplementation with SCM-198 rescued spindle/chromosomal structural defects during postovulatory aging. At suitable concentrations, SCM-198 is a new antioxidant with the same saving effects as melatonin or Coenzyme Q10 [34, 35].

Fertilization capacity is a key indicator of oocyte quality [36]. Previous studies have shown that SCM-198 promotes bovine oocyte maturation and enhances fertilization and embryonic development [16]. Therefore, we examined whether SCM-198 supplementation improves fertilization rates. These results indicate that SCM-198 supplementation could improve the fertilization ability of oocytes, which could be considered as a partial restoration of fertilization potential and promotion of subsequent embryonic development.

CGs are organelles unique to oocytes and are associated with sperm binding and the prevention of multiple sperm fertilization [37–39]. The postovulatory aged oocytes with decreased CGs results in the inability to undergo normal exocytosis of CGs during fertilization and reduces fertilization capacity. However, supplementation with SCM-198 effectively ameliorated the abnormal distribution of CGs and promoted cortical reactions, thereby improving the oocyte quality and promoting embryonic development. Furthermore, ovastacin, a CG metalloprotease, blocks sperm binding to the zona pellucida surrounding fertilized eggs [40]. The results of this study indicate that when oocytes are aged, owing to issues such as localization errors, they may cleave the zona pellucida protein ZP2. This can dramatically affect the recognition domain of the sperm and the binding between the sperm and zona pellucida [34]. Supplementation with SCM-198 may prevent the precocious exocytosis of ovastacin. The results show that SCM-198 could recover the sperm-binding capacity in aged oocytes.

After successful navigation of the zona pellucida, acrosome-reactive sperm could be found in the perivitelline space, which can fuse oocytes [41]. According to relevant studies, Juno, the oocyte receptor, can play a very important role in sperm-oocyte interaction and fusion [23]. After fertilization, Juno is shed quickly, allowing sperm and egg binding to proceed normally [25]. Because of its important role in the fertilization process, variations in Juno in aged oocytes and in oocytes supplemented with SCM-198 were studied. It is possible that cellular senescence can damage the location of Juno, and that the level of damage can be decreased by adding an appropriate amount of SCM-198. This may be the mechanism by which SCM-198 increases the fertilization ability of senescent oocytes.

Previous studies have shown that oocyte quality is affected by aging-related oxidative stress [42], which directly or indirectly leads to an increase in ROS levels and early apoptosis of oocytes [43]. SCM-198 has been found to exert a therapeutic effect on ischemic stroke by alleviating oxidative stress [44] and regulating oxidative stress by enhancing stress defenses in the serum and hepatic tissues [45].

Mitochondria are the most abundant cell organelles in oocytes, affecting various aspects of mammalian oocyte maturation and their function represents a pivotal determinant of oocyte quality [46]. Recent studies have highlighted mitochondrial dysfunction as a major contributor to oocyte aging because mitochondria coordinate numerous metabolic, epigenetic, redox, and calcium signaling processes [47]. Mitochondria are involved in many processes including oocyte maturation, fertilization, and early embryonic development [48]. When mitochondrial function is disrupted, it leads to an increase in ROS levels and a reduction in mtDNA copy number, and the oocyte may suffer damage from OS [49]. Abnormal mitochondrial function leads to excessive accumulation of ROS,

which can cause damage to oocytes. Over time, these lesions accumulate to the extent that they eventually trigger the program of early oocyte apoptosis [50]. Relatively high ROS levels can have a negative impact on oocytes because of mitochondrial dysfunction, spindle fragility, and abnormal chromosomes, which means that oocytes do not have a high fertilization ability and have a significant impact on embryonic development [51]. In addition, SCM-198 reduced the expression of pro-apoptotic genes, including Bax, to inhibit apoptosis [52] and exerts cardioprotective effects in mammalian systems [53]. Similarly, our research indicated that SCM-198 reduced the increase in ROS levels induced by aging, thereby inhibiting oocyte apoptosis caused by oxidative stress.

Conclusion

The results of this study showed that SCM-198 inhibited oxidative stress-induced oocyte apoptosis, thereby improving the quality of both postovulatory and maternally aged oocytes. This study provides a method to delay oocyte cellular senescence so that humans might have a higher success rate of ART. However, further clinical research is needed on the relationship between oocytes and SCM-198 to clarify their impact on the quality of human oocytes and subsequent embryonic development.

Accession codes

OVASTACIN: Q6HA09
JUNO: Q9EQF4

Supplementary Information

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Supplementary Material 1

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Author contributions
Xia Wang and Yizhun Zhu designed research; Wei Ma and Xue Wu analyzed data; Wei Ma, Xi Zhao, Qingxin Wang, Xue Wu, Tingting Yang, and Yuqi chen performed research; Wei Ma and Xi Zhao wrote the paper. All authors were involved in drafting and revising the manuscript.

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Data availability
Raw Data: https://www.jianguoyun.com/p/DUMJRd4QhdWHDBjmyaIFIAA.

Declarations

Competing interests
The authors declare no competing interests.

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