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Original Research Article

Zearalenone induces apoptosis and autophagy by regulating endoplasmic reticulum stress signalling in porcine trophectoderm cells

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ABSTRACT

Zearalenone (ZEA), a mycotoxin produced mainly by fungi belonging to *Fusarium* species in foods and feeds, causes a serious hazard to humans and animals. Numerous studies have revealed that ingesting ZEA can disrupt the reproductive function and impair the reproductive process in animals. This experiment was to investigate the toxicological effect and the mechanism of ZEA exposure on reproduction in pigs during early stages of pregnancy. In the present study, we treated with 0 to 80 μmol/L ZEA for 12 or 24 h in trophoblast ectoderm (pTr) cells. The results showed that ZEA had significantly decreased cell proliferation ($P < 0.05$), which was accompanied by DNA damage-related cell cycle arrest at G2/M phase, activation of the apoptosis and endoplasmic reticulum (ER) stress, as well as impairment of barrier function ($P < 0.05$). Western blot analysis and transmission electron microscopy (TEM) showed that exposure to ZEA can activation of autophagy in pTr cells. Importantly, pretreatment with chloroquine (CQ) or 3-methyladenine (3-MA) led to increased apoptosis in pTr cells. Interestingly, pTr cells pretreated with 4-phenylbutyric acid (4-PBA), an inhibitor of ER stress, resulted in reduced cell death in pTr cells, indicating a critical role for ER stress in the activation of autophagy. In conclusion, these results reveal that ZEA-triggered ER stress is critical for the cell fate decision of pTr cells during early porcine embryonic development. Application of small molecules with ability of blocking ER stress might be therapeutic option to reduce the deleterious effect of ZEA in pregnant animals.

1. Introduction

Zearalenone (ZEA), a common non-steroidal estrogenic mycotoxin mainly produced by several *Fusarium* species, is a kind of secondary metabolite. ZEA is frequently found in maize and other crops, such as wheat, barley, sorghum and rye (Alshannaq and Yu, 2017; Ropejko and Twaruzek, 2021). Due to its estrogenic effects and physiological toxicity, ZEA has been reported to cause severe reproductive disorders in animals and hyper estrogenic syndromes in humans (Rai et al., 2020; Zhang, 2018; Zhang et al., 2018b). Besides, multiple studies have shown that ZEA not only disrupts the estrus cycle of female animals, but also interferes with early pregnancy, including fertilization, embryo implantation and development (Kunishige et al., 2017; Yin et al., 2014; Zhao et al., 2014). Few reports on the toxicological effects and related mechanisms of ZEA in early stages of pregnancy of female animals are available despite substantial studies have shown that ZEA is harmful to reproductive function in animals.

In pigs, embryonic mortality in early stages of pregnancy (between d 12 and 18) can be as high as 20% to 40%, most of which occurs during the peri-implantation stage (Bazer et al., 2012; Kunishige et al., 2017). The peri-implantation stage is an important

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stage in early embryonic development, which is a critical period for trophoblastic elongation and placental attachment (Bazer and First, 1983). The process of pig embryo implantation is extremely complicated, a well-coordinated communication network between the conceptus and maternal uterus is critically valuable during the early stages of pregnancy (Jeong et al., 2016b). Trophectoderm, the first epithelium of blastocyst that contacts directly with the uterine fluid and implicated in the formation of the placenta, is responsible for transporting nutrients and therefore providing energy substrates for the inner cell mass (Luo et al., 2019). Accumulating evidence has suggested that the placenta is a target of ZEA. For instance, ZEA and its metabolites can pass through placentas and interfere with physiological placental estrogen signalling thereby disrupting the course of pregnancy (Bernhoft et al., 2001; Danicke et al., 2007; Huuskonen et al., 2015). Studies have shown that exposure of pregnant mice to ZEA between 5.5 and 13.5 d of gestation resulted in increased placental hemorrhage, impaired placental function, and decreased placental and fetal weight (Li et al., 2019). In addition, pigs are the most sensitive animal to ZEA (Zhang et al., 2018a). Therefore, it is of great significance to investigate the effects of ZEA on the development and toxicity of pig embryos in early stages of pregnancy.

In this study, we investigated the toxicity of ZEA and its mechanism through the effects of different concentrations of ZEA on cell proliferation, cell cycle, cell apoptosis, endoplasmic reticulum (ER) stress and autophagy related protein expression in trophoblast ectoderm (pTr) cells.

2. Materials and methods

2.1. Animal ethics, chemicals and reagents

The model in this study is a cell line. The study does not involve animal experiments, and no animals were used. Zearalenone (ZEA, #Z-2125), 3-methyladenine (3-MA, #189490), and chloroquine (CQ, #C6628) were purchased from Sigma–Aldrich Co. LLC., Shanghai, China. Fetal bovine serum (FBS; catalog no. 16000044), Dulbecco's modified Eagle's F12 Ham medium (DMEM-F12; catalog no. 12500096), and penicillin-streptomycin (catalog no. 15140122) were obtained from Gibco BRL (Carlsbad, CA, USA). 4-Phenylbutyric acid (CAS No. 1821-12-1) was purchased from MedChemExpress (MCE, Shanghai, China). Primary antibodies against were obtained from Cell Signaling Technology. The secondary antibodies were purchased from Sangon Biotech Co (Shanghai, China). The types and diluted concentrations of antibodies are shown in Supplemental Table S1. The Trizol reagent (#RN0402) was obtained from Aidlab Biotechnologies Co (Beijing, China).

2.2. Cell culture

The pTr cells were obtained from blastocysts of 15 to 19 d pregnant gilts as a heterogeneous monolayer and cultured as previously descried (Corps et al., 1990). In brief, monolayer cells were cultured and passaged in DMEM-F12 supplemented with 1% penicillin-streptomycin, 5 μg/mL insulin, and 10% FBS in a humidified incubator under an atmosphere of 5% CO_2 at 37 °C. The cells were treated with ZEA (0 to 80 μmol/L) after a confluency of 70% to 80% was reached. Subsequent treatment related to inhibitors is as follows: the pTr cells were pretreated with 5 mmol/L autophagy inhibitor 3-MA for 2 h or 100 μmol/L autophagy inhibitor CQ for 6 h, pretreated with 5 mmol/L ER stress inhibitor 4-phenylbutyric acid (4-PBA) for 1 h, respectively.

2.3. Cell viability and lactate dehydrogenase release assay

Cell Proliferation BeyoClick EdU-594 kit (#C0078S) and a cell-counting kit (#HY-K0301) were purchased from MedChemExpress (NJ, USA). Lactate dehydrogenase (LDH) was a marker of cell death for determination of cytotoxicity by measuring an LDH activity released from damaged cells. The absorbance of the solution was measured at 450 nm using a microplate meter (SpectraMax M3, Sunnyvale, CA, USA). All the operation steps were completed under the instruction of the manufacturer.

2.4. Flow cytometry analysis of apoptosis and cell cycle

Induction of apoptosis and cell cycle arrest in pTr cells triggered by ZEA was analyzed using a cell cycle and apoptosis analysis kit which purchased from Beyotime Biotechnology (Shanghai, China). Fluorescence intensity of FITC Annexin V and PI (propidium iodide) was analyzed using a flow cytometer (Beckman Coulter, Miami, FL, USA). Data of apoptosis and cell cycle were analyzed by using the CytExpert software.

2.5. Detection of mitochondrial membrane potential

The JC-1 is an ideal fluorescent probe to detect mitochondrial membrane potential ($\Delta\psi m$). After treating with ZEA for the indicated time periods, cells were stained by JC-1, a permeable cationic potentiometric vital dye, at 37 °C for 20 min. After successfully loading JC-1 probe, we observed the changes of green fluorescence in cells treated with ZEA by fluorescence microscope (TCS SPE, Lecia, Germany).

2.6. Determination of intracellular reactive oxygen species (ROS)

Cells (1.6×10^5/well) were seeded in a 6-well plate and treated with ZEA for the indicated time periods. Intracellular ROS production was determined using a ROS assay kit (#S0033M, Beyotime Biotechnology, Shanghai, China). All the operation steps are completed under the instruction of the manufacturer.

2.7. Western blot analysis

Western blot analysis of pTr cells treated with 0 to 80 μmol/L ZEA was performed for different periods of time. Total protein of cells was extracted using radio immunoprecipitation assay (RIPA) lysis buffer. Concentration of proteins extracted from the cells was measured by bicinchoninic acid (BCA) method. Proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride membrane. Primary and secondary antibodies were then incubated, and finally, a band containing the target protein was added the Image Quant LAS 4000 mini system (GE Healthcare, Piscataway, NJ, 52USA) and photographed.

2.8. Total RNA extraction and quantitative real-time PCR (qRT-PCR)

The Trizol reagent was used to extract the total RNA. Sequentially, cDNA was synthesized with a high-capacity cDNA archive kit. Gene expression levels were measured by using the ABI 7500 real-time PCR system (ABI 7500, Alameda, CA, USA). All the operation steps were completed under the instruction of the manufacturer. The *GAPDH* genes were used as the endogenous control for normalization. The primer sequences are shown in Supplemental Table S2.

2.9. Transmission electron microscopy (TEM)

After ZEA treatment, cells were collected and fixed with 3% glutaraldehyde for more than 2 to 4 h. After fixation, the cell masses were sent to the electron microscopy chamber for dehydration and encapsulation. The samples were cut into ultrathin slices about 50 nm thick with an ultrathin slicer. Uranyl acetate for 45 min and lead citrate for 30 min were electronically stained. Finally, the numbers of autophagic structure and the morphology of autophagosome in each group were observed under TEM (JEM-1400PLUS, Japan).

2.10. Statistical analysis

All data were statistically analyzed using SPSS statistical software (Version 25.0). Values were expressed as means ± standard error of the means (SEM). One-way analysis of variance (ANOVA) was used for comparison between multiple groups, $P < 0.05$ were considered statistically significant. Duncan's Multiple–Range test was used to test the differences between groups.

3. Results

3.1. ZEA inhibited cell proliferation and viability in pTr cells

We used cell counting kit-8 (CCK-8) assay to detect the effect of ZEA on pTr cell viability. As shown in Fig. 1A, ZEA had the inhibitory effects on viability of pTr cells in a concentration-dependent manner at both 12 and 24 h of treatment. At 20 μmol/L ZEA, pTr cells viability decreased by 7% ($P < 0.01$) and 16% ($P < 0.001$) after 12 and 24 h treatment, respectively. When pTr cells were treated with 160 μmol/L ZEA, cell proliferation was reduced by 51.5% at 12 h and 67.3% at 24 h, respectively ($P < 0.001$). In addition, we analyzed the expression of proliferating cell nuclear antigen (PCNA), which is an objective index to evaluate cell proliferation state as a DNA clamp. The results revealed that ZEA exposure led to the reduction of PCNA at both time points (Fig. 1B and D). Consistently, the release of LDH in pTr cells after 12 and 24 h exposure was significantly higher, compared to these of control (Fig. 1C). However, after treating pTr cells with 20 μmol/L ZEA for 12 h, the expression of PCNA was not significantly decreased compared with the control group (Fig. 1D). With the increase of ZEA treatment concentration, the number of EdU positive cells decreased gradually through fluorescence microscopy, indicating significantly reduced cell proliferation activity (Fig. 1E). This result is consistent with Fig. 1F. The number of EdU positive cells also decreased significantly at both 12 and 24 h ($P < 0.001$) (Fig. 1F). These results indicated that the cell viability of pTr cells significantly decreased after expose to ZEA for 12 h or 24 h, and the release of LDH may induce apoptosis, while the results of EdU suggested that ZEA may affect the cell cycle.

3.2. ZEA exposure resulted in cell cycle arrest in pTr cells

To further explore the cause of ZEA-induced decline in pTr cell viability, we measured the distribution of cell cycle. Flow cytometry analysis showed that the proportion of Sub-G1 phase of pTr cells increased with the increase of ZEA concentration after 12 and 24 h treatment. When treated with 80 μmol/L ZEA for 12 h, the proportion of Sub-G1 phase cells were significantly increased by 6.55% ($P < 0.001$) compared with the control group. After treatment for 24 h, the proportion of Sub-G1 phase cells increased by 14.2% ($P < 0.001$) compared with the control group (Fig. 2A–D). The increase of Sub-G1 phase cells suggested that the exposure to ZEA might induce apoptosis in pTr cells. On the other hand, the addition of ZEA significantly reduced the proportion of pTr cells in G1 phase and increased the proportion of pTr cells in G2/M phase. These results suggested that ZEA exposure can arrest pTr cells in the G2/M phase. We next investigated the effect of ZEA on the expression levels of cell cycle-related genes. As shown in Fig. 2E and F, pTr cells treated with ZEA for 12 and 24 h decreased expression levels of *PCNA*, *Cyclin A*, *Cyclin B* and *CDK1* with the increase concentration of ZEA. The expression levels of *P53* and *P21* were significantly increased by ZEA exposure in a dose- and time-dependent manners. In summary, the cell cycle was arrested in the G2/M phase after 12 h treatment with high concentration of ZEA. After 24 h treatment, both high and low concentrations of ZEA induced cell cycle arrest in the G2/M phase. The changes of Sub-G1 cells and cell cycle-related mRNA gene expressions at both time points indicated that ZEA exposure could impact the cell cycle progress in pTr cells.

3.3. ZEA induced apoptosis and depolarization of mitochondrial membrane potential in pTr cells

For the next step, we performed an annexin V-FITC and PI staining assay to determine whether ZEA induced apoptosis of pTr cells, and found that ZEA led to increase the percentage of apoptosis of pTr cells at different periods of time (Fig. 3A–D). Consistently, Western blot analysis showed that ZEA exposure resulted in increased expression levels of Bax, cleaved-Caspase-3, and cleaved-PARP at both time points, all of which are classical features of apoptosis. Moreover, the expressions of anti-apoptotic protein Bcl-2 and Bcl-xl were significantly reduced ($P < 0.05$) when treated with 80 μmol/L ZEA (Fig. 3E–H). Through the above it can be found that ZEA induced apoptosis of pTr cells in a time and dosage dependent manner. In addition, alteration in mitochondrial membrane potential (MMP) is an indicative of mitochondrial apoptotic signaling. Thus, we investigated whether ZEA disrupts MMP using JC-1 analyses. The results of fluorescence microscopy showed that the green signal of the JC-1 monomer was increased after treatment with ZEA at both 12 and 24 h (Fig. 3I). Depolarization of MMP can further lead to the release of pro-apoptotic factors, including Bax and cytochrome C. In agreement with that, Western blot analysis further confirmed this result (Fig. 3E–H).

3.4. ZEA inhibited PI3K/AKT pathway and activated MAPK signaling pathway in pTr cells

In order to further explore the interaction mechanism between ZEA and cell signaling pathways, we selected PI3K/AKT and MAPK pathways, which regulate cell apoptosis, growth and the expression of some important genes, and detected the protein expression levels of upstream and downstream molecules of the pathway. We found that phosphorylation of AKT, mTORC1, 4E-BP1 was significantly down-regulated by 80 μmol/L treatment in pTr cells at both time points (Fig. 4A–C). However, the expression of phosphorylated p70S6K and S6 was increased after ZEA exposure in pTr cells (Fig. 4D and E). On the other hand, ZEA treatment for 12 and 24 h both activated the expression of related proteins in MAPK signaling pathway in pTr cells. For example, pTr cells treated with 80 μmol/L ZEA for 12 and 24 h, had increased protein abundance of p-ERK1/2 by 2- and 6-fold ($P < 0.001$) at different time periods, compared to that in the control cells (Fig. 4F). Furthermore, the abundances of p-JNK1/2 and p-p38 MAPK gradually increased at both 12 and 24 h in response to ZEA exposure in pTr cells (Fig. 4G and H). Collectively, these results suggested that ZEA-induced cytotoxicity of pTr cells may be mediated by inhibition of PI3K/AKT pathway and activation of MAPK signaling pathway.

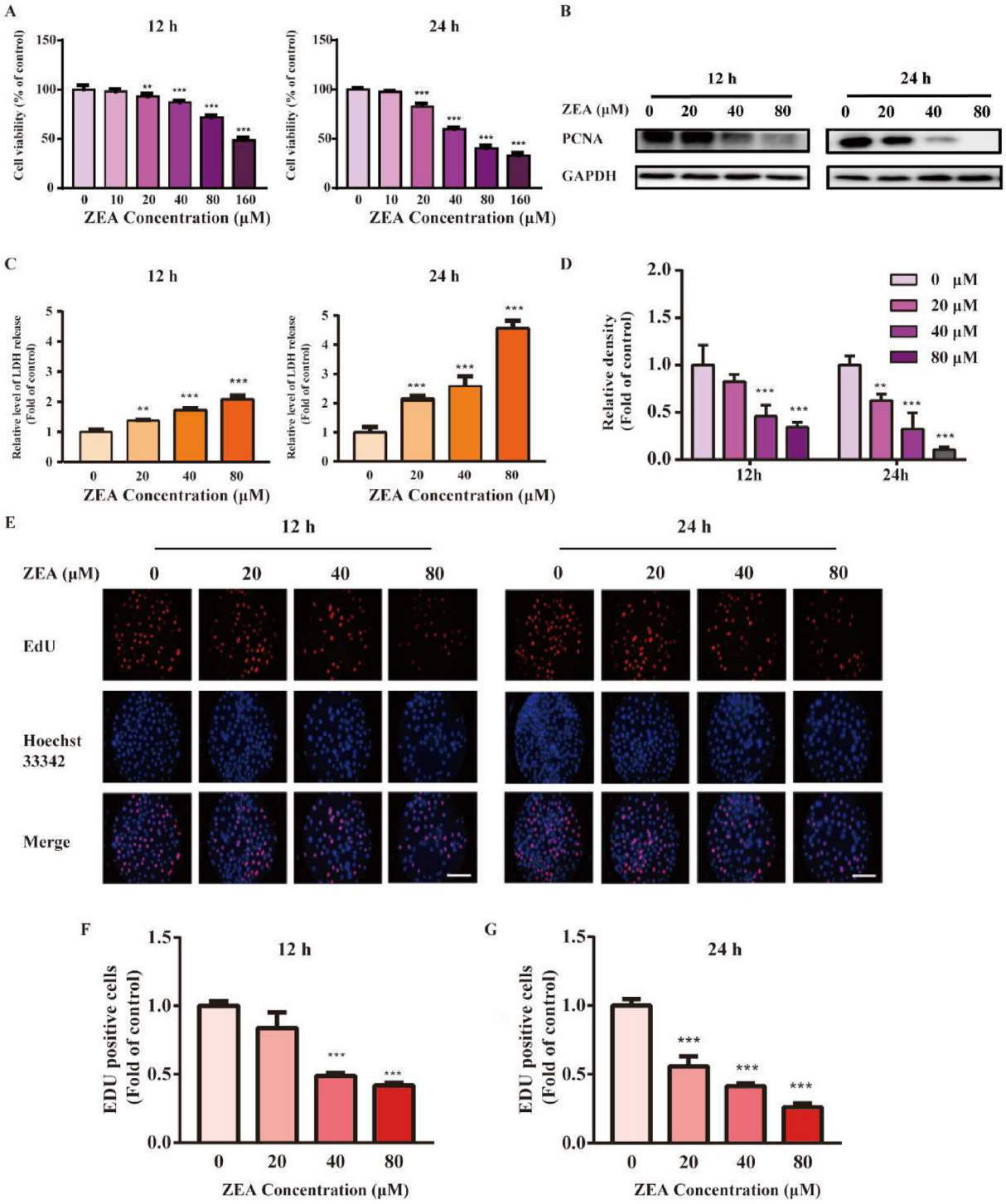

Fig. 1. Anti-proliferative and inhibited cell viability induced by ZEA in pTr cells for 12 or 24 h. (A) Concentration-dependent effects of ZEA on the proliferation of pTr cells were determined using proliferation assays, and data are presented as percentages relative to the control cells (100%). (B) Representative Western blot results for PCNA. GAPDH was used as a loading control. (C) Detection of LDH activity in pTr cells. (D) The columns showed the statistical analysis of protein abundance in (B). (E) Cell proliferation was determined by the EdU assay, and images were taken under a fluorescence microscope (magnification 400×). Scale bar represents 100 μm. (F) EdU positive cells was measured using ImageJ software. Data, expressed as the percent of control cells, are means ± SEM, $n = 6$ independent experiments. Asterisks indicate significant differences, compared to the vehicle-treated cells (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, pTr = porcine trophectoderm, PCNA = proliferating cell nuclear antigen, GAPDH = glyceraldehyde-3-phosphatedehydrogenase, LDH = lactate dehydrogenase, EdU = 5-ethynyl-2′-deoxyuridine.

3.5. Activation of the ER stress and autophagy pathway by ZEA in pTr cells

We next examined whether apoptosis of pTr cells induced by ZEA is associated with ER stress and autophagy. The GRP78 protein levels gradually increased compared to the control with the increase of ZEA concentration, although the 20 μmol/L ZEA did not significantly affect the ER stress proteins (Fig. 5A–D). Cells treated with ZEA had increased protein levels of CHOP in a dose-dependent manner.

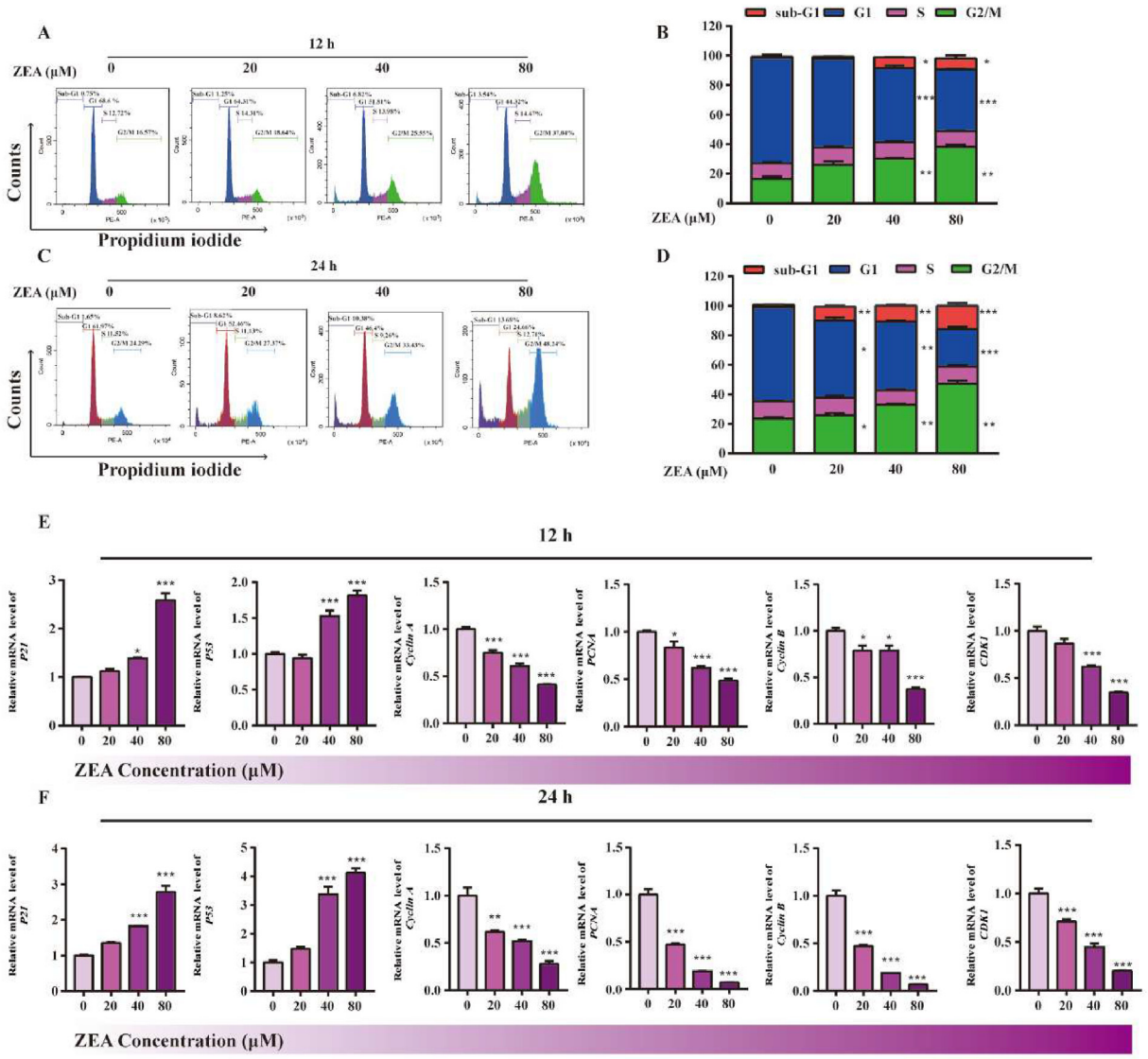

Fig. 2. Effects of ZEA on cell cycle progression in pTr cells for 12 or 24 h. (A–D) Cell cycle progression was analyzed by quantitation of DNA content using PI staining in pTr cells. The percentage of cells in each phase of the cell cycle was determined using flow cytometry. (E and F) Representative results for mRNA levels of p53, p21, cyclin A, PCNA, cyclin B and CDK1 after normalization to GAPDH. Asterisks indicate significant differences, compared to the vehicle-treated cells. (***$P < 0.001$, **$P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, pTr = porcine trophectoderm, p53 = tumor protein 53, p21 = cyclin-dependent kinase inhibitor 1A, PCNA = proliferating cell nuclear antigen, CDK1 = recombinant cyclin dependent kinase 1, GAPDH = glyceraldehyde-3-phosphatedehydrogenase, PI = propidium iodide.

ER stress can activate 3 transmembrane signaling proteins, including PERK, IRE1, and ATF6. Therefore, we examined the expressions of these proteins. The results showed that PERK-mediated signaling pathway and protein (p-eIF2α, ATF4) expressions increased significantly with the increase of exposure time and concentration of ZEA. The activation of ATF6 pathway protein only occurred after treatment with ZEA for 24 h. However, protein level of IRE1 was not affected by ZEA, regardless of treatment time and concentration (Fig. 5A–D). As shown in Fig. 5E and F, that addition of ZEA to culture medium resulted in the accumulation of LC3-II and reduction of p62, indicating the activation of autophagy. We also observed the formation of autophagosomes in ZEA-treated pTr cells by TEM, which further confirmed the occurrence of autophagy (Fig. 5G).

3.6. Intracellular reactive oxygen species was generated and barrier function impairment by ZEA in pTr cells

To detect the effect of ZEA on barrier function of pTr cells, we measured the expressions level of barrier functional proteins using Western blot. The results elucidated that the expressions of tight junction protein were significantly decreased by ZEA exposure in a dose- and time-dependent manners, including ZO-3 and claudin-3 (Fig. 6A–C). The expressions of tight junction proteins (ZO-1, ZO-2, claudin-1 and occludin) were significantly decreased ($P < 0.001$) at high concentration (40 to 80 μmol/L) only when pTr cells were treated with ZEA for 24 h. In addition, cells treated with ZEA had higher levels of ROS at both 12 and 24 h, as evidenced by flow cytometry analysis, with a peak shift to right, when compared with

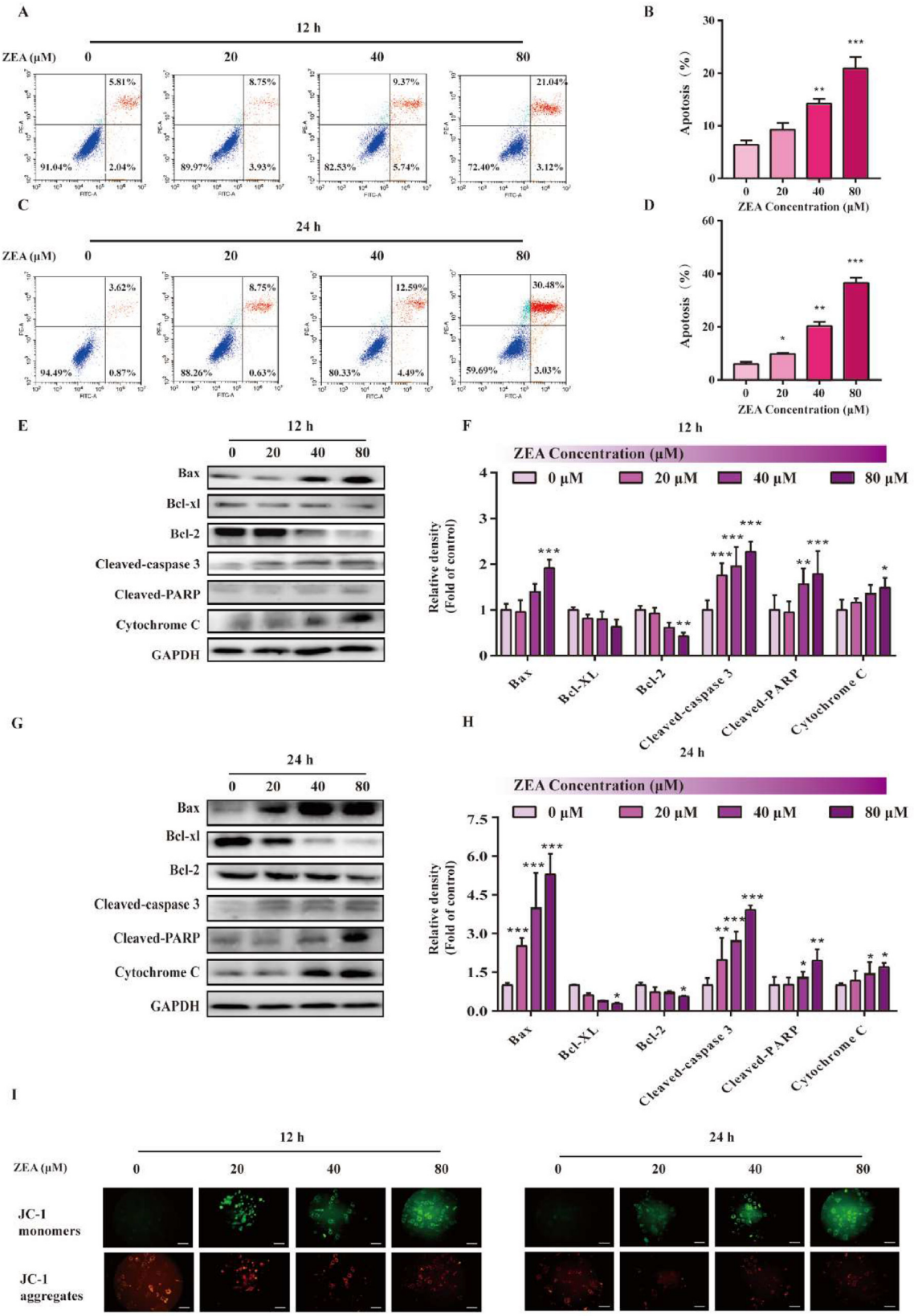

Fig. 3. Apoptosis and disruption of mitochondrial membrane potential (MMP) by ZEA-induced cellular stress in pTr cells for 12 or 24 h. (A–D) Detection of apoptosis in pTr cells after ZEA treatment. Annexin V and propidium iodide (PI) fluorescence was quantified using flow cytometry. (E–H) Representative Western blot results for protein levels of Bax, Bcl-xl, Bcl-2, cleaved-caspase-3, cleaved-PARP and cytochrome C. GAPDH was used as a loading control. (I) Detection of JC-1 signals in pTr cells by fluorescence confocal microscopy. The double staining of cells by JC-1 is visible either as green for J-monomers or red for J-aggregates. Scale bar represents 200 μm. Values are expressed as means ± SEM from 3 independent experiments and statistical significance is indicated in the bar chart. Asterisks indicate significant differences, compared to the vehicle-treated cells (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, pTr = porcine trophectoderm, Bax = Bcl2-associated X, Bcl-2 = B-cell lymphoma-2, Bcl-xl = B-cell lymphoma-extra large, GAPDH = glyceraldehyde-3-phosphatedehydrogenase, JC-1 = 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide.

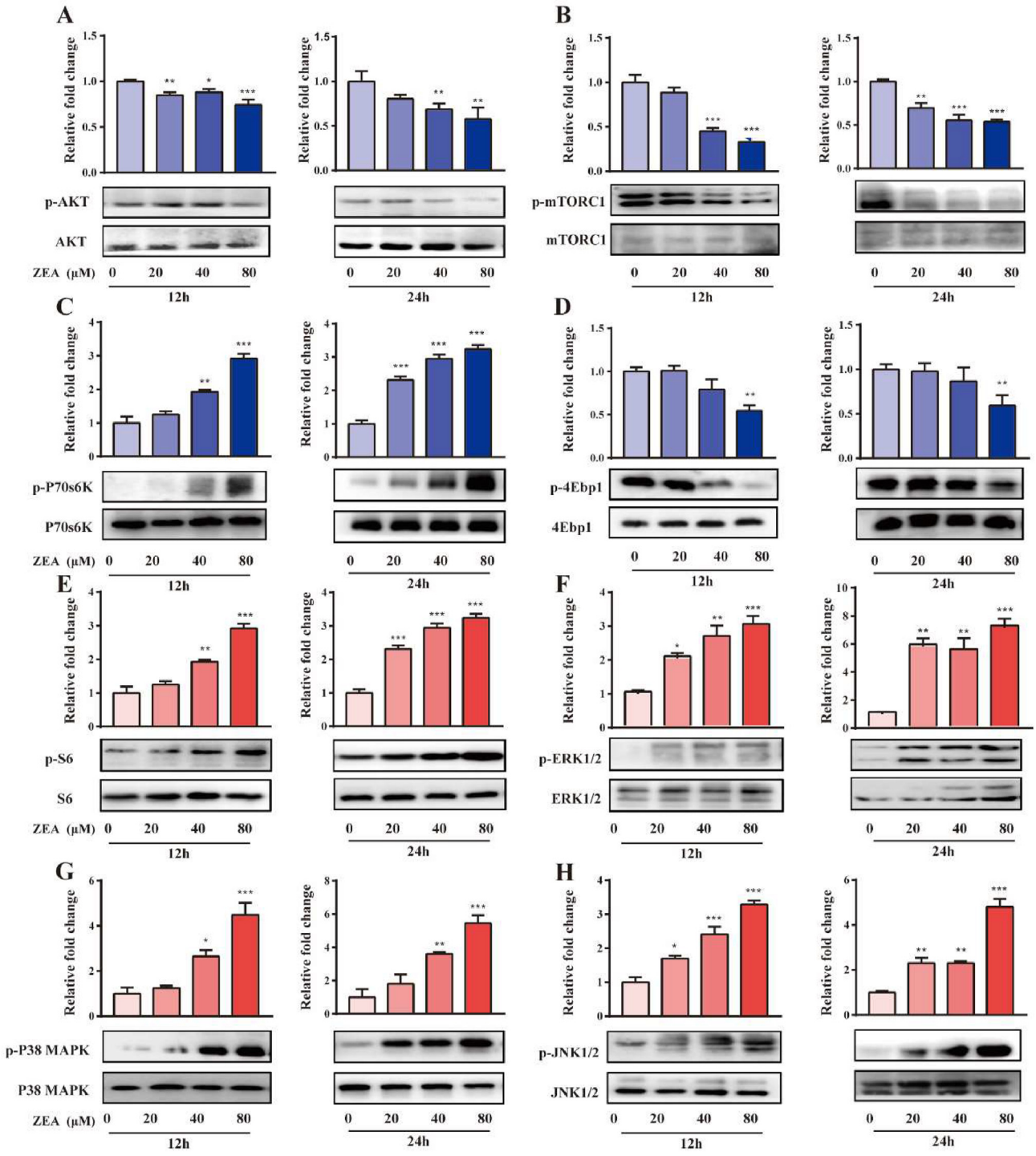

Fig. 4. Dose-dependent effects of ZEA on the phosphorylation of proteins in the PI3K/AKT, ERK1/2 MAPK, and JNK MAPK signaling pathways in pTr cells for 12 or 24 h. Phosphorylated (A) AKT, (B) mTOR, (C) P70S6K, (D) 4EBP1, (E) S6, (F) ERK1/2, (G) P38 MAPK, and (H) JNK1/2 expression levels were measured after treatment of different concentrations of ZEA (0, 20, 40, and 80 μM). Immunoblots were captured and quantified using Image J software, and then the normalized values were calculated and presented as ratios of phosphorylated proteins relative to total proteins. Asterisks indicate significant differences, compared to the non-treated cells (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, pTr = trophoblast ectoderm, PI3K = phosphatidylinos-itol-3 kinase, AKT = Protein Kinase B, ERK1/2 = extracellular signal-regulated kinases 1 and 2, MAPK = mitogen-activated protein kinases, JNK = c-Jun N-terminal kinase, pTr = porcine trophectoderm, mTOR = mechanistic targeting of the rapamycin, GAPDH = glyceraldehyde-3-phosphatedehydrogenase.

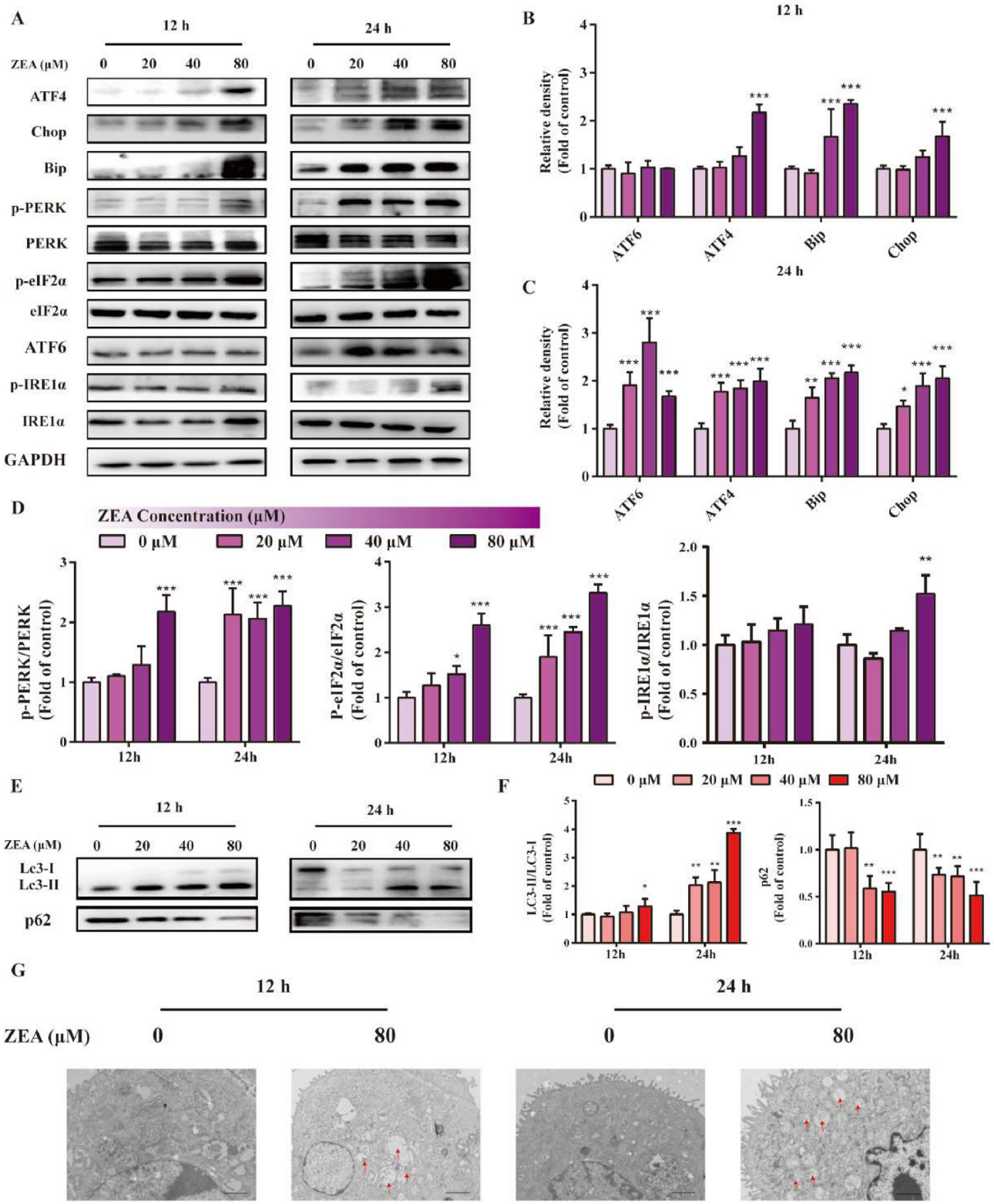

Fig. 5. ZEA triggered the endoplasmic reticulum stress and autophagy response in pTr cells for 12 or 24 h. (A) Representative Western blot results for ATF4, Chop, Bip, p-PERK, t-PERK, p-eIF2α, t-eIF2α, p-IRE1α, t-IRE1α, ATF6. GAPDH was used as a loading control. (B–D) The columns showed the statistical analysis of protein abundance in (A). (E) Representative Western blot results for LC3-I, LC3-II and p62. (F) The columns showed the statistical analysis of protein abundance in (E). Values are expressed as means ± SEM from 3 independent experiments and statistical significance is indicated in the bar chart. Asterisks indicate significant differences, compared to the vehicle-treated cells (*** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, pTr = trophoblast ectoderm, ATF4 = recombinant activating transcription factor 4, Bip/GRP78 = 78-kD glucose-regulated protein, Chop = C/EBP homologous protein, PERK = protein kinase R (PKR)-like endoplasmic reticulum kinase, eIF2α = eukaryotic initiation factor 2α, IRE1α = inositol-requiring enzyme-1α, ATF6 = activating transcription factor 6, LC3 = microtubule associated protein, p62 = sequestosome 1, GAPDH = glyceraldehyde-3-phosphatedehydrogenase.

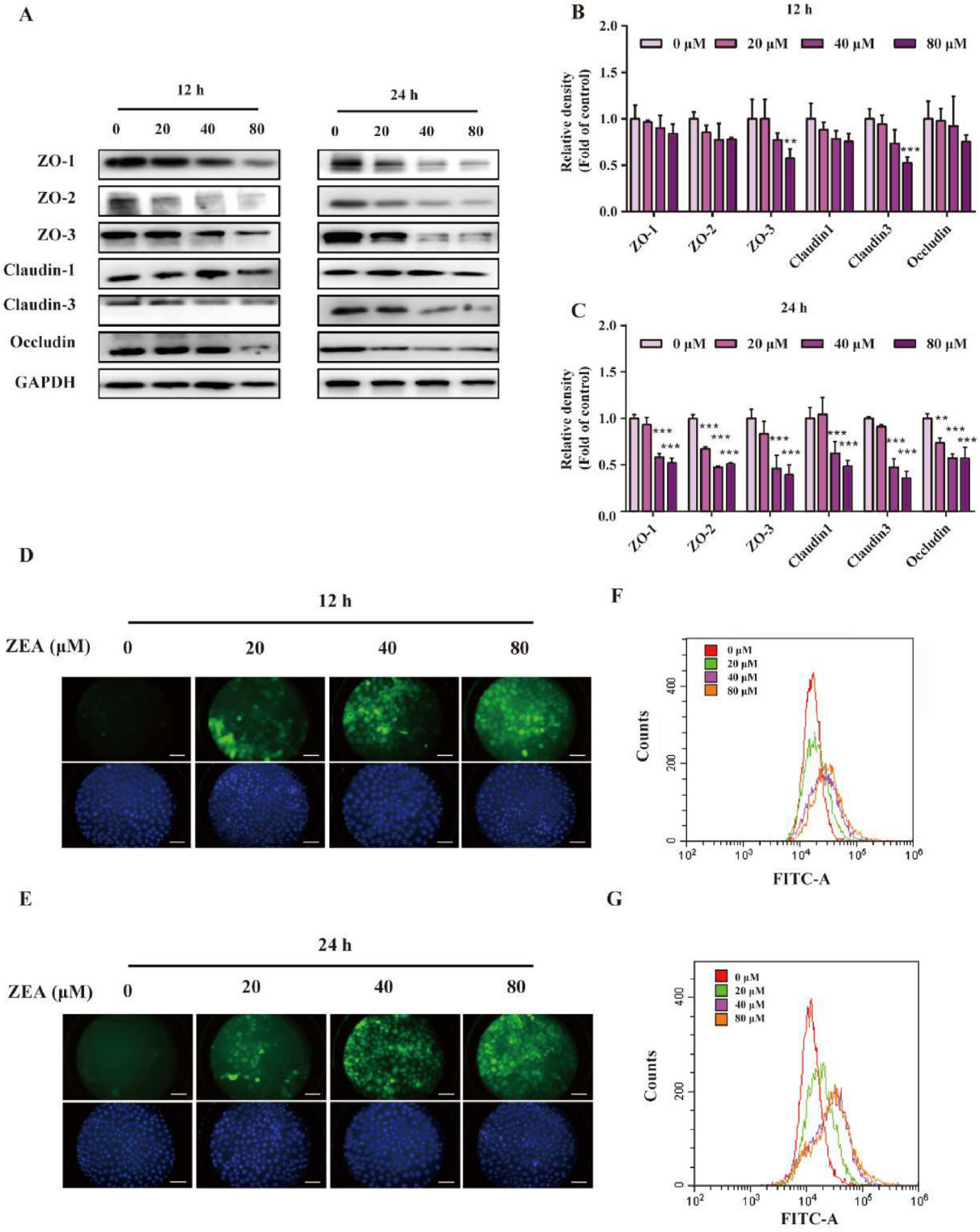

Fig. 6. ZEA induced barrier function impairment and ROS generation in pTr cells for 12 or 24 h. (A) Representative Western blot results for ZO-1, ZO-2, ZO-3, claudin-1, claudin-3, occludin. GAPDH was used as a loading control. (B and C) The columns showed the statistical analysis of protein abundance in A. (D and E) The images were taken under a fluorescence microscope. Scale bar represents 200 μm. (F and G) ROS content was determined by the DCFH-DA assay, and DCFH-DA positive populations were determined by the flow cytometry analysis. Values are expressed as means ± SEM from 3 independent experiments and statistical significance is indicated in the bar chart. Asterisks indicate significant differences, compared to the vehicle-treated cells (***$P < 0.001$, **$P < 0.01$, and * $P < 0.05$). ZEA = zearalenone, ROS = reactive oxygen species, pTr = trophoblast ectoderm, ZO-1 = zonula occludens-1, ZO-2 = zonula occludens-2, ZO-3 = zonula occludens-3, GAPDH = glyceraldehyde-3-phosphatedehydrogenase.

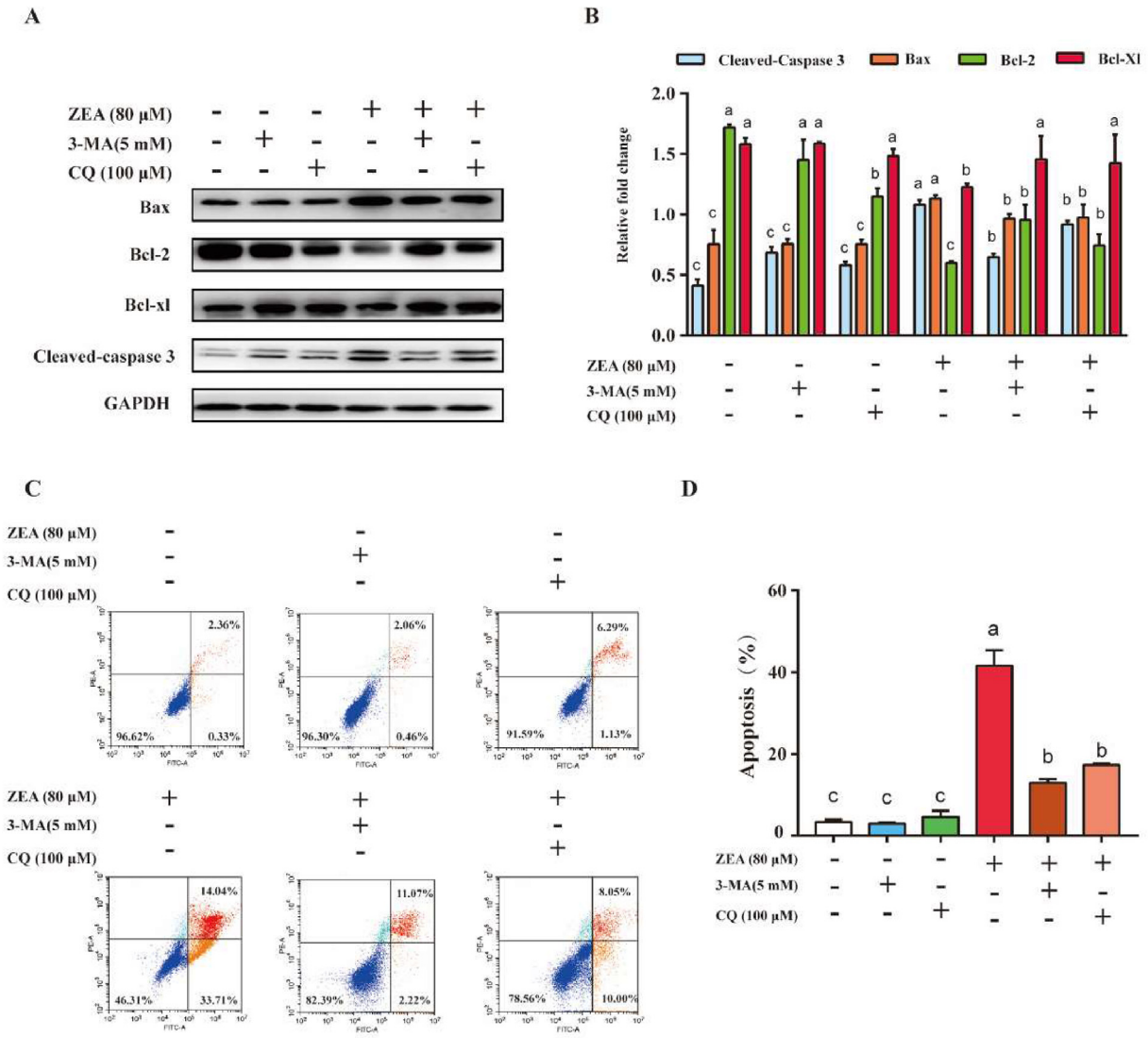

Fig. 7. Autophagy inhibitor 3-MA or CQ reversed ZEA-induced apoptosis of pTr cells for 12 or 24 h. The pTr cells were pretreated with 5 mM autophagy inhibitor 3-MA for 2 h or 100 μM autophagy inhibitor CQ for 6 h, respectively. (A) Representative Western blot results for Bax, Bcl-2, Bcl-xl, cleaved-caspase-3. GAPDH was used as a loading control. (B) The columns showed the statistical analysis of protein abundance in (A). (C and D) Detection of apoptosis in pTr cells after autophagy inhibitor treatment. Annexin V and propidium iodide (PI) fluorescence was quantified using flow cytometry. Results are presented as means ± SEM of 3 independent experiments. Columns that do not share the same superscript letter differ significantly ($P < 0.05$). ZEA = zearalenone, Bax = Bcl2-associated X, Bcl-2 = B-cell lymphoma-2, Bcl-xl = B-cell lymphoma-extra large, GAPDH = glyceraldehyde-3-phosphatedehydrogenase, CQ = chloroquine, 3-MA = 3-methyladenine.

the control. This effect of ZEA was confirmed by enhanced fluorescence intensity.

3.7. ZEA-induced apoptosis is alleviated by autophagy inhibitors in pTr cells

We further detected the apoptosis of pTr cells in the presence of 3-MA or CQ, to elucidate the relationship between apoptosis and autophagy in ZEA-induced toxicity. The results showed that the induction of apoptosis-related proteins, including Bax and Cleaved-caspase-3, following ZEA exposure was significantly reduced by 3-MA or CQ ($P < 0.05$). Besides, the decrease of Bcl-2 and Bcl-xl induced by ZEA exposure was significantly reversed (Fig. 7A and B). The results of flow cytometry demonstrated that the apoptotic rate significantly decreased after cotreatment with ZEA and 3-MA (12.93%) or ZEA and CQ (17.28%), when compared with ZEA alone treatment (41.6%) (Fig. 7C and D). In summary, ZEA induced autophagy and promoted apoptosis in an autophagy-dependent manner.

3.8. ZEA promotes apoptosis by inducing autophagy through endoplasmic reticulum stress in pTr cells

To further explore the functional role of ER stress on ZEA-induced autophagy, we pretreated the cells with ER stress inhibitor, and then determined apoptosis and cell viability in pTr cells.

As shown in Fig. 8A and B, the percentage of apoptosis of pTr cells co-treatment with 4-PBA and ZEA was significantly lower when compared with cells treated with ZEA alone. Western blot analysis showed that the expressions of autophagy and apoptosis-related proteins in the presence or absence of ER stress inhibitor 4-PBA. The results showed that the expression levels of proteins related to apoptosis and autophagy were significantly down-regulated when 4-PBA was added compared with ZEA alone

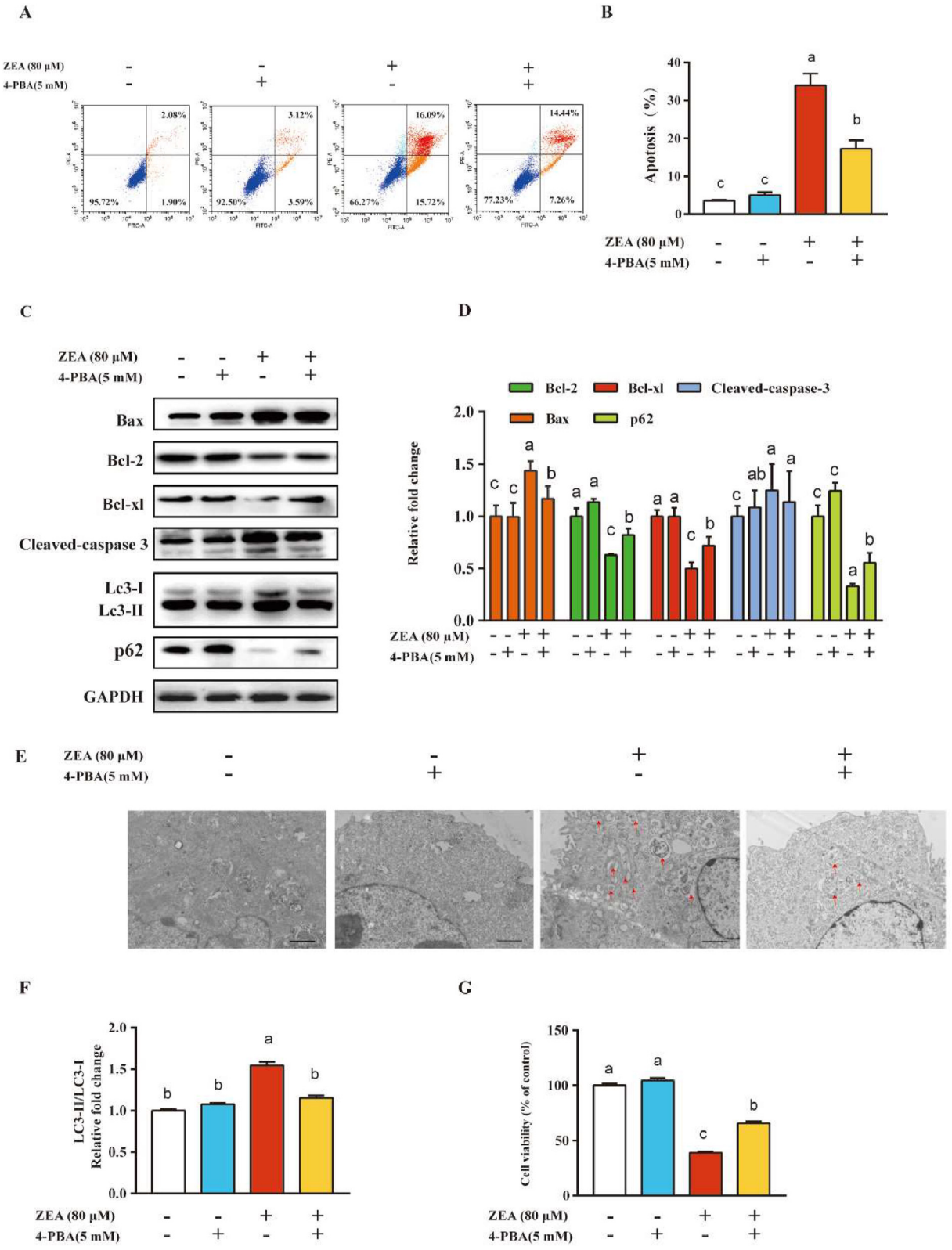

Fig. 8. Effects of 4-phenylbutyric acid (4-PBA) on apoptosis and autophagy in pTr cells for 12 or 24 h. The pTr cells were pretreated with 5 mM 4-PBA for 1 h. (A) Detection of apoptosis in pTr cells after 4-PBA treatment. Annexin V and propidium iodide (PI) fluorescence was quantified using flow cytometry. (B) The columns showed the statistical analysis of protein abundance in (A). (C) Representative Western blot results for Bax, Bcl-2, Bcl-xl, cleaved-caspase-3, LC3-I, LC3-II and p62. GAPDH was used as a loading control. (D and E) The columns showed the statistical analysis of protein abundance in (C). (F) Cell viability of pTr cells was examined after 4-PBA treatment, and data are presented as percentages relative to the control cells (100%). Results are presented as means ± SEM of 3 independent experiments. Columns that do not share the same superscript letter differ significantly ($P < 0.05$). ZEA = zearalenone, pTr = porcine trophectoderm, Bax = Bcl2-associated X, Bcl-2 = B-cell lymphoma-2, Bcl-xl = B-cell lymphoma-extra large, LC3 = microtubule associated protein, p62 = sequestosome 1, GAPDH = glyceraldehyde-3-phosphatedehydrogenase.

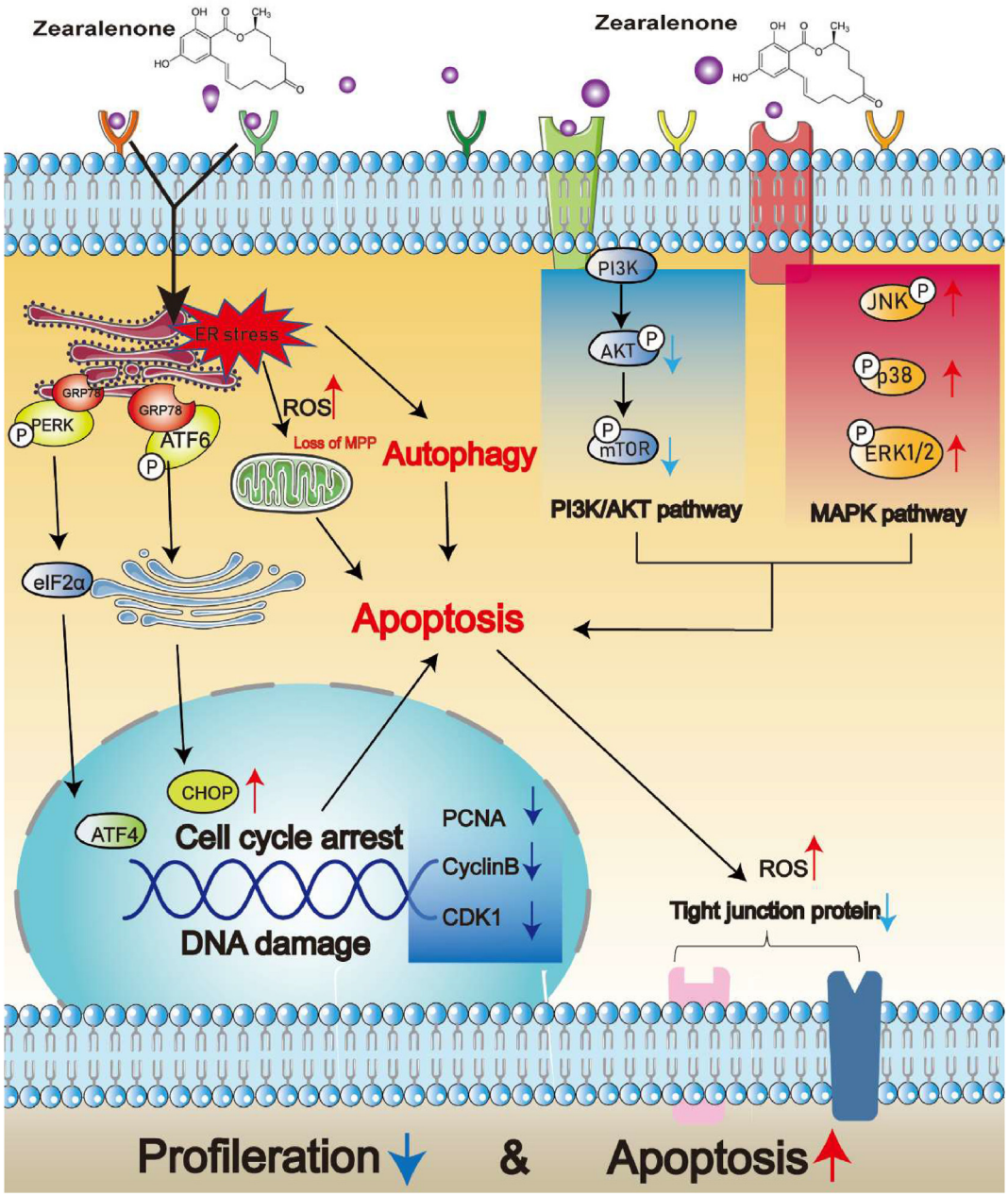

Fig. 9. Hypothetical schemes of mechanism regulated by zearalenone (ZEA) in decreasing proliferation and apoptotic cell death in trophoblast ectoderm (pTr) cells. ZEA reduced cell proliferation with attenuation of PCNA expression and induced cell cycle arrest at G2/M phase via inhibition of cyclin B and CDK1. In addition, ZEA depolarized the mitochondrial membrane potential leading to release of proapoptotic proteins. Moreover, ER stress-response proteins including GRP78, ATF6, PERK, and eIF2α were activated by ZEA. ZEA induced apoptosis of pTr cells through ER stress activated autophagy. Furthermore, ZEA altered the signal transduction pathway including PI3K/AKT and MAPK pathways. Consequently, ZEA induced disruption of cell physiology, which might lead to implantation failure in pTr cells. ROS = reactive oxygen species, MMP = mitochondrial membrane potential. AKT = protein kinase B, ATF4 = recombinant activating transcription factor 4, ATF6 = activating transcription factor 6, Bip/GRP78 = 78-kD glucose-regulated protein, CDK1 = recombinant cyclin dependent kinase 1, Chop = C/EBP homologous protein, ERK1/2 = extracellular signal-regulated kinases 1 and 2, eIF2α = eukaryotic initiation factor 2α, IRE1α = Inositol-requiring enzyme-1α, JNK = c-Jun N-terminal kinase, mTOR = mechanistic targeting of the rapamycin, PERK = Protein kinase R (PKR)-like endoplasmic reticulum kinase.

(Fig. 8C, D and F). Transmission electron microscope results also showed that the addition of 4-PBA reduced the number of autophagosomes in cells and alleviated the autophagy reaction caused by ZEA to a certain extent (Fig. 8E). Finally, CCK-8 kit was used to determine cell viability, and the results showed that pTr cells pretreated with 4-PBA significantly recovered cell viability compared with those treated with ZEA alone (Fig. 8G). In conclusion, the apoptosis of pTr cells induced by ZEA was through the mechanism of autophagy induced by the ER stress (Fig. 9).

4. Discussion

The results of a growing number of studies have consistently suggested that ZEA can cause damage to female reproductive system (Boeira et al., 2014; Del Fabbro et al., 2019). Although toxicity of ZEA has been reported in some animal models (Zhang et al., 2014; Zhao et al., 2013), its toxicological effects and underling mechanisms during embryonic development have been rarely reported. Besides, proliferation and migration of pTr cells, which constitute

the placenta, play a key role in embryo implantation (Chen et al., 2004; Park et al., 2020). Therefore, the pTr cell lines are invaluable as in vitro models for exploring the adverse effects and toxicological mechanisms of ZEA on embryonic development during early pregnancy in pigs (Geisert et al., 2015; Wang et al., 2000). In the present study, we revealed antiproliferative and pro-apoptotic effects of ZEA in pTr cells. Importantly, ZEA-induced autophagy and apoptosis were rescued by ER stress inhibitors, indicating a functional role of ER stress in ZEA-induced cell death in pTr cells.

Alterations of trophoblast functions cause adverse pregnancy outcomes including embryonic death, implantation failure, and premature delivery (Lunghi et al., 2007). In this experiment, pTr cells were treated with ZEA and its effect on cell proliferation was determined. The results showed that ZEA could significantly inhibit cell viability, which was accompanied by increased LDH release, an indicator of cell death due to cellular membrane damage (Fotakis and Timbrell, 2006). The results of the present study suggest that ZEA inhibits proliferation of pTr cells by inducing cell cycle arrest and apoptotic cell death. Accumulation studies also have shown that a high dose of ZEA can cause cell death and cell cycle arrest, oxidative stress, DNA damage, mitochondrial damage, as well as apoptosis (So et al., 2014; Tatay et al., 2016; Wang et al., 2014; Zheng et al., 2018). In the present study, we examined changes in cell cycle distribution of pTr cells treated with ZEA. As expected, the proportion of cells in the Sub-G1 and G2/M phase increased in a concentration-dependent manner, which was consistent with previous study with MTEC1 cells (Zhang et al., 2018b). Additionally, we found elevated p53 and p21 mRNA in pTr cells which indicating occurrence of DNA damage. It has been reported that activation of p53 and downstream target p21, can bind with PCNA, the cofactor of DNA polymerase δ, to inhibit the transcription of CDK1, resulting in G2/M arrest (Kotani et al., 2013; Wang et al., 2012). Our results indicated a regulatory effect of ZEA on p53/p21 signaling in pTr cells. The increased proportion of Sub-G1 phase cells indicated the occurrence of apoptosis following ZEA exposure. Moreover, ZEA damaged placental barrier function and produced ROS in pTr cells, reflecting the oxidative damage caused by ZEA to cells. These results suggested that ZEA may impair placental formation in early stages of pregnancy, thus affecting embryo development and reproductive function in animals.

We next investigated signal transduction pathways responsible for the antiproliferative effects of ZEA in pTr cells. Previous studies have shown PI3K/AKT signaling pathways can be activated by proliferation-promoting factors in pTr cells (Jeong et al., 2014, 2016a, 2017), and the JNK pathway is activated by stress stimuli (Kyriakis and Avruch, 2001). In the present study, we showed that ZEA treatment decreased the phosphorylation of most PI3K/AKT signaling proteins in pTr cells, including AKT, mTORC1 and 4E-BP1. However, other signaling proteins, including P70S6K, S6, ERK1/2, JNK1/2 and p38 MAPK, were activated after ZEA treatment. These results were agreement with previous study in RAW264.7 cells (Yu et al., 2011) or Sertoli cells (Wang et al., 2018). These results implied that the cytotoxic effects of ZEA on pTr cells may occur through inhibition of PI3K/AKT pathway and activation of MAPK signaling pathway.

The ER stress signaling can be activated in response to various stimuli to reduce translation and upregulate protein folding capacity (Navid and Colbert, 2017). In our study, we found that ZEA triggered ER stress in pTr cells through the PERK and ATF6 signaling pathways. This result was in line with previous studies (Yoon et al., 2020). In addition, we observed upregulated proteins of autophagy, such as LC3-II and p62, indicating activation of the autophagy (Chen et al., 2015).

Autophagy stabilizes the intracellular environment and maintains cell survival through balancing anabolism and catabolism (Takagi et al., 2007). Generally, autophagy is considered to be a pro-survival mechanism, but autophagy also contributes to cytotoxicity and cell death when the process exceeds a crucial threshold (Marino et al., 2014). In order to explore the effect of ZEA-activated autophagy and apoptosis, autophagy inhibitors 3-MA and CQ were used to detect the cross-talk between apoptosis and autophagy. Our results indicated that high dose of ZEA induced autophagy, while the apoptosis phenotype was reversed with the addition of autophagy inhibitors, suggesting that ZEA-induced autophagy promoted apoptosis. This result provided a theoretical basis for the effects of ZEA on embryonic development and reproductive function in early stages of pregnancy.

Another novel finding of the present study is that autophagy following ZEA exposure was mediated by ER stress in pTr cell. Prolong ER stress can lead to cell death through CHOP, a downstream signaling molecule of eIF2α (Meyerovich et al., 2016; Oyadomari and Mori, 2004). To investigate a functional role of ER stress signaling and its contribution to ZEA-induced apoptosis, pTr cells pretreated with or without 4-PBA, an ER stress inhibitor as previously described (Xiao et al., 2011), were subjected to ZEA treatment. As expected, apoptosis and autophagy induced by ZEA was effectively suppressed by 4-PBA, as evidenced by the expressions of Cleaved-caspase-3, Bax, Bcl-2 and Bcl-xl as well as autophagy-related proteins was reversed.

5. Conclusion

In conclusion, we found that ZEA has detrimental effects on pTr cells, as demonstrated by cell cycle arrest, mitochondria permeabilization, apoptosis induction, ROS accumulation, as well as ER stress. The deleterious effect of ZEA was associated with inhibition of PI3K/AKT pathway and activation of MAPK signaling pathway in pTr cells. Mechanistically, we proved that ZEA-induced apoptosis of pTr cells is through ER stress activated autophagy. These data provide new insight into the mechanistic pathways of ZEA toxicity on female reproductive development. Considering a prevalent common contaminant of ZEA in grain-related food products and its potential adverse effects on the health of both humans and animals, in vivo studies are required to verify the adverse effects of ZEA in fetal morphogenesis and placentation during pregnancy.

Author contributions

Jun Bai: data curation, writing—original draft preparation. **Jun Li** and **Ning Liu:** investigation. **Hai Jia** and **Xuemeng Si:** collected samples & implicated in data analysis. **Yusong Zhou** and **Zhian Zhai**: investigation. **Ying Yang**, **Fazheng Ren** and **Zhenlong Wu**: supervision, writing—review & editing.

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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Appendix supplementary data

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