Asiatic acid protects oocytes against *in vitro* aging-induced deterioration and improves subsequent embryonic development in pigs

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ABSTRACT

As a pentacyclic triterpene in *Centella asiatica*, asiatic acid (AA) is a powerful antioxidant with many bioactivities. In the present research, we investigated whether AA has the potential to rescue the decrease in porcine oocyte quality that occurs during *in vitro* aging (IVA). Mature porcine oocytes were collected and then continuously cultured for an additional 24 h or 48 h with or without AA in maturation medium as an IVA model. The results revealed that AA supplementation reduced the percentage of abnormal aged porcine oocytes during IVA. Furthermore, AA supplementation effectively maintained aged porcine oocyte developmental competence, both parthenogenetic activation and *in vitro* fertilization. The number of sperm that bound to the zona pellucida on aged porcine oocytes was higher in the AA-supplemented group than in the non-supplemented group. Moreover, AA supplementation not only blocked IVA-induced oxidative stress but also maintained intracellular GSH levels and reduced the percentage of early apoptosis aged porcine oocytes. Mitochondrial functions were disordered during the IVA process. The intracellular ATP levels and mitochondrial membrane potential in aged porcine oocytes were dramatically increased by AA supplementation. Therefore, AA has beneficial effects on porcine oocyte quality and developmental potential maintenance during IVA.

INTRODUCTION

Currently, the use of assisted reproductive technology (ART) is widespread [1–3]. It is inevitable for oocytes used in ART to undergo *in vitro* culture before fertilization. If oocytes are not fertilized at the optimal period, unfertilized oocytes show a time-dependent decline in quality; this phenomenon is called *in vitro* oocyte aging [4, 5]. Oocyte quality determines the developmental capacity of the embryo after fertilization [4–6]. Compared with high-quality oocytes, poor-quality oocytes have reduced antioxidation activity and weak developmental competence [7, 8].

Many studies point to a series of changes in aged oocytes compared with fresh oocytes, such as zona pellucida hardening [9], mitochondrial dysfunction [6, 10], spindle abnormalities [5, 11] and chromosome

aneuploidy [12]. Recent studies have shown that a decline in antioxidant capacity occurs during the oocyte aging process [13], which produces superfluous free radicals, including reactive oxygen species (ROS), inducing many cascades that influence oocyte quality, such as DNA damage, and apoptotic pathways [14–16]. Oocyte mitochondria are very vulnerable to oxidative stress and DNA damage, and dysfunctional mitochondria impair the production of adenosine triphosphate (ATP); moreover, an insufficient ATP supply is an important reason for decreased oocyte fertilization competence and altered embryo development [17]. Oxidative stress is also a factor that induces apoptosis and chromosome instability [18]. It is well known that antioxidants can partially counteract the negative effects of oxidative stress [19, 20]. Therefore, the balance between antioxidant and pro-oxidant substances is a critical factor influencing the quality of aged oocytes.

As a pentacyclic triterpene in *Centella asiatica* [21], asiatic acid (AA) has been shown to have many bioactivities, including antiapoptotic [22], anticancer [23], anti-inflammatory [24], neuroprotective [25], antifibrotic [26], and hypoglycemic action [27, 28]. In addition, AA effectively protects against oxidation [29], which could guard against the damage to the neuronal system resulting from quinolinic acid [30], alleviate seizures caused by kainic acid [31], and play an important role in preventing LPS/d-GalN-induced fulminant hepatic failure (FHF) by limiting oxidative stress [32]. Furthermore, our previous results suggested that AA exerts beneficial effects on porcine early embryonic development by ameliorating oxidative stress and preserving mitochondrial function [33]. It has been reported that AA might also be beneficial to the prevention or alleviation of brain aging [34].

Despite the well-known biological characteristics and physiological functions of AA and its beneficial protective effects, the protective effect and underlying mechanisms of AA on mammalian oocytes have not been defined. Thus, we hypothesized that AA could protect porcine oocytes from deterioration during the *in vitro* aging (IVA) process and could improve subsequent embryonic developmental competence. In the present research, we first explored the effects of AA supplementation during the IVA process on morphological changes and subsequent developmental competence of aged porcine oocytes. Second, we further analyzed the effects of AA on oxidative stress, apoptosis and mitochondrial function in porcine oocytes via the IVA model to identify the associated underlying mechanism. The results could shed light on the underlying beneficial effects of AA on aged oocyte quality.

RESULTS

AA reduces morphological defects during the IVA process of porcine oocytes

Abnormal morphology often results in morphological changes. Therefore, we first investigated whether AA supplementation promoted the maintenance of the morphology of aged porcine oocytes. After *in vitro* maturation (IVM), mature oocytes were collected for IVA, and morphological changes were recorded at 24 and 48 h (Figure 1A–1D). As shown in Figure 1E, the percentage of abnormal oocytes increased in a time-dependent manner (IVA 24 h, $3.70 \pm 0.81\%$, n=245; IVA 48 h, $37.52 \pm 2.17\%$, n=245; $p<0.05$). Compared with the non-AA-supplemented group, the group of *in vitro* aged porcine oocytes supplemented with 10 μM AA showed a decrease in the percentage of oocytes with abnormal morphology at 24 h ($0.85 \pm 0.50\%$, n=200; $3.70 \pm 0.81\%$, n=245; $p<0.05$). However, the morphological defects were not dramatically reduced by AA supplementation at 48 h of IVA ($36.41 \pm 1.97\%$, n=200; $37.52 \pm 2.17\%$, n=245; $p>0.05$).

AA supplementation improves the embryonic developmental potential of porcine *in vitro* aged oocytes after parthenogenetic activation

To extend our finding that supplementation with AA ameliorated the time-dependent deterioration of porcine oocyte quality, oocytes were parthenogenetically activated to evaluate subsequent embryonic development (Figure 2A). The results showed that AA supplementation during the IVA process ameliorated the subsequent declines in developmental competence. Although the percentage of activated oocytes that reached the 2-cell stage ($52.77 \pm 2.25\%$, n=159; $45.18 \pm 4.00\%$, n=162; $45.76 \pm 1.78\%$, n=160; $p>0.05$; Figure 2B) and 4-cell stage ($72.25 \pm 6.42\%$, n=159; $68.37 \pm 4.36\%$, n=162; $70.50 \pm 5.14\%$, n=160; $p>0.05$; Figure 2C) showed no obvious change, when *in vitro* aged porcine oocytes were supplemented with AA, the percentage of activated oocytes that reached the blastocyst stage was obviously improved compared with that not supplemented with AA ($50.19 \pm 2.85\%$, n=155; $12.27 \pm 2.91\%$, n=160; $27.19 \pm 4.94\%$, n=183; $p<0.05$; Figure 2D). Furthermore, the average total number of cells in blastocysts derived from AA-supplemented *in vitro* aged oocytes tended to be higher than that in blastocysts derived from non-supplemented *in vitro* aged oocytes (56.24 ± 1.13, n=41; 25.26 ± 1.18, n=39; 41.75 ± 2.96, n=40; $p<0.05$; Figure 2E). The average diameter of blastocysts derived from AA-supplemented *in vitro* aged oocytes was also decreased compared with that derived from non-supplemented *in vitro* aged oocytes (199.57 ± 5.86, n=49; 163.10 ± 4.18, n=41; 180.31 ± 3.51, n=42; $p<0.05$; Figure 2F).
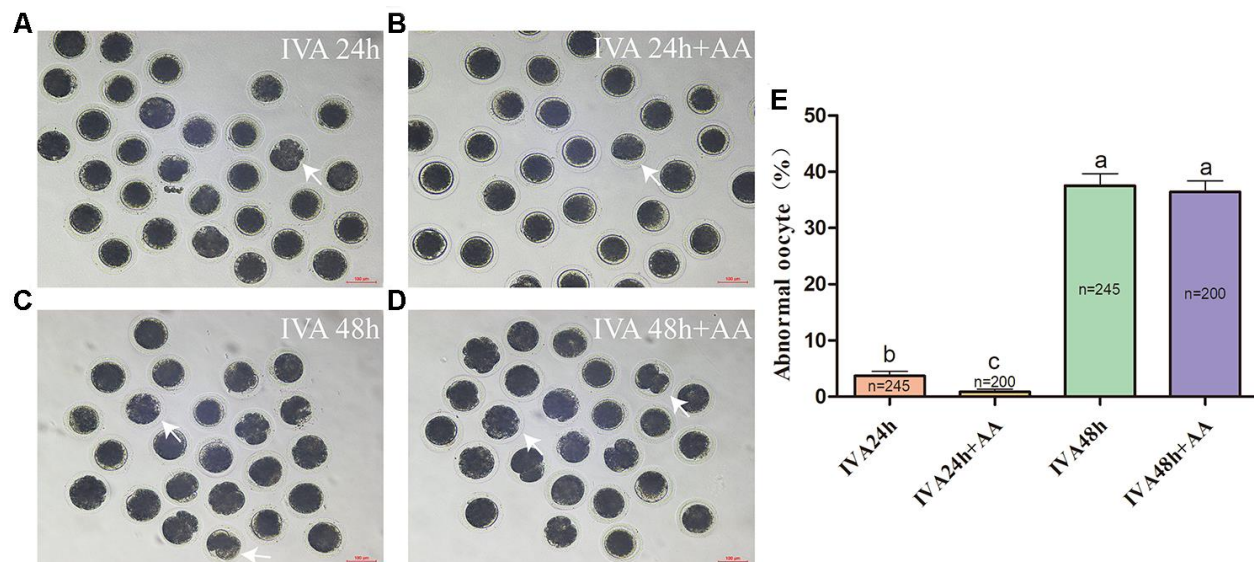
Figure 1. Changes in the morphology of *in vitro* aged porcine oocytes after AA supplementation. (**A**) 24-h *in vitro* aged oocytes; (**B**) 24-h *in vitro* aged oocytes supplemented with 10 μM AA; (**C**) 48-h *in vitro* aged oocytes; (**D**) 48-h *in vitro* aged oocytes supplemented with 10 μM AA; (**E**) morphological changes in porcine oocytes recovered at different culture time points from the AA supplementation groups. Representative morphologically abnormal *in vitro* aged oocytes (white arrows) were examined by optical microscopy. Scale bar=100 μm. The numbers of oocytes examined in each group are shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).
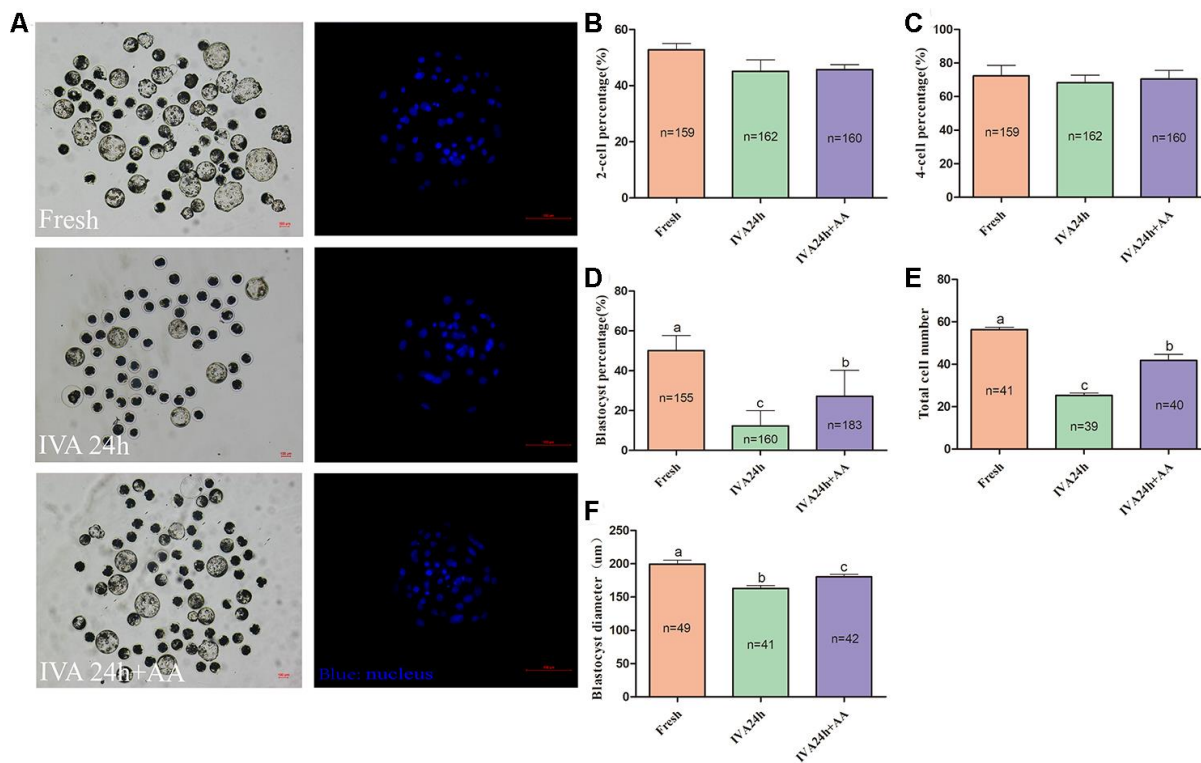

Figure 2. Effect of AA supplementation on the developmental competence of *in vitro* aged porcine oocytes after parthenogenetic activation. (**A**) Blastocyst formation at day 7 in each group (Left); and representative fluorescence images of blastocyst staining with Hoechst 33342 in each group (Right). Scale bar=100 μm. (**B**) Percentage of 2-cell embryos, (**C**) 4-cell embryos and (**D**) blastocyst formation in each group. (**E**) Total number of cells in blastocysts from each group. (**F**) Diameter of blastocysts from each group. The number of embryos examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).

AA supplementation ameliorates sperm the binding ability of porcine *in vitro* aged oocytes and subsequent embryonic developmental competence after *in vitro* fertilization

The results of the present research showed that the decreased oocyte developmental competence induced by IVA was ameliorated by AA supplementation after *in vitro* fertilization. Sperm-egg binding assay results suggested that the average number of sperm that bound to the zona pellucida of *in vitro* aged oocytes tended to be higher in the AA-supplemented group than the non-supplemented *in vitro* aged group (162.26 ± 7.53, n=35; 36.34 ± 2.85, n=38; 101.28 ± 6.83, n=38; *p<0.05*; Figure 3A and 3B). The above results showed that AA supplementation could improve the quality of porcine oocytes. Therefore, *in vitro* fertilization was carried out with fresh, *in vitro* aged and AA-supplemented *in vitro* aged porcine oocytes (Figure 4A). The results showed that 23.06 ± 0.72% (n=190) of fresh oocytes could develop into the blastocyst stage. For *in vitro* aged porcine oocytes, the percentage of oocytes that developed into the blastocyst stage decreased to 6.23 ± 0.48% (n=194). However, in the AA-supplemented *in*

vitro aged oocyte group, the percentage of oocytes that developed into the blastocyst stage was significantly increased (12.30 ± 0.67%, n=193). Similar to the blastocyst formation results, the average total number of cells in blastocysts derived from AA-supplemented *in vitro* aged oocytes was also increased compared with that of blastocysts derived from non-supplemented *in vitro* aged oocytes (45.68 ± 0.76, n=36; 25.22 ± 0.71, n=33; 34.85 ± 0.67, n=33; *p<0.05*; Figure 4C).

AA supplementation ameliorates oxidation resistance in porcine IVA oocytes

Because oxidative stress was associated with oocyte quality and subsequent embryonic developmental competence, we further assessed the free radical scavenging capacity of AA in *in vitro* aged porcine oocytes. The DCFH fluorescent reaction was used to analyze intracellular ROS levels in oocytes. As shown in Figure 5A and 5B, intracellular ROS levels were markedly increased in *in vitro* aged porcine oocytes compared with fresh oocytes, while AA supplementation effectively alleviated the increase in intracellular ROS levels during the IVA process of
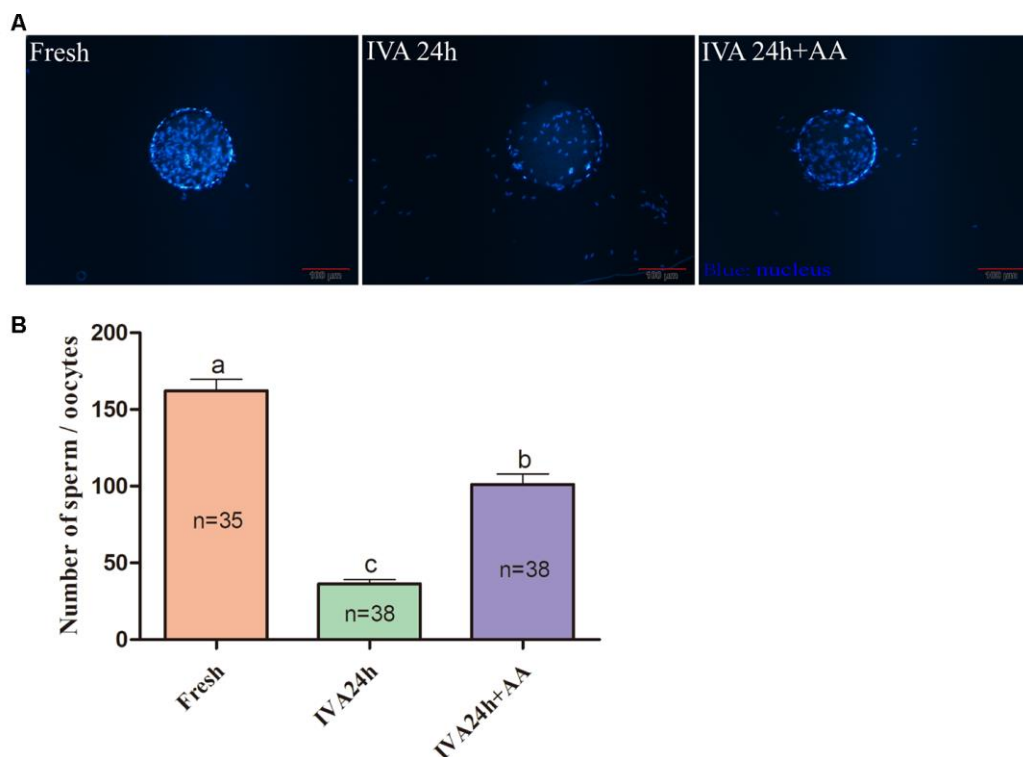

Figure 3. Effect of AA supplementation on sperm binding to the zona pellucida of porcine *in vitro* aged oocytes after *in vitro* fertilization. (**A**) Representative fluorescence images of sperm binding to the surface of the zona pellucida surrounding oocytes staining with Hoechst 33342 in each group. Scale-bar=100 μm. (**B**) Number of sperm binding to the surface of the zona pellucida surrounding oocytes in each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).
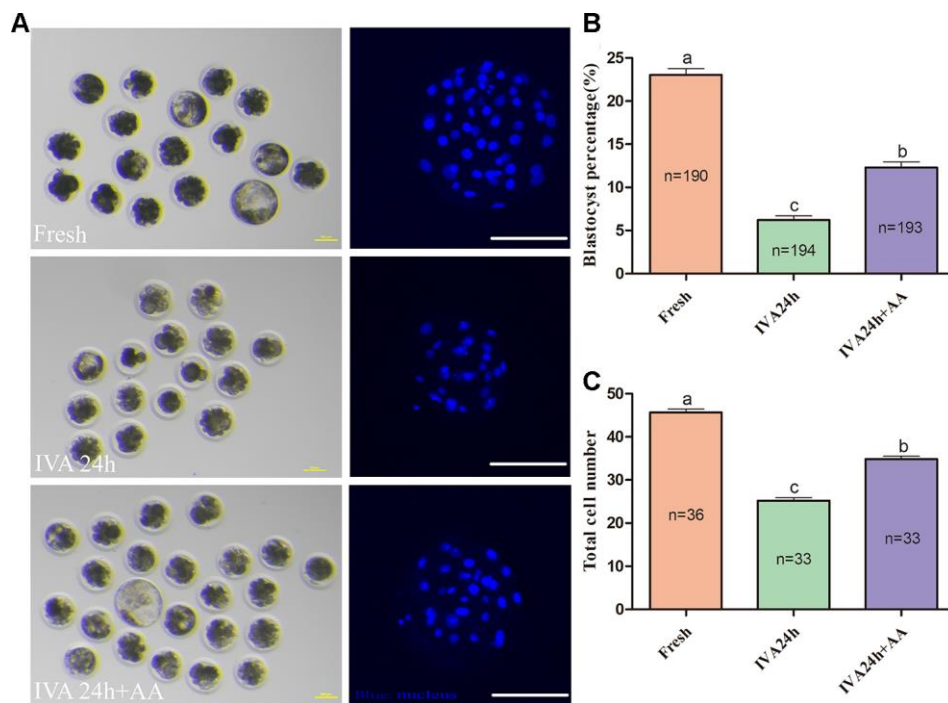
Figure 4. Effect of AA supplementation on the developmental competence of porcine *in vitro* aged oocytes after *in vitro* fertilization. (**A**) Blastocyst formation at day 7 in each group (Left); and representative fluorescence images of blastocyst staining with Hoechst 33342 in each group (Right). Scale bar=100 μm. (**B**) Percentage of blastocyst formation in each group. (C) Total number of cells in blastocysts from each group. The number of embryos examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).
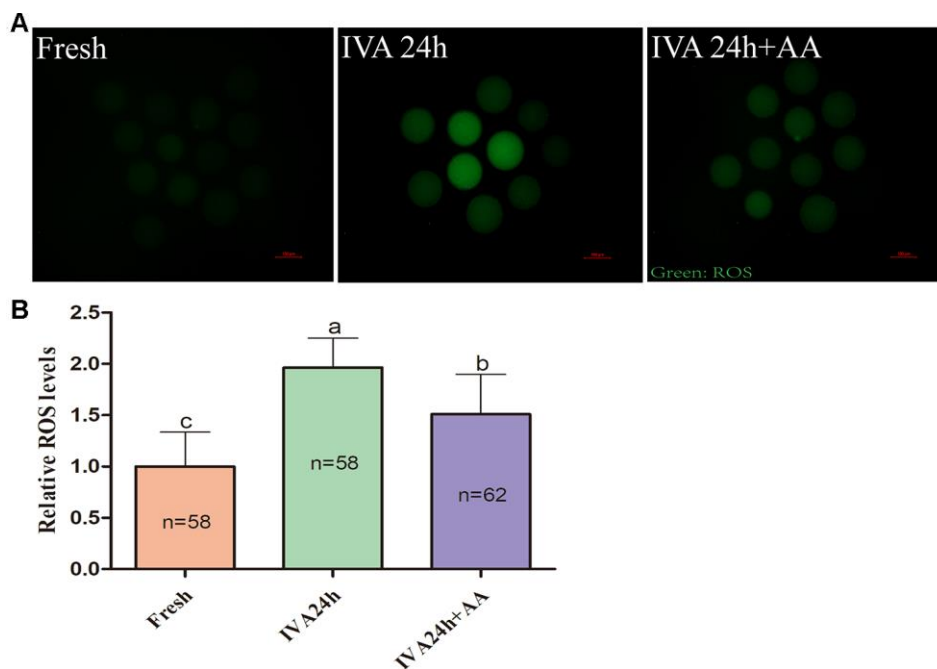

Figure 5. Effects of AA supplementation on the production of intracellular ROS in porcine IVA oocytes. (**A**) Representative fluorescence images of intracellular ROS in oocytes in each group. Scale bar=100 μm. (**B**) Relative intracellular ROS levels in oocytes in each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).

porcine oocytes. In accordance with the above results, we further assessed the intracellular GSH levels of *in vitro* aged porcine oocytes. As shown in Figure 6A and 6B, the intracellular GSH levels of *in vitro* aged porcine oocytes were markedly decreased compared with those of fresh oocytes, while AA supplementation effectively alleviated the decline in intracellular GSH levels in porcine oocytes during the IVA process.

AA supplementation decreased the early apoptosis of *in vitro* aged porcine oocytes

To further define the role of AA during the IVA process of porcine oocytes, the early apoptosis of *in vitro* aged porcine oocytes was monitored via the Annexin-V and TUNEL assays. As shown in Figure 7A and 7B, only $34.84 \pm 2.26\%$ (n=63) and $1.33 \pm 1.33\%$ (n=79) of fresh oocytes were positive for Annexin-V and TUNEL, respectively; however, the proportion of Annexin-V and TUNEL positive oocytes among *in vitro* aged oocytes were increased to $67.78 \pm 3.98\%$ (n=65) and $10.74 \pm 1.03\%$, respectively (n=74); $p<0.05$. In contrast, in oocytes supplemented with AA during the process of IVA, the proportion of Annexin-V and TUNEL positive oocytes was reduced to $50.14 \pm 2.00\%$ (n=67) and $3.89 \pm 2.04\%$, respectively (n=68); $p<0.05$.

AA supplementation alleviated mitochondrial function in porcine IVA oocytes

Mitochondria play important roles in the regulation of cellular energy metabolism, free radical generation and apoptosis. Therefore, we evaluated ATP levels and mitochondrial membrane potential (MMP) in porcine IVA oocytes. As shown in Figure 8A and 8B, the ATP levels of *in vitro* aged porcine oocytes were markedly decreased compared with those fresh oocytes, while AA supplementation effectively alleviated the decline in ATP levels during the IVA process of porcine oocytes. The MMP of *in vitro* aged porcine oocytes was evaluated using the JC-1 fluorescent dye (Figure 9A). Quantification showed that the MMP of *in vitro* aged porcine oocytes was markedly decreased compared with that of fresh oocytes, while AA supplementation effectively alleviated the decrease in MMP during the IVA process of porcine oocytes (Figure 9B).

DISCUSSION

The IVM of pig oocytes is used not only for understanding the event of meiosis but also for helping breed, clone and genome edit pigs for animal production. Previous research has shown that compared with that of other species oocytes, the maturation time of pig oocytes is prolonged [35]. During IVM, porcine oocytes are particularly susceptible to aging, leading to disturbances in metabolism, stress response and cell cycle regulation [36]. These changes reduce the quality of oocytes [13] and impact later embryonic development [7, 37]. In the present research, we found that AA could delay the process of pig oocyte IVA and subsequently improve embryonic developmental potential.
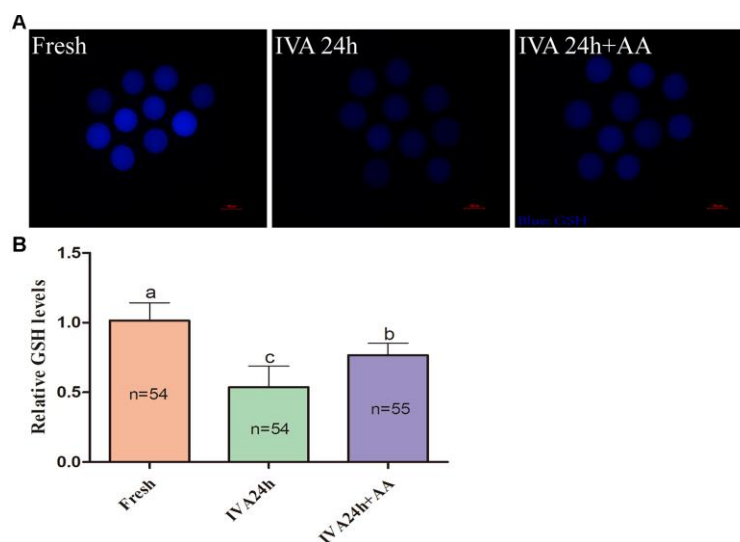

Figure 6. Effects of AA supplementation on the production of intracellular GSH in porcine IVA oocytes. (**A**) Representative fluorescence images of intracellular GSH in oocytes in each group. Scale bar=100 μm. (**B**) Relative intracellular GSH levels in oocytes in each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).

Fertilization is a complex process of sexual reproduction in which the female and male gametes are mixed to generate new individuals [38]. Therefore, fertilization ability is one of the indicators used to evaluate oocyte quality. Our results show that AA can alleviate the decreased fertilization ability of aged oocytes, which is consistent with our results that AA could improve the parthenogenetic activation of developing embryos. In the reproduction process of animals, a particular sperm needs to arrive at, attach to, and mix with the egg plasma membrane; hence, it has to attach to and pass through the zona pellucida [39–41]. In other words, fertilization begins with the binding of the sperm to the zona pellucida. We found that AA could increase the number of sperm that bind to aged oocytes. Early studies suggested that the process of oocyte aging is usually accompanied by zona pellucida changes [5]. Thus, we suspect that AA may have other potential mechanisms to mitigate fertility declines due to aging by altering certain components of the zona pellucida.
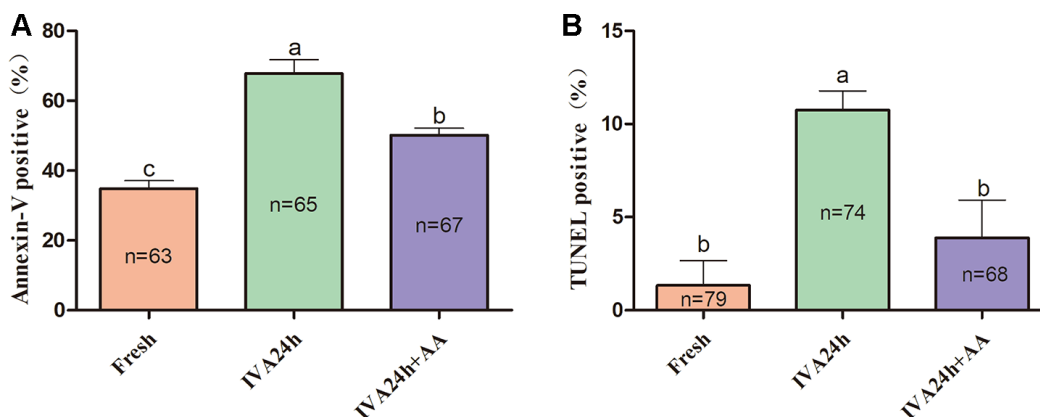

Figure 7. Effects of AA supplementation on the early apoptosis of *in vitro* aged porcine oocytes. (**A**) Percentage of Annexin-V and (**B**) TUNEL positive oocytes in each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).
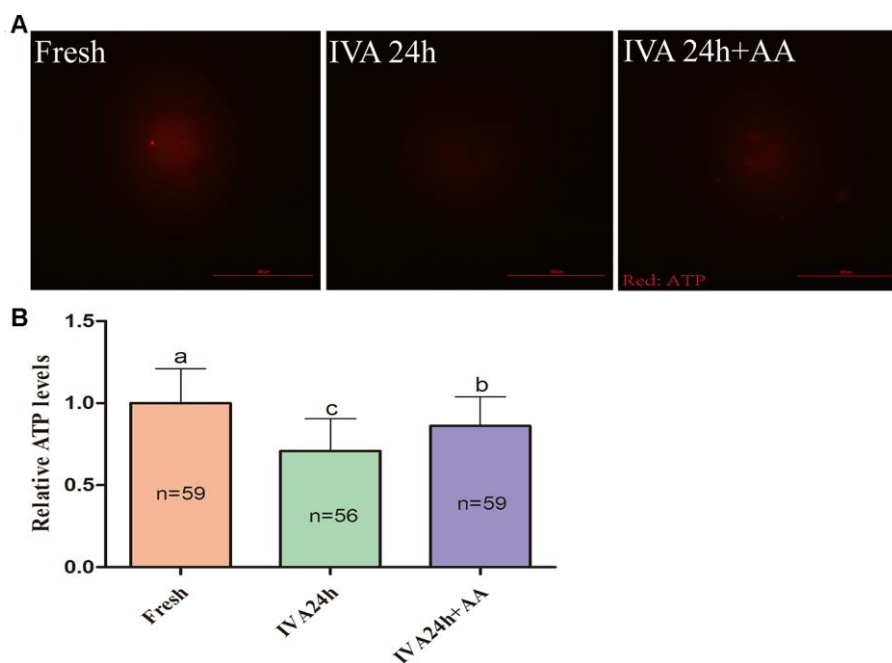

Figure 8. Effects of AA supplementation on the ATP production of *in vitro* aged porcine oocytes. (**A**) Representative fluorescence images of ATP staining in oocytes from each group. Scale bar=100 μm. (**B**) Relative ATP levels in oocytes from each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).

Previous studies have shown that the quality of oocytes decreases gradually during the IVA process and that aged oocytes are sensitive to oxidative stress [37, 42]. Thus, this process of oocyte aging easily results in oxidative stress via the accumulation of free radicals. The increase in intracellular ROS could reduce the developmental potential of oocytes [43]. Mammalian cells have a wide range of antioxidants that have the ability to clear ROS, including non-enzymatic antioxidants [44] and several enzymatic antioxidants [45]. GSH, as an intrinsic antioxidant in oocytes, plays an important role in maintaining the redox state in oocytes [46]. GSH deficiency could lead to increased intracellular oxidative stress [47], germ cell apoptosis [48], and widespread mitochondrial damage [49], and a high intracellular GSH content could improve the quality of the oocytes [50]. In addition, a lack of GSH synthesis is the basis of oxidative stress during aging [51]. Our results demonstrate that AA supplementation clearly ameliorated oxidative stress because it not only decreased intracellular ROS levels but also maintained intracellular GSH levels during the process of IVA. Similarly, AA was reported to reduce intracellular ROS levels in microglial cells [52], HepG2 cells [53], and SH-SYS5Y cells [54] and increase superoxide dismutase activity and intracellular GSH production in

spinal cord injury in rats [55]. Therefore, we hypothesized that AA might reduce oxidative stress by removing intracellular ROS and increasing GSH synthesis, thus delaying the decline in porcine oocyte quality during the process of IVA.

Functional mitochondria play an important role in normal oocyte function, since within both oocytes and early embryos, these organelles are the major source of ATP production [56]. Proton pumps, expressed on both sides of the mitochondrial membrane, regulate the potential of the mitochondrial membrane by the production of ATP [57]. An insufficient supply of ATP affects oocyte quality, leading to many problems, such as arrested cell division, abnormal cytokinesis, fragmentation [58–60], maturation and fertilization failure [17]. In mammals, the number of mitochondria increases dramatically with the growth of oocytes [61, 62], and after fertilization, the number of mitochondria remains constant from the early stages of embryo development through embryo implantation [62, 63]. Furthermore, abnormal MMP is closely related to fertilization, oocyte maturation, calcium homeostasis maintenance and apoptosis [17, 37, 64]. Therefore, it is very important for oocytes to have sufficient properly functioning mitochondria to ensure appropriate
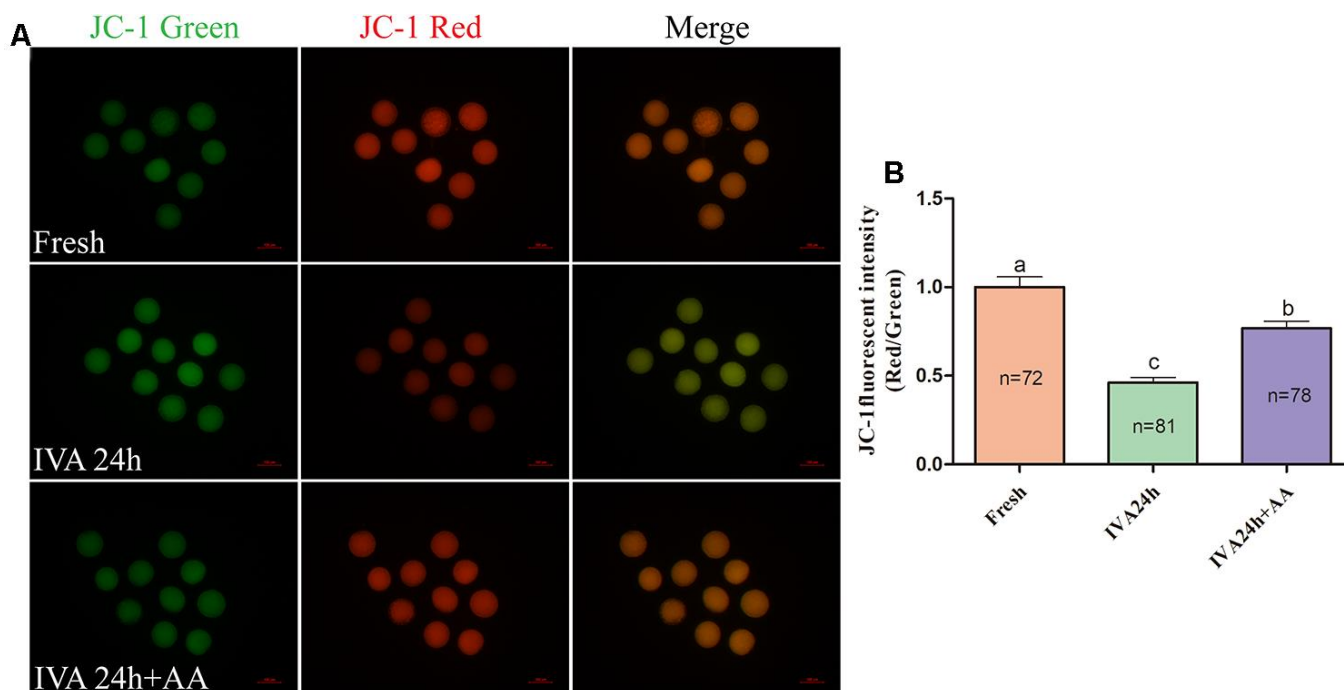

Figure 9. Effects of AA supplementation on the mitochondrial function of *in vitro* aged porcine oocytes. (**A**) Representative fluorescent images of JC-1 stained oocytes from each group. Scale bar=100 µm. (**B**) Relative JC-1 fluorescence intensity (red/green) in oocytes from each group. The number of oocytes examined in each group is shown by the bars. Statistically significant differences are indicated by different letters (*p<0.05*).

embryonic development. Increasing data have proven that changes in mitochondrial function are inextricably linked with stress-induced damage to oocyte developmental capacity [65]. Recent research shows that this impairment decreases mitochondrial ROS production, impairs the ability of mitochondria to sufficiently meet the cellular ATP demand, and induces mitochondrial-mediated apoptosis [66]. Moreover, mitochondria are considered to be a key element in the process of oocyte aging, which is greatly impacted by mitochondrial dysfunction [67, 68]. Our study shows that AA reduced the level of intracellular ROS and increased ATP activity and the red/green fluorescence ratio of JC-1, suggesting that mitochondrial dysfunction and oxidative stress were improved by AA supplementation during the process of porcine oocyte IVA. Since the findings in these experiments are in line with previous studies, it can be concluded that mitochondrial dysfunction can be well reduced by AA in various cells, including murine BV2 microglia cells, rat cortical neuronal cells [69, 70] and human A549 cells [71], and these results further confirm our hypothesis.

Since cell death via apoptosis is the last stage of oocyte aging [72, 73], it is hypothesized that AA can inhibit the activation of the apoptotic pathway of aged oocytes. We found that AA could delay the commencement of apoptosis in aged oocytes by Annexin-V and TUNEL assays. This function of AA is consistent with the results of other studies, which highlighted that AA protects against lactate-induced apoptosis through controlling the lactate signaling cascade and mediating mitochondria-dependent caspase activation and oxidative stress [71]; moreover, because of its anti-oxidant, mito-protective and antiapoptotic properties, pretreatment with AA before rotenone exposure in SH-SY5Y cells prevented marked ROS overproduction, mitochondrial dysfunction and apoptosis [54]. In early studies, the aging of humans and mammals was shown to be closely related to an increased level of ROS in mitochondria with age, which subsequently triggered apoptosis [74]. The problems associated with IVA appear to have a similar mechanism to the body's response to aging, which needs further study.

In summary, we found that AA could effectively improve the quality of *in vitro* aged porcine oocytes by relieving oxidative stress, reducing apoptosis, improving mitochondrial function, and promoting subsequent embryonic development (Figure 10). In other words, as an outstanding antioxidant that can be extracted from natural products, AA is expected to prevent the *in vitro* aging-induced decrease in oocyte quality.
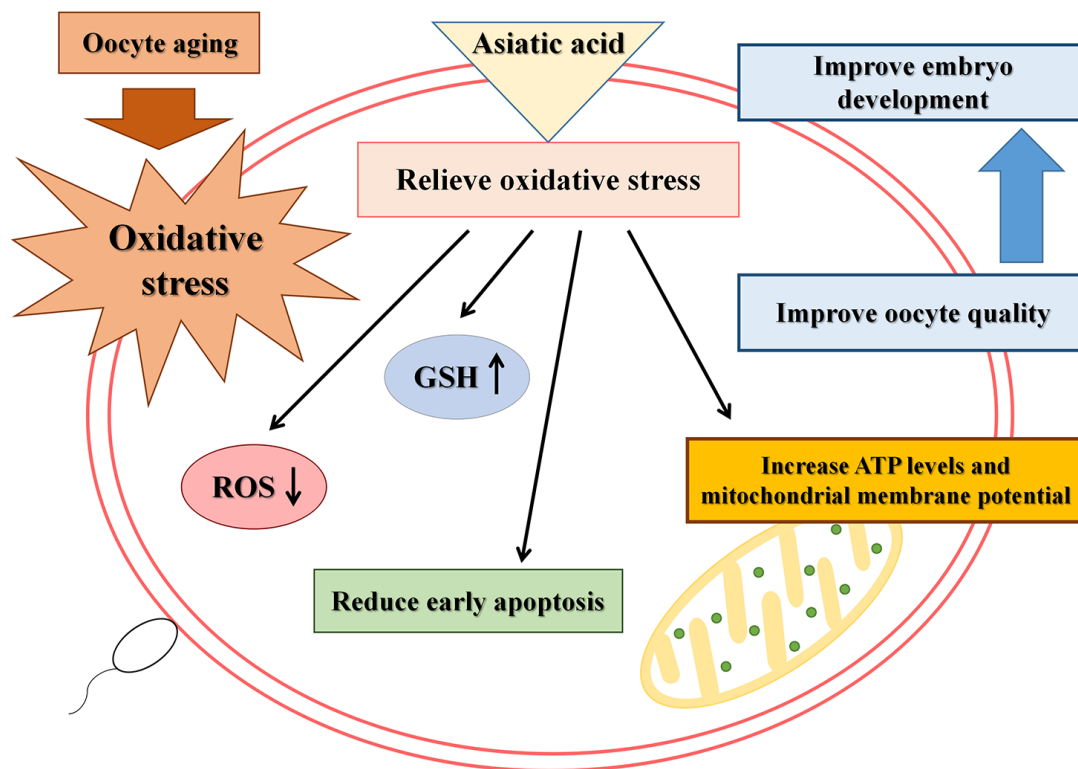

Figure 10. Schematic representation of the protective action of AA on porcine IVA oocytes.

MATERIALS AND METHODS

All chemicals used in this experiment were obtained from Sigma-Aldrich, a company based in St. Louis, America, except for those that are specifically defined.

Oocyte collection and IVM

Porcine ovaries were collected from a local abattoir and transported to the laboratory in 0.9% saline at 30-35° C. Follicular fluid was aspirated from 3-8 mm antral follicles using a syringe needle. Only evenly distributed cumulus oocyte complexes (COCs) with more than three layers of cumulus cells were collected under a stereomicroscope (S22-LGB, Nikon). After that, the COCs were washed three times in Tyrode's lactate-HEPES buffered medium and were cultured in IVM medium (tissue culture medium 199 supplemented with 0.6 mM L-cysteine, 0.91 mM Na pyruvate, 10 ng/mL EGF, 10% porcine follicular fluid, 10 IU/mL follicle-stimulating hormone, 10 IU/mL luteinizing hormone and 75 mg/mL kanamycin) for 42 h at 38.5° C in an atmosphere of 5% CO_2 and 100% humidity covered with mineral oil.

IVA of porcine oocytes

At the end of IVM, the expanded cumulus cells were removed from COCs by treatment with 0.1% hyaluronidase, and only matured oocytes with normal morphology were collected for IVA. The oocytes were then cultured in IVM medium, treated or not treated with 10 μM AA, covered with mineral oil in a damp environment of 38.5° C and 5% CO_2 and cultured for 24 or 48 h. The final concentration of AA used in the present research was selected according to our preliminary experiment.

Parthenogenetic activation and *in vitro* fertilization

The parthenogenetic activation and *in vitro* fertilization procedures were performed as described in our previous research [33]. After parthenogenetic activation and *in vitro* fertilization, activated or fertilized oocytes were washed in porcine zygote medium (PZM)-3 and then transferred into four-well plates containing 500 μL PZM-3 covered with mineral oil and cultured for 7 days at 38.5° C in an atmosphere of 5% CO_2 and 100% humidity without changing the medium. Blastocyst rates and diameters were analyzed on day 7, and blastocyst diameters were analyzed via NIH ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Assessment of the total number of cells in blastocysts

To quantifying the total number of cells contained in blastocysts, day 7 blastocysts derived from embryos that underwent parthenogenetic activation and *in vitro* fertilization were fixed in PBS-PVA with 4% PFA for 30 min at room temperature. Then, the blastocysts were washed with PBS-PVA three times and incubated with 10 μg/mL Hoechst 33342 for 5 min to label the nuclei. Next, blastocysts were gently mounted onto glass slides and examined under a fluorescence microscope (IX73, Olympus, Tokyo, Japan). NIH ImageJ software was used to analyze the total number of nuclei.

Sperm binding assay

Frozen boar sperm samples were used in this research, and the percentage of motile spermatozoa after thawing was 70%-90%. Approximately 15-20 oocytes were transferred into 60 μl droplets of mTBM for 30 min and then co-incubated with 20 μl diluted sperm for 1 h at 38.5° C in an atmosphere of 5% CO_2 and 100% humidity. Afterwards, the oocytes were fixed in PBS-PVA with 4% PFA for 30 min at room temperature and stained with 10 μg/mL Hoechst 33342. Next, the oocytes were gently mounted onto glass slides and examined under a fluorescence microscope. The number of sperm bound to each oocyte was analyzed via NIH ImageJ software.

Evaluation of intracellular ROS and GSH levels in oocytes

The intracellular ROS and GSH levels in fresh and aged oocytes were measured using the fluorescent probes 2',7'-dichlorodihydrofluorescein diacetate (10 μM, Thermo Fisher Scientific, C400) and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (10 μM, Thermo Fisher Scientific, C12881), respectively. Oocytes from each treatment group were incubated in the dark for 25 min at 38.5° C in an atmosphere of 5% CO_2 and 100% humidity. After incubation, the oocytes were washed three times with PBS-PVA, and 10 μL droplets were removed. The fluorescence signal was captured using an epifluorescence microscope, and the fluorescence intensity of oocytes was analyzed using NIH ImageJ software.

Evaluation of oocyte apoptosis

The apoptosis of fresh and aged oocytes was measured via Annexin-V and TUNEL assays. Staining procedures were performed according to the product manual. For the Annexin-V assay, the oocytes were incubated in 100 μL Annexin-V binding solution with 5 μL Annexin-V and fluorescein isothiocyanate (FITC) conjugate for 30 min in the dark at room temperature. For the TUNEL assay, the oocytes were incubated with 50 μL TUNEL reaction mixture for 1 h at 37° C in the dark. The fluorescence signal was captured using a confocal microscope.

ATP measurement in oocytes

The ATP content of individual oocytes was measured using a fluorescent antibody (Abcam, 198367). Briefly, oocytes were collected, washed twice in PBS-PVA and then fixed in PBS-PVA with 4% PFA for 10 min at room temperature. Afterwards, the oocytes were washed three times with ice-cold PBS-PVA and then incubated with PBS-PVA containing 0.1% Triton X-100 for membrane permeabilization for 1 h. After being blocked for 30 min in 1.5% bovine serum albumin prepared in PBS-PVA (blocking solution), the oocytes were incubated with the antibody diluted in blocking solution (1:100, v/v) overnight at 4° C. After thoroughly washing with PBS-PVA, the fluorescence signal was captured using an epifluorescence microscope, and the fluorescence intensity of oocytes was analyzed using NIH ImageJ software.

JC-1 staining

To measure MMP, fresh and aged oocytes were washed three times with PBS-PVA. They were measured using the fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (2 μM, JC-1, Dojindo, China) for 30 min. After incubation, the oocytes were washed three times with the solution in the kit. The red and green fluorescence signals were captured using a fluorescence microscope connected to a digital camera. The fluorescence intensity of each oocyte was analyzed using NIH ImageJ software.

Statistical analysis

SPSS software version 11.0 (IBM, USA) was used to analyze all the data collected. The data from the two groups were compared with Student's t-test, the results are presented as the mean ± standard error of the mean, and considerable discrepancies are indicated by different letters ($p<0.05$).

AUTHOR CONTRIBUTIONS

Shuang Liang, Bo-Xing Sun, Xiao Xia Li and Wei-Yi Hu conceived the experiment. Wei-Yi Hu, Xiao Xia Li and Yun Fei Diao performed the experiments. Wei-Yi Hu and Xiao Xia Li analyzed the data and wrote the article. Jia-Jia Qi and Da-Li Wang helped with the analysis of the results and revised the figures. Shuang Liang, Bo-Xing Sun and Jia-Bao Zhang assisted in the analyses of the results and helped revise the article.

CONFLICTS OF INTEREST

All the authors declare that they have no conflicts of interest.

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