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RESEARCH ARTICLE

# Long-term copper exposure promotes apoptosis and autophagy by inducing oxidative stress in pig testis

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## Abstract

Copper (Cu) is a heavy metal which is being used widely in the industry and agriculture. However, the overuse of Cu makes it a common environmental pollutant. In order to investigate the testicular toxicity of Cu, the pigs were divided into three groups and were given Cu at 10 (control), 125, and 250 mg/kg body weight, respectively. The feeding period was 80 days. Serum hormone results showed that Cu exposure decreased the concentrations of follicular stimulating hormone (FSH) and luteinizing hormone (LH) and increased the concentration of thyroxine (T4). Meanwhile, Cu exposure upregulated the expression of Cu transporter mRNA (Slc31a1, ATP7A, and ATP7B) in the testis, leading to increase in testicular Cu and led to spermatogenesis disorder. The Cu exposure led to an increased expression of antioxidant-related mRNA (Gpx4, TRX, HO-1, SOD1, SOD2, SOD3, CAT), along with increase in the MDA concentration in the testis. In LG group, the ROS in the testis was significantly increased. Furthermore, the apoptotic-related mRNA (Caspase3, Caspase8, Caspase9, Bax, Cytc, Bak1, APAF1, p53) and protein (Active Caspase3) and the autophagy-related mRNA (Beclin1, ATG5, LC3, and LC3B) expression increased after Cu exposure. The mitochondrial membrane potential in the testicular tissue decreased, while the number of apoptotic cells increased, as a result of oxidative stress. Overall, our study indicated that the Cu exposure promotes testicular apoptosis and autophagy by mediating oxidative stress, which is considered as the key mechanism causing testicular degeneration as well as dysfunction.

**Keywords** Oxidative stress · Pig · Testis · Copper · Toxicity

## Introduction

Copper (Cu) is an important heavy metal being used in the industry and agriculture (Eheliyagoda et al. 2019). In animal production system, Cu is used as food additive that promote animal growth and also has an antimicrobial activity (Yang et al. 2017a, b; Villagómez-Estrada et al. 2020). However, the excessive use of Cu is polluting the land and water sources Vázquez-Blanco et al. (2020). The animals can regulate the Cu homeostasis in vivo through Cu transporters (Petris et al. 2002; Kim et al. 2013). The recommended intake of Cu for adult animal is 2.00 mg/day. Oral doses higher than 200.00 mg/kg body weight (bw) can cause death (Li et al. 2020). The cytotoxicity of Cu is similar to iron and is associated with production of reactive oxygen species (ROS) leading to peroxidation of the membrane lipids, protein oxidation, and nucleic acid breakage (Tapiero et al. 2003).

To meet the meat consumption needs of the society, copper is widely used as a feed additive to enhance the production performance of the animals (He et al. 2016; Liu et al. 2018). Unlike classical antimicrobial growth promoters, Cu is inert and non-degradable in the environment, which poses challenges to the environmentalists (Jensen et al. 2016). The unreasonable addition of Cu in the feed causes environmental pollution, and long-term exposure to high Cu in the feed reverses the effect of promoting livestock growth into an inhibitory effect, even it has a toxic effect Li et al. (2010).

Yuanliang Li and Hanming Chen have contributed equally to this work and shared as the co-first author.

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The reproductive performance of livestock is important aspect of production (Schulze et al. 2020). The testis serves as the site of spermatogenesis but is vulnerable to oxidative damage due to the presence of large amount of highly unsaturated fatty acids and the presence of ROS Aitken and Roman (2008). Some studies indicated that the Cu could cause decrease in the antioxidant capacity and testicular spermatogenesis (Zhao et al. 2019; Zebral et al. 2019; Lin et al. 2020). Furthermore, Cu exposure upregulates the expression of genes of oxidative phosphorylation pathway (such as Cytc, SOD1, and GST) in the testis to induce ROS production, which is related with the toxicity of Cu in the testis (Zhao et al. 2019). These results reflect the disruption of balance between antioxidant and oxidant after Cu exposure, and it causes oxidative damage in the testis. The ROSs are one of the byproducts of normal mitochondrial metabolism and homeostasis, and the ROSs have biphasic effects, as the appropriate level of ROS has a physiological role in the cell differentiation and sperm capacitation, while excessive ROS can cause oxidative stress in the testis (Zorov et al. 2014). Excessive ROS can cause damage to the electron transport chain and then lead to mitochondrial dysfunction, which many studies have shown that oxidative stress may cause apoptosis through mitochondria-dependent and mitochondria-independent pathways (Indo et al. 2007; Sinha et al. 2013). These findings have indicated a link between oxidative stress and apoptosis, and thus it contributes to the toxicity by Cu. It has been found that Cu indeed is toxic to the organs by mediating oxidative stress and apoptotic pathways (Guo et al. 2017; Yang et al. 2017a, b; Yang et al. 2019a, b; Modanloo and Shokrzadeh 2019).

Previous studies found that long-term exposure to Cu leads to accumulation of Cu in the form of residues in the visceral organs, leading the upregulation of mitochondrial apoptotic pathway-related genes and the class P13K autophagy-related genes, which further promote damage in the tissues (Kheirandish et al. 2014; Chen et al. 2020; Shao et al. 2019). Autophagy is a process of cellular homeostasis, which plays an important role in the cell survival and maintenance through the degradation of cytoplasmic organelles, proteins, and macromolecules and is related with the recycling of breakdown products Levine and Kroemer (2019). Autophagy occurs mainly through the PI3K complex pathway. The nutrition, energy, and apoptotic states, all affects the progress of the autophagy. However, autophagy disorder may also promote cytopathy (Glick et al. 2010). Apoptosis, the programmed death of cells, is an essential component of various processes, including metabolism and disease development. The initiation of apoptosis mainly proceeds through the mitochondrial pathway by activation of the Akt, a serine/threonine kinase, extracellular signal-regulated kinases (ERK1/2), p38, as well as c-Jun NH2-terminal kinase (JNK) pathway, and the endoplasmic reticulum stress pathway (Elmore 2007; Kerr et al. 1972). Although apoptosis and autophagy represent two distinct auto-degradation processes, there are also certain connections between them, reflected by the fact that molecularly the expression pathways of the two exist in many common genes (Zhao et al. 2015). To the best of our knowledge, there is no report indicating that Cu could induce testicular toxicity in vivo through oxidative stress-induced cell apoptosis and autophagy.

Although previous studies have evaluated the reproductive toxicity of Cu, there is no study to verify that Cu poisoning mediates testicular apoptosis and autophagy disorder through oxidative stress mechanism. Thus, the purpose of this study was to investigate the mechanisms related with Cu exposure leading to testicular toxicity and the association between oxidative stress, apoptosis, and autophagy in this mechanism.

## Materials and methods

### Animals and treatments

Total of 12 pigs (30-day-old, procured from a pig farm in Shaoguan, Guangdong) were acclimatized for the experimental conditions before staring the treatment, and they were raised in the experimental animal center of South China Agricultural University during treatment. Before raising, pig houses were cleaned and disinfected with formaldehyde. All the feed and tools were disinfected and delivered to the pig houses after being irradiated by ultraviolet lamp in the transmission window. Pigs were wiped with povidone-iodine (1:200) for disinfection and then delivered to the pig houses after being irradiated by ultraviolet lamp in the transmission window. All pigs were fed twice a day, drinking freely, and a 12-h light/dark cycle was set. Experimental animal treatment was performed according to the guidelines of the Institutional Animal Care and Use Committee of South China Agricultural University (IACUC, SCAU).

### Experimental design

In the study, 12 pigs were divided into three experimental groups; each group had four pigs. The Cu used was anhydrous copper sulfate ($CuSO_4$) as a Cu source. The dose of Cu was selected on the basis of a previous study by Yang et al. (2016). The control group pigs were fed on normal diet (with 10 mg/kg bw Cu) throughout the study and served as control group; the LG (low dose group) pigs were similar to the control group except additionally were fed with 125mg/kg anhydrous copper sulfate (CuSO4); the HG (high-dose group) pigs were additionally fed with 250mg/kg $CuSO_4$ to that of control group. After feeding pigs for 80 days, the pigs were properly restrained to collect the blood from the jugular vein, then sacrificed with sodium pentobarbital, and collected the testes (Oskam 1989). The left side of the testis was fixed in 4% paraformaldehyde solution, and the right side was cut off and digested with trypsin partly. The rest of the testicular

tissue was frozen by liquid nitrogen and stored at −80 °C in an ultra-low temperature refrigerator (Thomas Scientific, Swedesboro, NJ).

## Testicular coefficient evaluations

All pigs were weighed before sacrifice, and both testicles were removed and weighed quickly after sacrifice. The testicular coefficient was calculated as:

$$\text{Testicular coefficient} = \frac{\text{Testis weight(KG)}}{\text{Pig weight(KG)}} * 100\%.$$

## ICP-MS analysis testicular $Cu^{2+}$ concentration

The 20 mg of testicular tissue was treated with 1mL of aqua regia and 1 mL of hydrogen peroxide. All samples were transferred to the microwave-assisted digestion using MARSXpress (maximum power: 1200 W) laboratory microwave digestion system (CEM, Matthews, NC, USA). After the samples were cooled, added deionized water, and diluted to 10 mL, and the 0.22-μm diameter filter (TJJinteng, China) was used to filter the solution. The amount of $Cu^{2+}$ in the testis was measured by Agilent 1260/7700X ICP-MS (Agilent, USA). The concentration of heavy metal was calculated by 5-point calibration curve of ICP-MS, and the linearity of each target element was greater than 0.99.

## Hormone analysis

The serum estradiol (E2), testosterone (T), luteinizing hormone (LH), parathyroid hormone (PTH), thyroxine (T4), and follicle-stimulating hormone (FSH) were measured by using the Synergy HTX Multi-Mode Reader (BioTek, America) according to the manufacturer's protocol.

## Assessment of content of MDA and ROS in the testes

Partial fresh testicular tissues were used to evaluate the oxidative stress-related indexes. According to the instructions provided by the manufacturer, using an ELISA kit (Beyotime Biotechnology Co., Ltd, Shanghai) to determine MDA level in the testes. About ROS, the instruction manuals provided by the manufacturer of the kit (Beyotime Biotechnology Co., Ltd., Shanghai) were followed. Fresh testicular tissue was made into cell suspension. The cell suspension was treated according to the guidance provided in the reactive oxygen species (ROS) assay kit (Beyotime Biotechnology Co., Ltd., Shanghai) and then used flow cytometry (Beckman Coulter Co., Ltd) to measure the ROS level in the tissue.

## Testicular pathology and apoptosis assessment

After the pigs were sacrificed, the testicles and epididymis were removed, fixed in 4% paraformaldehyde solution (Shanghai EKEAR Biotech Co., Ltd, Shanghai) overnight, embedded in paraffin, sectioned at a thickness of 5 μm, and then stained with hematoxylin and eosin stain (H&E) (Anhui Leagene Biotechnology Co., Ltd., Anhui). The stained sections were then analyzed by using a Leica DM1000 light microscope (Leica, German). As described in previous studies, to detect the apoptosis in the testis, paraffin sections of the testis were subjected to TUNEL assay staining (Beyotime Biotechnology Co., Ltd., Shanghai) using immunohistochemistry (Liu et al. 2018).

## Indirect immunofluorescence analysis

To analyze the effects of Cu on the autophagy gene LC3B expression, indirect immunofluorescence staining of the testis sections was performed according to previously reported method (Chen et al. 2015). Briefly, the testicular sections were deparaffinized and processed through xylene (Guangzhou Chemical Reagent Factory, Guangzhou) and graded concentrations of ethanol (Guangzhou Chemical Reagent Factory, Guangzhou) and then followed the sodium citrate method to sequester the testicular tissue antigens; thereafter, treated with 0.5% Triton X-100/1×PBS (Takara Biotechnology Co., Ltd., Anhui) and then blocked with 5% skimmed milk, incubated with LC3B primary antibodies (Ruiying Biotech Co., Ltd., Jiangsu, China) for over 16 h at 4 °C, and then incubated with FITC secondary antibodies (Beyotime Biotechnology Co., Ltd., Shanghai); and finally, used DAPI (Beyotime Biotechnology Co., Ltd., Shanghai) blocking reagent, and then by using Leica fluorescence microscope (Leica, German), the image was recorded.

## RT-PCR analysis

The expression of mRNA for Cu transporter, oxidative stress, apoptosis, and autophagy-related genes in the testicular tissues were studied by RT-qPCR. Total RNA was harvested from testis samples with the TRIzol reagent (Takara Biotechnology Co., Ltd., Anhui) following the manufacturer's instructions. Synthesis of the first strand (cDNA) was performed with HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme Biotech Co., Ltd., Nanjing). Primers were designed with Primer 3.0 (http://bioinfo.ut.ee/primer3-0.4.0/) according to the gene sequence of the pig to produce an amplification product and tested on NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). All the designed primer sequences were obtained (Sangon Biotech (Shanghai) Co., Ltd., Guangzhou). The primers used to amplify the genes are shown in Table 1. The GAPDH was used as a

**Table 1** Specific primers used for real-time PCR analyses

| Gene name | Forward primer (3′-5′) | Reverse primer (3′-5′) |
|---|---|---|
| GAPDH | ACCCAGAAGACTGTGGATGG | AAGCAGGGATGATGTTCTGG |
| SOD1 | CAGGGCACCATCTACTTCG | TCACCTTCAGCCAGTCCTTT |
| SOD2 | GCTGGAAGCCATCAAACG | TTAGAACAAGCGGCAATCTG |
| SOD3 | CTGACGCTGCTCTGTGCTTA | TCAACTCCTGCCAGATCTCC |
| CAT | AGAGGAAACGCCTGTGTGAG | GTCCAGAAGAGCCTGAATGC |
| Gpx4 | ATTCTCAGCCAAGGACATCG | TTTGACGTTGTAGCCAGCAG |
| HO-1 | GCTGAGAATGCCGAGTTCAT | GGAAGTAGAGGGGCGTGTAG |
| TRX | TTCCAATGTCGTGTTCCTTG | ACCCACCTTCTGTCCCTTTT |
| Caspase3 | GCAGTTTTATTTGCGTGCTTC | TCCGTCTCAATCCCACAGTC |
| p53 | CCTCACCATCATCACACTGG | CACAAACACGCACCTCAAAG |
| Caspase8 | ACCTGGCTTCCCTCAAGTTC | TCCATCTCCTCCTCATTGGT |
| Caspase9 | GACCTCCATCCTGTGGTGAT | GCATTTCCCTTGGCTCTGT |
| Bax | AAGCGCATTGGAGATGAACT | GGCCTTGAGCACCAGTTTAC |
| Cytc | AAGACTGGTCCAAACCTCCA | ACGCCATCAGTGTCTCCTCT |
| APAF1 | TGGATGCAAAAGCTCGAAAT | TGCTCGTTGGCATTGAGTAG |
| Bak1 | ATGACATCAACCGGCGATAC | TTGATGCCACTCTCGAACAG |
| p62 | TCCAGCACAGAGGACAAGTG | ATGGGTCCAGTCATCGTCTC |
| Beclin1 | GATAGTGGCGGAAAATCTCG | CATCTGGGCATAACGCATCT |
| ATG5 | GCCATCAATCGGAAACTCAT | TGAAGCCACAGGACGAAAG |
| LC3 | CCTTCTTCCTGCTGGTGAAC | GGGAGGCGTAGACCATGTAG |
| LC3B | TGCAGCTCAATGCTAACCAA | CTTCATCCTTCTCGCTTTCG |
| Slc31a1 | TGGCGGTGTTTTTACTAGCC | CTGCCCAACTGTTTTGTGTG |
| ATP7a | AAGCTGTGGGCTTTCCAGTA | TCCTTTGTGGTGATCCTTCC |
| ATP7b | TAGAAGGCAGGCTCAGGAAA | TCAATAGGTCCCAGGCTCAC |
| ATOX1 | TCAACAAGCTGGGAGGAGTT | CAGGGTCTCCAGCAGAGTGT |

housekeeping gene to normalize the target gene transcript level, and the relative expression level of the selected gene was calculated by $2^{-\Delta\Delta ct}$ method.

### Active Caspase3 protein expression analysis

Follow the previously described method, the quantitative immunoblotting was carried out (Chen et al. 2020). After homogenizing the testis samples, the protein concentration of each sample was measured by bicinchoninic acid (BCA) method (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were separated on 12% SDS-PAGE gels and then transferred onto the polyvinylidene difluoride membranes (Millipore, USA). Incubations were performed using the Active Caspase3 (1:1000) primary antibody (Zen-bioscience Co., Ltd., Chengdu) for over 16 h at 4 °C with subsequent secondary antibody which was used at a dilution of 1:5000. Western blots were visualized using the commercial ECL solution (Millipore, USA). The signal was detected by X-ray film (TransGen Biotech Co., Beijing). Protein levels were then analyzed by ImageJ software. All the protein measurements were normalized to GAPDH, and data were expressed relative to the values in control group.

### Statistical analysis

In the study, all the data are expressed as mean ± SD. The groups were compared by one-way ANOVA applying Tukey's test using IBM SPSS Statistics 25.0 Statistical Software (SPSS Inc., Chicago, IL, USA). Probability (P) values of $< 0.05$ indicated that the difference is statistically significant. GraphPad prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used to visualize the data.

## Results

### Effects of Cu toxicity on Cu transport carrier gene expression

To verify that Cu exposure caused additional Cu transport into the pig testis, we determined the testicular Cu content and the levels of Cu transporter genes. In LG and HG groups, the levels of Cu transporter genes Slc31a1, ATP7a, ATP7b, and ATOX1 were significantly increased in a dose-dependent manner compared to the control group ($P < 0.05$) (Fig. 1A to E). In parallel, the LG and HG groups showed increase in

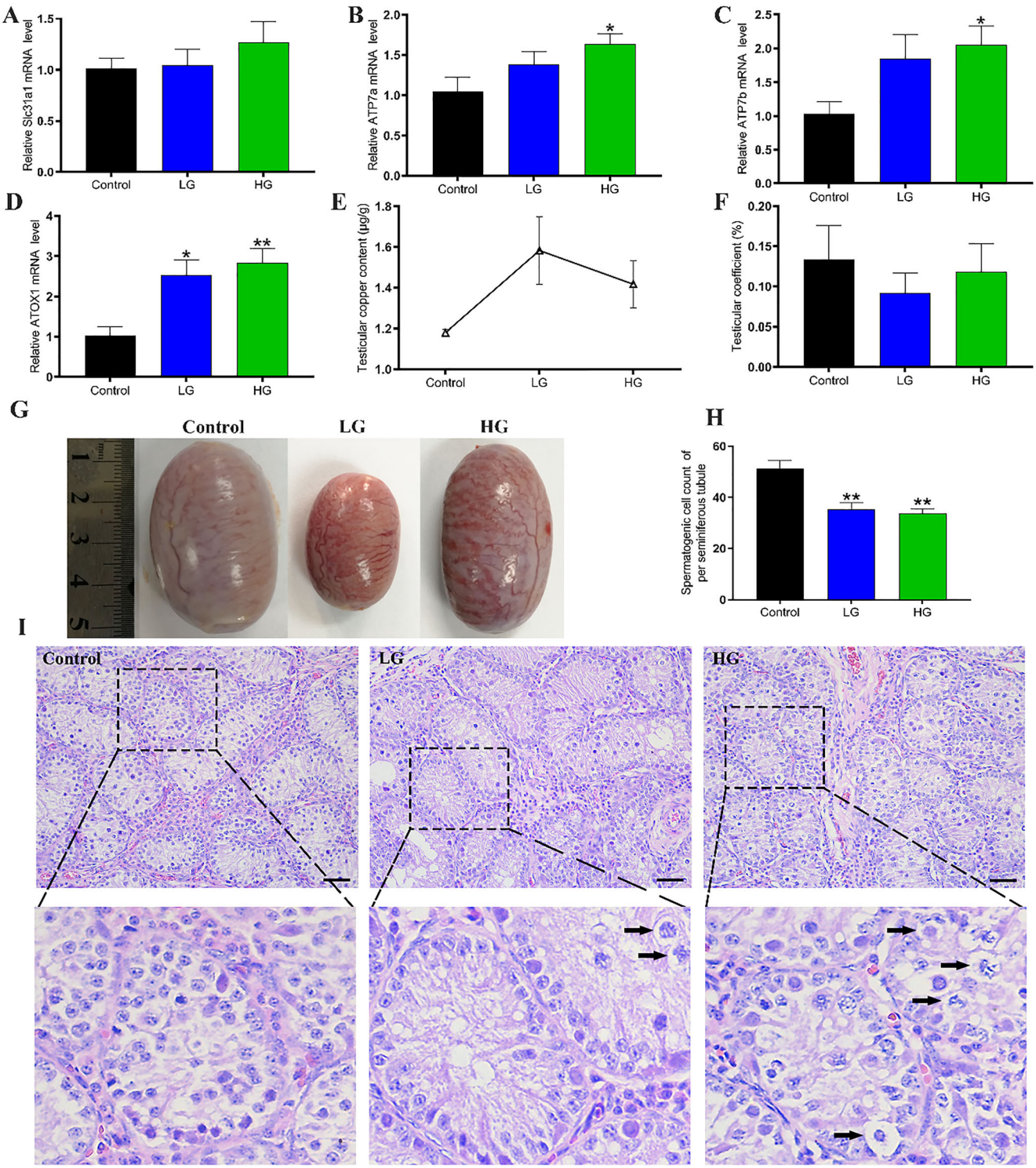



**Fig. 1** Cu enters the testes via copper transporter and causes pathological changes in the testes. **A**, **B**, **C**, and **D** The expression level of Slc31a1, ATP7a, ATP7b, and ATOX1 mRNA in the testes of the control, LG, and HG groups. **E** Testicular Cu content in the testes of the control, LG, and HG groups. **F** Testicular coefficient in the testes of the control, LG, and HG groups. **G** Testicular appearance in the testes of the control, LG, and HG groups. **H** Spermatogenic cell count of per seminiferous tubule. **I** Histological cross sections of testicular samples. Representative H&E staining images are shown. The copper-exposed boar testes show spermatocyte sloughing (arrow) and vacuolar degeneration in the seminiferous tubules (star). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

testicular Cu contents, with no significant difference compared to the control group ($P > 0.05$) (Fig. 1E).

Then, to investigate the effects of Cu exposure on the morphology and function of testis, the testicular indices were

calculated. We found that after Cu exposure, the testicular coefficient in LG and HG group appeared decreased; however, the difference was not significant ($P > 0.05$) (Fig. 1F). In morphological results, compared with the control group, the testicular volume of LG group was lower, while the testicular volume of HG group did not change significantly. However, compared with the control group, the testis of LG group and HG group showed expansion of blood vessel, i.e., congestion (Fig. 1G). Besides, Cu exposure resulted in a decrease in spermatogenic cells, as well as induced spermatogonia and spermatocytes sloughing (arrow) and vacuolar degeneration (star) in the seminiferous tubules (Fig. 1 H and I). Moreover, Cu exposure changed the serum hormone levels in pigs. Among them, the levels of E2, T4, and T in the LG and HG group were increased, while the levels of LH and FSH in LG and HG group were decreased, compare with control group, but the PTH showed no change after Cu exposure. The change in LH, T4, and FSH were significant ($P < 0.05$) (Table 2). All the results suggested that the Cu exposure does affect the testis through change in the levels of serum hormones and the expressions of Cu transport carrier genes, as well as caused damage to testicular tissue.

## Cu exposure induce testicular oxidative stress

Spermatogenesis is a process of rapid cell division, which means a high rate of oxygen consumption. Maintaining testicular homeostasis and avoiding oxidative stress can ensure normal spermatogenesis. The results of the study suggest that Cu exposure led to a high concentration of Cu in testes, and the spermatogenesis was reduced, and the testicular tissue showed degenerative changes. Therefore, to investigate the role of oxidative stress in testicular toxicity of Cu, we determined whether the testicular oxidation and antioxidant balance could be affected after Cu exposure. Frist, RT-PCR was used to determine the relative mRNA expression levels of antioxidant factors in the testis. Results showed that, the relative mRNA expression levels of Gpx4, TRX, HO-1, SOD1, SOD2, SOD3, and CAT increased after Cu treatment. Compared with the control group, the change of the relative mRNA expression levels of antioxidant factors in LG and HG groups were significantly different ($P < 0.05$) (Fig. 2A). Then, we used flow cytometry to determine the testicular ROS level, and we found that the level of ROS in the testes increased after Cu treatment. The increase of ROS in LG group was significantly different ($P < 0.05$), but in the HG group was not ($P > 0.05$) (Fig. 2B and C). Furthermore, we found that the content of MDA in the testes increased in the LG and HG groups, but the difference between control group and treatment groups were not significant ($P > 0.05$). These results suggest that Cu exposure disrupts the balance of oxidants and antioxidants in the testes, resulting the oxidative stress in testes.

## Oxidative stress that Cu mediated promotes apoptosis and autophagy in the testes

Considering that oxidative stress will induce oxidative damage to DNA, protein, and lipids. Therefore, we evaluated the apoptosis in the testis. Results showed that, all the apoptosis-related factors that we measured were increased in a dose-dependent manner. In short, the relative mRNA expression levels of Caspase3, Caspase8, Caspase9, APAF1, p53, Bax, Bak1, and Cytc increased after Cu exposure. Meanwhile, compared with control group, the expressions of Caspase8, Caspase9, and Cytc in LG group and the expression of Caspase3, Caspase8, Caspase9, APAF1, p53, Bax, Bak1, and Cytc in HG group were significantly different ($P < 0.05$) (Fig. 3A). Next, we found that Cu exposure caused increased expression of Active Caspase3 protein in a dose-dependent manner, and the difference between control and HG groups were significant ($P < 0.05$) (Fig. 3B and C). Moreover, we measured the mitochondrial membrane potential of testes by using JC-1 fluorescent probe, and the results showed that, the testicular JC-1 aggregates decreased in a dose-dependent manner. Compared with the control group, JC-1 aggregates in LG and HG groups decreased significantly ($P < 0.05$), indicated that the testicular mitochondrial membrane potential decreased after Cu exposure (Fig. 3D and E). Furthermore, the TUNEL staining was used to detect apoptosis in testicular tissue, and we found that compared with the control group,

**Table 2** Effects of dietary copper exposure on serum hormone levels in pigs.

| Measurement | Control | LG | HG |
|---|---|---|---|
| Estradiol (pmol/L) | $88.62 \pm 4.49^{a}$ | $96.43 \pm 5.16^{a}$ | $98.12 \pm 3.77^{a}$ |
| Testosterone (nmol/L) | $88.74 \pm 0.74^{a}$ | $85.02 \pm 1.83^{a}$ | $100.81 \pm 8.93^{a}$ |
| Luteinizing hormone (ng/L) | $7.20 \pm 1.13^{a}$ | $2.63 \pm 0.23^{b}$ | $2.84 \pm 0.32^{b}$ |
| Parathyroid hormone (pg/mL) | $323.32 \pm 9.01^{a}$ | $330.23 \pm 9.17^{a}$ | $321.59 \pm 4.00^{a}$ |
| Thyroxine (pmol/L) | $514.27 \pm 35.68^{a}$ | $540.00 \pm 9.18^{ab}$ | $618.58 \pm 38.50^{b}$ |
| Follicle-stimulating hormone (U/L) | $3.55 \pm 0.33^{a}$ | $2.57 \pm 0.27^{b}$ | $2.80 \pm 0.16^{ab}$ |

Data are the mean with S.E. (n = 4). Within rows, values with different superscript letters differ significantly ($P < 0.05$, one-way ANOVA)

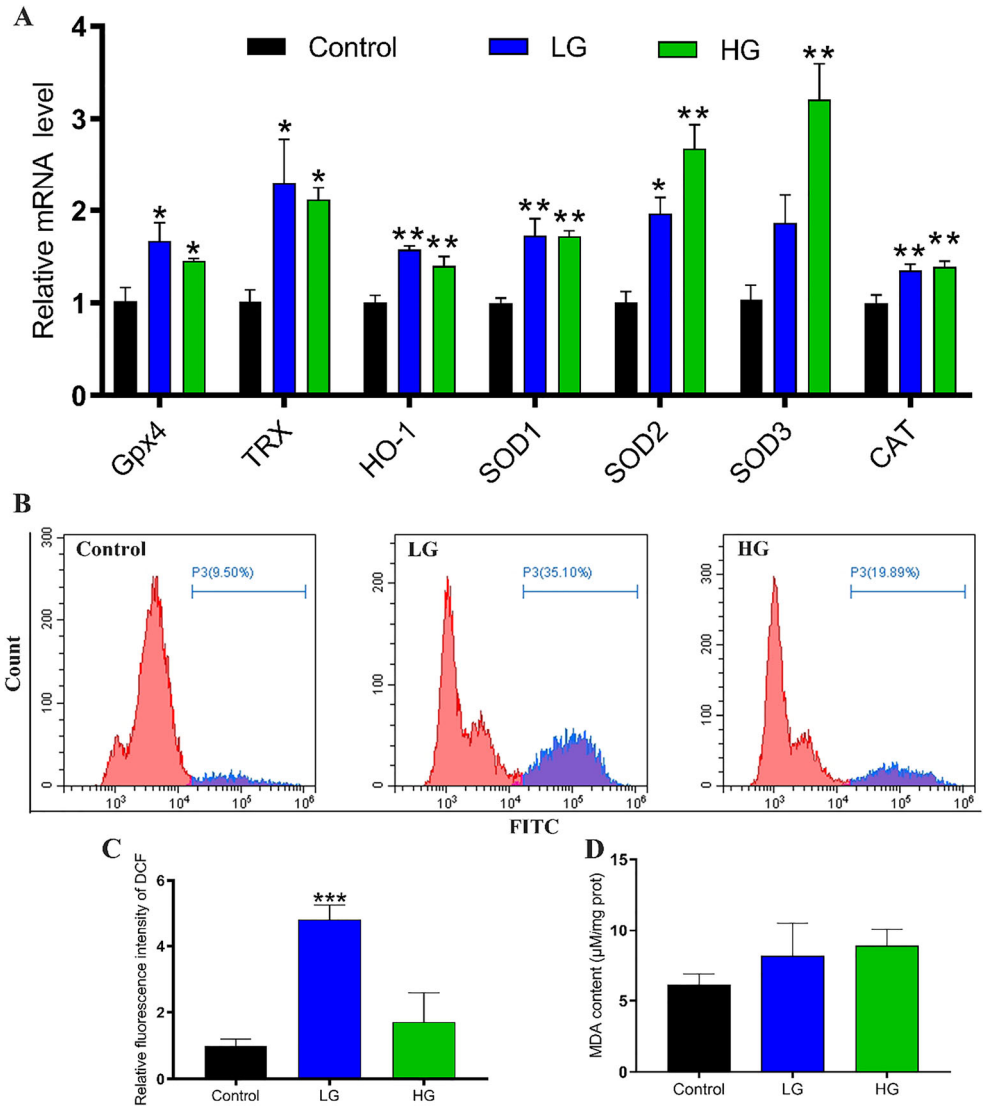


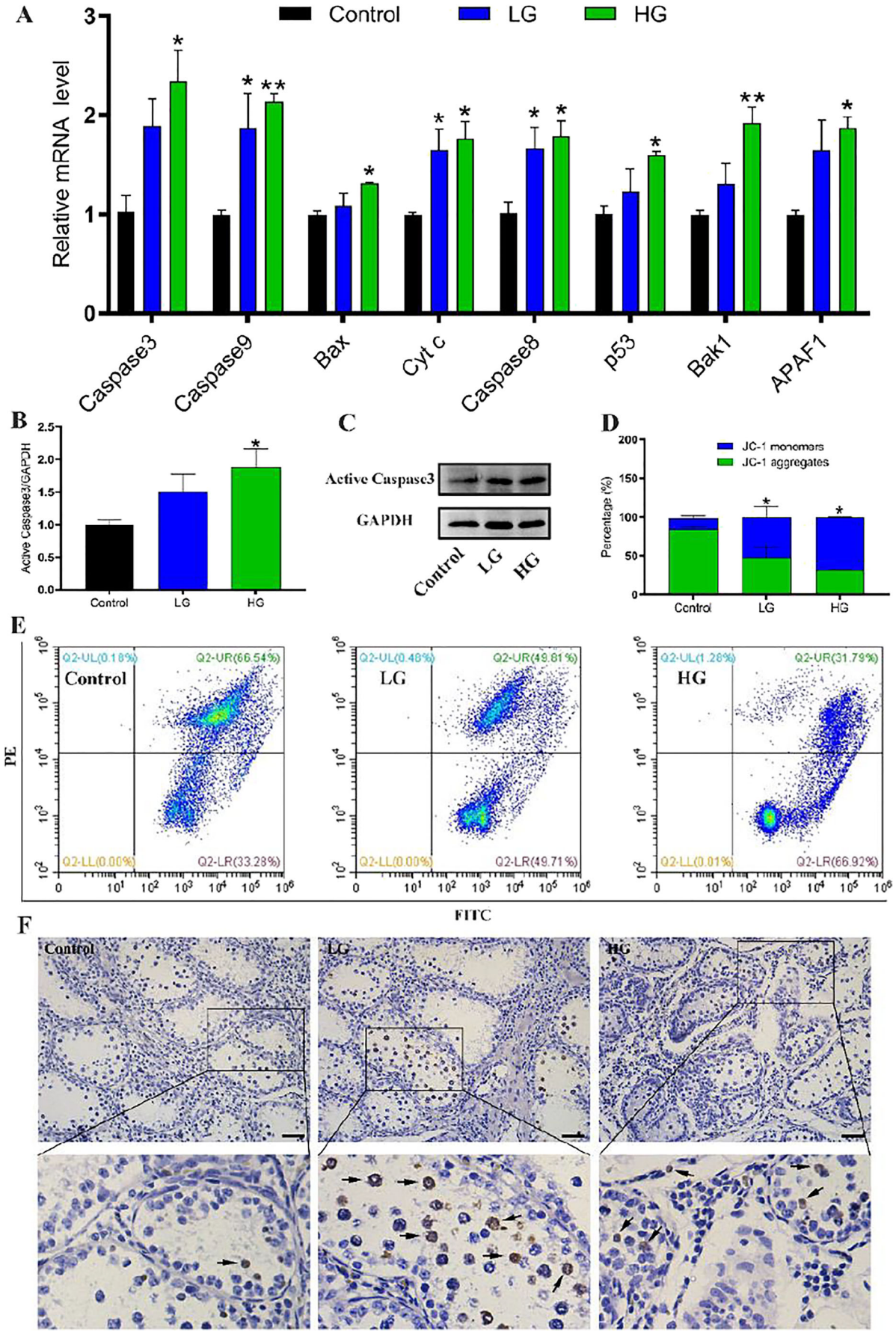

**Fig. 2** Cu exposure induced oxidative stress. **A** The relative expression levels of antioxidants mRNA in the testes of the control, LG, and HG groups. **B** The levels of ROS in the testes of the control, LG, and HG groups. **C** Relative fluorescence intensity of DCF of the control, LG, and HG groups. **D** Testicular MDA content in the control, LG, and HG groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

the number of TUNEL-positive spermatocytes (arrow) in the testes in LG and HG groups increased. All the results showed that oxidative stress caused decrease in mitochondrial function, promoted the apoptosis in testis, and induced the spermatogenesis disorder by damaging sperm DNA.

Autophagy is not only playing a role in homeostasis of cellular nutrients but also is involved in cell death. Considering that decreased mitochondrial function triggers cellular autophagy, leading to the degradation of mitochondria by autophagosomes. The expressions of autophagy-related factors were determined. The results showed that, the relative mRNA expressions of autophagy-related factors like Beclin1, p62, ATG5, LC3, and LC3B were increased after Cu exposure, and compared with control group, the expressions of ATG5 and LC3B in LG group were significantly increased ($P < 0.05$), while in HG group, the expressions of LC3, ATG5, LC3B, and Beclin1 were significantly increased ($P < 0.05$) (Fig. 4A). In addition, the LC3B-positive cells in testes increased after Cu exposure, and the LC3B mean optical density in LG and HG groups was significantly increased compared with the control group ($P < 0.05$) (Fig. 4B and C). All the results suggested that the testicular oxidative stress promotes autophagy in testis via Beclin1/ATG5/LC3 autophagy signals.

## Discussion

The Cu is widely used in industrial and agricultural production system, but the long-term application of copper causes environmental pollution. The environmental pollution can lead to chronic exposure of copper in animals by ingesting water and plants with high copper contents. One of the effects can be on the testis in animals which is a potential danger. As previous studies have reported, copper can accumulate in the testes via CRT1 and can cause toxic damage to the testes (Ghaffari et al.


A
Control
LG
HG
Relative mRNA level
Caspase3
Caspase9
Bax
Cyt c
Caspase8
p53
Bak1
APAF1
B
Active Caspase3/GAPDH
Control
LG
HG
C
Active Caspase3
GAPDH
Control
LG
HG
D
JC-1 monomers
JC-1 aggregates
Percentage (%)
Control
LG
HG
E
Control
Q2-UL(0.18%)
Q2-UR(66.54%)
Q2-LL(0.00%)
Q2-LR(33.28%)
LG
Q2-UL(0.48%)
Q2-UR(49.81%)
Q2-LL(0.00%)
Q2-LR(49.71%)
HG
Q2-UL(1.28%)
Q2-UR(31.79%)
Q2-LL(0.01%)
Q2-LR(66.92%)
PE
FITC
F
Control
LG
HG

◀ **Fig. 3** Cu treatment induced increased apoptosis through mitochondrial apoptosis pathway in the testis. **A** The expression levels of apoptotic-related mRNA in testes of the control, LG, and HG groups. **B** and **C** The relative expression levels of Active Caspase3 protein in testes of the control, LG, and HG groups. **D** The percentage of JC-1 monomers and JC-1 aggregates in the testes of the control group, LG group, and HG group. **E** Expression of results of both green and red JC-1 fluorescence intensities in control group, LG group, and HG group. **F** TUNEL staining was performed on testicular sections of the three groups. The apoptosis of spermatocytes by Cu inducing was observed under microscope (arrow)

2019; Lee et al. 2001). Some researchers have found that the copper causes testicular toxicity, through oxidative stress and thus leads to disorders of spermatogenesis (Kheirandish et al. 2014; Chen et al. 2020). However, due to the complexity of the testicular internal environment and function, the potential mechanisms of copper-induced testicular toxicology remained obscured.

In this study, we firstly observed the effect of Cu exposure on the testes and verified the Cu accumulation in the testis by determining the testicular Cu contents and testicular Cu transporter-related gene expression. The results showed that Cu accumulate in testis through upregulation of Cu transporters Slc31a1 (CTR1) ATP7a, ATP7b, and ATOX1. The Cu ion entry into cells is mediated by Cu transporters (CTR), and 80% of Cu ion is transported into the cell by CTR1, which is encoded by Slc31a1 gene (Ogórek et al. 2017). The intracellular Cu ions are supplied to SOD1 and metallothionein (MT), and excessive Cu ions will bind to the ATOX1 protein and then be transported to ATP7A and ATP7B, which can transport Cu ions to outside the cell or into the blood, respectively (Herman et al. 2020). The upregulated expression of the testicular copper transporter gene indicated that the concentrations of Cu in the testicular cells was beyond the range of tolerance in testicular cells; for that, Cu transporter gene expression was elevated in order to maintain testicular cell homeostasis. Moreover, in this study, the rising Cu content of testis in LG and HG groups also proved the above perspective. Interestingly, we observed that the testicular Cu content was higher in the LG group than in the HG group, which may be related to the elevated expression of ATP7A and ATP7B in the testes, and similar results have been reported in other studies (Li et al. 2021). Then, Cu exposure caused the decrease in testicular coefficient, testicular degeneration, and sloughing of spermatocytes in the testicular tissue, and these results were similar to previous reports (Zhang et al. 2016; Chen et al. 2020). Therefore, we speculate that the Cu exposure caused testicular toxicology probably because the high concentrations of Cu caused upregulated expression of testicular Cu transporters, resulting in Cu to accumulate in the testicular tissue, which damages the testis.

Besides, the hormones play an important role in spermatogenesis and testicular growth; serum hormones can also represent whether testicular function is normal (Grande et al. 2019). The results that we found showed that the levels of FSH and LH decreased significantly; conversely, the serum E2 and TH levels increased, though the results of E2 were not significant. Interestingly, serum T and PTH levels did not change in response to treatment with different concentrations of Cu. In other studies, serum T levels were significantly reduced by Cu treatment; however, it has also been found that Cu exposure does not affect serum T levels (Zhang et al. 2016; Chen et al. 2020). The LH and FSH can respectively induce proliferation of testicular Leydig cell and Sertoli cell, and LH additionally supports a normal increase in the testicular size (Wells et al. 2013). The TH is important for growth, development, and metabolism, and testicular development is also highly dependent on TH status (Hernandez 2018). The E2 is expressed in male testes and regulates the survival and apoptosis of germ cells, and excessive or insufficient estrogen levels can affect the testicular function (Correia et al. 2015; Sierens et al. 2005). Combined with all the results, we found that the changes in TH, E2, FSH, and LH levels affected the growth and function of the testicular tissue of the pig after Cu exposure.

As important antioxidants in cell, SOD, HO-1, CAT, Gpx4, and TRX function to eliminate the oxidation products and antioxidants (Liu et al. 2021b; Zhang et al. 2020; Wang et al. 2021). The MDA content reflects the severity of body exposure to free radicals, and ROS as a common oxidation product in cells, excessive ROS cause oxidative stress Yang et al. (2019a, b). After Cu exposure, to avoid the oxidations induced oxidative damage to the testes, the expression of antioxidants increased. Therefore, we observed that the expressions of antioxidant-related genes (SOD1, SOD2, SOD3, HO-1, CAT, Gpx4, and TRX) were increased in the testes, as well as the ROS and MDA levels. The Cu exposure is known to cause a decrease in activity of antioxidants (Wan et al. 2020). In order to avoid the adverse effects of excessive oxidants, the overexpression of antioxidants is to resist the oxidant's activity. Interestingly, with the increase of Cu dose, the level of ROS in the testes decreased; we speculate that the increased expression of antioxidants enhances the antioxidant capacity of the testis and enables the testis to clear excessive ROS. Nevertheless, the content of MDA in the testes did not decrease, and increased continuously, which reflected that the testes were still in the imbalance state that oxidation capacity was higher than the antioxidant capacity, which means the overexpression of antioxidants still does not decrease the oxidant production. Altogether, results suggested that the oxidation and antioxidant balance dysregulation may be a key mechanism in Cu-induced testicular toxicity (Liao et al. 2019).

Next, we further observed that the Cu exposure promoted apoptosis and autophagy in the testes, and it was associated with oxidative stress. The Cu exposure upregulated apoptotic signals. Apoptosis is characterized by caspase activation,

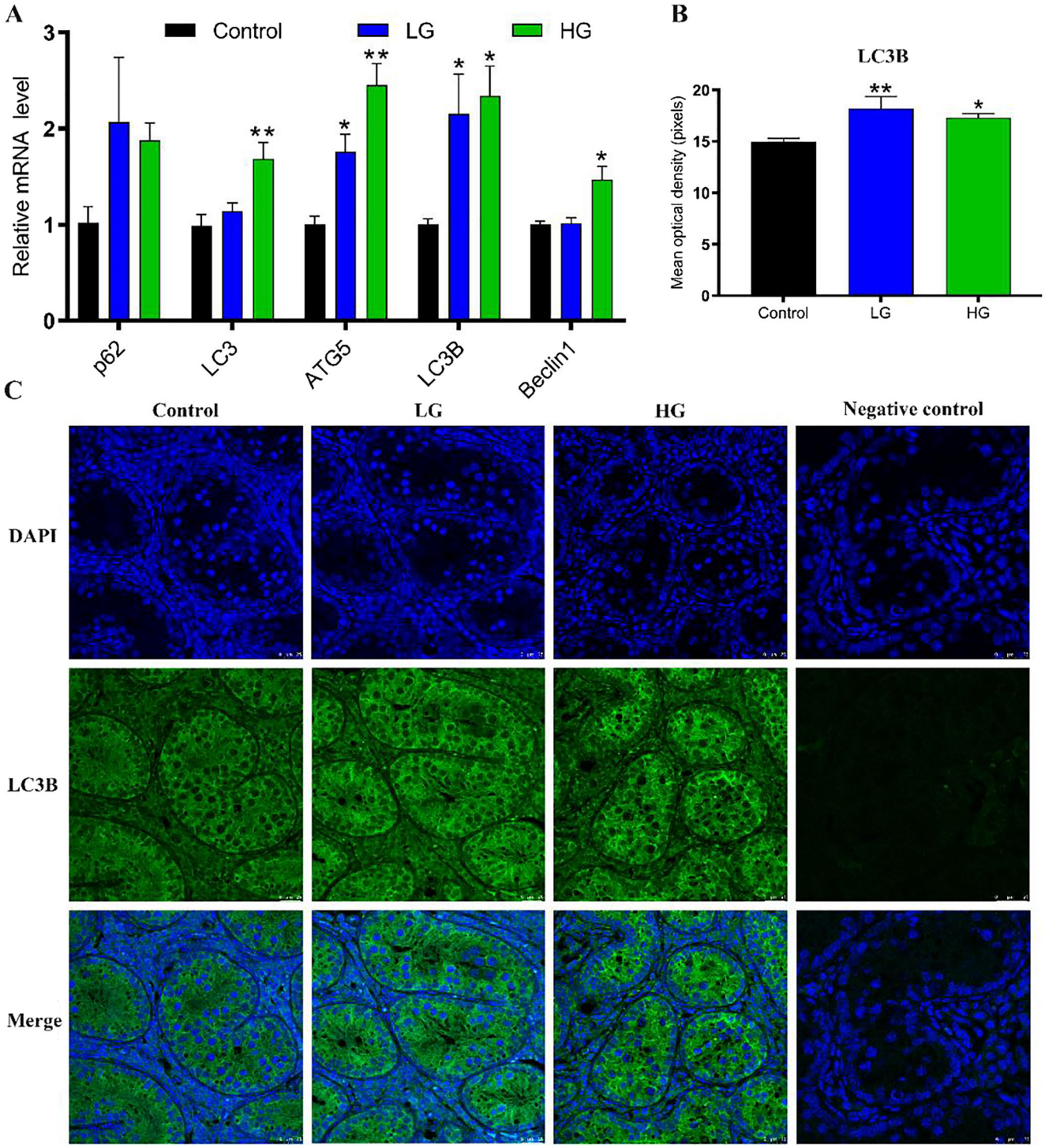



**Fig. 4** The increase of autophagy induction by Cu in the testes. **A** The relative expression levels of autophagy-related mRNA in the testes after treated pigs with 9 mg/kg bw, 125 mg/kg bw, and 250 mg/kg bw Cu. **B** The LC3B mean optical density in the testes after treated pigs with 9 mg/kg bw, 125 mg/kg bw, and 250 mg/kg bw Cu. **C** Expression and location of LC3B was detected by immunofluorescence staining for evaluate the testes autophagy. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

which is playing a central role in it; each of the apoptotic pathways activates the apoptosis initiator caspase via specific triggering signals, which ultimately activates the apoptosis executioner Caspase3 (Elmore 2007). Mitochondria are important organelles for oxidative phosphorylation as well as ATP production and consumption in eukaryotic cells Yang et al. (2019a, b). The impairment of mitochondria can induce an increase in oxidative products, which in turn can

further damage the cell, and induce apoptosis Sun et al. (2020). In this study, at first, the ROS upregulated the expression of Caspase8 by suppressing anti-inflammatory-related signals of the extrinsic pathway, and ROS further promoted the expression of Bak1, Bax, and Cytc signaling, which regulate the mitochondrial apoptotic pathway (Sinha et al. 2013; Tummers and Green 2017). Meanwhile, p53 also promotes the expressions of Bax and Bak1 in mitochondrial apoptotic pathway by interacting with Bcl-2 family of proteins (Estaquier et al. 2012; Wang et al. 2021). And then Cytc binds to the apoptotic factor APAF1, activates Caspase9, and finally promotes Caspase3 expression with Caspase8 signaling, eventually inducing apoptosis (Sinha et al. 2013; Tummers and Green 2017; Liu et al. 2021a). The decrease in proportion of JC-1 aggregates indicated decreased mitochondrial membrane potential in the testis, which is a sign of the early stage of apoptosis (Savitskiy et al. 2003). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect DNA fragmentation by labeling the free 3'- hydroxy terminal. In short, TUNEL is a method of staining cells by labeling broken DNA, which is often used to determine cells in the early stage of apoptosis Mirzayans and Murray (2020). To further demonstrate the association among oxidative stress, mitochondria, and apoptosis, we also measured the mitochondrial membrane potential and TUNEL-positive cells in testes. Not unexpectedly, Cu exposure caused decrease in mitochondrial membrane potential and increase in TUNEL-positive cells in the testes. Spermatogenesis is a process of vigorous cell division, which causes a lot of oxygen consumption. When testicular antioxidant level is decreased, spermatocytes are vulnerable to oxidative damage, and thus our results were similar as that most TUNEL-positive cells in this study were spermatocyte (Kang et al. 2021).

Overall, Cu-mediated oxidative stress is associated with testicular apoptosis. The oxidative stress promotes testicular apoptosis by reducing mitochondrial function, regulating molecules of apoptosis pathway, and damaging the DNA.

Oxidative stress affects chromatin structure and consequently mediates many cellular changes, including regulation of gene expression, cell death, survival, and mutagenesis, and the oxidative stress can crosstalk with autophagy machinery (Kreuz and Fischle 2016; Filomeni et al. 2015). This study determined the expression of autophagy-related genes in the testes and found a significant increase in expression of autophagy-related genes like ATG5, LC3, LC3B, and Beclin1 after Cu exposure. Then we performed LC3B localization and semi-quantitative analysis of pig testis sections by using immunofluorescence staining and observed that LC3B mean optical density was enhanced in the pig testicular seminiferous tubules. Previous reports have suggested that oxidative stress induces increase in levels of LC3B, and overexpression of LC3B would promote the formation of autophagosome, which is consistent with our results (Satyavarapu et al. 2018). The p62 is an autophagy receptor protein that mediates the selective autophagy of ubiquitinated cargos, and if autophagy is promoted through this pathway, p62 will be largely recruited and then downregulated Sánchez-Martín et al. (2019). We have not found that the expression of p62 has changed significantly after Cu exposure, suggesting that Cu does not promote autophagy by regulating p62. The Beclin1, as one of the components of PI3K complex, is responsible for initiating the formation of autophagy, and oxidative stress can promote the expression of Beclin1 (Shi et al. 2019). The upregulation of Beclin1 positively regulates the autophagy by promoting PI3K complex synthesis (Van Erp et al. 2017). Once autophagy is started, autophagosome membrane elongation is regulated by LC3 and ATG5 (Van Erp et al. 2017). Altogether, our findings indicated that the oxidative stress mediated by Cu promotes autophagy in testes by regulating Beclin1/ATG5/LC3 signaling.

Still, due to the limited antibodies from pigs, we only measured the expression of oxidative stress, apoptosis, and autophagy-related genes at the gene level. To address this deficiency, we continue to investigate the testicular toxicity mechanism of Cu based on the results of this study in the future. In addition to investigate whether Cu exposure can promote the expression of gene-related proteins that we have detected in this study, the gene expression of upstream-related pathways of apoptosis and autophagy should be further explored.

## Conclusions

In this study, we determined the pig testicular Cu toxicity and found a complex relationship between oxidative stress, autophagy, and apoptosis in Cu-exposed testes. The Cu exposure induces testicular oxidative stress and further damages testis by promoting apoptosis and autophagy, and apoptosis decreases testicular antioxidant capacity through mitochondrial pathway. These findings provide a new perspective into the mechanism toxicity by Cu in testis.

**Availability of data and materials** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Author contribution** Y.L.L. and H.M.C. made equal contributions in conducting experiments. Z.X.T. and Y.L. provided the research idea. H.M.C., J.Z.L., K.L.C., N.Q., Q.W.Z., B.X.L., and J.N.Y. contributed reagents, materials, and analysis tools; Y.L.L. and M.T.J. wrote the manuscript. All the authors participated in manuscript writing and reviewing.

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## Declarations

**Ethics approval** All animal experiments were reviewed and approved by the Animal Ethics Care Committee of the South China Agriculture University.

**Consent to participate** All authors informed consent to participate in this study.

**Consent for publication** All authors agree to publish the article.

**Conflict of interest** The authors declare no competing interests.

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