

# The Scutellarin Protects Mouse Ovarian Granulosa Cells from ZEA-Induced Injury

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## Research

**Keywords:** ZEA, scutellarin, ovarian granulosa cells, apoptosis, livestock

**Posted Date:** October 13th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-90807/v1>

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# Abstract

## Background

The zearalenone (ZEA) contained in the animal grain feeds is produced by *Fusarium fungi* and this toxin targets ovarian granulosa cells (GCs) to cause reproductive disorders in female animals. Current research on drugs that can rescue ZEA-induced GCs damage is limited. The purpose of this study was to explore the effect of scutellarin (Scu) on ZEA-induced apoptosis of mouse ovarian GCs and its mechanism.

## Results

In one set of experiments, the primary cultured mouse ovarian GCs were co-treated with ZEA and scutellarin for 24 h. The results showed that Scu significantly alleviated ZEA-induced cell damage, restored cell cycle arrest, and inhibited apoptosis by reducing the ratio of cleaved-caspase-3, cleaved-PARP, and Bax/Bcl-2. In other set of experiments, six weeks old mice were intragastrical administered with 40 mg/kg ZEA for 2 h, followed by 100 mg/kg Scu for 3 d. It was shown that Scu inhibited ZEA-induced apoptosis and positive signal expression of cleaved-caspase-3 in the ovarian granulosa layer, with the involvement of mitochondrial apoptotic pathway.

## Conclusion

Scu attenuated ZEA-induced reproductive toxicity by targeting mouse ovarian GCs, mainly affecting cell cycle phase distribution and apoptosis via mitochondrial apoptotic pathway *in vitro* and *in vivo*. These data provide strong evidence that Scu can be further developed as potential new therapeutic drug for preventing or treating reproductive toxicity caused by animal exposure to ZEA found in the grains of animal feeds.

## Background

Zearalenone (ZEA) is a secondary metabolite produced by some *Fusarium* species, naturally exists in cereals, such as corn, barley, and sorghum <sup>1</sup>. Due to its high resistance to heat and widespread existence, ZEA contained in the grains is difficult to be eliminated <sup>2</sup>. Exposure of humans and animals to ZEA poses a great threat to humans and animals health as the ZEA can cause severe systemic toxicity in many organs <sup>3,4</sup>. The main target organs are those in the female reproductive system and ZEA induced toxicity can cause reproductive disorders in pigs, cattle, horses, and mice. The disorders include follicular development problem, ovulation abnormalities, and follicular atresia, leading to great economic losses to livestock and poultry industry <sup>5-7</sup>.

Granulosa cells (GCs) being one type of the important somatic cells of ovarian follicles play a key role in the growth and development of the follicles. GCs secrete steroid hormones and provide nutritional support for oocyte development <sup>8-10</sup>. Studies have shown that apoptosis occurred in 10% or more of GCs population will cause follicle atresia, which results in reproductive disorders <sup>11-13</sup>. ZEA is shown to cause

ovarian GCs apoptosis as well as steroid hormone secretion disorders and reproductive disorders<sup>14,15</sup>. ZEA inhibited the proliferation of GCs in a dose-dependent manner, induce apoptosis and necrosis of GCs through the mitochondrial apoptosis pathway<sup>16</sup>. The ER stress and autophagy were also involved in the mechanism of the action of ZEA on GCs<sup>17</sup>. Therefore, inhibiting GCs apoptosis may alleviate the reproductive toxicity caused by ZEA.

The adsorption of ZEA using physical and chemical methods and its degradation using microbial enzyme are used to eliminate ZEA from *Fusarium* contaminated grains<sup>18</sup>. However, these methods have only achieved limited results. Thus, ZEA can still reach animal feed and human diets, and it is becoming important to explore therapies including drugs to protect tissue cells from their injuries caused by ZEA toxicities. Developing a drug for a therapy would be a pragmatic step to take in this end. Specifically, the ZEA being a mycotoxin induces ovarian GCs apoptosis and affect the secretion of steroid hormones. Therefore, it is of great significance to develop drugs that could ameliorate ZEA-induced GCs apoptosis.

Reports have shown that proanthocyanidins, saffron, lycopene can protect tissue cells from ZEA-induced damage<sup>19-21</sup>. Scutellarin (Scu) is a flavonoid compound derived from plants such as *Erigeron breviscapus*, *Scutellaria barbata*, and *Scutellaria baicalensis*. These herbs are widely used in the treating cardiovascular and cerebrovascular diseases, diabetes, metabolic disorders, and other related diseases in clinical practice<sup>22,23</sup>. The pharmacological studies have shown that Scu has anti-inflammatory, antioxidant, anti-apoptotic, and antibacterial properties. Studies have reported that Scu can protect kidney from damaged due to cisplatin by inhibiting the expression of inflammatory factors and pro-apoptosis related proteins<sup>24</sup>. Based on these findings, we hypothesize that the Scu may protect tissue cells against ZEA-induced reproductive toxicity and does so via modulating apoptotic pathway.

In this study, we aimed to test this hypothesis by observing the effect of Scu on ZEA-induced ovarian GCs apoptosis *in vitro* and *in vivo* through the mitochondrial apoptotic pathway. Furthermore, the effect of Scu to relieve ZEA-induced GCs S-phase arrest was also checked. The findings of this study suggested that Scu could be further developed as a new effective drug for the treatment of reproductive toxicity caused by ZEA.

## Materials And Methods

### Reagents and antibodies

ZEA (Z2125) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregnant mare serum gonadotropin (PMSG) from Shu Sheng Hormone (Ningbo, China); Dulbecco's modified Eagles medium with Hams F-12 nutrient mixture (DMEM/F12, 1:1) and fetal bovine serum (FBS) were obtained from Biological Industries (Israel). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Solarbio Life Science (Beijing, China). Cell cycle detection kit (using PI/RNase A) was obtained from Keygen Biotechnology (Jiangsu, China). Cleaved-

caspase-3 (ab214430), Bax (ab32503) and Bcl-2 (ab182858) antibodies were from Abcam (Cambridge, MA, USA), and cleaved-PARP (94885) was purchased from Cell Signaling Technology (Boston, MA, USA)

Scu shown in Fig 1 was obtained from National institutes for Food and Drug Control (Beijing, China), and the purity was 91.7%.

### **Isolation and culture of mouse GCs**

The mouse GCs were collected and cultured according to our previously describe study<sup>25</sup>. Briefly, three-week-old female Kunming mice were intraperitoneally injected with 5 IU PMSG, and were euthanized 46 h later. Bilateral ovaries were collected and punctured with 26 gauged# needles under a stereomicroscope to isolate GCs and passed through 0.074 mm sieve and centrifuged (1000 rpm, 5 min). After washing three times with PBS, the cells were resuspended in DMEM/F12 medium supplemented with 10% FBS and 1% streptomycin-penicillin, and were incubated at 37°C with 5% CO<sub>2</sub>.

### **Determination of cell viability**

Isolated cells were seeded in 96-well plates at a density of  $1 \times 10^5$  cells/mL. After 24 h of culture, the cells were treated with different concentrations (2000, 1000, 500, 250, 125, 62.5 and 31.25  $\mu\text{g} / \text{mL}$ ) of Scu for 24 and 48 h; or treated with different concentrations of ZEA (10, 30, 60, 90 and 120  $\mu\text{M}$ ) for 24 h; or treated with 60  $\mu\text{M}$  ZEA for different time points (6, 12, 24, 36 and 48 h); or treated with combinations of different concentrations (2000, 1000, 500  $\mu\text{g} / \text{mL}$ ) of Scu with ZEA (60  $\mu\text{M}$ ) for 24 h. Cell viability was detected using MTT assay. Briefly, the medium was discarded, and cells were incubated with 25  $\mu\text{L}$  MTT at 37°C for 4 h. 150  $\mu\text{L}$  DMSO was added to dissolve the formazan crystals at 37°C for 30 min. The OD<sub>490</sub> values were obtained using a microplate reader.

### **Cell cycle distribution analysis**

Cells were seeded in 6-well plates at a density of  $1 \times 10^6$  cells/mL. After reaching 80-90% confluency, the cells were treated with the medium containing different concentrations of Scu (2000, 1000 and 500  $\mu\text{g}/\text{mL}$ ) with or without ZEA (60  $\mu\text{M}$ ) for 24 h. Cells were collected and fixed with cold 70% ethanol for 2 h. After being washed with PBS, cells were incubated with PI/RNase A in dark for 30 min, then the cell cycle distribution was detected using flow cytometry.

### **Animals and Treatments**

Five-week-old female Kunming mice were provided by Charles River (Beijing, China), and were housed under standard laboratory conditions of room temperature (22-24°C) and relative humidity (50-60%), with a 12 h light-dark cycle. The mice were allowed free access to full rodent food and water. The overview of the experimental protocol is shown in Fig 2. The mice were allowed to acclimatize for 1 week. The mice were then randomly divided into control, model, scutellarin groups with eight mice in each group. The model and scutellarin groups were both treated with a single intragastric administration of ZEA dissolved

in corn oil at 40 mg/kg. After 2 h, Scu group was intragastrically given 100 mg/kg Scu in PBS (PH=7.4) for 3 days. The control group was intragastric administered with corn oil and then 2 h later with PBS (Fig.2). All mice were weighed and sacrificed post 72 h of ZEA administration, and the ovaries were collected for further study. These procedures in the protocol was performed by conforming the regulations and guidelines of ethical committee of Shanxi Agricultural University (Taigu, China).

### **In situ TUNEL fluorescence staining assay**

Apoptotic cells were detected using deadend<sup>TM</sup> fluorometric TUNEL system according to the manufacturer's protocol (Promega, Germany). Briefly, after treated with medium containing different concentrations of Scu (2000, 1000, and 500 µg/mL) with ZEA (60 µM) for 24 h, the cells were fixed with 4% paraformaldehyde at 4°C for 25 min. After two washes with PBS, the cells were incubated with 0.2% Triton X-100 for 5 min. After washing, cells were equilibrated with 100 µL equilibration buffers at room temperature for 8 min. The equilibration buffer was discarded and 50 µL rTdT incubation buffer was added to the cells on a 5cm<sup>2</sup> area on a tissue slide, and incubated at 37°C for 60 min. The cells were then incubated with 2×SSC for 15 min. After being washed, the slides were sealed with mounting medium with DAPI, and analyzed under a fluorescence microscope.

Apoptosis in the ovarian tissues was also investigated by TUNEL staining. Briefly, 4% paraformaldehyde-fixed, paraffin-embedded sections were deparaffinized, rehydrated, treated with 20 µg/mL proteinase K for 8 min at room temperature, and then re-fixed with 4% paraformaldehyde for 5 min. After washing, the sections were treated with 100 µL equilibration buffers at room temperature for 8 min, and then incubated with rTdT incubation buffer at 37°C for 1 h in a humidified chamber away from light. After reaction, the sections were washed with 2×SSC for 15 min. Finally, the sections were sealed with mounting medium with DAPI, and analyzed under a fluorescence microscope.

### **Western blotting analysis**

Total proteins were extracted from cells or tissues using a total protein extraction kit (KeyGen, China), and protein concentrations were measured using BCA assay. Proteins were separated via SDS-PAGE and then transferred to a PVDF membrane. After being blocked with 5% non-fat dry milk for 2 h at room temperature, the membranes were subsequently incubated with primary antibodies to β-actin, Bax, Bcl-2, cleaved-caspase-3 or cleaved-PARP) 4°C overnight. After three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three final washes with TBST, the protein bands were observed using an ECL kit (CW BIO, China), and the band intensities were analyzed using Image J software.

### **Immunohistochemistry**

Immunohistochemical staining was conducted according to the manufacturers' instructions. Briefly, 4% paraformaldehyde-fixed, paraffin-embedded sections were deparaffinized, rehydrated, and treated with H<sub>2</sub>O<sub>2</sub> for 10 min. After washing with PBS, the sections were blocked with 5% BSA for 10 min. Then 40 µL

cleaved-caspase-3 antibody (1:50) was added to each section and incubated at 4°C overnight. After rinsing with PBS, the sections were incubated with a biotinylated secondary antibody for 10 min at room temperature. After being washed, the sections were added the streptavidin labeled with catalase. Subsequently, the DAB was used for color development. The sections were stained with hematoxylin for tissue morphology. After being dehydrated with gradient ethanol and cleared with xylene, the sections were sealed with neutral resin. The signals were observed under a fluorescence microscope and photographed.

## **Statistical analysis**

The data were presented as mean  $\pm$  standard deviation (SD). All statistical analysis procedures were performed in GraphPad Prism<sup>TM</sup> 5 software (GraphPad Software Inc., La Jolla, CA, USA), and one-way analysis of variance (ANOVA) was used to determine significant differences among groups. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## **Results**

### **ZEA reduces GCs viability**

As shown in Fig. 4A, starting from 10  $\mu\text{M}$  of ZEA, the cell viability was decreased in a dose-dependent manner, and decreased significantly from 30  $\mu\text{M}$ . At 60  $\mu\text{M}$ , 50% of cell viability was remained. To further evaluate that the suppressive effect was time-dependent, the cells were treated with 60  $\mu\text{M}$  ZEA for 6, 12, 24, 36 and 48 h. The results confirmed that the inhibitory effect was time-dependent with the longer time of treatment producing more severe inhibitory effects (Fig. 4B). Based on these two results, 60  $\mu\text{M}$  concentration of ZEA with 24 h duration treatment was therefore selected as the positive control to establish a GCs damaged model for subsequent experiments.

### **Scu promotes GCs proliferation**

Scu with concentrations ranged from 31.25 to 2000  $\mu\text{g}/\text{mL}$  showed no cytotoxic effect on GCs. It promoted cell proliferation in a dose-dependent manner (Fig.3A). Therefore, high concentrations (2000, 1000 and 500  $\mu\text{g}/\text{mL}$ ) of Scu were chosen for the following studies. The mechanism of Scu on GCs proliferation was explored by treating the cells with 2000, 1000 and 500  $\mu\text{g}/\text{mL}$  of Scu for 24 h, and the cell cycle distribution was detected using flow cytometry. The results showed that all these different doses of Scu reduced the number of cells in the S phase, and the difference between 2000 and 1000  $\mu\text{g}/\text{mL}$  group was significant. 1000  $\mu\text{g}/\text{mL}$  Scu significantly increased the number of cells in the G2/M phase as shown in Fig.3B, and C. These results revealed that Scu could affect the cell cycle via promoting the transformation of cells from S-phase to G2/M-phase.

### **Scu rescues ZEA-induced GCs injury**

In order to investigate whether Scu protects GCs from ZEA-induced cytotoxicity, GCs were cultured with medium containing Scu and ZEA. The results demonstrated that ZEA markedly decreased the cell viability, which was significantly reversed by Scu treatment (Fig. 5A). The cell morphology of ZEA treated cells became rounded with destroyed of cells adhesion (Fig. 5B). These changes were restored by Scu treatment as shown in Fig. 5B. These results demonstrated that Scu could significantly rescue ZEA-induced GCs injury as indicated by the restoration of cell viability and morphology.

### **Scu prevents ZEA-induced S-phase arrest**

As shown in Fig.6, compared with control group, ZEA significantly decreased the number of cells in G0/G1 phase, and increased the number of cells in the S phase and no change in the number of cells in G2/M phase. These results revealed that ZEA induced S phase arrest. The co-treatment of Scu (2000,1000 and 500 µg/mL) and ZEA significantly increased the number of cells in G0/G1 phase while decreased the number of cells in S phase compared to the model. These data indicated that Scu prevented ZEA-induced S-phase arrest.

### **Scu rescues ZEA-induced GCs injuries via mitochondrial apoptotic pathway**

In order to explore the protective effect of Scu on ZEA-induced GCs injuries, the cells were co-treated with different doses (2000,1000 and 500 µg/mL) of Scu and ZEA (60 µM) for 24 h. The results of TUNEL assay demonstrated that ZEA markedly increased the apoptotic rate (35%) comparing to control group (Fig.7A, B). In contrast, Scu significantly reversed the effect of ZEA. These data showed that Scu could inhibit the apoptosis of GCs induced by ZEA.

To investigate the anti-apoptosis mechanism of Scu in GCs under ZEA treatment, western blot analysis was used to detect the protein expressions of Bax, Bcl-2, cleaved-caspase-3 in apoptotic pathway and the apoptosis hallmark protein, the cleaved-PARP. As shown in Fig.7C, ZEA exposure remarkably increased the level of cleaved-caspase-3, cleaved-PARP, and the ratio of Bax/Bcl-2. All these levels were decreased by Scu treatment. These results demonstrated that Scu had anti-apoptotic effect against the ZEA-induced GCs apoptosis through regulating mitochondrial apoptotic pathway.

### **Scu rescues ZEA-induced the apoptosis of mouse ovary *in vivo***

To investigate the effect of ZEA and the protective effect of Scu on ZEA-induced apoptosis of ovary in mice, apoptosis in ovarian tissues of each groups was detected by TUNEL staining. The results demonstrated that ZEA exposure has increased the ovarian tissues apoptosis. The apoptosis occurred predominantly in follicle granulosa layers (white arrows, Fig.8). These findings confirmed that 40 mg/kg ZEA induced granulosa cells apoptosis in mice. The numbers of apoptotic cells in Scu treated group was significantly decreased. This result indicated that Scu is capable of preventing or rescuing ZEA-induced granulosa cells apoptosis.

### **Anti-apoptotic mechanism of Scu *in vivo***

To investigate whether Scu exerts a protective role in *in vivo* through the mitochondrial apoptotic pathway, the localization and expression of cleaved-caspase-3 was measured by immunohistochemistry and western blot analyses. The immunohistochemistry study in Fig.9A and B showed that, compared with control group, the expression of cleaved-caspase-3 was markedly increased in model. And the positive cells were mainly located in follicle granulosa layers and to a lesser extent in theca layer (black arrow) and ovary stroma (thick white arrow). The expression of cleaved-caspase-3 was significantly decreased in Scu group, especially in follicle granulosa layer, and positive tissue cells of cleaved-caspase-3 in theca layer and ovary stroma were also decreased.

The apoptosis related proteins were measured using western blot showed that ZEA treatment significantly increased the ratio of Bax/Bcl-2 and the expression of cleaved-PARP, while Scu treatment significantly reversed these changes (Fig.9B). These data suggested that Scu attenuated ZEA-induced apoptosis in granulosa layer via mitochondrial apoptotic pathway *in vivo*.

## Discussion

Present study was aimed to assess if Scu can protect the ovarian GCs from ZEA-induced cytotoxicity. It was found that Scu can rescue ZEA-induced apoptosis in the GCs.

Using *in vitro* cell culture methods, we established a cellular model of ZEA-induced injuries on GCs isolated from mouse. It was found that 60  $\mu\text{M}$  ZEA for 24 h led to 50% viability reduction of GCs in the cell culture model. This is a new model in addition to porcine GCs model where the 90  $\mu\text{M}$  ZEA for 24 h caused 50% viability reduction<sup>16</sup>. Yang reported that the 25  $\mu\text{M}$   $\beta$ -zearalenol treatment for 24 h reduced 50% viability to build the bovine GCs model. We further found that ZEA inhibited the proliferation of GCs in a dose- and time-dependent manner that is consistent to the findings by Chen<sup>17</sup>. At the same time, we established that Scu promoted GCs proliferation and affected cell cycle distribution. These findings suggested that Scu may play an important role on female egg growth.

Ben Salem and Yang used a dose of ZEA that could inhibit 50% of cells viability as a treatment condition for subsequent experiments<sup>15,26</sup>. Hence, using the newly established ZEA toxicity mode, i.e.-60  $\mu\text{M}$  ZEA treatment for 24 h in the present study, we found that different dose of Scu significantly alleviated the ZEA-induced cell damage.

In the ensuing experiment we assessed the protective effect of Scu against ZEA-induced injuries from cellular model to a mouse model. First, a ZEA-induced mouse ovarian damage model was established by intragastric administration of 40 mg/kg ZEA. Researches have reported that ZEA exposure caused abnormal and hypertrophy of female ovaries, as well as an obvious toxicity on the gametogenesis and embryonic development of female or male mice<sup>5,27,28</sup>. After treated with a single intragastric administration of 40 mg/kg ZEA, obvious damage was observed in the testis of male mice<sup>19</sup>. Our results showed that 40 mg/kg ZEA markedly induced the ovarian tissues apoptosis, indicated the model was built successfully to meet our objective. According to dose of the reference<sup>29</sup>, we chose 100 mg/kg Scu

for continuous intragastric administration for 3 days, and the results showed that Scu attenuated ZEA-induced TUNEL-positive cells in ovary. All data demonstrated that Scu has protection against ZEA-induced ovary injury *in vivo* and *in vitro*. It could be used as a candidate drug for follow-up research. And more dose of Scu is suggested to be used in the further research animal study to provide full evidence for the clinical application of Scu.

We then explored the molecular mechanism as how Scu exerts the protection of GCs against the ZEA induced injuries on follicles from the perspective of cell cycle distribution and apoptosis. We found that ZEA facilitated cell cycle progression from the G0/G1 phase to S phase. Li reported that ZEA has promoted the transition from G0/G1 phase to S cell cycle phase, and cells are arrested in S phase<sup>30</sup>. This is consistent with our results. However, there was no obvious change in cells number at G2/M phase. The data suggested that ZEA exerted its cytotoxic effect via redistribution of cell cycle phases with a speculation of DNA damage in G0/G1 phase.

Mitochondria are an important organelle in cells which are considered as the main cellular location of the apoptosis pathway. The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 play a critical role in the regulation of apoptosis. For example, Bcl-2 family proteins are involved in the regulation of the apoptosis by controlling membrane permeability. Caspase-3 is a major executor of apoptosis<sup>31</sup>. Poly (ADP-ribose) polymerase (PARP) which is involved in DNA repair and genome stability is a major target of caspases<sup>32-33</sup>. We found that ZEA significantly increased the ratio of Bax/Bcl-2, the expression of cleaved-caspase-3 and cleaved-PARP. This finding is supported by previous studies that ZEA could induce GCs apoptosis via mitochondrial pathway with an increase in Bax and caspase-3<sup>34-36</sup>.

Scu has significantly alleviated the injury induced by ZEA, and has affected the cell cycle phase distribution of normal GCs. Hence, we speculated that Scu could attenuate the cytotoxicity caused by ZEA via affecting cell cycle distribution. Our results of relieving ZEA-induced S phase arrest by Scu showed that it could play a protection role in this end, but the mechanism still needs to be further studied.

Researches have shown that ZEA induces apoptosis in different types of cells such as bovine GCs, primary Leydig cells<sup>15, 37-39</sup>. With these preceding results, we suspected that the protective mechanism of Scu against ZEA-induced GCs injury could be through apoptosis. Indeed, we found that Scu treatment alleviated apoptosis induced by ZEA both *in vitro* and *in vivo* models. These results showed that Scu significantly alleviated ZEA-induced cell apoptosis via decreasing the ratio of Bax/Bcl-2, and the expression of cleaved-caspase-3 and cleaved-PARP *in vivo* and *in vitro*. And it is worth noting that the positive signal of cleaved-caspase-3 in ZEA group was significant in follicle granulosa layers, theca layer and ovary stroma. While the protein expression levels of cleaved-caspase-3, especially in follicle granulosa layers, were decreased in Scu treated mouse. These findings indicated that Scu could block ZEA-induced GCs apoptosis in mice via mediating mitochondrial related proteins *in vivo* and *in vitro*. The apoptosis in theca layer and ovary stroma induced by ZEA and rescued by Scu is a new found in the current study and still need to further explore as our mainly research was focused on the apoptosis of GCs.

## Conclusion

our data demonstrated that 1), *in vitro* and *in vivo* animal models of ZEA-induced GCs injuries were established successfully. 2), Scu attenuated ZEA-induced reproductive toxicity by targeting mouse ovarian GCs, mainly affecting cell cycle phase distribution and apoptosis via mitochondrial apoptotic pathway *in vitro* and *in vivo*. 3), the Scu can be further developed as potential new therapeutic drug for preventing or treating reproductive toxicity caused animals exposure to ZEA found in the grains of animal feed.

## Abbreviations

ZEA: Zearalenone; GCs: granulosa cells; Scu: scutellarin; PARP: Poly (ADP-ribose) polymerase; PMSG: Pregnant mare serum gonadotropin; DMEM/F12: Dulbecco's modified Eagles medium with Hams F-12 nutrient mixture; FBS: fetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO: dimethylsulfoxide; SD: standard deviation.

## Declarations

### Ethics approval and consent participate

The experimental protocol was approved by conforming the regulations and guidelines of ethical committee of Shanxi Agricultural University.

### Consent for publication

All authors critically revised the manuscript for important intellectual contents and approved the final manuscript.

### Availability of data and material

The datasets analyzed in the present study are available from the corresponding author on reasonable request.

### Conflict of interest

The authors declare that there are no conflicts of interest in this research project.

### Funding

This research was supported by the National Key R&D Program of China (Grant Number 2017YFD0501500).

### Author contribution

**YYY:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft. **WSX:** Investigation, Formal analysis, Data curation, Writing - original draft. **KA:** Writing - review & editing. **WSY:** Writing - review & editing, Visualization. **GJH:** Writing - review & editing, Visualization. **ZXZ:** Writing - review & editing, Visualization. **LHQ:** Conceptualization, Supervision. **SN:** Conceptualization, Supervision, Project administration, Investigation.

## References

1. Yang, D. C.; Jiang, X. W.; Sun, J. X.; Li, X. S.; Jiao, R.; Peng, Z. Y.; Li, Y. Q.; Bai, W. B. Toxic effects of zearalenone on gametogenesis and embryonic development: a molecular point of review. *Food Chem Toxicol.* 2018; 119: 24-30.
2. Kong, W. J.; Shen, H. H.; Zhang, X. F.; Yang, X. L.; Qiu, F.; Ou-yang, Z.; Yang, M. H. Analysis of zearalenone and  $\alpha$ -zearalenol in 100 foods and medicinal plants determined by HPLC-FLD and positive confirmation by LC-MS-MS. *J Sci Food Agr.* 2013; 93: 1584-1590.
3. Rai, A.; Das, M.; Tripathi, A. Occurrence and toxicity of a fusarium mycotoxin, zearalenone. *Crit Rev Food Sci Nutr.* 2019; 60: 1-20.
4. Tatay, E.; Espín, S.; García-Fernández, A.; Ruiz, M. Oxidative damage and disturbance of antioxidant capacity by zearalenone and its metabolites in human cells. *Toxicol In Vitro.* 2017; 45: 334-339.
5. Gao, X.; Sun, L. H.; Zhang, N. Y.; Li, C.; Zhang, J. C.; Xiao, Z. H.; Qi, D. S. Gestational zearalenone exposure causes reproductive and developmental toxicity in pregnant rats and female offspring. *Toxins.* 2017; 9: 21.
6. Minervini, F.; Dell Aquila, M. E. Zearalenone and Reproductive Function in Farm Animals. *Int J Mol Sci.* 2008; 9: 2570-2584.
7. Zhang, G. L.; Feng, Y. L.; Song, J. L.; Zhou, X. S. Zearalenone: a mycotoxin with different toxic effect in domestic and laboratory animals' granulosa cells. *Front Genet.* 2018; 9: 667.
8. Su, Y. Q.; Sugiura, K.; Eppig, J. J. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin Reprod Med.* 2009; 27: 032-042.
9. Kidder, G. M.; Vanderhyden, B. C. Bidirectional communication between oocytes and follicle cells: ensuring oocyte developmental competence. *Can J Physiol Pharm.* 2010; 88: 399-413.
10. Li, R.; Albertini, D. F. The road to maturation: somatic cell interaction and self-organization of the mammalian oocyte. *Nat Rev Mol Cell Biol.* 2013; 14: 141-152.
11. Matsuda, F.; Inoue, N.; Manabe, N.; Ohkura, S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *J Reprod Develop.* 2012; 58: 44-50.
12. Girard, A.; Dufort, I.; Douville, G.; Sirard, M., Global gene expression in granulosa cells of growing, plateau and atretic dominant follicles in cattle. *Reprod Biol Endocrin.* 2015; 13: 17.
13. Almeida, C. P.; Ferreira, M. C. F.; Silveira, C. O.; Campos, J. R.; Borges, I. T.; Baeta, P. G.; Silva, F. H. S.; Reis, F. M.; Del Puerto, H. L. Clinical correlation of apoptosis in human granulosa cells-A review. *Cell Biol Int.* 2018; 42: 1276-1281.

14. Zhang, G. L.; Zhang, R. Q.; Sun, X. F.; Cheng, S. F.; Wang, Y. F.; Ji, C. L.; Feng, Y. Z.; Yu, J.; Ge, W.; Zhao, Y.; Sun, S. D.; Shen, W.; Li, L. RNA-seq based gene expression analysis of ovarian granulosa cells exposed to zearalenone in vitro: significance to steroidogenesis. *Oncotarget*. 2017; 8: 64001-64014.
15. Yang, F. X.; Li, L.; Chen, K. L.; Li, C. M.; Wang, Y. R.; Wang, G. L. Melatonin alleviates  $\beta$ -zearalenol and HT-2 toxin-induced apoptosis and oxidative stress in bovine ovarian granulosa cells. *Environ Toxicol Phar*. 2019; 68: 52-60.
16. Zhu, L.; Yuan, H.; Guo, C. Z.; Lu, Y.; Deng, S. J.; Yang, Y.; Wei, Q.; Wen, L. X.; He, Z. P. Zearalenone induces apoptosis and necrosis in porcine granulosa cells via a caspase-3- and caspase-9-dependent mitochondrial signaling pathway. *J Cell Physiol*. 2012; 227: 1814-1820.
17. Chen, F. L.; Wen, X.; Lin, P. F.; Chen, H. T.; Wang, A. H.; Jin, Y. P. HERP depletion inhibits zearalenone-induced apoptosis through autophagy activation in mouse ovarian granulosa cells. *Toxicol Lett*. 2019; 301: 1-10.
18. Rogowska, A.; Pomastowski, P.; Sagandykova, G.; Buszewski, B. Zearalenone and its metabolites: Effect on human health, metabolism and neutralisation methods. *Toxicol*. 2019; 162: 46-56.
19. Boeira, S. P.; Funck, V. R.; Borges Filho, C.; Del Fabbro, L.; Gomes, M. G. D.; Donato, F.; Royes, L. F. F.; Oliveira, M. S.; Jesse, C. R.; Furian, A. F. Lycopene protects against acute zearalenone-induced oxidative, endocrine, inflammatory and reproductive damages in male mice. *Chem Biol Interact*. 2015; 230: 50-57.
20. Long, M.; Yang, S. H.; Zhang, Y.; Li, P.; Han, J. X.; Dong, S.; Chen, X. L.; He, J. B. Proanthocyanidin protects against acute zearalenone-induced testicular oxidative damage in male mice. *Environ Sci Pollut Res. Int*. 2017; 24: 938-946.
21. Ben Salem, I.; Boussabbeh, M.; Helali, S.; Abid-Essefi, S.; Bacha, H. Protective effect of Crocin against zearalenone-induced oxidative stress in liver and kidney of Balb/c mice. *Environ Sci Pollut Res. Int*. 2015; 22: 19069-19076.
22. Wang, L. P.; Qiang, M. Clinical benefits and pharmacology of Scutellarin: A comprehensive review. *Pharmacol Ther*. 2018; 190:105-127.
23. Chledzik, S.; Strawa, J.; Matuszek, K.; Nazaruk, J. Pharmacological effects of scutellarin, an active component of Genus Scutellaria and Erigeron: a systematic review. *Am J Chin Med*. 2018; 46: 19-337.
24. Sun, C. Y.; Nie, J.; Zheng, Z. L.; Zhao, J.; Wu, L. M.; Zhu, Y.; Su, Z. Q.; Zheng, G. J.; Feng, B. Renoprotective effect of scutellarin on cisplatin-induced renal injury in mice: impact on inflammation, apoptosis, and autophagy. *Biomed Pharmacother*. 2019; 112: 108647.
25. Yi, Y. Y.; Wan, S. X.; Hou, Y. X.; Cheng, J.; Guo, J. H.; Wang, S. Y.; Khan, A.; Sun, N.; Li, H. Q. Chlorogenic acid rescues zearalenone induced injury to mouse ovarian granulosa cells. *Ecotox Environ Safe*. 2020; 194: 110401.
26. Ben Salem, I.; Prola, A.; Boussabbeh, M.; Guilbert, A.; Bacha, H.; Abid-Essefi, S.; Lemaire, C. Crocin and quercetin protect HCT116 and HEK293 cells from zearalenone-induced apoptosis by reducing endoplasmic reticulum stress. *Cell Stress and Chaperones*. 2015; 20: 927-938.

27. Zhao, F.; Li, R.; Xiao, S.; Diao, H. L.; El Zowalaty, A. E.; Ye, X. Q. Multigenerational exposure to dietary zearalenone (ZEA), an estrogenic mycotoxin, affects puberty and reproduction in female mice. *Reprod Toxicol.* 2014; 47: 81-88.
28. Ahmad, B.; Shrivastava, V. K.; Saleh, R.; Henkel, R.; Agarwal, A. Protective effects of saffron against zearalenone-induced alterations in reproductive hormones in female mice (*Mus musculus*). *Clin Exp Reprod Med.* 2018; 45: 163-169.
29. Liu, Y. G.; Wang, J.; Zhang, X. R.; Wang, L.; Hao, T.; Cheng, Y. L.; Wang, D. Scutellarin exerts hypoglycemic and renal protective effects in db/db Mice via the Nrf2/HO-1 Signaling Pathway. *Oxid Med Cell Longev.* 2019; 2019: 1-12.
30. Li, Y. J.; He, X. Y.; Yang, X.; Huang, K. L.; Luo, Y. B.; Zhu, L. Y.; Li, Y. Z.; Xu, W. T. Zinc inhibits the reproductive toxicity of Zearalenone in immortalized murine ovarian granular KK-1 cells. *Sci Rep.* 2015; 5: 14277.
31. Slee, E. A.; Adrain, C.; Martin, S. J., Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem.* 2001; 276: 7320-7326.
32. Agarwal, A.; Mahfouz, R. Z.; Sharma, R. K., Sarkar, O.; Mangrola, D.; Mathur, P. P. Potential biological role of poly (ADP-ribose) polymerase (PARP) in male gametes. *Reprod Biol Endocrinol.* 2009; 7: 143-162.
33. Weat J. D.; Ji, C.; Marnett, L. J. Modulation of DNA Fragmentation Factor 40 Nuclease Activity by Poly(ADP-ribose) Polymerase-1. *J Biol Chem.* 2005; 280: 15141-15147.
34. Zheng, W. L.; Wang, B. J.; Wang, L.; Shan, Y. P.; Zou, H.; Song, R. L.; Wang, T.; Gu, J. H.; Yuan, Y.; Liu, X. Z.; Zhu, G. Q.; Bai, J. F.; Liu, Z. P.; Bian, J. C. ROS-mediated cell cycle arrest and apoptosis induced by zearalenone in mouse sertoli cells via ER stress and the ATP/AMPK pathway. *Toxins.* 2018; 10: 24.
35. Banjerdpongchai, R.; Kongtawelert, P.; Khantamat, O.; Srisomsap, C.; Chokchaichamnankit, D.; Subhasitanont, P.; Svasti, J. Mitochondrial and endoplasmic reticulum stress pathways cooperate in zearalenone-induced apoptosis of human leukemic cells. *J Hematol Oncol.* 2010; 3: 50.
36. Xu, M. L.; Hu, J.; Guo, B. P.; Niu, Y. R.; Xiao, C.; Xu, Y. X. Exploration of intrinsic and extrinsic apoptotic pathways in zearalenone-treated rat sertoli cells. *Environ Toxicol.* 2016; 31: 1731-1739.
37. Wang, Y. L.; Zhou, X. Q.; Jiang, W. D.; Wu, P.; Liu, Y.; Jiang, J.; Wang, S. W.; Kuang, S. Y.; Tang, L.; Feng, L. Effects of dietary zearalenone on oxidative stress, cell apoptosis, and tight junction in the intestine of juvenile grass carp (*ctenopharyngodon idella*). *Toxins.* 2019; 11: 333.
38. Yuan, H.; Deng, Y. T.; Yuan, L. Y.; Wu, J.; Yuan, Z. H.; Yi, J.; Zhang, M.; Guo, C. Z.; Wen, L. X.; Li, R. F.; Zhu, L.; He, Z. P. *Gynostemma pentaphyllum* protects mouse male germ cells against apoptosis caused by zearalenone via Bax and Bcl-2 regulation. *Toxicol Mech Methods.* 2010; 20: 153-158.
39. Wang, Y. J.; Zheng, W. L.; Bian, X. J.; Yuan, Y.; Gu, J. H.; Liu, X. Z.; Liu, Z. P.; Bian, J. C. Zearalenone induces apoptosis and cytoprotective autophagy in primary Leydig cells. *Toxicol Lett.* 2014; 226: 182-191.

## Figures

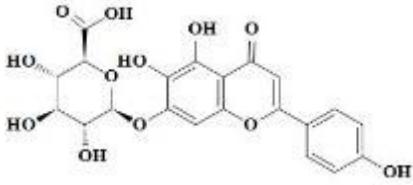


Figure 1

Chemical structure of Scu

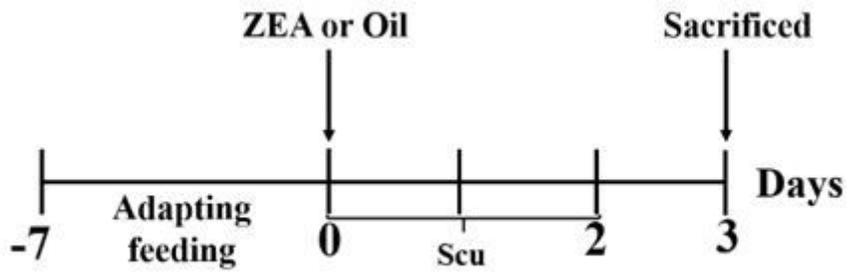
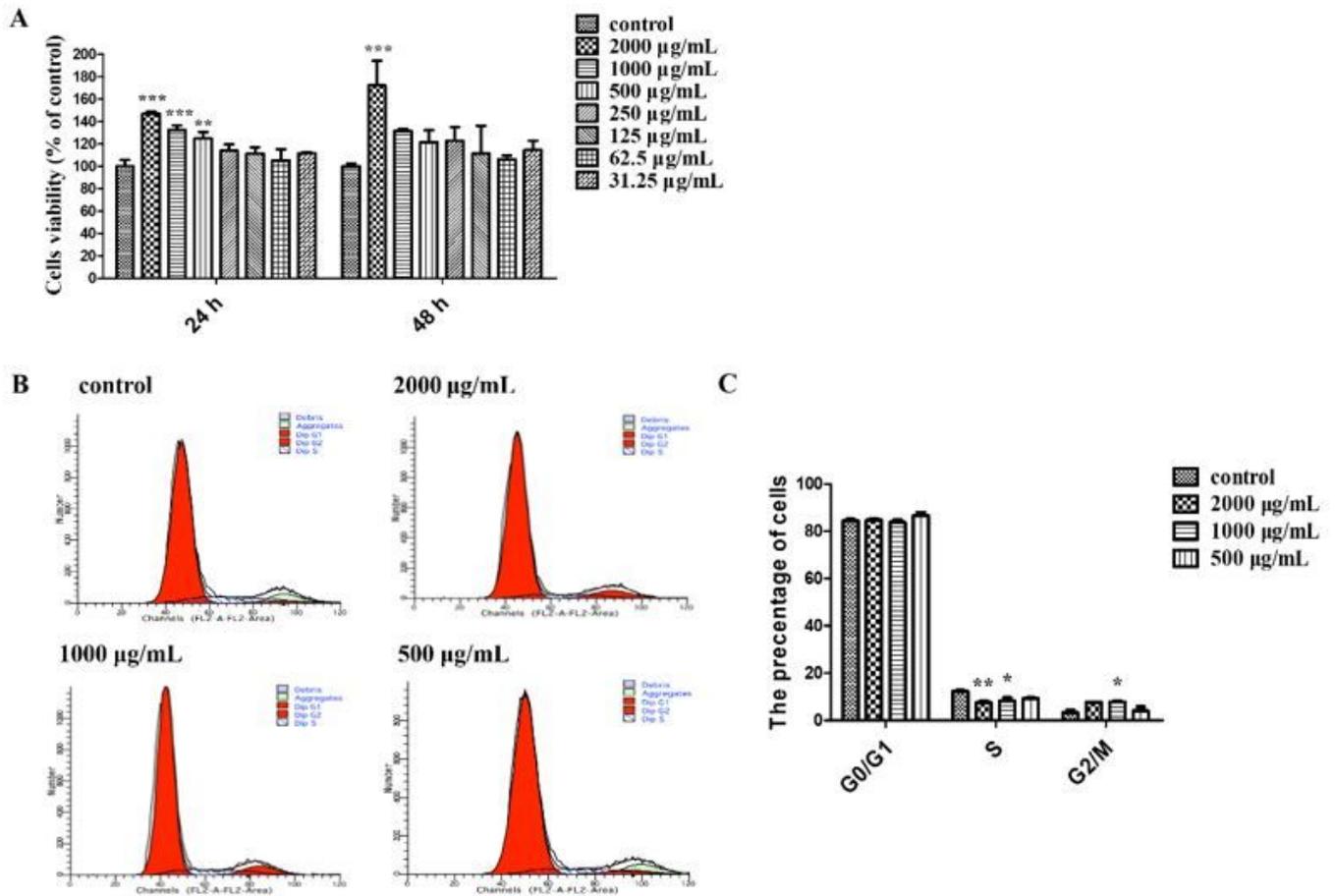


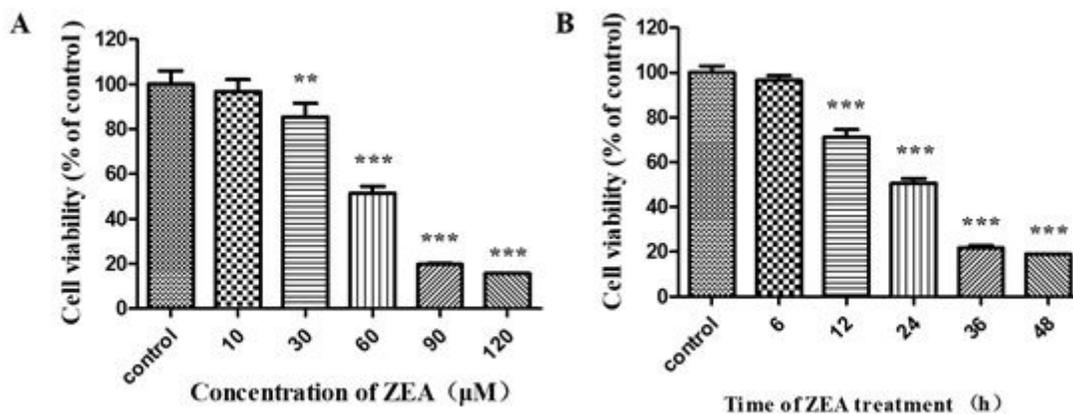
Figure 2

Schematic presentation of the experimental protocol



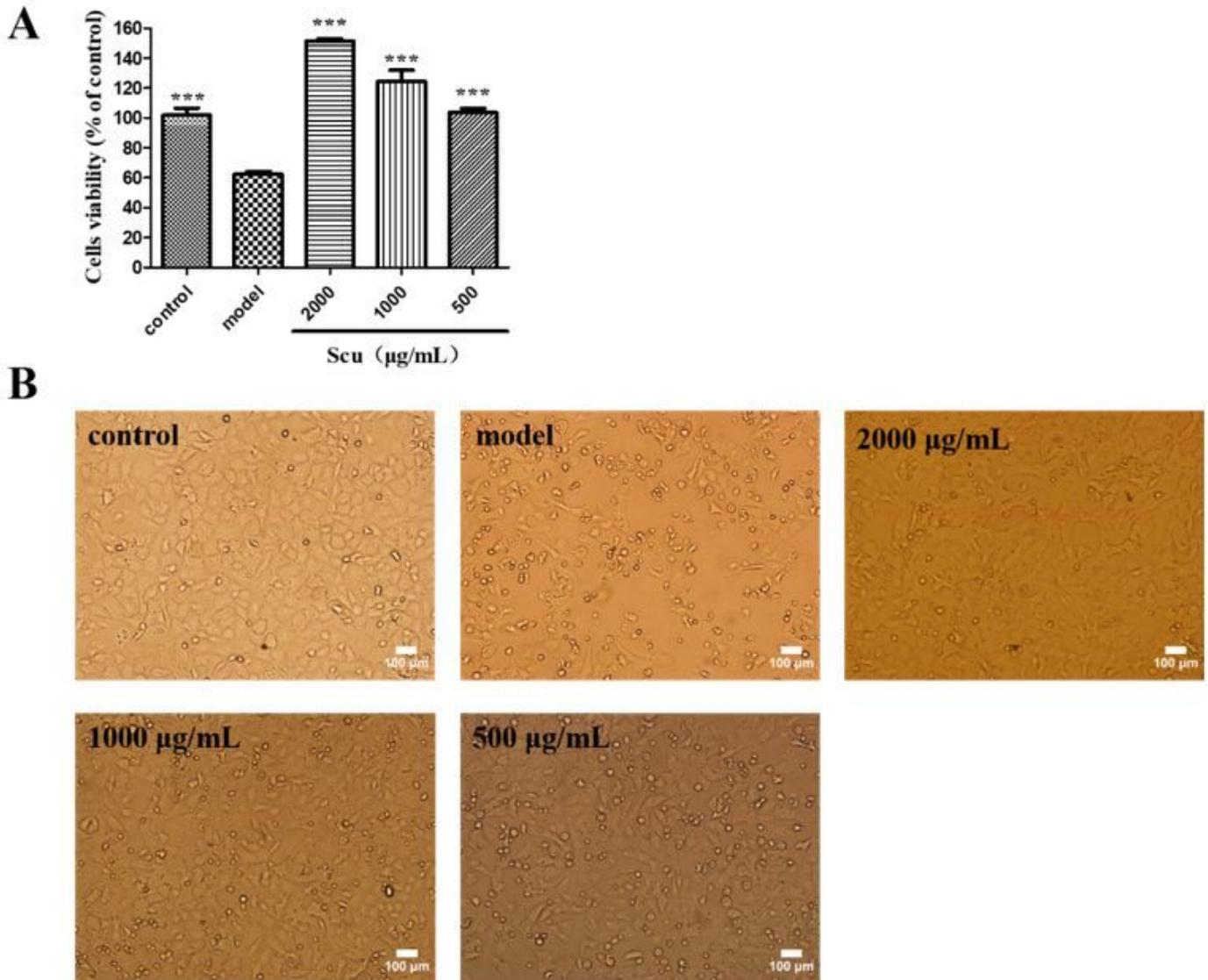
**Figure 3**

Effect of Scu on the cell viability of GCs. A. After treated with different concentrations of Scu for 24 and 48 h, cells viability was measured via MTT assay. B. GCs were treated with 2000, 1000 and 500 µg/mL Scu for 24 h, the cell cycle distribution was detected by flow cytometry. C. The analysis of cells numbers on different phases. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control.



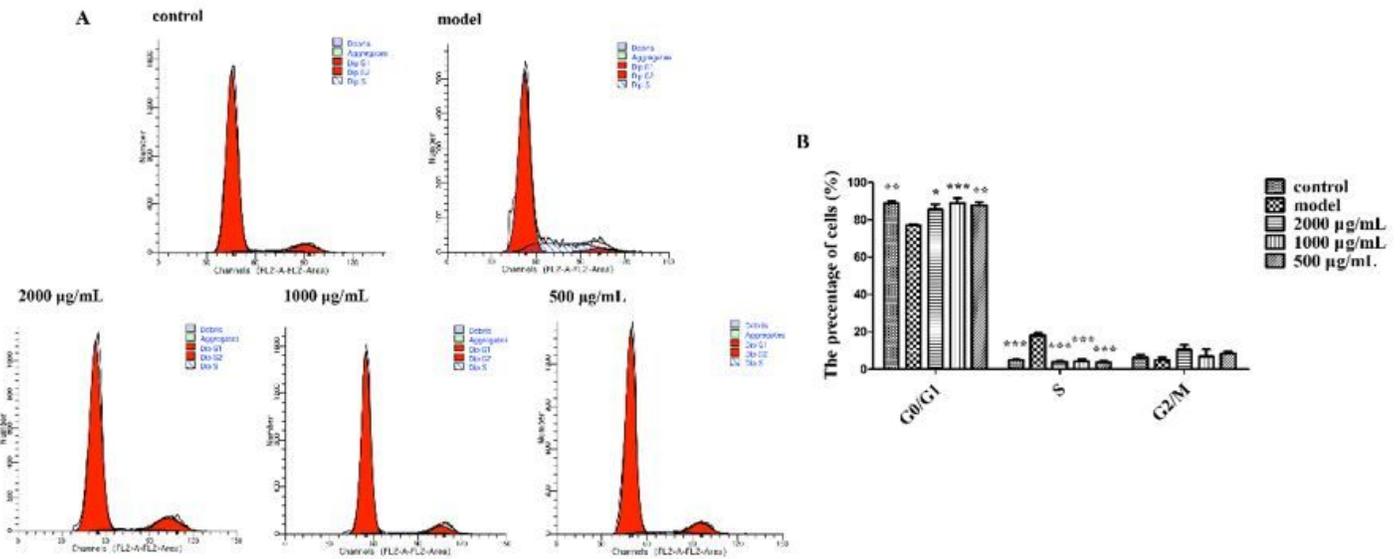
**Figure 4**

The effect of ZEA on ovarian GCs viability. A. GCs were treated with various concentrations of ZEA (0-120  $\mu\text{M}$ ) for 24 h. B. GCs were treated with 60  $\mu\text{M}$  ZEA for different time periods and the cell viability was analyzed using MTT assay. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. control.



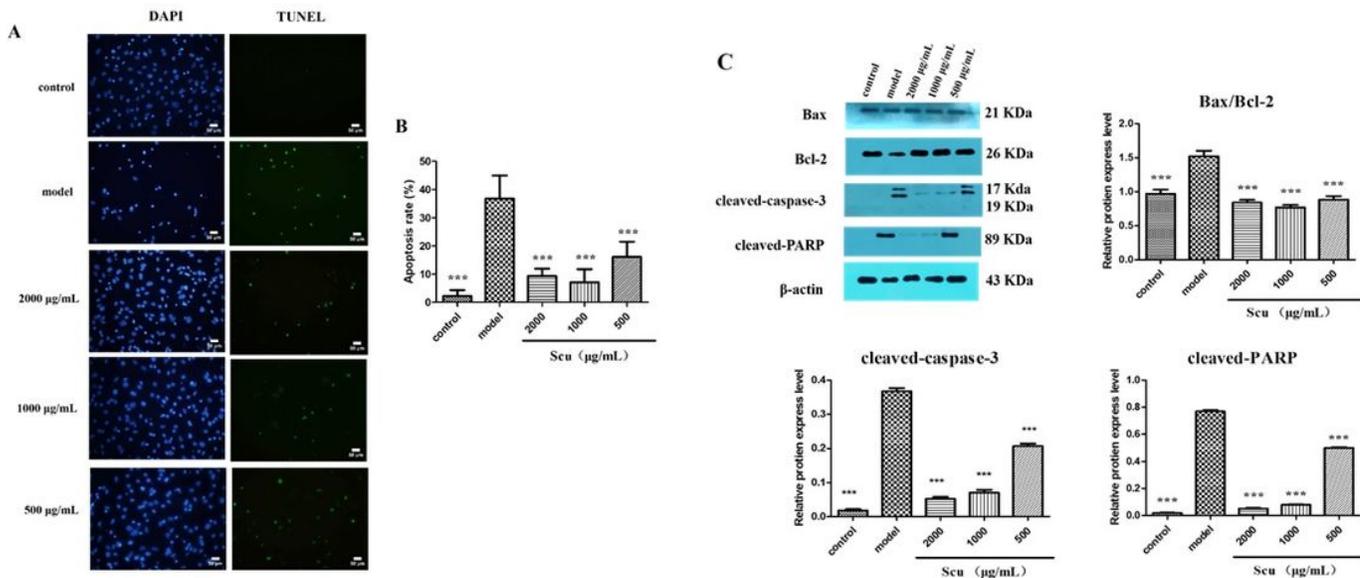
**Figure 5**

Protective effect of Scu on ZEA-induced GCs injury. A. The cells were co-treated with Scu and ZEA (60  $\mu\text{M}$ ) for 24 h, then MTT assay was used to detect cell viability. \*\*\* $p < 0.001$  vs. model. B. Cell morphology was observed under different conditions. Scale bar: 100  $\mu\text{m}$ .



**Figure 6**

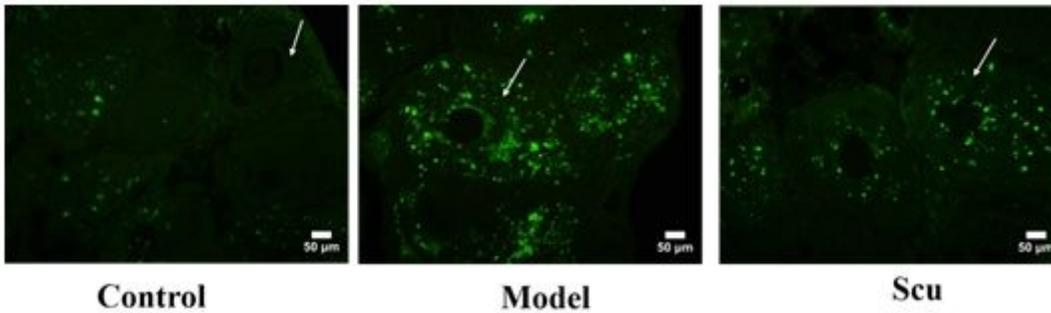
Effect of Scu on ZEA-induced cell cycle distribution. A. Cells were respectively co-treated with different dose (2000,1000, and 500 µg/mL) of Scu and ZEA (60 µM) for 24 h, and then cell cycle distribution was detected using flow cytometry. B. The analysis of cell numbers on different phases. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. model.



**Figure 7**

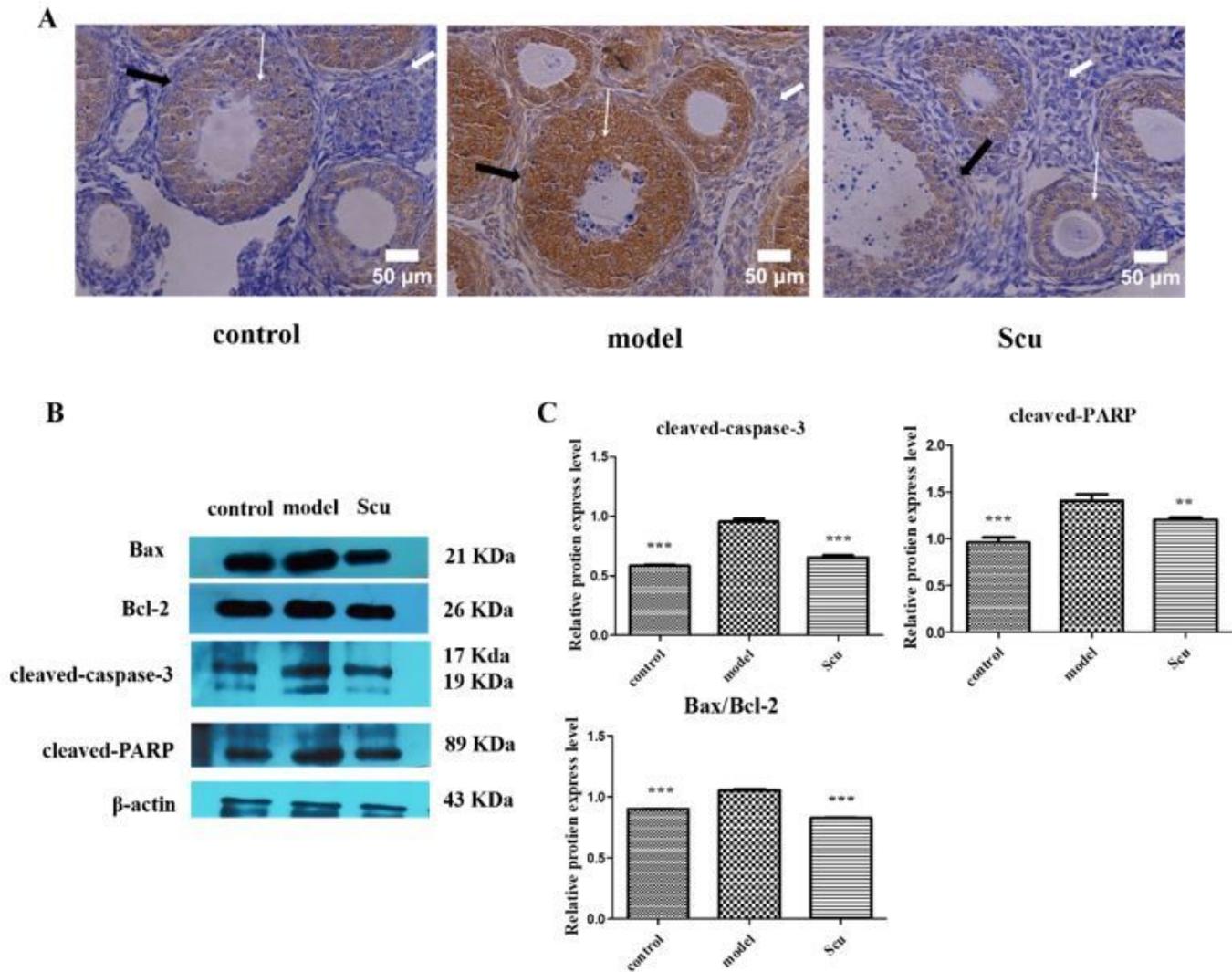
Protective effect of Scu on ZEA-induced GCs apoptosis. A. Cells were respectively co-treated with different dose of Scu (2000,1000, and 500 µg/mL) and ZEA (60 µM) for 24 h, and then key proteins in the apoptosis were detected using TUNEL staining. B. Quantification of apoptosis rate from TUNEL assay. C. The protein expression levels of Bax, Bcl-2, cleaved-caspase-3, cleaved-PARP, and β-actin were detected by

western blot analysis. D. The relative bands of the representative protein were analyzed and compared to that of  $\beta$ -actin. \*\*\* $p < 0.001$  vs. model.



**Figure 8**

Effect of Scu on ZEA-induced apoptosis of mouse ovarian tissues. The model group and scutellarin group were both treated with a single intragastric administration of ZEA dissolved in corn oil at 40 mg/kg. After 2 h, Scu group was given PBS (pH=7.4) containing 100 mg/kg/d Scu for 3 days. The control group was administered corn oil and PBS. All ovaries were collected post 72 h of ZEA administration. “white arrows” indicate the follicle granulosa layers.



**Figure 9**

Effect of Scu on ZEA-induced mitochondrial related proteins in ovarian tissues. A. The location of cleaved-caspase-3 was observed by immunohistochemistry. Brown indicates the apoptotic cells; “thin white arrow” indicates the follicle granulosa layer, “black arrow” indicates theca layer, “thick white arrow” indicates the ovary stroma. Scale bar: 50 μm; B. The expression levels of mitochondria related proteins were analyzed using western blot, and the bands of the representative protein were quantified and normalized with β-actin. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs. model.