Isorhamnetin protects zearalenone-induced damage via the PI3K/Akt signaling pathway in porcine ovarian granulosa cells

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Abstract

Zearalenone (ZEA) is widely derived from moldy cereal grain, which has adverse effects on animal reproduction. In particular, pigs are more sensitive to ZEA-induced toxicity than other animals. Isorhamnetin has extensive pharmacological activity. However, the role of isorhamnetin in ZEA-induced cytotoxicity remains unclear. This study was designed to investigate the therapeutic effect of isorhamnetin on ZEA-induced damage in porcine ovarian granulosa cells and elucidate its molecular mechanism. Two experiments were conducted, where a minimum of 3 biological replicates were used for each treatment. In Exp. 1, ovarian granulosa cells were treated with different concentrations of isorhamnetin (1, 5, 10, 20 and 30 μmol/L) and ZEA (0, 10, 30, 60, 90 and 120 μmol/L) for 24 h. Our results indicated that 60 μmol/L ZEA (half-maximal inhibitory concentration value) and 20 μmol/L isorhamnetin (the most effective concentration against ZEA-induced cytotoxicity) were optimum concentrations. In Exp. 2, ovarian granulosa cells were treated with isorhamnetin (20 μmol/L) for 2 h, before treatment with ZEA (60 μmol/L) for 24 h. Apoptosis, endoplasmic reticulum stress, oxidative stress, proliferation and hormone secretion of ovarian granulosa cells were detected. Our findings showed that isorhamnetin suppressed (P < 0.05) ZEA-induced apoptosis by altering mitochondrial membrane potential and apoptosis-related proteins (B-cell lymphoma-2 [Bcl-2], Bcl2-associated x [Bax] and cleaved caspase-3 [C-Casp3]). Changes in intracellular Ca2+ levels and C/EBP homologous protein (CHOP), recombinant activating transcription factor 6 (ATF6), glucose regulated protein78 kD (GRP78) indicated that isorhamnetin rescued (P < 0.05) ZEA-induced endoplasmic reticulum stress. Furthermore, isorhamnetin prevented (P < 0.05) ZEA-induced oxidative stress via the mitogen-activated protein kinase (P38) signaling pathway. Isorhamnetin also recovered (P < 0.05) ZEA-induced steroidogenesis disorder by regulating steroidogenic enzyme gene and proteins (follicle-stimulating hormone receptor [FSHR] and cytochrome P450 family 19 subfamily a member 1 [CYP19A1]). Collectively, these findings show that isorhamnetin protects ovarian granulosa cells from ZEA-induced damage, which promotes proliferation, alleviates apoptosis, endoplasmic reticulum stress, oxidative stress, and steroidogenesis disorder.

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1. Introduction

Zearalenone (ZEA), a non-steroid mycotoxin, is derived from Fusarium strains (Stanciu et al., 2018). ZEA can contaminate livestock feed and human food worldwide, such as cereals, corn, wheat and their products (Huang et al., 2019; Schatzmayr and Streit, 2018).
ZEA is also an airborne mycotoxin (Huang et al., 2019). In general, ZEA is heat stable and insoluble in water, which may cause ZEA-infected crops and soils to contaminate the aquatic environment through drainage and rainwater (Ryu et al., 2003; Waskiewicz et al., 2012). In addition, ZEA can induce to irreversible toxic damage to the development of embryos and the reproductive system (Zhang et al., 2018). Specifically, pigs are more sensitive to ZEA-induced damage than other animals (Liu et al., 2018). ZEA may accumulate in the meat products along the food chain, which may cause serious consequences and might be harmful to humans (Yang et al., 2021). Accumulating evidence shows that exposure to ZEA could damage the reproductive ability and interfere with the development of oocytes and sperm in animals and humans (Adibnia et al., 2016; Takagi et al., 2008; Yousef et al., 2017). Thus, it is of great significance to explore effective methods to alleviate the toxicity of ZEA.

In mammalian ovaries, ovarian granulosa cells (GC) play a vital role in follicular growth, oocyte maturation, ovulation and steroidogenesis (Guler and Zik, 2018). Ovarian GC are indispensable for folliculogenesis. A previous report demonstrated that ovarian GC affected follicular development by releasing follicular growth and maturation factors (Lai et al., 2017). Ovarian GC also provides nutrients for oocytes and allows full developmental ability (Jiang et al., 2010; Sugiiura et al., 2005). Over 99% of follicles will undergo atresia during follicular development. Most follicular atresia is caused by ovarian GC apoptosis (Inoue et al., 2011). ZEA increases undergo atresia during follicular development. Most follicular atresia is of great significance to explore effective methods to alleviate the toxicity of ZEA.

Isorhamnetin (a natural flavonoid) is commonly distributed in vegetables and fruits (Gong et al., 2020). Studies have indicated that isorhamnetin possesses a wide range of pharmacological effects, including anti-oxidant, anti-inflammatory, anti-tumor and cardiovascular protection (Gong et al., 2020). Furthermore, our previous results suggested that isorhamnetin promoted estrogen secretion and proliferation in porcine ovarian GC (Li et al., 2021). However, it is unclear whether isorhamnetin can improve the reproductive system of animals, specifically in ZEA-induced ovarian GC damage. Thus, it is necessary to investigate the effects of isorhamnetin on ZEA-induced damage in ovarian GC.

In this study, we hypothesized that isorhamnetin could alleviate the toxic effects of ZEA on ovarian GC. In addition, we attempted to identify the signaling pathways and potential mechanisms responsible for isorhamnetin inhibition of ZEA damage. Our findings suggest that isorhamnetin can be used as a natural flavonoid additive to resist the ZEA-induced toxicity in porcine ovarian GC. Moreover, these findings may also benefit human reproduction and health.

2. Materials and methods

2.1. Animal ethics

All animal care and procedures were performed in accordance with institutional and national guidelines and approved by the Utilization Committee of Northwest A&F University (Yangling, China).

2.2. Chemical reagents

Isorhamnetin (Cat#: N1358) was obtained from APEXBio. ZEA (Cat#: 17924-92-4) was obtained from SIGMA. Phosphatidylinositol 3 kinase (PI3K) antagonist LY294002 (Cat#: S737) and mitogen-activated protein kinase (P38) antagonist SB203580 (Cat#: S1863) were purchased from Beyotime. Sodium phenylbutyrate (4-PBA) (Cat#: 1716-12-7) is an endoplasmic reticulum inhibitor obtained from Santa Cruz Biotechnology. The antibodies used in this study were as follows: anti-microtubule-associated protein 1 light chain 3 (anti-LC3) (Cat#: 192890) was purchased from ABCAM; anti-β-actin (Cat#: 66009-1-1g) and the seques-}

2.3. Culture and treatment of ovarian GC

The mature female pigs (Duroc, Landrace and Yorkshire cross-breeds) used in the experiment were 7 months old and weighed 120 ± 1.89 kg. Porcine ovaries were obtained from a local abattoir. Porcine ovaries were transported to the lab within 1 h in 37 °C phosphate buffered solution (PBS) containing penicillin (100 IU/mL) and streptomycin (100 mg/mL). Ovarian GC were separated from follicles using a syringe. Then, the ovarian GC were washed twice with cell culture medium. After washing, ovarian GC were cultured in 24-well plates (10^6 in 1 mL). The DMEM/F12 medium was supplemented with 1% streptomyacin–penicillin and 8% fetal bovine serum (SERA-PRO, Germany). Ovarian GC were cultured at 37 °C in 5% CO2 for 36 to 44 h. Then, ovarian GC were replaced with isorhamnetin and ZEA, and incubated for 24 h in DMEM/F12 medium containing transferrin (2.5 mg/mL), 0.5% bovine serum albumin (wt/vol), sodium bicarbonate (10 mmol/L), insulin (50 ng/mL), sodium selenite (5 mg/mL), follicle-stimulating hormone (FSH) (0.1 IU/mL), penicillin (100 U/mL), streptomycin (100 mg/mL) and nonessential amino acid mix (1×) (Chen et al., 2019). Following this, ovarian GC were collected for protein and mRNA extraction. The media was harvested for an ELISA assay.

2.4. 5-ethyl-2'-deoxyuridine (EdU) and cell counting kit (CCK-8) evaluates

The proliferation of ovarian GC was detected by EdU-488 (Beyotime, China). Cells were cultured with Edu 1:1,000 for 5 h incubation. Follow the reagent instructions for the rest of the steps. After Edu incubation, the fluorescence microscopy was used to take images.
CCK-8 (Beyotime, China) was used to test the viability of ovarian GC. The 96-well plate was seeded with ovarian GC. After, ovarian GC were mixed with CCK-8 (10 μL) at 37 °C for 2 h. Then, the optical density was determined using a microplate reader.

2.5. Real-time quantitative PCR (RT-qPCR)

The RNA of ovarian GC was extracted using Trizol reagent (TAKARA, Japan) based on the manufacturer’s descriptions. The cDNA was synthesized on ice by cDNA synthesis kit (TAKARA, Japan). RT-qPCR was carried out with master mixes (Vazyme, China) in a 20 μL reaction system. All steps are based on manufacturer’s protocol. β-actin was used as the housekeeping gene. Then, the relative normalized expression was calculated by the 2^−∆∆CT. The primer sequence is displayed in Table 1.

2.6. Western blot analysis

The total protein from ovarian GC was extracted with RIPA lysis buffer (Beyotime, China). BCA kit (Beyotime, China) was used to measure protein concentration. The protein (15 to 20 μg) was separated by 10% to 15% SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane (Millipore, Billerica, MA, USA). Then, the membrane was sealed with QuickBlock Western (Beyotime). The membrane was incubated with the primary antibodies overnight at 4 °C. Cleaning with TBST, the membrane was sealed with secondary antibodies at room temperature for 1 h. The protein band was detected by X-ray and band intensity was quantified using ImageJ software.

2.7. Flow cytometry assay

For cell cycle distribution, cells were fixed at 4 °C. Then, cells were mixed with propidium iodide (PI) for 30 min at 37 °C. Subsequently, ovarian GC were tested using flow cytometry. The results were analyzed by ModFit software.

2.8. Measurement of mitochondrial membrane potential

Ovarian GC were treated with Mito-Tracker Red CMXRos (200 nmol/L, Beyotime) at 37 °C for 25 min. After washing 2 times with PBS. Fluorescence microscopy was used to take images.

2.9. Detection of reactive oxygen species (ROS) and steroid hormones production

Cells were cultured with fluorescent dye 20, 70-dichlorodihydrofluorodihydrol-60-fluorescein diacetate DCFH-DA (Beyotime, China) at 37 °C for 20 min. Then, ovarian GC were washed 3 times with PBS. ImageJ was used to analyze fluorescence intensity.

Superoxide dismutase assay kit, MDA assay kit and glutathione peroxidase (GSH-PX) assay kit were bought from Nanjing Jiancheng Bioengineering Institute. The GSH-PX, SOD and MDA were performed according to assay kit descriptions. Briefly, ovarian GC lysates were mixed with detection reagent. Data were analyzed via a microplate reader.

2.10. Measurement of Ca^{2+} level

Fura-2 AM (Beyotime) was used to detect intracellular Ca^{2+} content. Ovarian GC were mixed with Fura-2 AM (5 μmol/L) for 30 min at 37 °C. Data were analyzed via a microplate reader.

2.11. Measurement of malondialdehyde (MDA) production and antioxidant enzyme activity

To investigate whether ZEA exposure affects ovarian GC growth and apoptosis. Ovarian GC were treated with ZEA (0, 10, 30, 60, 90 and 120 μmol/L) for 24 h. It was found that ZEA significantly decreased ovarian GC viability in a dose-dependent manner and the half maximal inhibitory concentration value of ZEA was 60 μmol/L (P < 0.05; Fig. 1B). Pretreatment withisorhamnetin (Fig. 1A) at dosage of 1, 5, 10, 20 and 30 μmol/L increased the viability of ovarian GC exposed to 60 μmol/L ZEA for 24 h (Fig. 1C). Particularly, the 20 μmol/L isorhamnetin was the most effective in resisting ZEA-induced cytotoxicity (P < 0.05; Fig. 1C). Hence, the dosage of 60 μmol/L ZEA and 20 μmol/L isorhamnetin were used in this study. Subsequently, Western blot analysis indicated that the protein expression of Bax and C-Casp3 were significantly increased in ZEA-treated group (P < 0.05), but the Bcl-2 was significantly.

Table 1

| Genes   | Primer sequences (5’−3’)            | NCBI accession number | Product size, bp |
|---------|-------------------------------------|-----------------------|------------------|
| Beta-actin | Forward: ATCAAGATCATCGCGCCCTCC     | XM-003124280.5        | 169              |
|          | Reverse: AATGCACTAACATCGGCGCCCT   |                       |                  |
| CYP19A1  | Forward: TCGCAATGACTGGGCTAC        | NM-21449.1            | 103              |
|          | Reverse: GCTTCTTCGGCTCATGCCC      |                       |                  |
| ER1      | Forward: GCTACATCATCTGCCTCCCCT    | NM-21220.1            | 115              |
|          | Reverse: ACTTCAGGGTGCTGGACAGA     |                       |                  |
| ER2      | Forward: TCCTTTAGGCTCATGCCC       | XM-021081392.1        | 214              |
|          | Reverse: TCTGACAGCATACTGCCC       |                       |                  |
| FSHR     | Forward: TTCAAGATCACGCCCTTTCCC    | XM-021085884.1        | 152              |
|          | Reverse: CACGCACAGATGACCAAAAA     |                       |                  |

CYP19A1 – cytochrome P450 family 19 subfamily a member 1; ER1 – estrogen receptor 1; ER2 – estrogen receptor 2; FSHR – follicle-stimulating hormone receptor.
decreased compared with the control group \((P < 0.01; \text{Fig. } 1\text{D, E, F, and G})\). As expected, isorhamnetin pretreatment could inhibit the changes of pro-apoptotic proteins \((\text{Fig. } 1\text{D, E, F, and G})\). Next, the mitochondrial quality was detected. The Mito-Tracker Red CMXRos results showed that ZEA-treated group significantly decreased the fluorescence signal and membrane potential of mitochondrial compared with the control group \((\text{Fig. } 1\text{H}; P < 0.05)\). These data suggest that ZEA-treated group leads to apoptosis of ovarian GC. Conversely, ZEA-induced mitochondrial impairment was partially recovered by isorhamnetin pretreatment \((\text{Fig. } 1\text{H}; P < 0.05)\). Similarly, the Western blot analysis revealed a lower protein expression of PGC-1 (regulatory proteins of mitochondrial biogenesis) in ZEA-treated ovarian GC \((P < 0.05; \text{Fig. } 1\text{I, and J})\). This effect was restored by isorhamnetin treatment \((\text{Fig. } 1\text{I, and J})\). To determine whether ZEA is involved in autophagy, autophagy-associated \((\text{LC3 and P62})\) proteins were examined. We observed that ZEA treatment significantly increased the protein expression of LC3 and P62 in ovarian GC, whereas isorhamnetin therapy effectively relieved this ZEA-induced upregulation \((P < 0.05; \text{Fig. } 1\text{K, L and M})\). Overall, these findings suggest that isorhamnetin can inhibit ovarian GC apoptosis induced by ZEA.

### 3.2. Isorhamnetin relieved ZEA-induced endoplasmic reticulum stress (ERS)

Given that ERS could induce apoptosis, we next studied the changes of ERS-related apoptotic markers in ovarian GC. We found that the protein expression of Casp12 and CHOP were significantly increased in ZEA-treated ovarian GC \((P < 0.05)\), which was abolished by isorhamnetin treatment \((\text{Fig. } 2\text{A, B, and C})\). Additionally, ZEA treatment alone clearly increased the protein expression of ATF6, IRE1, XBP1 and GRP78 \((P < 0.05)\), suggesting that ERS happened in ZEA-exposed ovarian GC \((\text{Fig. } 2\text{F, G, H, I, and J})\). But, 4-PBA (an endoplasmic reticulum inhibitor) effectively reduced the protein expression of ATF6, IRE1, XBP1 and GRP78 in ZEA-treated ovarian GC \((\text{Fig. } 2\text{F and G, H, I, and J})\). Consistent with the effect of 4-PBA, the changes in ERS proteins could be restored by isorhamnetin treatment \((\text{Fig. } 2\text{F and G, H, I, and J})\). Considering that ERS can lead to intracellular calcium imbalance, calcium-associated proteins in ovarian GC were detected. We found that the protein expression of calcineurin A and CaMKII \(\alpha\) were significantly increased in ZEA-treated ovarian GC \((P < 0.05)\), which was then recovered by isorhamnetin treatment \((\text{Fig. } 2\text{A, D, and E})\).
Interestingly, pretreatment with isorhamnetin or 4-PBA clearly inhibited the level of Ca\(^{2+}\) in ovarian GC exposed to ZEA (\(P < 0.01\); Fig. 2K). The data show that isorhamnetin can block ZEA-induced ERS and maintain ER homeostasis.

3.3. Isorhamnetin inhibited oxidative stress caused by ZEA

High levels of ROS also lead to ovarian GC apoptosis. We next measured the P-P38 signal in ZEA-treated ovarian GC, which was believed to be engaged in antioxidative stress. Western blot analysis indicated that an effective activation of P-P38 occurred in the ZEA-treated ovarian GC (\(P = 0.03\); Fig. 3A, and E). Conversely, the protein expression of SOD2, GPX1 and SOD1 were significantly decreased in ZEA-treated ovarian GC, which was partially recovered by isorhamnetin treatment (\(P < 0.05\); Fig. 3A, B, C, and D). In line with this, the content of MDA was significantly increased, whereas the activity of SOD and GSH-PX were significantly decreased in ZEA-treated ovarian GC (\(P < 0.05\); Fig. 3F, G, and H). However, ZEA-induced oxidative stress was relieved by isorhamnetin therapy (\(P < 0.05\); Fig. 3A, B, C, D, E, F, G, and H). Moreover, ZEA significantly increased the level of ROS in ovarian GC, and isorhamnetin inhibited ROS accumulation via its antioxidant activity (\(P = 0.003\); Fig. 3I, and J). Similarly, the ROS generation induced by ZEA treatment was partially alleviated when the ovarian GC were treated with SB203580 (Fig. 3I, and J). These results reveal that isorhamnetin can inhibit ROS in ovarian GC induced by ZEA via down-regulating P-P38/P38.

3.4. Isorhamnetin recovered ovarian GC proliferation caused by ZEA in a PI3K/Akt dependent manner

We further investigated how isorhamnetin alleviated the loss of ovarian GC caused by ZEA. Cell proliferation marker related proteins were detected. Western blot results showed that ZEA treatment significantly decreased the protein expression of PCNA, C-myc, PI3K and P-Akt/Akt in ovarian GC (\(P < 0.05\); Fig. 4A and B, C, D, and E). This action was recovered by isorhamnetin therapy (Fig. 4A, B, C, D, and E). Consistently, the proliferation of ovarian GC treated with ZEA was highly significant inhibited by Edu staining, which was alleviated by isorhamnetin (\(P = 0.007\); Fig. 4F, and G); this beneficial action was abolished by LY294002 (PI3K antagonist).
Similarly, we found that a marked decrease in the protein expression of cyclin D and cyclin A in ZEA-treated ovarian GC ($P < 0.05$; Fig. 4H, I, and J). However, co-treatment with isorhamnetin significantly increased the expression of cycle-related proteins in ovarian GC (Fig. 4H, I, and J). Additionally, flow cytometry data showed that ZEA treatment highly significantly improved the ratio of cells in the G0/G1 phase ($P = 0.007$; Fig. 4K, and L), whereas the ratio of cells in the S phases were significantly decreased ($P = 0.003$; Fig. 4K, and M). As expected, the decrease of S phase cells was recovered when ovarian GC were treated with isorhamnetin. Interestingly, isorhamnetin-induced cell division was abolished by LY294002 (Fig. 4K, L, M, and N). These data manifest that isorhamnetin depends on the PI3K/Akt signaling pathway, which recovers ovarian GC proliferation in response to ZEA-stimulated apoptosis.

### 3.5. Isorhamnetin prevented ZEA-induced steroid secretion disorder in a PI3K/Akt-dependent manner

To explore the effect of isorhamnetin on ovarian GC steroidogenesis in response to ZEA treatment, we next measured the indicators related to hormone secretion. Western blot analysis showed ZEA treatment significantly decreased protein expression of FSHR and CYP19A1 in ovarian GC ($P < 0.05$; Fig. 5A and B, and C). This action was restored by isorhamnetin pretreatment (Fig. 5A and B, and C). Consequently, ZEA-treated ovarian GC significantly inhibited the mRNA expression of CYP19A1, FSHR, estrogen receptor 2 (ER2) and ER1, which was recovered by isorhamnetin administration ($P < 0.05$; Fig. 5E and F, G, and H). However, isorhamnetin significantly increased the secretion of estrogen in ovarian GC, which was abolished by LY294002 ($P = 0.007$; Fig. 5D). The secretion of estrogen in ovarian GC was consistent with the expression trend of estrogen-synthesized mRNA (Fig. 5D and E, F, G, and H). Based on these results, we can speculate that isorhamnetin alleviates ZEA-induced steroid secretion disorder through the PI3K/Akt.

### 4. Discussion

ZEA is widely found in moldy cereal grain, which is harmful to the growth of gametogenesis and embryo in animals and humans (Yang et al., 2018). ZEA can cause poisoning to animals and damage the reproductive system of animals (Zhao et al., 2013). In particular, pigs are more sensitive to ZEA-induced toxicity than other animals (Liu et al., 2018). Thus, it is of great significance to explore effective methods to alleviate the damage of ZEA to porcine ovarian GC. Isorhamnetin, a flavonoid compound, has a wide range of
pharmacological effects, including anti-oxidation, anti-inflammation, anti-bacterial, anti-virus and anti-tumor (Teng and Luan, 2016). However, it’s still uncertain whether isorhamnetin attenuates ZEA-induced damage in ovarian GC. Here, we provided direct evidence that isorhamnetin inhibited the damage of ovarian GC caused by ZEA.

To elucidate the beneficial mechanism of isorhamnetin on ZEA-induced apoptosis, ovarian GC were utilized. The Bcl-2 family regulates the cytochrome c release and thereby starts the caspase cascade, a well-known apoptotic pathway (Woo et al., 2018). Published data have suggested that 60 μmol/L ZEA could significantly reduce the cells viability of mouse ovarian GC and induce apoptosis (Yi et al., 2020). Consistently, we found that ZEA (60 μmol/L) activated the protein expression of Bax and C-Casp3 in ovarian GC. However, isorhamnetin could rescue the mitochondrial apoptosis pathway induced by ZEA. Strong evidence indicated that isorhamnetin could alleviate the apoptosis of H2O2-treated PC12 cells by inhibiting caspase-3 protein expression (Hwang et al., 2009). Likewise, isorhamnetin has a protective effect on endothelial cell apoptosis caused by oxidized low-density lipoprotein (Bao et al., 2010). Mitochondrial dysfunction has been considered as a major factor inducing cell death (Marra and Svegliati-Baroni, 2018). A published study has indicated that ZEA could damage mitochondrial functions (Adibnia et al., 2016), Zhu et al. (2012) reported that ZEA caused the loss of mitochondrial transmembrane potential in porcine ovarian GC. Consistently, we found that ZEA-treated group significantly decreased mitochondrial membrane potential and PGC-1 protein expression. However, isorhamnetin could recover the expression of PGC-1 to alleviate the mitochondrial dysfunction caused by ZEA. Similar to our studies, Bao et al. (2010) demonstrated that isorhamnetin could significantly inhibit mitochondrial membrane potential alteration in endothelial cells induced by oxidized low-density lipoprotein. Furthermore, isorhamnetin prevented obesity-associated mitochondrial dysfunction in 3T3-L1 cells by promoting the expression of PGC-1 mitochondrial biosynthesis gene (Mak-Soon and Yangha, 2018). It is considered that autophagy and apoptosis can regulate each other. We further studied that isorhamnetin alleviated the autophagy of ovarian GC induced by ZEA. Indeed, isorhamnetin can directly inhibit autophagy and protect acute fulminant hepatitis caused by concanavalin A in mice (Lu et al., 2018). It is conceivable that isorhamnetin rescues the ZEA-induced apoptosis by regulating apoptosis-related proteins, mitochondrial function and autophagy of ovarian GC.

When the ER function is badly impaired, the expression of ERS related markers and the organelles changes, which leads to apoptosis (Li et al., 2014). Our results showed that isorhamnetin decreased the protein expression of Casp12 and CHOP, which was associated with ERS. Subsequently, the changes of ER membrane sensors-related proteins ATP6, IRE1, XBP1 and GRP78 showed that isorhamnetin relieved ZEA-induced ERS. Consistently, previous study confirmed that isorhamnetin could inhibit ERS-induced apoptosis in N2a cells (Qiu et al., 2017). ERS can stimulate...
intracellular calcium overload (Qiu et al., 2017). Conversely, the published data have shown that isorhamnetin inhibited Ca\(^{2+}\) overload in N2a cells (Qiu et al., 2017). In addition, pretreatment with isorhamnetin could directly decrease the level of Ca\(^{2+}\) in H\(_{2}\)O\(_{2}\)-treated PC12 cells (Hwang et al., 2009). Consistent with previous results, we found that isorhamnetin alleviated the increase of Ca\(^{2+}\) in ZEA-induced ovarian GC by reducing the protein expression of calcineurin A and CaMKII\(\alpha\). Our data show that isorhamnetin may alleviate ZEA-induced ovarian GC toxicity by inhibiting ERS.

Data from the present study indicated that oxidative stress and ERS, as closely related events, played a vital role in cellular apoptosis (Xu et al., 2020). A previous study showed that ZEA-induced ROS-mediated cell cycle arrest and apoptosis in mouse sertoli cells (Tatay et al., 2017). Likewise, Pan et al. (2020) reported that ZEA impaired the placental function of rats by stimulating MDA level and inhibiting the expression of Sod2, Sod1, Cat and Gpx1 proteins. Consistently, we found that isorhamnetin alleviated the increase of Ca\(^{2+}\) in ZEA-induced ovarian GC by reducing the protein expression of calcineurin A and CaMKII\(\alpha\). Our data show that isorhamnetin may alleviate ZEA-induced ovarian GC toxicity by inhibiting ERS.

Data from the present study indicated that oxidative stress and ERS, as closely related events, played a vital role in cellular apoptosis (Xu et al., 2020). A previous study showed that ZEA-induced ROS-mediated cell cycle arrest and apoptosis in mouse sertoli cells (Tatay et al., 2017). Likewise, Pan et al. (2020) reported that ZEA impaired the placental function of rats by stimulating MDA level and inhibiting the expression of Sod2, Sod1, Cat and Gpx1 proteins. Consistently, we found that ZEA stimulated MDA level and decreased the protein expression of SOD2, GPX1 and SOD1 by activating the P-P38 pathway. Fortunately, isorhamnetin played a protective role in response to ZEA-induced oxidative stress through P-P38 inactivation. Indeed, previous research shown that isorhamnetin inhibited the content of MDA and increased the activity of SOD and GSH-Px in APAP-induced injured L02 cells (Jiang et al., 2018). Additionally, published data indicated that isorhamnetin suppressed P-P38 to against oxidative stress in PC12 cells (Hwang et al., 2009). Furthermore, isorhamnetin could inhibit ConA-induced autophagy and apoptosis via the P38/ peroxisome proliferators-activated receptor \(\alpha\) (PPAR-\(\alpha\)) pathway in mice (Lu et al., 2018). These results suggest that isorhamnetin can against ZEA-induced oxidative stress through the P-P38 signaling pathways.

Cell cycle progression is a vital physiological process in cell life activities, and interference to cell cycle often leads to aging, degeneration or apoptosis (Pack et al., 2019). The changes of cell cycle related proteins are crucial in regulating cell cycle progression (Lim and Kaldis, 2013). Strong evidence indicated that ZEA regulated the expression of cyclin B and cyclin D proteins, leading disruption of the cell cycle and reducing cell viability of sow ovarian GC (Zhang et al., 2018). A recent study found that the cell cycle of ZEA-treated porcine ovarian GC was arrested in the G2/M phase (Li et al., 2020). Interestingly, we found that the S phase of DNA synthesis was decreased in ZEA-treated ovarian GC. Furthermore, ZEA inhibited the protein expression of cyclin A and cyclin D in ovarian GC, which was consistent with the results of flow cytometry analysis. On the contrary, isorhamnetin stimulated the protein expression of cyclin A and cyclin D.
expression of cyclin D and PCNA, and the proportion of EdU positive cells, which recovered ovarian GC proliferation in response to ZEA-induced damage. Subsequently, isorhamnetin increased the S phase of DNA synthesis in ovarian GC, which was suppressed by the PI3K/Akt inhibitor. Consistent with our previous studies, isorhamnetin can increase the proliferation of porcine ovarian GC via the PI3K/Akt dependent manner (Li et al., 2021). Similarly, Jiang et al. (2012) found that isorhamnetin blocked cells from entering S phase of DNA synthesis, and finally inhibited the proliferation of HepG-2 cells. These findings demonstrate that isorhamnetin restores ovarian GC proliferation against ZEA-induced damage via the PI3K/Akt signaling pathway.

Healthy follicles have higher concentrations of estrogen (Chen et al., 2019; He et al., 2016). Thus, the steroidogenesis of ovarian GC is essential for atresia and follicular development. Studies have shown that estrogen synthesis was controlled by steroidogenic enzymes, including CYP19A1 and hormone receptor (Xu et al., 2018; Wang et al., 2020). Growing evidence has revealed that ZEA altered the production of steroid hormones in porcine ovarian GC (Zhu et al., 2012; Zhang et al., 2017). In vitro results suggested that ZEA (20 mg/kg) reduced the concentration of estrogen in serum of pregnant rats (Gao et al., 2017). Likewise, diets with ZEA (2 mg/kg) reduced serum estrogen level in female pigs (Wang et al., 2010). Consistently, our data demonstrated that ZEA suppressed estrogen secretion of ovarian GC. Strikingly, isorhamnetin can regulate the expression of steroid-related genes and proteins to alleviate the hormone balance disturbed by ZEA-treated ovarian GC. Subsequent experiments confirmed that the protective actions of isorhamnetin on ZEA-induced hormonal disorders was abolished by PI3K inactivation. Indeed, isorhamnetin increased estrogen secretion in porcine ovarian GC via the PI3K/Akt signaling pathway (Li et al., 2021). Additionally, isorhamnetin has a possible protective action inhibiting oxidative stress in human RPE cells by activating the PI3K/Akt signaling pathway (Wang et al., 2018). In brief, isorhamnetin recovers ZEA-induced steroid secretion disorder through PI3K/Akt activation.

5. Conclusion
In conclusion, we found that a novel role of isorhamnetin in alleviating the damage caused by ZEA in ovarian GC. We first demonstrated that isorhamnetin protected ovarian GC from ZEA-induced damage by reducing apoptosis, ERS and oxidative stress. Additionally, isorhamnetin recovered the proliferation and estrogen secretion of ovarian GC disturbed by ZEA in a PI3K/Akt dependent manner. These results suggest that isorhamnetin can effectively reduce the damage caused by ZEA in porcine ovarian GC, which shows great promise for applications of isorhamnetin in alleviating ZEA-associated reproductive toxicity; allowing humans and animals to be healthier, and protection for food and feed products.

Author contributions
Xiaoya Li: conceptualization; methodology; data curation; writing-Original draft preparation. Huali Chen, Zelin Zhang and Jiaxin Duan: sample collection; software. Rongmao Hua, Li Yang and Jianyong Cheng: visualization and investigation. Xiaodi Li: writing-review and editing. Qingsheng Li: supervision; writing-reviewing and editing; project administration; funding acquisition.

Declaration of competing interest
We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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