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Effects of exogenous spermidine on autophagy and antioxidant capacity in ovaries and granulosa cells of Sichuan white geese

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Abstract

Autophagy can inhibit ovarian senescence induced by oxidative stress and regulate follicle development and atresia, but its mechanism is still unclear. Exogenous spermidine can induce autophagy and scavenge reactive oxygen species (**ROS**). In this experiment, oxidative stress in Sichuan white geese ovaries and follicular granulosa cells (**GCs**) was caused by 3-nitropropionic acid (**3-NPA**) and spermidine was added to explore the effect of exogenous spermidine inducing autophagy and inhibiting oxidative stress in vivo and in vitro. Research results showed that putrescine, spermidine and spermine contents in goose ovaries in the group treated with spermidine combined with 3-NPA were 2.70, 1.94, and 1.70 times higher than those in the group treated with 3-NPA, respectively ($P < 0.05$). The contents of spermidine and spermine in GCs were 1.37 and 0.89 times higher in the spermidine in combination with the 3-NPA group than in the 3-NPA group, respectively ($P < 0.05$). LC3 and p62 were mainly expressed in the follicular granulosa layer. The LC3-II/I ratio and p62 level in GCs in the spermidine combined with 3-NPA treatment group were 1.37 and 0.77 times higher than that of the 3-NPA treatment group, respectively ($P < 0.05$). 3-NPA treatment significantly increased ROS level and the apoptosis rate in GCs, while the combined treatment of spermidine and 3-NPA reversed this change ($P < 0.05$).

In conclusion, spermidine alleviated the oxidative damage induced by 3-NPA by improving the antioxidant capacity of ovaries and follicular GCs of Sichuan white geese and may be alleviated by inducing autophagy in GCs.

Lay summary

This study investigated the effects of exogenous spermidine on oxidative stress induced by 3-nitropropionic acid (**3-NPA**) in ovaries and granulosa cells of Sichuan white geese. In ovarian tissue, spermidine can reduce malondialdehyde accumulation induced by 3-NPA by increasing antioxidant enzyme activity, thus alleviating the oxidative damage induced by 3-NPA. In addition, spermidine can also improve the morphological structure of follicles and alleviate the structural damage caused by 3-NPA. Our results showed that autophagy-associated proteins are mainly concentrated in the granulosa layer of follicles and spermidine can alter their expression. Subsequently, we found that spermidine could induce autophagy and reduce the accumulation of reactive oxygen species and apoptosis rate induced by 3-NPA in granulosa cells. Therefore, we speculate that spermidine can alleviate oxidative stress induced by 3-NPA by inducing autophagy in granulosa cells.

In conclusion, spermidine can relieve oxidative stress induced by 3-NPA by increasing the activity of antioxidant enzymes, and may also relieve oxidative stress by inducing autophagy.

Keywords: autophagy, goose ovaries, granulosa cells, oxidative stress, spermidine

Abbreviations: 3-NPA, 3-nitropropionic acid; AG, amino guanidine; APAO, acetyl-polyamine oxidase; BAX, B-cell lymphoma-2-associated X protein; BCL-2, B-cell lymphoma-2; CASPASE3, cysteinyl aspartate-specific proteinase-3; CASPASE8, cysteinyl aspartate-specific proteinase-8; CASPASE9, cysteinyl aspartate-specific proteinase-9; CAT, catalase; DAB, diaminobenzidine; ddH_2O doble distilled water; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCs, granulosa cells; GPX, glutathione peroxidase; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; ODC, ornithine decarboxylase; PBS, phosphate buffer saline; RIPA, radio-immunoprecipitation assay; ROS, reactive oxygen species; SMO, spermine oxidase; SOD, superoxide dismutase; SPDS, spermidine synthase; SPMS, spermine synthase; SSAT, spermidine/spermine-N1-acetyltransferase

Introduction

Granulosa cells (GCs) are important functional cells of follicles and participate in the secretion of follicle growth stimulating factors, such as estradiol and progesterone (Chowdhury et al., 2016), and their apoptosis can induce follicular atresia (Tilly et al., 1991; Jiang et al., 2003). Oxidative stress induces the apoptosis of GCs, which leads to a decrease in ovarian reserve capacity (An et al., 2021). Meanwhile, oxidative stress in GCs is an important factor leading to ovarian senescence and fertility degradation (Lin et al., 2022). Oxidative stress induced by heat stress in chicken ovarian GCs activates the death receptor pathway and induces cell apoptosis (Li et al., 2020). Inducing oxidative stress can intensify GCs apoptosis and decrease the laying rate (Zhou et al., 2020). Therefore,

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inhibiting oxidative stress-induced follicular GCs apoptosis is a useful method for improving ovarian function.

Autophagy can reduce oxidative stress by engulfing the damaged organelles (Yun et al., 2020), which plays a unique role in follicular development and oxidative stress (Zhou et al., 2019). For example, autophagy regulates follicular development by mediating the HIF-1α/BNIP3 pathway and prevents GCs oxidative stress induced by follicular hypoxia (Tang et al., 2021). Moreover, follicle-stimulating hormone inhibits mTOR phosphorylation by mediating HIF-1α, thus inducing autophagy in mouse follicular GCs and blocking hypoxia-induced follicular atresia (Zhou et al., 2017). This suggests that autophagy may inhibit ovarian GCs oxidative stress and relieve follicular atresia. Spermidine is considered a reproductive nutrient (Lefevre et al., 2011), free radical scavenger (Rider et al., 2007), and autophagy inducer (Madeo et al., 2019). Recent studies have reported that spermidine is beneficial for the antioxidant system (Chai et al., 2019; Jiang et al., 2021a). Exogenous spermidine increases the expression of sirtuin-1, mitochondrial transcription factor A, and nuclear respiratory factors 1 and 2, and reduces the production of reactive oxygen species (**ROS**), thus protecting H_2O_2-treated cardiomyocytes (Wang et al., 2020). Spermidine pretreatment attenuated 3-nitropropionic acid (**3-NPA**)-induced changes in oxidative stress, neuro-inflammatory markers, and striatum neurotransmitters in rats (Jamwal and Kumar, 2016). Simultaneously, spermidine, as an autophagy inducer, is conducive to regulating the acetylation and phosphorylation of autophagy-associated proteins (Madeo et al., 2010, 2018). For example, spermidine protects nucleus pulposus cell apoptosis by upregulating the LC3-II/I ratio and downregulating p62 protein expression, and 3-methyladenine (autophagy inhibitor) reverses the positive effect of spermidine on cell apoptosis (Zheng et al., 2018). Autophagy induced by spermidine may be critical for ovarian development and oxidative stress. However, the specific mechanism has not been elucidated. In this study, 3-NPA was employed to induce oxidative stress, and related indices of autophagy and oxidative stress were detected after 3-NPA was combined with spermidine treatment to explore the effect of exogenous spermidine in regulating oxidative stress and autophagy in the Sichuan white goose ovaries and GCs.

Materials and Methods

Animals and collection of ovaries and GCs

This experiment was completed in the College of Animal Science and Technology of Sichuan Agricultural University (DKY-B2018202006). All feeding and use requirements were in line with the Sichuan Agricultural University animal practices, and approved by the Welfare Management Committee.

Sichuan white geese at the age of 220 ± 5 d were randomly selected under the same environment. To screen the optimal concentration of spermidine treatment in vivo, eight Sichuan white geese were randomized into four groups (n = 2): control group, 3-NPA treatment group (10 mg/kg), and spermidine (10 or 15 mg/kg) combined with the 3-NPA treatment group, respectively. Ovarian tissue was collected. Subsequently, another 60 Sichuan white geese were randomized into four groups (n = 15): control group, 3-NPA treatment group (10 mg/kg), spermidine (10 mg/kg) treatment group, and spermidine combined with the 3-NPA treatment group. These geese were weighed before the test. On the first and second day of the experiment, geese were given spermidine and poultry saline by intragastric administration at 08:00 and 20:00 each day according to the following procedures. Geese in the control group and 3-NPA group were given 3 mL of poultry saline, and geese in the spermidine treatment group and spermidine combined with the 3-NPA treatment group were given 2 mL of spermidine and 1 mL of poultry saline. From the third day of the experiment, based on the above treatments, geese were intraperitoneally injected with 2 mL of 3-NPA or poultry saline at 08:30 and 20:30. The control group and spermidine treatment group were intraperitoneally injected with 2 mL of poultry saline, and the 3-NPA treatment group and spermidine combined with the 3-NPA treatment group were intraperitoneally injected with 2 mL of 3-NPA. After 7 d, geese were weighed, and goose serum and whole blood were collected from wing vein and then placed on ice. The goose ovaries were collected and weighed, part of the ovary was fixed with 4% paraformaldehyde, and the rest of the ovary was frozen in liquid nitrogen after transfer to −80 °C refrigerator.

In vitro experiments, Sichuan white geese reared under the same environment and at the peak of egg production were selected. F2 to F5 hierarchical follicles were collected quickly after slaughter and follicular GCs were isolated and cultured.

Cell culture

During the laying period, follicles of Sichuan white geese were collected, and the granulosa layer was separated and placed in a clean beaker. GCs were digested and collected using collagenase II (Sigma, Shanghai, China). Cells were cultured in DMEM/F12 (HyClone, USA) with fetal bovine serum (Gibco, USA) at a concentration of 10% in a 37 °C/5% CO_2 incubator after adjusting the cell density to 1×10^6. When the cell density per well reached more than 80%, the cells were treated in accordance with the experimental requirements. After completion, cells were collected and frozen for experimentation.

Hematoxylin and eosin (H&E) staining

A 4% formaldehyde solution was used to fix the ovarian tissue, embedded in paraffin and sectioned at 4-μm thickness. Hematoxylin and eosin were used to stain tissue sections according to the general procedure (Wuhan Service Biotechnology Co., Ltd., Wuhan, China). Light microscopy was used to observe the morphologies of the ovary and follicle.

Immunohistochemistry

The dewaxed sections were stained with 3% methanol/hydrogen peroxide. After repeatedly washing with phosphate buffer saline (**PBS**) solution three times, the slices were soaked in 0.01 mol/L citric acid buffer, heated after boiling, and allowed to cool at room temperature naturally, and then repeatedly heated to boiling. After the slices were completely cooled, the slices were washed with PBS solution. The primary antibody was incubated with the slices overnight at 4 °C after the sections were blocked with goat serum at room temperature. Then, the secondary antibody was incubated at 37 °C for 30 min. The slices were washed with PBS three times; the diaminobenzidine (**DAB**) reagent was mixed according to the instructions, and the sections were stained with DAB reagent and observed by optical microscopy. After reaching the requirements, the DAB reagent was washed with double distilled water (**ddH_2O**). Hematoxylin dehydrates the slices after re-staining, and the second formaldehyde is transparent.

After it was transparent, we used neutral gum for sealing. Microscopic observation and analysis of the distribution of target proteins were then performed.

Cell viability assay

GCs were seeded and treated in 96-well cell culture plates. After 24 h, the medium was discarded, and 100 μL DMEM/F12 containing 10% CCK-8 reagent (Beyotime) (set zeroing holes) was added to each well and incubated for 2 to 4 h at 37 °C. A multifunctional microplate reader was used to measure the OD value at 475 nm, and then, the data were entered into the following formula to calculate the cell activity.

$$\text{cell viability} = \frac{(\text{OD treatment group} - \text{OD zeroing well})}{(\text{OD control group} - \text{OD zeroing well})}$$

SOD enzyme activity assay

Approximately 50 mg of ovarian tissue was weighed, and a 1:10 (mg/mL) superoxide dismutase (**SOD**) sample preparation solution was added. The sample was ground on ice using an electric homogenizer and centrifuged. The sample to be tested was the supernatant. The protein concentration of the sample was detected according to the instructions of the BCA detection kit (Beyotime). SOD sample diluent was used to dilute the subsequent experimental samples to 20 to 100 μg. The WST-8 working solution and reaction-starting solution were prepared according to the SOD instructions (Beyotime), and samples were treated to detect and calculate the SOD content in the tissues.

MDA level detection

Samples were lysed on ice with radio-immunoprecipitation assay (**RIPA**) buffer (containing PMSF), and the supernatant was collected by centrifugation at 12,000 r/min. Then, the BCA detection kit (Beyotime) was used to determine the protein concentration. The reagents were prepared according to the malondialdehyde (**MDA**) instructions (Beyotime), and the standard curve was prepared. The samples were treated according to the MDA instructions, and the MDA content in tissues was measured and calculated.

Determination of polyamine content

The polyamine content in ovaries and GCs were detected using high-performance liquid chromatography (**HPLC**). The steps were as follows. Ovarian tissue: After weighing the ovary, the ovary was ground with a glass homogenizer. Cells: After collecting cells, we shook and mixed. Both were placed in 5% $HCLO_4$. 10 μL of 1, 6-hexamethylenediamine (Sigma) was added to the sample and vortexed, and ultrasonic treatment was performed. The supernatant was collected after centrifugation. Benzoyl chloride was added after 2 mol/L NaOH was used to adjust the sample pH to basic. The samples were derivatized in a 40 °C water bath for 1 h under dark conditions. After the derivatization, the pH value was adjusted to neutral with 6 mol/L HCl and 2 mol/L NaOH. Then, 100% chromatographic methanol and a ddH_2O-activated HyperSep-C18 solid phase extraction column (Thermo Fisher Scientific, USA) for derivative fluid extraction were used. The ddH_2O and 15% chromatographic methanol eluted inorganic and organic impurities, and they were blow dry. Then, 100% chromatographic methanol was added to collect the components to be measured. Impurities were removed using a 0.22 μm filter for detection.

Real-time fluorescence quantitative PCR

Total RNA from the ovaries and GCs were extracted by the traditional TRIzol (Takara, Dalian, China) method, and the degradation was detected by 1% to 1.5% agarose gel electrophoresis. Following the instructions of the reverse transcription kit (Takara), cDNA was reverse transcribed from the extracted total RNA. According to the SYBR Green q-PCR kit (Takara), the optimum temperature was determined by amplification efficiency and the dissolution curve, and target gene relative expression was calculated using glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) as an internal reference. Primer information is shown in Table 1.

Protein expression assay

Tissue and cell samples were lysed with RIPA buffer (including 0.1% PMSF), and the supernatant was collected after centrifugation. Then, the BCA detection kit (Beyotime) was used to determine the protein concentration, and the protein was diluted to 2 μg/μL. Afterward, 4 × protein loading buffer (10% β-Mercaptoethanol) of the 1/4 system was added and mixed well in a metal bath for 10 min. Then, 10% polypropylene gel electrophoresis was used to separate the target protein, and the protein was transferred onto a nitrocellulose membrane (Bio-Rad, Shanghai, China). The nitrocellulose membrane was incubated with primary antibody at 4 °C overnight after blocking with 5% skimmed milk at room temperature. The relevant primary antibodies and dilution ratios were as follows: anti-LC3 (1:1000, Proteintech, China), anti-p62 (1:1000, Proteintech) and mouse anti-β-Actin (1:2000, TransGen Biotech, Beijing, China). The membrane was washed using TBST (Beyotime) and then incubated with goat anti-rabbit immunoglobulin (IgG) (1:1000, Beyotime) or goat anti-mouse immunoglobulin (1:1000, Beyotime). The membrane was washed again. Finally, the target band was visualized by ECL (Beyotime) using a gel imaging system. The relative protein content was calculated after analyzing the optical density value by using Image Lab (Bio-Rad).

Determination of apoptosis rate

In accordance with the instructions of the apoptosis detection kit (BD Bioscience, USA), the medium was added to an EP tube, and PBS and trypsin (Solarbio) were used to wash and digest the cells. The digestion was terminated using the spare medium, and the cells were transferred to the EP tube after blowing to a single state. About 500 g centrifuged for 5 min and washed with PBS. Then, 100 μL of 1 × binding buffer was added to each tube, blown until floating and filtered. The apoptosis rate was analyzed by using flow cytometry (BD Biosciences).

Determination of ROS level

The cell medium to be detected was removed, and the cells were digested with trypsin (Solarbio). After digestion termination with fetal bovine serum (Gibco), cells were collected after centrifugation and double washing. The DCFH-DA working solution was prepared according to the instructions (Beyotime), incubated with DMEM/F12 containing cells for 20 min at 37 °C, and mixed upside down after every few minutes. After incubation, the cells were washed twice to remove excess probes. Finally, the level of ROS in GCs was detected by flow cytometry (BD Bioscience).

Table 1. Primer sequences used in real-time fluorescence quantitative PCR

Gene	Primer sequences(5′ to 3′)	Annealing temperature (°C)	Product length (bp)
ODC[1]	F: TTGACTGCCACATCCTTG R: GCTCTGCTATCGTTACACT	58.0	199
SPDS[2]	F: ACCAGCTCATGAAGACAGCACTCA R: TGCTACACAGCATGAAGCCGATCT	60.0	189
SPMS[3]	F: TTCGGGTGACTCAGTTCCTGCTAA R: AACGGAGACCCTCCTTCAGCAAAT	60.0	199
APAO[4]	F: AGTCTTCACATGTGCTCTGTGGGT R: TGGCAATTGTGGGTTTCCTGTCAC	59.0	131
SSAT[5]	F: TGCCGGTGTAGACAATGACAACCT R: TAAAGCTTTGGAATGGGTGCTCGC	59.0	114
SMO[6]	F: CTACCCACGGTGCTGTGCTTT R: TTGAGCCCACCTGTGTGTAGGAAT	59.0	129
CAS3[7]	F: CTGGTATTGAGGCAGACAGTGG R: CAGCACCCTACACAGAGACTGAA	60.0	158
CAS8[8]	F: GGTGTCGCAGTTCAGGTA R: CATTGTAGTTTCAGGGCTT	57.0	127
CAS9[9]	F: CGAAGGAGCAAGCACGACAG R: CCGCAGCCCTCATCTAGCAT	61.4	130
BCL-2[10]	F: GATGACCGAGTACCTGAACC R: CAGGAGAAATCGAACAAAGGC	62.0	114
BAX[11]	F: GAAGCATTTACAGTTGCCATTACAG R: CCACAAGCAAGCAAAGAGCC	55.0	162
GPX[12]	F: AACCAATTCGGGCACCAG R: CCGTTCACCTCGCACTTCTC	60.0	122
SOD[13]	F: AAATGGGTGTACCAGCGCAG R: TCTTCTATTTCTACTTCTGCCACTCC	61.4	138
CAT[14]	F: ATACAGTTCGTGACCCTCG R: CCAGAAGTCCCATACCAT	55.8	188
GAPDH[15]	F: GTGGTGCAAGAGGCATTGCTGAC R: GCTGATGCTCCCATGTTCGTGAT	65.0	86

[1]Ornithine decarboxylase.
[2]Spermidine synthase.
[3]Spermine synthase.
[4]Acetyl-polyamine oxidase.
[5]Spermidine/spermine-N1-acetyltransferase.
[6]Spermine oxidase.
[7]Cysteinyl aspartate-specific proteinase-3.
[8]Cysteinyl aspartate-specific proteinase-8.
[9]Cysteinyl aspartate-specific proteinase-9.
[10]B-cell lymphoma-2.
[11]B-cell lymphoma-2-associated X protein.
[12]Gutathione peroxidase.
[13]Superoxide dismutase.
[14]Catalase.
[15]Glyceraldehyde-3-phosphate dehydrogenase.

Data analysis and statistical methods

The data were processed by the STATISTICAL ANALYSIS SYSTEM for one-way ANOVA and multiple comparisons. The collected experimental data were represented as the mean ± SEM and plotted using GraphPad Prism 9. $P < 0.05$ was considered statistically significant.

Results

Screening of spermidine concentration in vivo

3-NPA treatment and 15 mg/kg spermidine combined with 3-NPA treatment significantly increased MDA level ($P < 0.05$), whereas 10 mg/kg spermidine in combination with 3-NPA treatment did not affect MDA level ($P > 0.05$, Figure 1A). The combined treatment of spermidine and 3-NPA significantly reduced MDA level, but the effect of 10 mg/kg spermidine in combination with 3-NPA treatment was more significant as compared to the 3-NPA treatment group ($P < 0.05$). This suggested that 10 mg/kg spermidine is more beneficial to relieve oxidative stress induced by 3-NPA. Meanwhile, 3-NPA treatment significantly increased SOD activity ($P < 0.05$), while spermidine in combination with 3-NPA treatment reversed the increase in SOD activity caused by 3-NPA ($P < 0.05$). Therefore, 10 mg/kg spermidine was used to feed Sichuan white geese in subsequent experiments.

Effects of exogenous spermidine on MDA level and antioxidant enzyme activities in serum

To explore the effect of exogenous spermidine on the antioxidant indices of geese, MDA level and the activities of SOD and GPX were detected. 3-NPA significantly increased MDA

level ($P < 0.05$), whereas the MDA level of ovaries in the spermidine treatment group and the spermidine combined with 3-NPA treatment group were not significantly different from those in the control group ($P > 0.05$, Figure 2A). This result indicated that combined treatment of spermidine and 3-NPA significantly alleviated the increase of MDA induced by 3-NPA ($P < 0.05$). As shown in Figure 2B and C, SOD and GPX activities in the ovary of geese in the spermidine treatment and spermidine combined with 3-NPA treatment groups had no significant variation ($P > 0.05$), whereas 3-NPA significantly decreased SOD and GPX activities ($P < 0.05$). Compared with 3-NPA treatment, combined treatment with spermidine and 3-NPA significantly increased SOD and GPX activities ($P < 0.05$). These results suggested that exogenous spermidine affected antioxidant enzyme activities and relieved oxidative stress induced by 3-NPA.

Effects of exogenous spermidine on polyamine content and expression of polyamine anabolism genes in ovaries

To further study whether spermidine affected polyamine metabolism, HPLC and RT-qPCR were employed to determine polyamine contents and the expression of polyamine anabolism genes (Figure 3). Spermidine had no significant effect on the spermidine and spermine contents ($P > 0.05$), but significantly reduced the putrescine content of the ovaries ($P < 0.05$). 3-NPA treatment had no effect on spermidine content ($P > 0.05$), but significantly reduced the putrescine

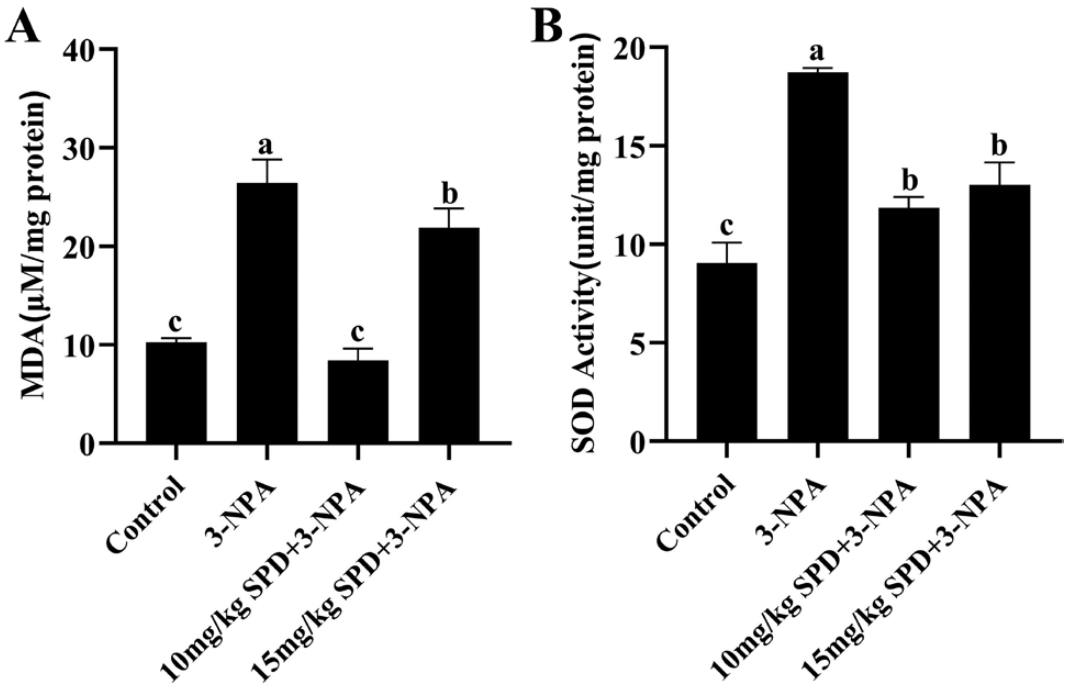

Figure 1. Effects of different concentrations of spermidine on ovarian antioxidant indices. (A) MDA level. (B) SOD activity. The experimental data were represented as the mean ± SEM. Different letters indicate a significant difference between the corresponding group and the other groups ($P < 0.05$). SPD: spermidine; 3-NPA: 3-nitropropionic acid (the same as below).

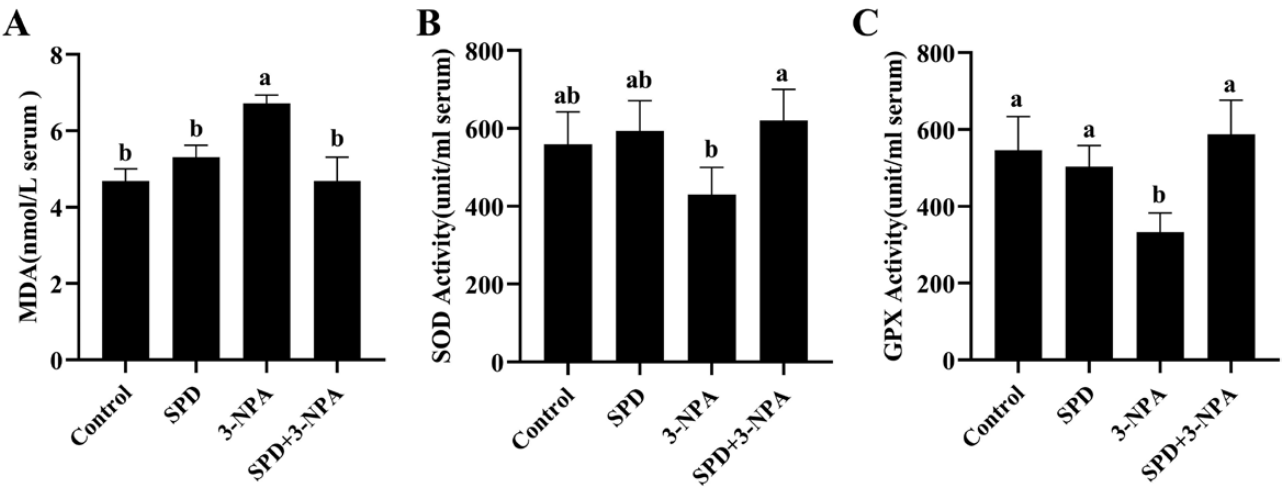

Figure 2. Effects of exogenous spermidine on antioxidant indices in serum. (A) MDA level. (B) SOD activity. (C) GPX activity. The experimental data were expressed as the mean ± SEM.

and spermine contents in ovaries ($P < 0.05$). In comparison with the 3-NPA treatment group, spermidine in combination with 3-NPA treatment increased putrescine, spermidine, and spermine contents ($P < 0.05$). Spermidine and 3-NPA treatments significantly decreased the gene expression of *SPDS* and *SMO* ($P < 0.05$, Figure 3B). *ODC*, *SPMS*, *SPDS*, *SSAT*, *APAO*, and *SMO* mRNA expression in the spermidine combined with 3-NPA treatment group was significantly increased as compared with the 3-NPA treatment group ($P < 0.05$).

Effects of exogenous spermidine on the morphology of ovaries and follicles

The shrunken follicles refer to the granulosa layer detachment from the basement membrane, inward depression, and further necrosis and disintegration. 3-NPA increased the number of shrunken follicles, whereas the spermidine had no significant effect. In addition to compared with the 3-NPA treatment group, spermidine combined with 3-NPA treatment reduced the number of shrunken follicles (Figure 4). This result indicated that spermidine relieved follicular damage caused by 3-NPA.

Effects of exogenous spermidine on antioxidant-related indexes in ovaries

As shown in Figure 5A, spermidine and spermidine combined with 3-NPA treatment had no effect on MDA level ($P > 0.05$), whereas 3-NPA significantly increased MDA level ($P < 0.05$). Meanwhile, the MDA level in ovaries of the spermidine combined with 3-NPA treatment group was significantly lower than that of the 3-NPA treatment group ($P < 0.05$). SOD activity in the spermidine treatment group had no significant variation compared with the control group ($P > 0.05$), but increased significantly in the 3-NPA treatment group and spermidine combined with the 3-NPA treatment group ($P < 0.05$, Figure 5B). As shown in Figure 5C, 3-NPA treatment significantly decreased *SOD* mRNA expression and increased *CAT* mRNA expression compared with the control group ($P < 0.05$). *SOD* and *GPX* mRNA expression level in the group treated with spermidine combined with 3-NPA were significantly higher than those in the group treated with 3-NPA ($P < 0.05$). This result indicated that exogenous spermidine improved the antioxidant capacity by affecting SOD activity and antioxidant enzyme gene expression.

Effects of exogenous spermidine on autophagy-related proteins in ovaries

To determine the effect of spermidine on autophagy in ovaries, the distribution and expression of autophagy proteins were determined by using immunohistochemistry and Western Blot. Immunohistochemical results showed that LC3 and p62 proteins were mainly distributed in the granulosa layer of ovarian follicles. Spermidine, 3-NPA, and spermidine combined with 3-NPA treatment significantly increased p62 protein expression and the LC3-II/I ratio ($P < 0.05$, Figure 6D). However, there were no significant changes in p62 and the LC3-II/I ratio in ovaries of the 3-NPA treatment group and the spermidine combined with 3-NPA treatment group ($P > 0.05$).

Concentration screening of spermidine, 3-NPA, and AG in vitro

As shown in Figure 7A, GCs were treated for 24 h with 2 to 9 mmol/L 3-NPA, and cell viability decreased significantly. Cell death increased sharply with increasing 3-NPA concentration. Therefore, GCs in the following experiments were treated for 24 h with 6.0 mmol/L 3-NPA. After GCs were treated with 10 to 100 μmol/L spermidine for 24 h or 48 h, the viability of GCs decreased sharply ($P < 0.05$, Figure 7B). Specifically, compared with 24 h, the activity of GCs in the control group at 48 h was significantly decreased, which may be caused by spermidine catabolism during cell culture. Therefore, GCs were treated with amino guanidine (**AG**, polyamine breakdown inhibitor) combined with spermidine to inhibit the catabolism of spermidine. GCs treated with 0.2 to 0.9 mmol/L AG for 24 h had no effect on the viability of GCs (Figure 7C). After treatment with 0.6 mmol/L AG combined with 6.0 mmol/L 3-NPA or 20.0 to 80.0 μmol/L spermidine for 24 h, the GCs viability was not affected ($P > 0.05$, Figure 7D and E). Consequently, in the following experiments, 0.6 mmol/L AG for 24 h was employed to treat GCs. As shown in Figure 7F, the GCs

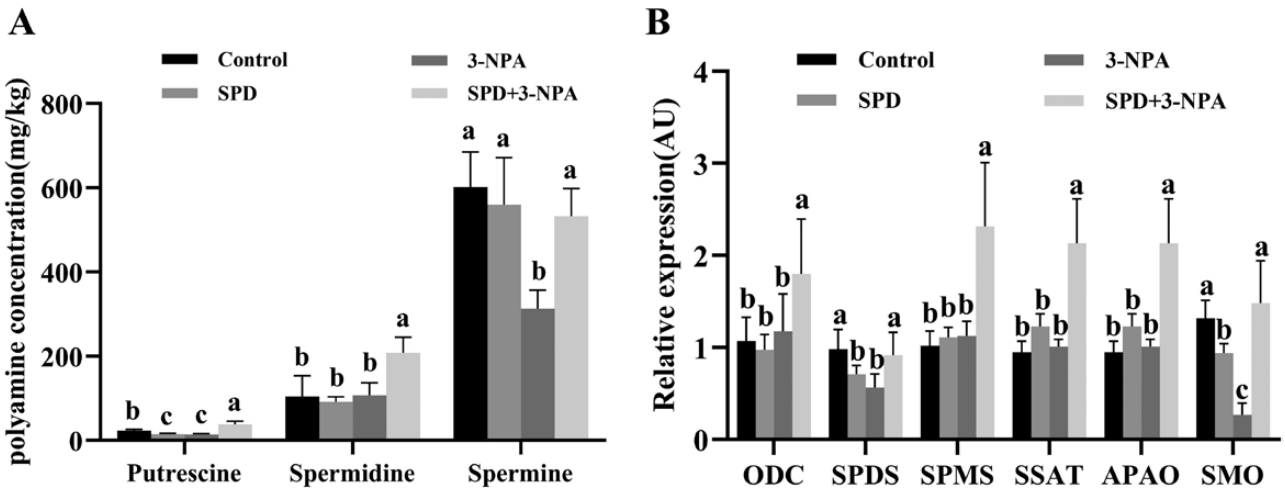

Figure 3. Effects of exogenous spermidine on polyamine content and polyamine anabolism genes in ovaries. (A) Content of putrescine, spermidine and spermine. (B) Relative expression level of polyamine anabolism genes. The experimental data were expressed as the mean ± SEM.

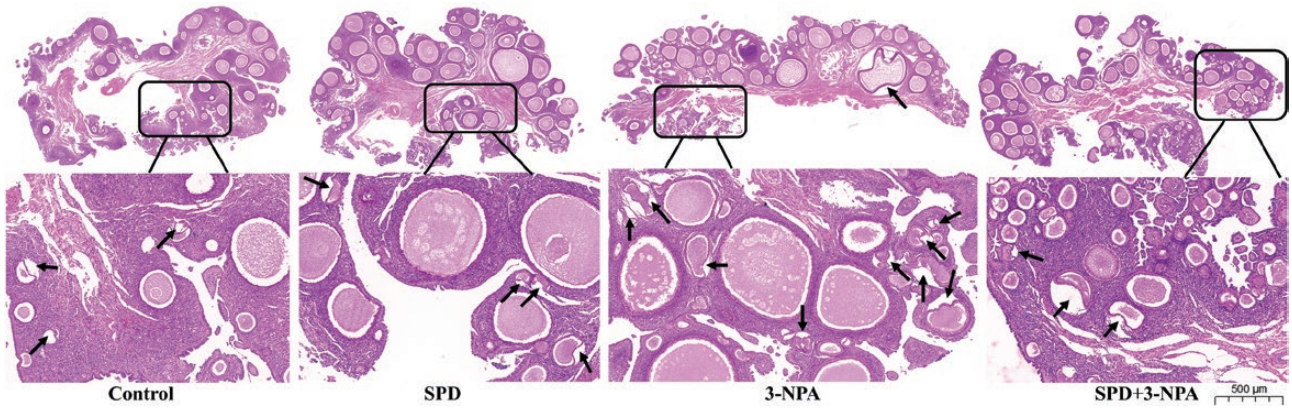

Figure 4. Effects of exogenous spermidine on the morphology of ovaries and follicles. The black arrow points to the follicles that shrunk. (scale bar: 500 μm).

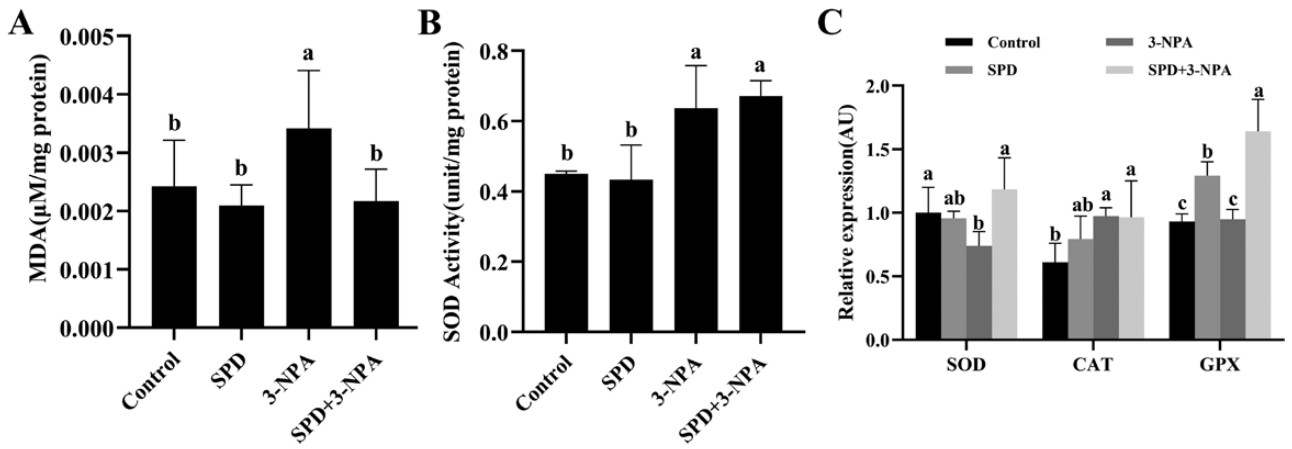

Figure 5. Effects of exogenous spermidine on antioxidant indices in ovaries. (A) MDA level. (B) SOD activity. (C) Expression level of antioxidant-related genes. The experimental data were expressed as the mean ± SEM.

viability gradually recovered after treatment for 24 h with 0 to 80 μmol/L spermidine, 0.6 mmol/L AG, and 6 mmol/L 3-NPA. Moreover, cell viability was 1.16 times that in the control group ($P < 0.05$). Therefore, spermidine at 80 μmol/L for 24 h was employed to treat GCs in the following experiments.

Effects of exogenous spermidine on polyamine content and expression of polyamine anabolism genes in GCs

As shown in Figure 8A, spermidine significantly decreased putrescine content and increased spermidine content ($P < 0.05$). 3-NPA treatment did not affect the putrescine content ($P > 0.05$), but significantly decreased the spermidine content and increased the spermine content ($P < 0.05$). As compared with the 3-NPA group, spermidine in combination with 3-NPA treatment significantly increased the spermidine content and significantly decreased the spermine content in GCs ($P < 0.05$). 3-NPA significantly increased *ODC*, *SPDS*, *SSAT*, *APAO*, and *SMO* gene expression ($P < 0.05$). Spermidine had no effect on the expression level of all genes detected in the experiment ($P > 0.05$). Spermidine in combination with 3-NPA treatment increased *SPDS*, *APAO*, and *SMO* mRNA expression in GCs compared with those in the 3-NPA treatment group ($P < 0.05$), whereas there were no significant changes in *ODC*, *SPMS*, or *SSAT* expression ($P > 0.05$).

Effects of exogenous spermidine on autophagy-related proteins in GCs

As shown in Figure 9, spermidine significantly decreased p62 protein expression and increased the LC3-II/I ratio ($P < 0.05$). 3-NPA treatment significantly increased p62 protein expression ($P < 0.05$), but did not affect the LC3-II/I ratio ($P > 0.05$). In comparison with the 3-NPA treatment group, the p62 protein level in the spermidine combined with 3-NPA treatment group was significantly decreased ($P < 0.05$), whereas the ratio of LC3-II/I was significantly increased ($P < 0.05$). This result indicated that exogenous spermidine may relieve oxidative stress caused by 3-NPA in GCs by inducing autophagy.

Effects of exogenous spermidine on antioxidant-related indexes in GCs

To determine the effect of spermidine on antioxidant-related indexes in GCs, ROS level was measured by using flow cytometry and the relative expression of genes related to antioxidant enzymes in GCs was measured by using RT–qPCR (Figure 10). Spermidine had no effect on ROS level ($P > 0.05$). 3-NPA treatment significantly increased ROS level ($P < 0.05$), whereas spermidine in combination with 3-NPA treatment reduced the increase in ROS level ($P < 0.05$). As shown in Figure 10C, spermidine had no effect on *SOD*, *CAT*, and *GPX* expression level ($P > 0.05$), whereas 3-NPA led to a significant increase in

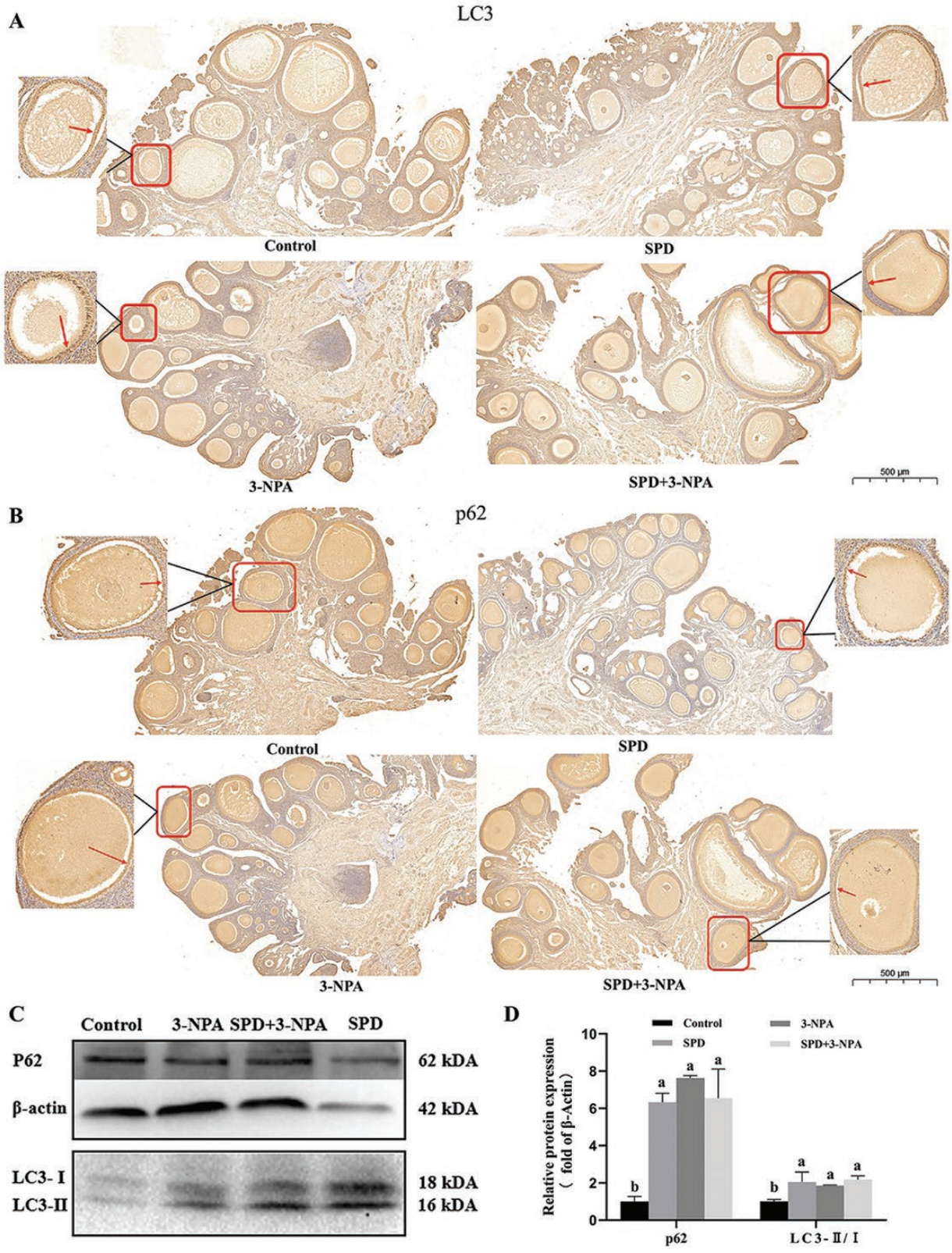

Figure 6. Effects of exogenous spermidine on autophagy-related proteins in ovaries. (A and B) The representative protein distribution of LC3 and p62 in ovaries was detected by immunohistochemistry. The arrow in the figure refers to the granulosa layer of the follicle. (C) p62 and LC3-II/I protein expression bands. (D) Normalized expressions of p62 and LC3-II/I. The quantification of p62 was performed with β-Actin as an internal reference, and the LC3-II band was normalized to LC3-I, respectively. The experimental data were expressed as the mean ± SEM.

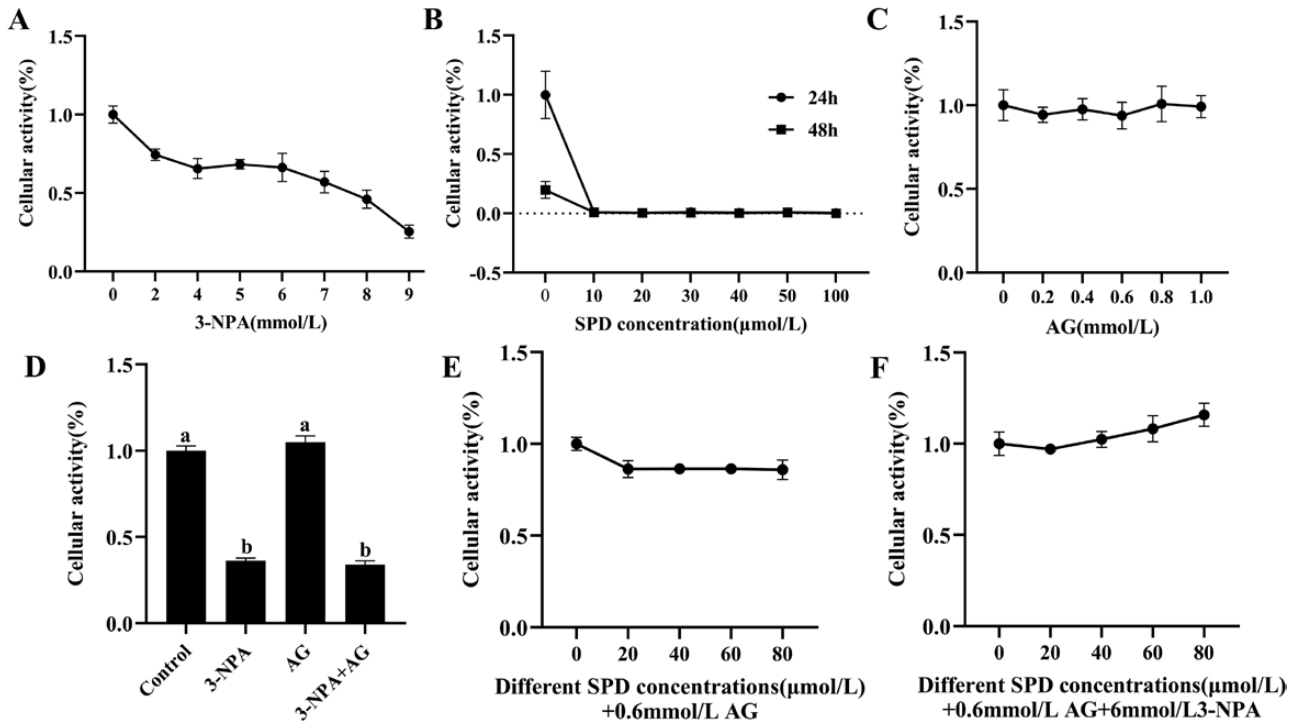

Figure 7. Granulosa cell viability under different treatment conditions. (A) Cell viability after 24 h of treatment with different concentrations of 3-NPA. (B) Cell viability after 24 h or 48 h of treatment with different concentrations of spermidine. (C) Cell viability after 24 h of treatment with different concentrations of AG. (D) Cell viability after 24 h of treatment with 6.0 mmol/L 3-NPA and 0.6 mmol/L AG. (E) Cell viability after 24 h of treatment with different concentrations of spermidine combined with 0.6 mmol/L AG. (F) Cell viability after 24 h of treatment with different concentrations of spermidine combined with 6.0 mmol/L 3-NPA and 0.6 mmol/L AG. The experimental data were expressed as the mean ± SEM.

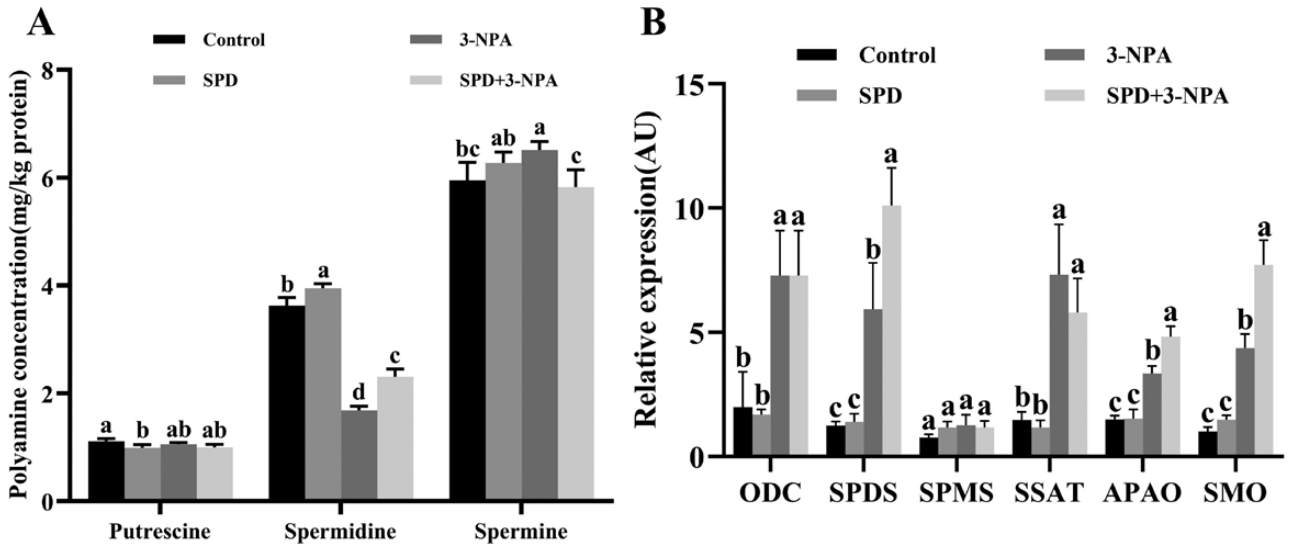

Figure 8. Effects of exogenous spermidine on polyamine content and polyamine anabolism genes in GCs. (A) Putrescine, spermidine and spermine contents. (B) Changes in polyamine anabolism gene expression. The experimental data were expressed as the mean ± SEM.

the expression of these three genes ($P < 0.05$). *CAT* and *GPX* mRNA expression level in GCs in the spermidine and 3-NPA combined treatment group were significantly higher than those in the 3-NPA group ($P < 0.05$), whereas *SOD* expression level was not significantly changed ($P > 0.05$).

Effects of exogenous spermidine on apoptosis of GCs

To determine the effect of spermidine on apoptosis of GCs, the apoptosis rate of GCs was measured by using flow cytometry, and the expression of apoptosis genes in GCs was measured by using RT-qPCR (Figure 11). Spermidine had no effect on the apoptosis rate of GCs ($P > 0.05$), whereas 3-NPA increased the apoptosis rate ($P < 0.05$). Furthermore, the combined treatment of spermidine and 3-NPA significantly alleviated the increase in the apoptosis rate induced by 3-NPA ($P < 0.05$). Spermidine had no effect on the gene expression level of *Caspase8*, *Caspase9*, and *Bcl-2/Bax* ($P > 0.05$) and significantly increased *Caspase3* expression ($P < 0.05$). The

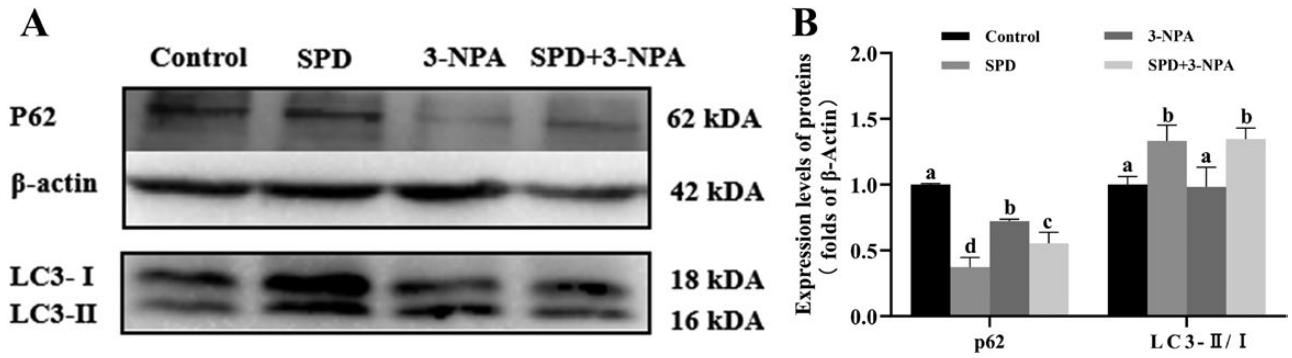

Figure 9. Effects of exogenous spermidine on autophagy-related proteins in GCs. (A) Representative p62 and LC3-II/I protein expression bands. (B) Normalized expressions of p62 and LC3- II/I. The quantification of p62 was performed with β-Actin as an internal reference, and the LC3- II band was normalized to LC3-I, respectively. The experimental data were expressed as the mean ± SEM.

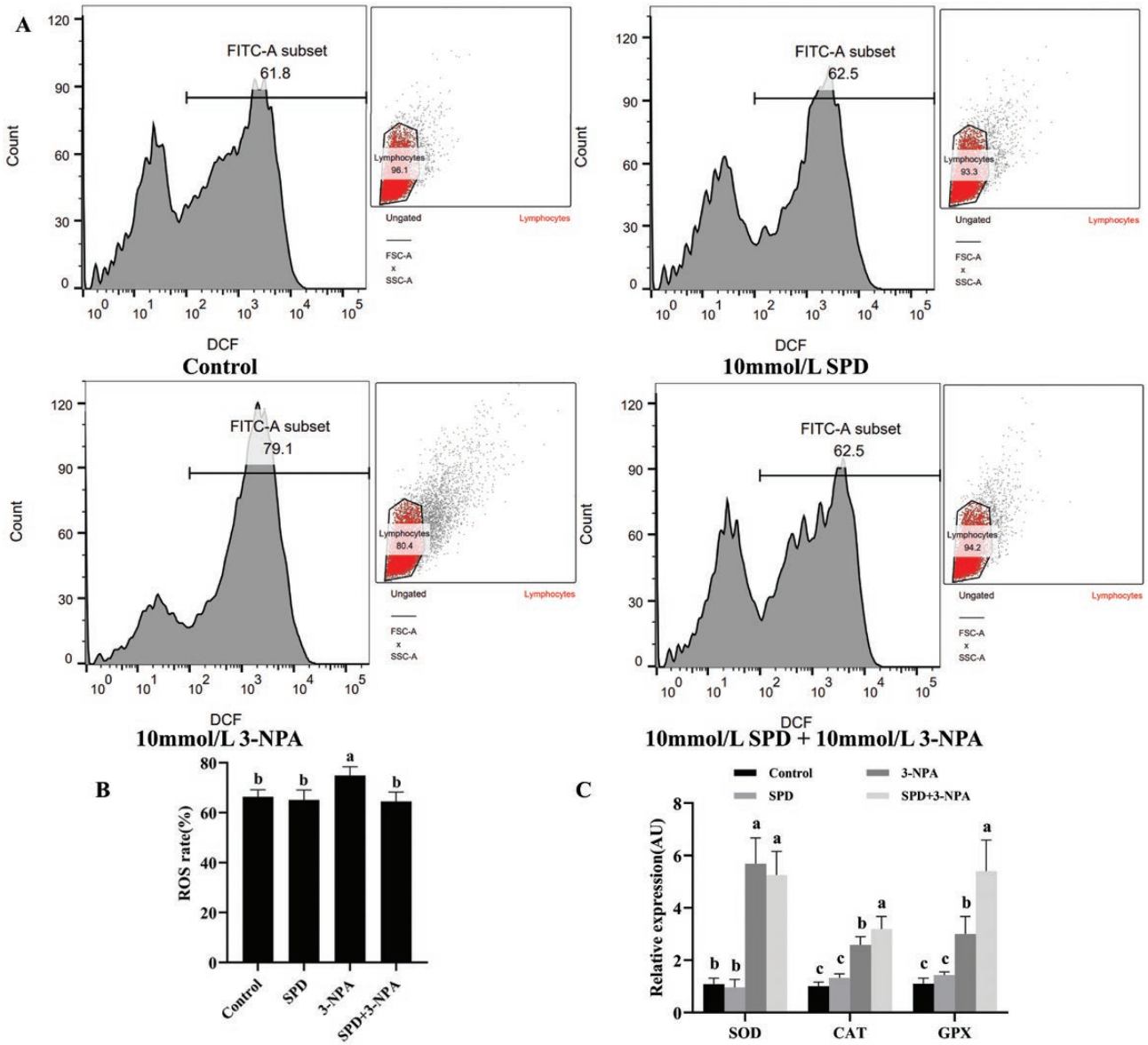

Figure 10. Effects of exogenous spermidine on antioxidant indices in GCs. (A and B) ROS level. (C) Relative expression of antioxidant enzyme-related genes. The experimental data were expressed as the mean ± SEM.

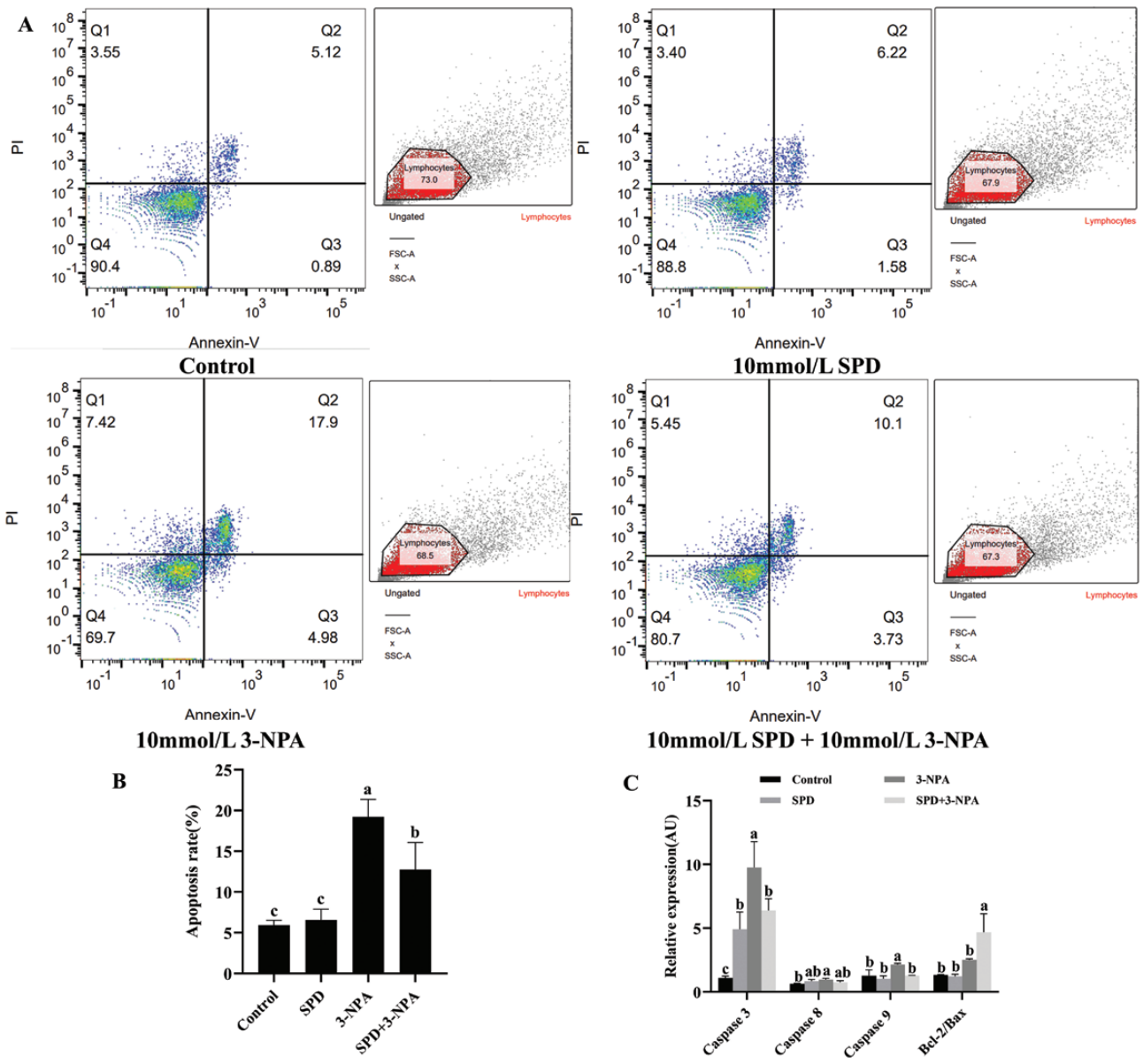

Figure 11. Effects of exogenous spermidine on apoptosis of GCs. (A and B) The apoptotic rate of GCs. (C) Expression of apoptosis-related genes. The experimental data were expressed as the mean ± SEM.

expression level of *Caspase3*, *Caspase8*, and *Caspase9* in GCs decreased significantly after 3-NPA treatment ($P < 0.05$). The spermidine in combination with 3-NPA treatment group significantly reduced *Caspase3* and *Caspase9* expression compared with the 3-NPA group, but the *Bcl-2/Bax* ratio increased ($P < 0.05$).

Discussion

Under physiological conditions, intracellular polyamine levels are regulated by polyamine synthesis and metabolism systems and transmembrane transport systems, and the homeostasis of polyamine play a critical role in the all living organisms (Casero et al., 2018). In our past study, treatment with spermidine significantly increased the contents of putrescine and spermine, while increased the level of MDA and H_2O_2 in mouse ovaries after 24 h (Jiang et al., 2021b). In this study, administration with 10 mg/kg spermidine significantly decreased the contents of putrescine and the spermidine synthesis genes expression in ovaries. Meanwhile, exposure of GCs to 80 μmol/L spermidine for 24 h caused the content of putrescine decreased, the *GPX* gene expression and content of spermidine increased. These findings suggested that exogenous spermidine may inhibit spermidine synthesis and promote polyamine metabolism to maintain their homeostasis in ovaries.

Super-physiological doses of ROS can induce oxidative stress and cause oxidative damage, and scavenging ROS is an important way to protect cells from oxidative toxicity. 3-NPA treatment can induce oxidative stress in ovaries (Kukurt and Karapehlivan, 2022) and cells (Kang et al., 2018). In this study, intraperitoneal injection of 10 mg/kg 3-NPA significantly increased the level of MDA in serum and ovaries and increased the SOD activity. Treatment of GCs with 6 mmol/L 3-NPA for 24 h significantly increased ROS level and apoptosis rate. This indicates that 3-NPA is an oxidative inducer of ovaries and GCs and enhances the antioxidant level of ovaries. It has

been reported that SMO induces oxidative damage in gastritis and prostatitis by oxidizing spermine to 3-amidopropanal and H_2O_2 (Goodwin et al., 2008; Gobert and Wilson, 2017). In this experiment, intraperitoneal injection of 10 mg/kg 3-NPA significantly reduced the mRNA expression of *SMO* in ovaries, reduced the content of putrescine and spermine, and increased the MDA level. The results suggested that 3-NPA reduces the antioxidant capacity of ovaries by reducing the putrescine and spermine contents and aggravates the oxidative stress of ovaries by regulating *SMO* mRNA expression. In addition, after the GCs were treated with 6 mmol/L 3-NPA for 24 h, spermidine content decreased, whereas spermine content, *SSAT*, *APAO,* and *SMO* mRNA expression, ROS level and apoptosis rate increased. These results suggested that 3-NPA decreased the antioxidant capacity of GCs by reducing spermidine content, whereas after spermidine transformed into spermine, spermine further decomposed through the SMO and SSAT/APAO pathways to produce excess ROS and induce oxidative stress. In summary, 3-NPA reduces antioxidant capacity by affecting polyamine content; however, it induces oxidative stress by activating polyamine catabolism to produce toxic substances (H_2O_2 and ROS).

Spermidine has been reported to play a dual role in maintaining redox balance and can be used as a free radical scavenger to scavenge ROS and as a substrate for serum amine oxidase to catalyze ROS. AG, an inhibitor of amine oxidase can significantly inhibit the catabolism of spermidine (Schiller et al., 2005). Therefore, in this experiment, AG was added to inhibit the activity of amine oxidase in the medium to avoid the decomposition of spermidine to produce oxidative toxic substances. Vitamin E treatment inhibited oxidative stress and alleviated H_2O_2-induced apoptosis of GCs through regulation of *SOD*, *CAT*, *GPX* expression, and ROS level (Hou et al., 2023). In this study, under the protection of 0.6 mmol/L AG pretreatment, 80 μmol/L spermidine posttreatment of GCs, *SOD*, *CAT,* and *GPX* mRNA expression, the ROS production and apoptosis rate did not change significantly. Pretreatment with spermidine decreased ROS induced by lipopolysaccharide in RAW 2644.7 cells (Jeong et al., 2018). Treatment with spermidine reversed the SOD and CAT activities during the myocardial cell aging (Wang et al., 2020). The study found that 10 mg/kg spermidine given orally increased serum SOD and GPX activities and upregulated *SOD* and *GPX* gene expression in ovaries, but decreased the level of MDA in serum and ovaries induced by 3-NPA. These results suggested that spermidine can improve the antioxidant capacity of ovaries by upregulating the expression of antioxidant genes and antioxidant enzyme activities. Furthermore, 80 μmol/L spermidine treatment further increased the upregulation of *CAT*, *GPX,* and *Bcl-2/Bax* mRNA expression induced by 3-NPA, reversed the increase in *Caspase9* and *Caspase3* mRNA expression, and reduced the ROS level and apoptosis rate. Spermidine may prevent GCs oxidative stress caused by 3-NPA by regulating antioxidant genes, reducing ROS production, and reducing the apoptosis rate. In conclusion, exogenous spermidine plays a major role in the antioxidative stress process of ovaries and follicular GCs.

Many studies have found that spermidine, as an autophagy inducer, plays a momentous role in improving oxidative stress induced by diseases and aging in cardiovascular (Abdellatif et al., 2018; Yamaguchi, 2019), liver (Yue et al., 2017) and nerves (De Risi et al., 2020; Stacchiotti and Corsetti, 2020). In addition, an increasing number of studies have shown that autophagy mediates follicular development and atresia (Zhou et al., 2019) and inhibits ovarian aging and disease induced by oxidative stress (Li et al., 2018; Peters et al., 2020). SQSTM1 (p62) is a ubiquitin-binding scaffold protein that interacts with phagophores through the microtubule-associated protein 1A/1B-light chain 3 (LC3)-interacting domain and degrades ubiquitinated protein aggregates (Islam et al., 2018). Although autophagy can degrade p62 (Liu et al., 2016), under the condition of autophagy substrate accumulation, the transcription of *p62* is also significantly increased (Lamark et al., 2017). Moreover, the p62 promoter contains oxidant reaction elements, which increase the expression level under oxidative stress and activate the transcription of Nrf2 factor to play the antioxidant function (Jain et al., 2010). Oxidative stress-activated NF-κB phosphorylation, which in turn upregulated p62 and increased autophagy to promote the survival of retinal pigment epithelial cells (Song et al., 2017). The study found that spermidine treatment significantly increased p62 and the radio of LC3-II/I in ovaries. This result indicated that spermidine increases ovarian autophagy, which may be induced by ROS produced by polyamine catabolism. This study also found that intraperitoneal injection of 3-NPA increased p62 protein expression and the radio of LC3-II/I. 3-NPA may induce ovarian autophagy by increasing ROS production but increases ubiquitinated proteins and upregulates p62 protein expression by protein damage induced by 3-NPA on the other hand. GCs supplemented with 80 μmol/L spermidine (combined with AG) did not increase ROS level and *SOD*, *CAT,* and *GPX* mRNA expression but increased the LC3-II/I ratio and decreased p62 protein expression. This result further confirmed that spermidine is a natural inducer of autophagy. In addition, 6 mmol/L 3-NPA promoted the accumulation of ROS in GCs and increased p62 protein expression, while the ratio of LC3-II/I remained unchanged, suggested that the ubiquitination process after protein damage induced by 3-NPA upregulated p62 protein expression. Moreover, spermidine combined with 3-NPA treatment not only reversed the increase in p62 protein expression, ROS level and apoptosis rate induced by 3-NPA but also increased the ratio of LC3-II/I. Thus, spermidine may protect GCs from 3-NPA-induced oxidative damage by inducing autophagy to remove ROS and damaged proteins. The above studies have shown that spermidine can improve the autophagy level of ovaries and GCs, improve the oxidative stress state of the body, and alleviate the oxidative stress induced by 3-NPA.

In summary, 3-NPA induces oxidative stress in ovaries and GCs by affecting polyamine metabolism to reduce polyamine content and causing the accumulation of MDA and ROS. Exogenous spermidine alleviates ovarian oxidative stress by increasing gene expression related to antioxidant enzymes and SOD and GPX activities to enhance antioxidant capacity. It also alleviates GCs oxidative stress by increasing *CAT* and *GPX* mRNA expression and reducing ROS level. In addition, exogenous spermidine may alleviate oxidative stress by increasing the LC3-II/I ratio and reducing p62 protein expression to induce autophagy in GCs.

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Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

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