



Protective effects of selenium against zearalenone-induced apoptosis in chicken spleen lymphocyte via an endoplasmic reticulum stress signaling pathway

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Abstract

Selenium (Se), an antioxidant agent, provides significant protection from reactive oxygen species (ROS)-induced cell damage in vivo and in vitro. However, it is unclear whether Se can protect against zearalenone (ZEN)-induced apoptosis in chicken spleen lymphocyte. In this study, we investigated the underlying mechanism of the apoptosis induced by ZEN in chicken spleen lymphocyte and further evaluated the protective mechanism of Se on ZEN-induced apoptosis. The results show that ZEN induced an increase in ROS generation and lipid peroxidation, and a decrease in levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione (GSH). The results of apoptosis morphologically from acridine orange/ethidium bromide (AO/EB) fluorescent staining and flow cytometry analysis show apparent apoptosis in the ZEN-treated group, and was confirmed by the upregulation of caspase-3, -12 and downregulation of Bcl-2. Meanwhile, ZEN activated the endoplasmic reticulum (ER) stress by upregulating ER stress-related molecular sensors (GRP78, ATF6, ATF4, IRE). However, co-treatment with Se effectively blocked ROS generation, improved antioxidative capacity, and reversed apoptosis and ER stress-related genes and protein expression. Taken together, these data suggest that oxidative stress and ER stress play a vital role in ZEN-induced apoptosis, and Se had a significant preventive effect on ZEN-induced apoptosis in chicken spleen lymphocyte via ameliorating the ER stress signaling pathway.

Keywords Zearalenone · Endoplasmic reticulum stress · Apoptosis · Spleen lymphocyte · Chicken

Abbreviations

ZEN	Zearalenone	GRP78	Glucose-regulated protein 78
ROS	Reactive oxygen species	ATF4	Activating transcription factor 4
ER	Endoplasmic reticulum	Se	Selenium
IRE	Inositol requiring enzyme	SOD	Superoxide dismutase
PERK	Protein kinase RNA (PKR)-like ER kinase	GSH-Px	Glutathione peroxidase
ATF 6	Activating transcription factor 6	GSH	Glutathione
		MDA	Malondialdehyde
		CAT	Catalase
		AO/EB	Acridine orange/Ethidium bromide

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Introduction

Zearalenone (ZEN) [6-(10-hydroxy-6-oxo-trans-1-undecenyl)-bresorcyclic acid lactone], also known as F-2 toxin, is a non-steroid estrogen mycotoxin. It is mainly produced by *Fusarium* species, such as *Fusarium cerealis*, *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium equiseti*, and exists widely in many foods and feedstuffs (Richard 2007). The structures of ZEN and its metabolites are similar

to that of 17β -oestradiol, and thus ZEN and its metabolites can competitively bind to estrogen receptors, disturbing steroid metabolism and causing functional changes in reproductive organs in farm animals and human (Olsen et al. 1981; Turcotte et al. 2005). Besides reproductive toxicity, ZEN also exhibits hepatotoxicity, cytotoxicity, genotoxicity, and immunotoxicity (Abid-Essefi et al. 2003; Tiemann et al. 2008; Zinedine et al. 2007). For instance, ZEN increased reactive oxygen species (ROS) production, leading to lipid peroxidation, DNA damages and immunosuppression (Abid-Essefi et al. 2003; Lin et al. 2015; Marin et al. 2011). In chicken, ZEN induced inflammation, oxidative stress, and calcium (Ca^{2+}) imbalance (Gresakova et al. 2012; Wang et al. 2012a, b). Furthermore, ZEA could induce apoptosis via an endoplasmic reticulum (ER) stress-dependent signaling pathway in mouse Leydig cells (Lin et al. 2015).

The endoplasmic reticulum (ER) is a main site for protein synthesis, protein folding, and intracellular calcium (Ca^{2+}) storage in eukaryotic cell, which plays a regulatory role in the cellular stress response (Todd et al. 2008). ER stress can be caused by various factors, such as hypoxia, hunger, calcium imbalance, free radical invasion, or drugs (Schröder and Kaufman 2005), and leads to an accumulation ROS, inflammation, and apoptosis (Guan et al. 2009). In ER stress, unfolded protein response (UPR) is a crucial signal transduction pathways, that is, sensed and activated by three upstream signaling proteins IRE (inositol requiring enzyme), PERK (protein kinase RNA (PKR)-like ER kinase), and ATF 6 (activating transcription factor 6) (Ron 2002). At normal conditions, the three ER stress transducers are in an inactive configuration by binding to the chaperone GRP78 (glucose-regulated protein 78). But chronic or excessive ER stress may break the balance between unfolded proteins and chaperones, and ultimately triggers apoptosis (Choi et al. 2010). As a specific player in the UPR, activated PERK also phosphorylates the α -subunit of the translation initiation factor eIF2 (eukaryotic translation initiation factor-2), increases the expression of ATF4 (activating transcription factor-4), and regulates apoptosis (Jiang et al. 2013). Previous studies have shown the ER stress-mediated cell death pathways in ZEN-treated various cells or tissues (Ben Salem et al. 2015; Lin et al. 2015; Long et al. 2016; Ren et al. 2017). However, little is known about the involvement of ER stress in ZEN-induced apoptosis in chicken.

According to the toxic effects of ZEN, researchers have investigated many chemical and/or biological substances with different properties to eliminate the adverse effects of mycotoxins. Several antioxidants like crocin, quercetin, and vitamin E have a strong protective effect against ZEN-induced toxicity (Abid-Essefi et al. 2003; Ben Salem et al. 2015). As an essential trace element, selenium (Se) plays an important role in the health and performance of animals. Se is involved in the protective effects of cells against excess ROS, and regulation of

the immune and reproductive systems due to its antioxidant properties (Long et al. 2016; Peng et al. 2010; Zhou et al. 2009). Se inhibited ultraviolet radiation-induced apoptosis in primary human keratinocytes (Rafferty et al. 2010). In LLC-PK1 cells, Se produced a significant protection against ROS-mediated apoptosis via mitochondrial dysfunction (Zhou et al. 2009). Also, in ZEN-caused reproductive system damage, high levels of Se improved antioxidant ability, and inhibited reproductive cell apoptosis (Long et al. 2016). In chicken, Se could ameliorate cadmium or lead-induced cytotoxicity, oxidative stress, ER stress, and apoptosis in the splenic lymphocytes, kidney, testis, ovary, and liver (Chen et al. 2012; Liu et al. 2015b; Wan et al. 2018; Wang et al. 2018). Moreover, a Se-deficient diet can cause the occurrence of oxidative stress and hepatocyte apoptosis (Yao et al. 2015), but dietary supplementation with Se reduced germ cells apoptosis in the testis during spermatogenesis in chicken (Song et al. 2015).

There are many studies for the toxic effects of ZEN and the protective effects of Se on many organs. In animals, spleen lymphocytes play a crucial role in immune system. Therefore, the disruption of spleen lymphocytes function can adversely affect immunologic function and disease resistance (Hemmi and Ishida 1980). The immune system is a potential target for ZEN, because various cells of the immune system have estrogen receptors (Sakazaki and Ueno 2002). The cytotoxicity of ZEN on spleen lymphocytes has been observed in different species (Chen et al. 2017; Ren et al. 2017). In chicken spleen lymphocytes, ZEN induced intracellular calcium imbalance and changed immune-related gene expression of IL-2, IL-6, and IFN- γ (Wang et al. 2012a, b). However, little is known about its effects on apoptosis and the molecular and cellular mechanisms in chicken spleen lymphocytes. In the current study, we evaluated the potential toxicity mechanism of ZEN in primary chicken spleen lymphocytes and further investigated the protective effects of Se on ZEN-induced cytotoxicity.

Materials and methods

Materials

ZEN, RPMI1640 medium, dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), acridine orange/ethidium bromide (AO/EB), and sodium selenite were purchased from Sigma (St Louis, Missouri, USA). Fetal bovine serum (FBS) was ordered from Gibco (Grand Island, NY, USA). Cell culture plates were supplied by Mediatech, Inc. (Cornig, Manassas, VA, USA). Annexin V-FITC and propidium iodide (PI) were purchased from ACTGene Inc., (Piscataway, NJ, USA). Quantitative real-time PCR (qPCR) kits were obtained from TaKaRa Bio, Inc. (Dalian, China). Anti-GRP78 antibody, anti-Bcl-2 antibody, anti-caspase-3 antibody, anti-

caspase-12 antibody, anti-ATF4 antibody, anti-ATF6 antibody, anti-IRE antibody, and anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (USA). All other reagents were purchased from Sigma, and all the used chemicals were of analytical grade.

Cell culture and treatment

All chickens in the test were obtained from Laboratory Animal Center, College of Veterinary Medicine, Northeast Agricultural University, China. Fifty 60-day-old Hyline cocks were fed food and water ad libitum. This study followed good laboratory practices (GLP), and the use of animal was approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

Chicken spleen lymphocytes were prepared and cultured according to the methods of Wang et al. (2012b). Briefly, spleen was taken into a petri dish, washed with sterile cooled PBS, and then ground at low temperature. The mixture was filtered through a 200-mesh sieve to collect spleen cell suspension. The lymphocytes were then collected by centrifuging (400×g, 15 min) in Histopaque 1077 (Sigma) at room temperature and washed twice with cooled PBS. The cells were re-suspended in RPMI 1640 medium (containing 10% FBS and 1% antibiotic-antimycotic solution) and counted using a hemocytometer. When the lymphocytes viability greater than 95% as tested by trypan blue exclusion, the cells were used for in vitro experiments.

The cells were adjusted to a density of 10^6 cell/mL and cultured in 6-well microplates at 2 mL/well under 5% CO₂ at 41.5 °C. After 24-h incubation, the cells were distributed into four treatment groups as follows: (1) control (without ZEN and Se), (2) treated with ZEN alone (5.0 µg/mL), (3) treated with Se alone (10^{-7} mol/mL), and (4) treated with Se (10^{-7} mol/mL) + ZEN (5.0 µg/mL). Each treatment was performed in quadruplicate and incubated at 41.5 °C in a humidified 5% CO₂ environment for 24 h. At the end of experiment, the cells were collected for biochemical and molecular assays, respectively. All experiments were repeated independently three times.

Determination of ROS level

The cells were collected after the treatments and the ROS levels in intracellular were determined using commercial kit (Sigma). In brief, the cells in each group were collected, washed three times with PBS, and then incubated with 2',7'-dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 30 min. The DCF fluorescence distribution of these cells was detected under an excitation wavelength of 488 nm and an emission wavelength of 530 nm using FACS Calibur flow cytometry (Becton-Dickinson, Franklin Lakes, USA).

Determination of antioxidant status

The cells antioxidant status was estimated by measuring the amount of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione (GSH), malondialdehyde (MDA), and catalase (CAT) using diagnostic kits obtained from Nanjing Jiancheng Bioengineering Research Institute (Zhao et al. 2010). Protein concentration of the cells was measured via the method reported by Bradford (1976).

Cell apoptosis assay by dual AO/EB staining and flow cytometry

Dual AO/EB fluorescent staining

After treatments with ZEN and/or Se, the cells were collected and washed with PBS by centrifugation (1500 r/min, 3 min). The cells were then stained with fluorescent staining solution (1 µL) containing 100 µg/mL AO (acridine orange) and 100 µg/mL EB (ethidium bromide) according to the method described by Liu et al. (2015a). The morphology of apoptotic cells was examined and 500 cells in each group were counted within 10 min using a fluorescent microscope (OLYMPUS, Japan).

Flow cytometry

After treatments with ZEN and/or Se, the cells were collected and washed with PBS by centrifugation (1500 r/min, 3 min). The cells were then stained with 10 µL Annexin V-FITC and 5 µL PI according to a previously described procedure (Eray et al. 2015). The fluorescent signal of the cells was measured with FACS Calibur flow cytometry (Becton-Dickinson).

Expression of genes

The total RNA in chicken spleen lymphocyte was isolated using Trizol (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. Isolated RNA was dissolved in RNase-free water (TaKaRa Bio, Inc., Dalian, China). The RNA concentration and purity were estimated by measuring absorbance at 260 and 280 nm on a spectrophotometer (Eppendorf, Inc., Hamburg, Germany). The 260/280 ratio of all samples was between 1.8 and 2.0. Purified RNA was subjected to reverse transcription to cDNA by FastKing RT Kit (With gDNase) (Tiangen Biotech, Beijing, China) following the manufacturer's instructions (Dai et al. 2015).

The gene expression was analyzed by qPCR using synthesized cDNA. QPCR experiments were performed with ABI PRISM 7500 Detection System (Applied Biosystems, USA). Briefly, for each gene, the qPCR reaction (total volume of 20 µL) contains 2 µL of cDNA, 10 µL of SYBR Premix Ex Taq, 0.4 µL of Rox Reference Dye II, 0.4 µL of forward and

reverse primer (10 μ M), and 6.8 μ L RNase-free water. Cycling conditions was as follows: a denaturation step at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 34 s. The specificity of qPCR product and quality of primer were confirmed by melting curve. All qPCRs were performed at least in triplicate. The data was normalized using GAPDH as the internal control gene and the relative gene expression was quantified using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The specific primers used for genes expression analysis are listed in Table 1.

Western blot analysis

Following treatment, cells for each group were washed with PBS, and total cell lysates were prepared as the method described by Pozio et al. (2002). The protein concentration was determined using a bicinchoninic acid (BCA) assay (Smith et al. 1985). Equal amounts of protein samples were separated by 12% SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred onto PVDF (polyvinylidene fluoride) membranes that were blocked with 5% dehydrated skim milk at room temperature for 2 h. Then, the membranes were incubated with the primary antibodies for GRP78 (1:1000), Bcl-2 (1:500), caspase-3 (1:100), caspase-12 (1:500), ATF4 (1:500), ATF6 (1:500), IRE (1:500), and GAPDH (1: 2000) overnight at 4 °C that was followed by incubation with horseradish peroxidase-conjugated secondary antibodies against rabbit IgG (1:1000) at room temperature for 1 h. The blots were visualized with enhanced chemiluminescence using ECL-plus reagent (GE Healthcare, Buckinghamshire, UK). The GAPDH was used

Table 1 Specific primers used for gene expression analysis by qPCR

Genes	Primer sequence (5'-3')
GPR78	F: GAATCGGCTAACACCAGAGGA R: CGCATAGCTCTCCAGCTCATT
ATF4	F: TCACCCAATGACAACCCG R: TCACCTTGCTGACGCTACC
ATF6	F: CGTCGTCTGAACCACTTACTGA R: CCTTCTTCTTAACAGCCACAC
Caspase-12	F: AATAGTGGGCATCTGGGTCA R: CGGTGTGATTAGACCCGTAAGAC
IRE	F: CTACAGGTGCTCCTCACATC R: ATCAGTCCTTCTGCTCCCATCT
Caspase-3	F: CTGAAGGCTCCTGGTTTA R: TGCCACTCTGCGATTTAC
Bcl-2	F: ATCGTCGCCTTCTTCGAGTT R: ATCCATCCTCCGTTGTCTT
GAPDH	F: ACCGCAAATGCTTCTAAACC R: CCAATCTCGTCTTGTTTTATGC

as an internal index. The optical density was quantified using Gel-pro Analyzer software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

The results are shown as the mean \pm standard error of the mean (SEM). The differences between different groups were analyzed using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Different lowercase letters indicate that there were statistical significance ($P < 0.05$) among different groups. Statistical analyses were carried out using SPSS version 20.0 software (SAS Institute Inc., Cary, NC).

Results

Level of ROS in chicken spleen lymphocyte

As shown in Fig. 1, ZEN induced a marked increase in the ROS production (39.7%) compared with control group (0.5%), whereas, co-treatment with Se significantly inhibited ROS formation that was reduced by 14.9% ($P < 0.05$). Se itself had no effect on the intracellular level of ROS ($P > 0.05$).

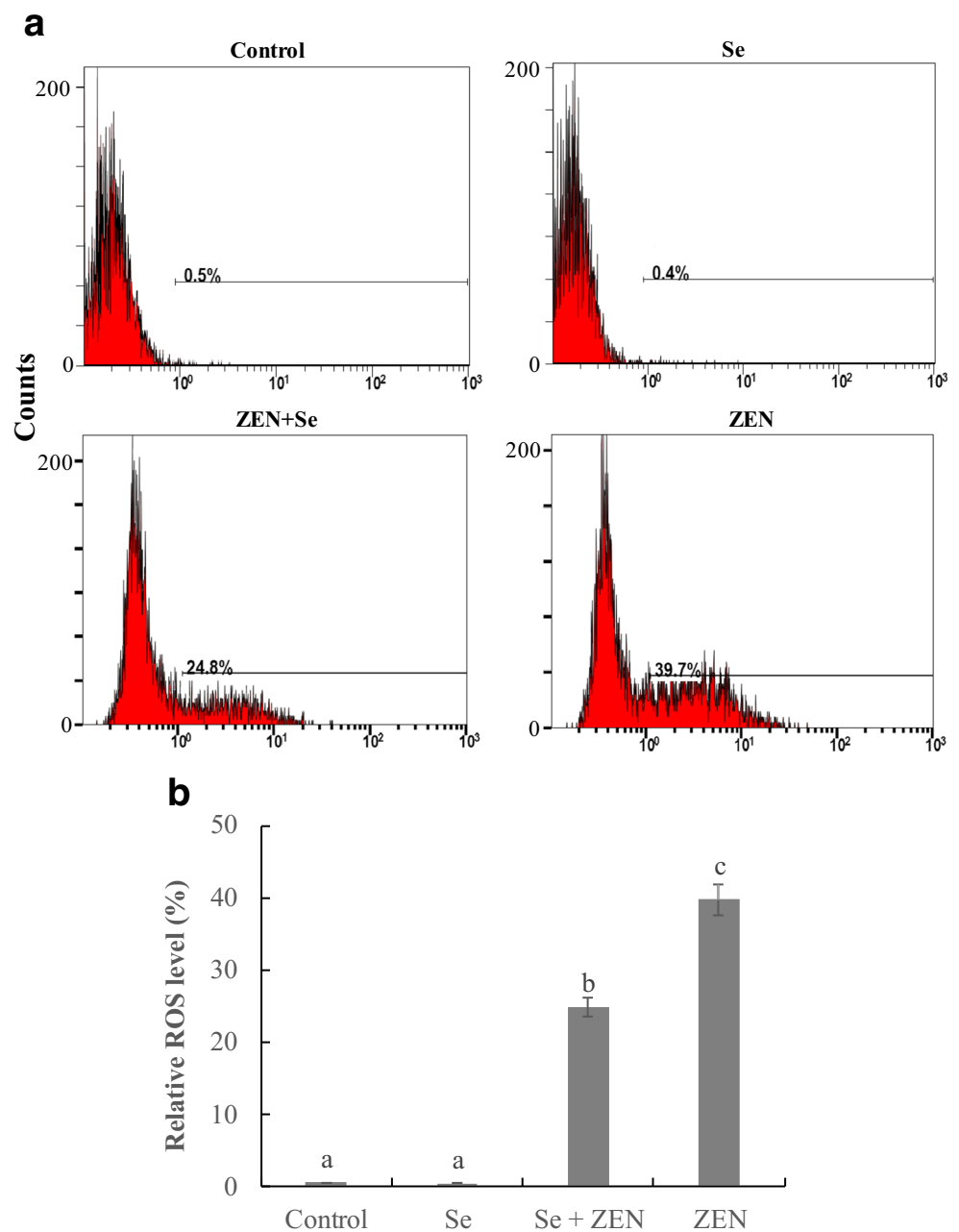
Antioxidant status in chicken spleen lymphocyte

The antioxidant status in chicken splenic lymphocyte was assessed by measuring SOD, GSH-Px, GSH, CAT, and MDA, and the results were depicted in Fig. 2. Compared to control group, treatment with ZEN alone resulted in a significant decrease in the levels of SOD, GSH-Px, GSH, and CAT, and a significant increase in formation of MDA in chicken splenic lymphocyte ($P < 0.05$). However, treatment with Se in combination with ZEN significantly suppressed the adverse changes of these parameters induced by ZEN alone ($P < 0.05$). Cells treated with Se alone showed a normal level in these parameters compared to control ($P > 0.05$).

Nuclear staining for assessment of apoptosis

After treatment with ZEN and/or Se for 24 h, the apoptotic cells were observed by AO/EB staining (Fig. 3a). The apoptotic cells had green or red-orange stained condensation nucleus. In the control group and Se-treated group, no obvious apoptosis was observed, but in treatment with ZEN alone, the number of apoptotic cells significantly increased. Moreover, the cell count showed that the apoptosis rate in treatment with Se + ZEN ($37.7 \pm 5.42\%$) was significantly lower than that in treatment with ZEN alone ($52.17 \pm 4.33\%$, $P < 0.05$) (Fig. 3b).

Fig. 1 The changes of ROS level in chicken splenic lymphocyte treated with Se and ZEN. **a** Cells were treated with control (untreated), Se, Se + ZEN, ZEN. **b** Relative ROS level, the data from three independent experiments represent the mean \pm SEM, different lowercase letters indicate that there are statistically significance ($P < 0.05$) among different groups



Flow cytometry for assessment of apoptosis

To further confirm the ZEN-induced apoptosis and anti-apoptosis of Se in chicken splenic lymphocyte, cells were treated with Se and ZEN and detected with flow cytometry-based annexin V-FITC/PI double staining. As shown in Fig. 4, more than 94% of the cells were living cells in control group and Se-treated group. Compared with the control group, the apoptosis cells were significantly increased to 42.1% and mainly showed late apoptosis (II) in treatment with ZEN alone ($P < 0.05$). But in the group treated with Se in combination with ZEN, the percentage of apoptotic cells was significantly decreased by 10.5% ($P < 0.05$).

The expression of apoptosis-related genes and proteins

As shown in Fig. 5, compared with the control group, the treatment with ZEN alone significantly upregulated the expression of caspase-3 and caspase-12 genes and proteins, and downregulated the Bcl-2 gene and protein expression. In contrast, when the splenic lymphocyte were co-treated with Se and ZEN, the mRNA and protein levels of caspase-3 and caspase-12 were decreased, and the mRNA and protein levels of Bcl-2 was increased compared with that in the ZEN-treated groups ($P < 0.05$).

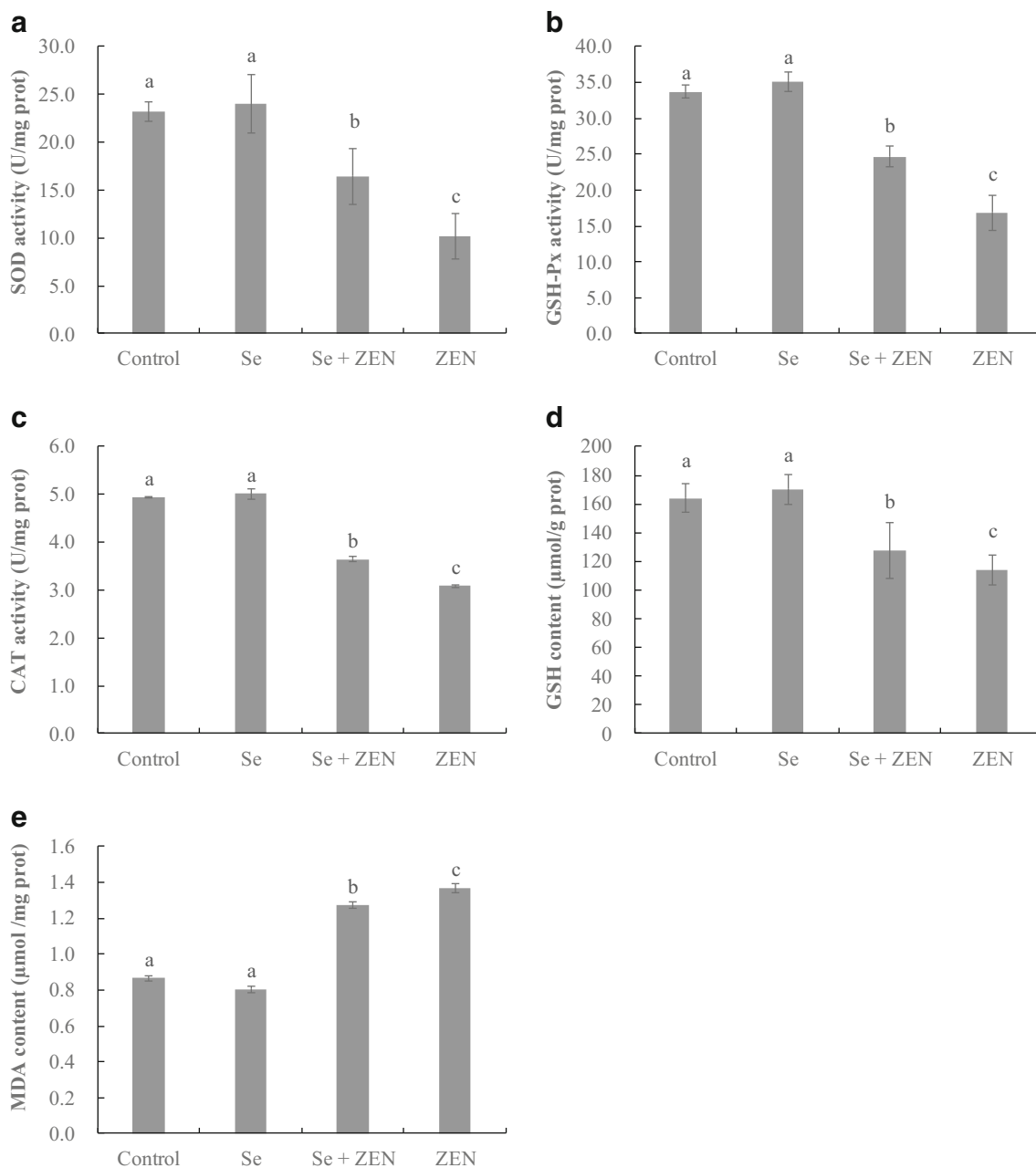


Fig. 2 a–e The changes of antioxidant capacity and lipid peroxidation in chicken splenic lymphocyte treated with Se and ZEN. The data represent the mean \pm SEM ($n = 4$); different lowercase letters indicate that there are statistically significance ($P < 0.05$) among different groups

The expression of ER stress-related genes and proteins

To examine the participation of the ER stress response in chicken splenic lymphocyte apoptosis, the mRNA and protein levels of GRP78, ATF4, ATF6, and IRE were analyzed by qPCR and Western Blot (Fig. 6). The mRNA and protein levels of GRP78, ATF4, ATF6, and IRE significantly increased in treatments with ZEN alone compared to control groups ($P < 0.05$), whereas the upregulation of the four genes and proteins was significantly suppressed in treatment with Se

+ ZEN ($P < 0.05$). Moreover, there was no difference between control and Se-treated group.

Discussion

ZEN, as a fusariotoxin, is harmful to animal health including immunotoxicity, oxidative stress and apoptosis (Zinedine et al. 2007). However, there are few reports of the effects of ZEN on apoptosis in chicken spleen lymphocyte. This study extends the knowledge of ZEN toxicity on chicken.

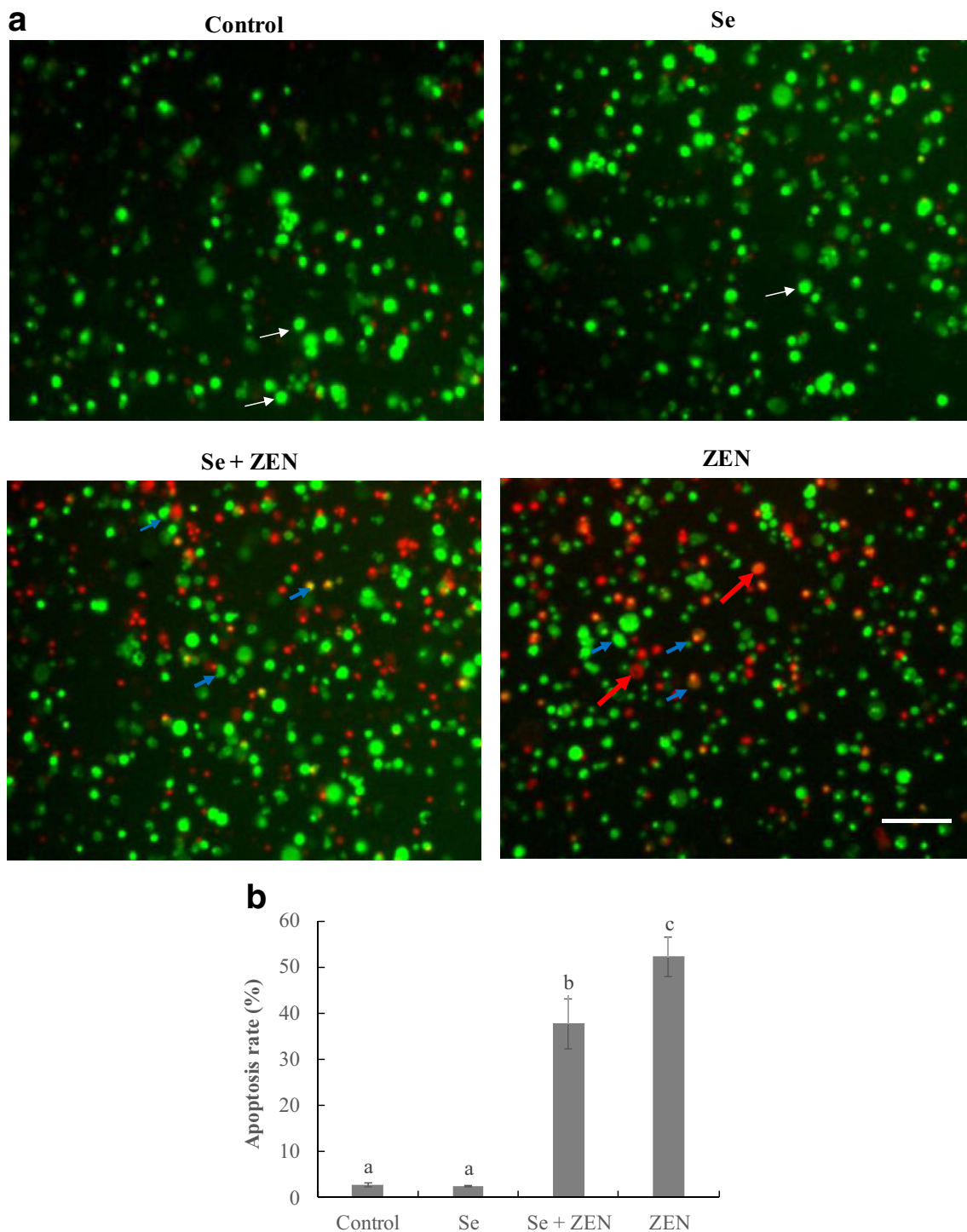


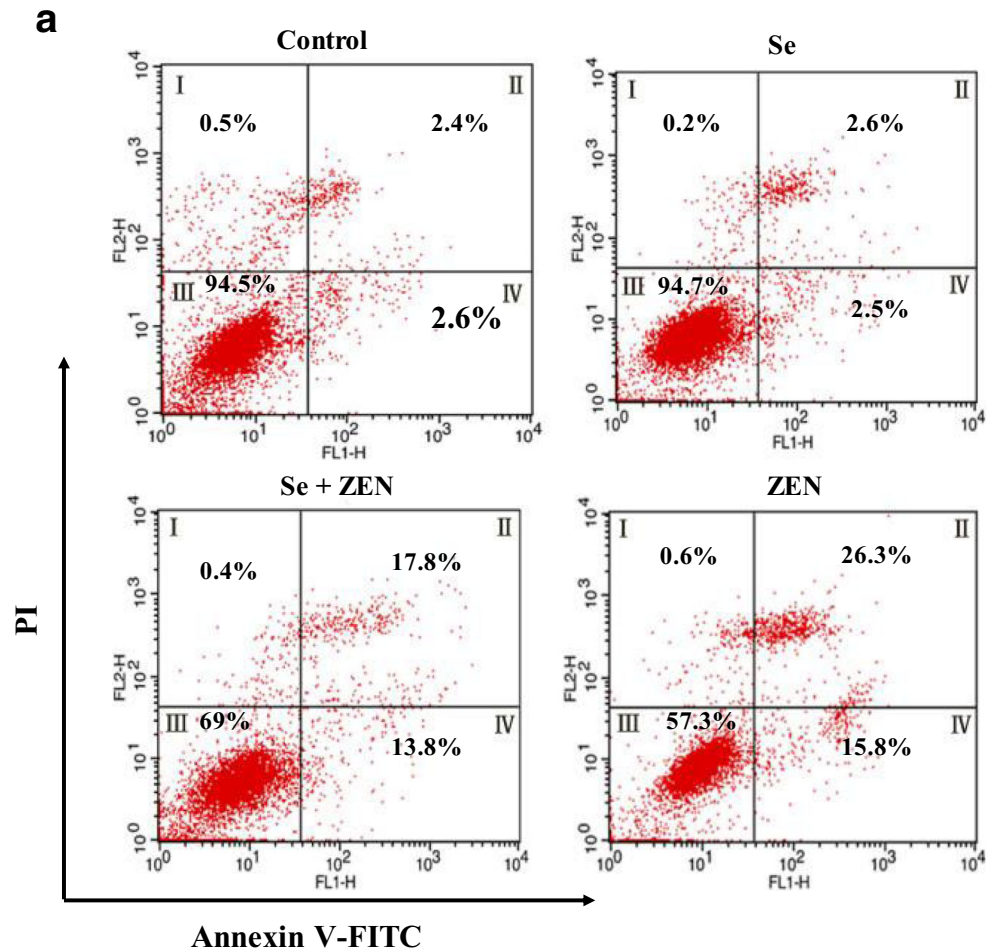
Fig. 3 Observation of morphological apoptosis in chicken splenic lymphocyte treated with ZEN and Se. **a** Fluorescence images determined by staining with acridine orange and ethidium bromide

ZEN is a potent inducer of ROS in animals (Ben Salem et al. 2017; Wang et al. 2014). Excessive ROS can consume antioxidant components (SOD, CAT, GPx, and GSH), and reduce antioxidant capacity (Newsholme et al. 2016). In the present study, we demonstrate that ZEN triggered oxidative

(AO/EB). The cells were treated with control (untreated), Se, Se + ZEN, and ZEN. White indicate arrows live cells; blue arrows indicate apoptotic cells; red arrows indicate necrotic cells

stress in spleen lymphocytes as evidenced by the overproduction of intracellular ROS. Meanwhile, the levels of antioxidant components (SOD, CAT, GPx, and GSH) were remarkably depressed in treatment with ZEN alone. ZEN-induced oxidative stress also attacked polyunsaturated fatty

Fig. 4 Changes of apoptosis in chicken splenic lymphocyte treated with ZEN and Se. Apoptosis was evaluated by flow cytometry using annexin V/PI staining. **a** The cells were treated with control (untreated), Se, Se + ZEN and ZEN. Quadrants: I, necrotic cells; II, late apoptosis cells; III, living cells; and IV, early apoptosis cells. **b** Columns, mean of three experiments, and the data represent the mean \pm SEM; different lowercase letters indicate that there are statistically significance ($P < 0.05$) among different groups



acids and induced lipid peroxidation that resulted in damage to cell structures (Jia et al. 2015). In our work, the increased MDA level indicated a lipid peroxidation formation in ZEN-treated group. These results are consistent with the findings of increased lipid peroxidation level with the concomitant decrease in antioxidative capacity showed a pronounced

oxidative stress, and the decrease of GPx level may be due to the conjugation with ZEN or its metabolites (Borutova et al. 2008). Moreover, ZEN could impair glutathione system balance that resulted in decrease of GSH level in a time and concentration-dependent manner (Hassen et al. 2007).

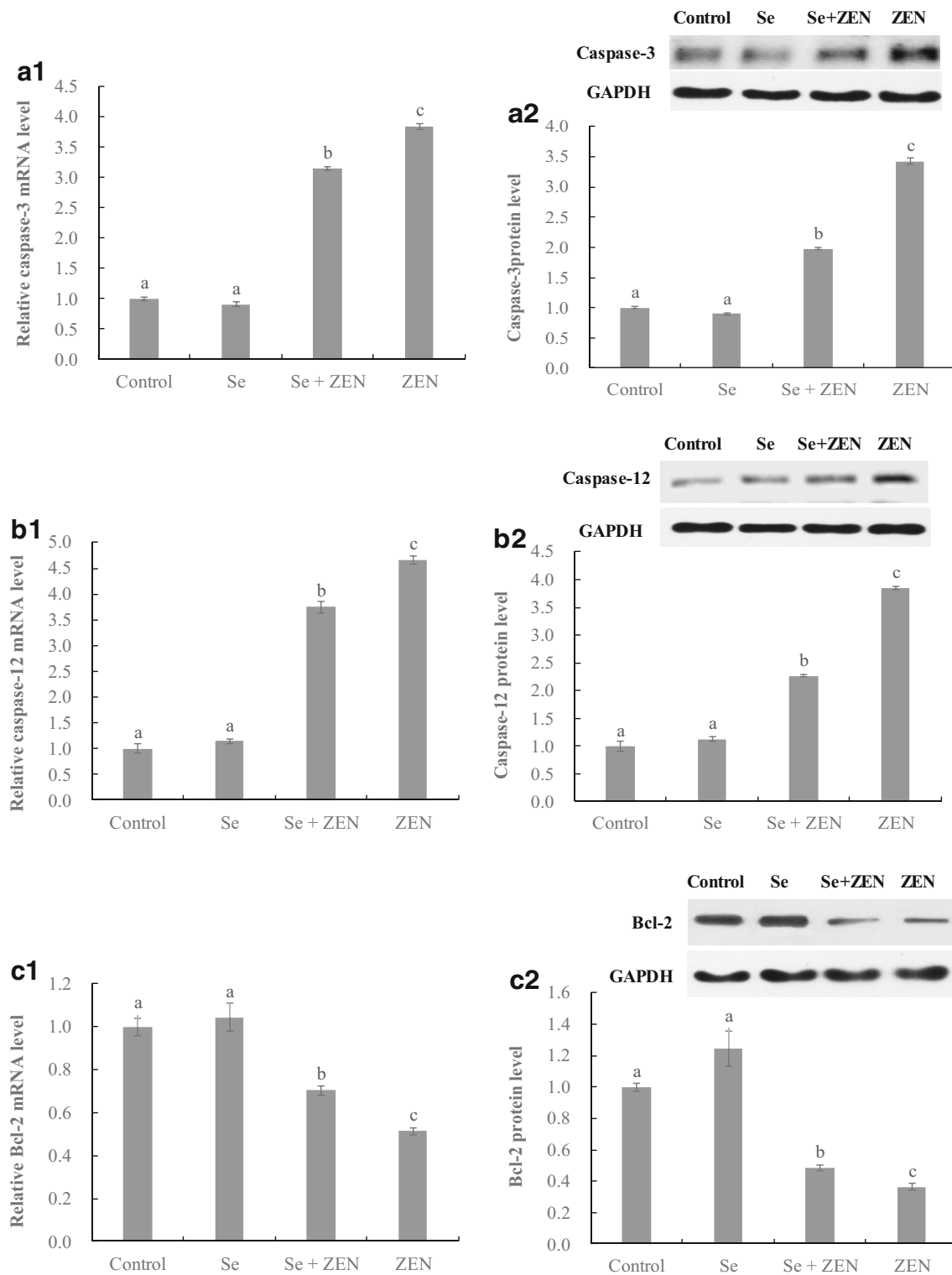


Fig. 5 The expression of apoptosis-related genes and proteins in chicken splenic lymphocyte treated with ZEN and Se. Data with different letters are significantly different ($P < 0.05$) among treatments. The values are normalized to control values and expressed as mean \pm SEM ($n = 4$)

The activation of apoptosis is an important molecular consequence of ZEN toxicity (Shen et al. 2015). In the present study, the results of apoptosis morphologically from AO/EB fluorescent staining and flow cytometry analysis showed that

ZEA-induced apoptosis in primary chicken spleen lymphocytes. Similar results were also reported in other ZEN-treated porcine splenic lymphocytes, cardiac cells, and Leydig cells (Ben Salem et al. 2017; Ren et al. 2017; Wang

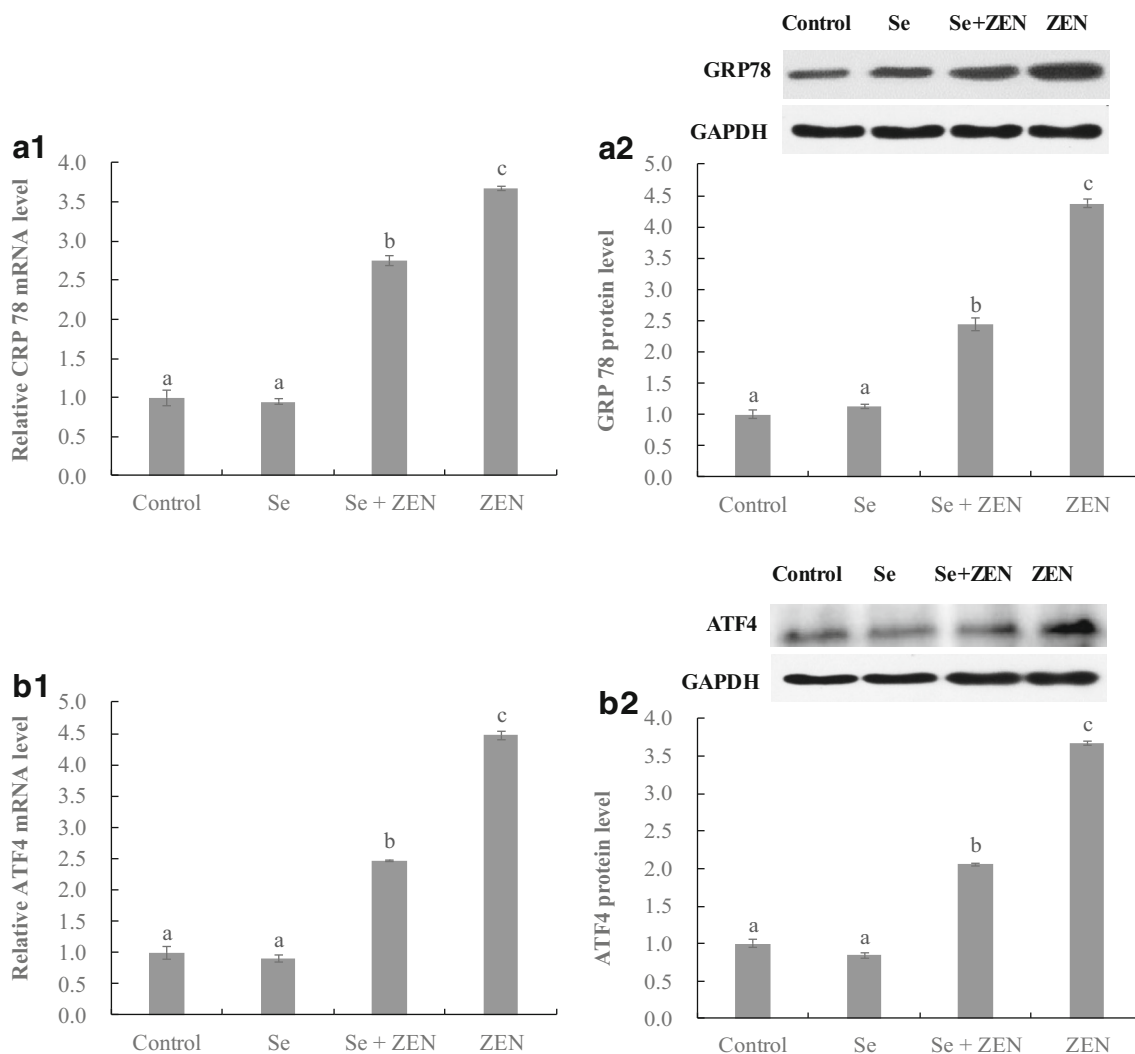


Fig. 6 The expression of ER stress-related genes and proteins in chicken splenic lymphocyte treated with ZEN and Se. Data with different letters are significantly different ($P < 0.05$) among treatments. The values are normalized to control values and expressed as mean \pm SEM ($n = 4$)

et al. 2014). In apoptotic responses of cells (mitochondrial-mediated or death receptor-dependent), caspases play crucial roles. Caspase-3 is the major executioner caspase in apoptosis, and caspase-12 mainly mediates ER-specific apoptosis (Earnshaw et al. 1999). Many investigations have demonstrated that ZEN could trigger apoptosis via caspase-dependent pathway in some cells. Caspase-3, -9 were activated in ZEN-toxicated rat Leydig cells, indicating that ZEN participated in the activation of the caspase cascade to induce apoptosis (Wang et al. 2014). Caspase-3, and -8 protein levels in ZEN-treated porcine splenic lymphocytes led to the activation of caspases and initiation of cell apoptosis (Ren et al. 2017). In the present study, we also discovered the upregulation of caspase-3, and -12 upon ZEN-treatment, suggesting that ZEN could induce caspase-dependent apoptosis in chicken spleen lymphocytes, and the apoptosis pathway might be related to ER stress.

Bcl-2, as an effector molecule in the apoptotic response, is a potent anti-apoptotic protein. Bcl-2 was suppressed in many types of cell apoptosis induced by different stimuli, such as oxidative stress, drugs, or virus (Liao et al. 1997; Takahashi et al. 2004). Similarly, Bcl-2 was involved in ZEN-induced apoptosis, and the level was decreased in rat Leydig cells (Lin et al. 2015). In vivo, chronic ZEN administration downregulated the Bcl-2 gene expression in mice reproductive system (Long et al. 2016). In line with previous reports, our study showed that ZEN downregulated Bcl-2 gene and protein expression that triggered apoptosis in chicken spleen lymphocytes. Our results also suggest that the balance of Bcl-2 level might be a key step for ZEN induction of apoptosis and eventually cell death.

Growing evidence suggests that oxidative stress leads to excessive oxidative modifications of proteins that induce ER stress and UPR activation (Anantharam et al. 2008; Verfaillie et al. 2012). Generally, the UPR is an adaptive cellular

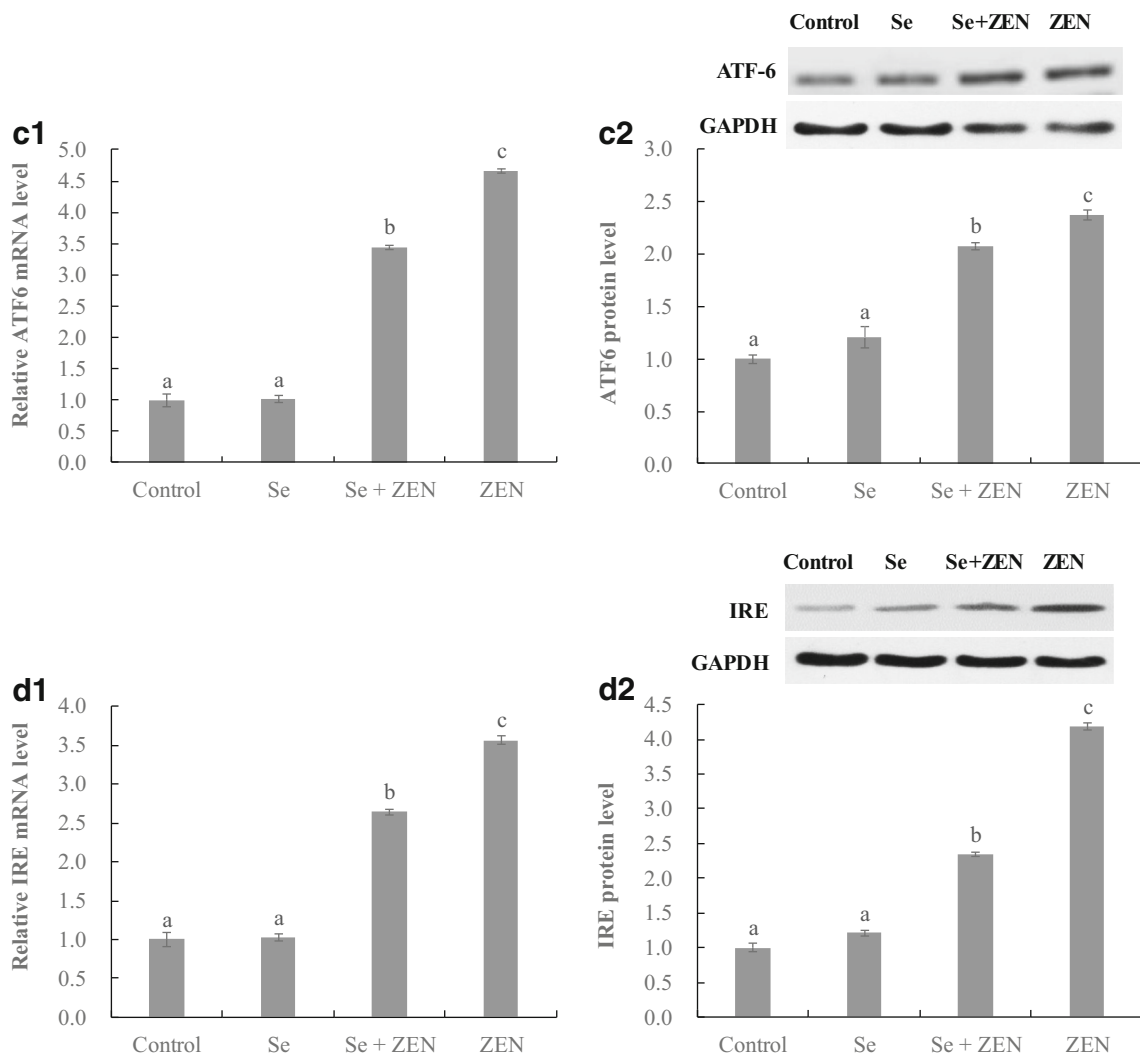


Fig. 6 continued.

response and can restore homeostasis in the ER by expressing numerous chaperone proteins (e.g., GRP78) and transcription factors (e.g., ATF 4 and ATF6). But, prolonged or overloading ER stress restrains the UPR scavenging capacity, and eventually leads to cell apoptosis (Bánhegyi et al. 2010). Here, we observed the activation of ER stress as evidenced by upregulation of molecular sensors including GRP78, IRE, ATF6, and ATF4 after treatment with ZEN, and correspondingly, the caspase-12 was also upregulated and apoptosis occurred. These results suggested that the ER stress signaling pathway played a vital role in the regulation of ZEN-induced apoptosis in chicken spleen lymphocytes. Similar results were reported in other studies. ER stress was the mechanism involved in ZEN-induced apoptotic death in human leukemic cells, where ZEN upregulated GRP78 and calreticulin, and then triggered apoptosis (Banjerdpongchai et al. 2010). HCT116 cells treated with ZEN resulted in induction of ER stress as demonstrated by increase of GRP78, ATF6, and ATF4 protein and mRNA levels (Ben Salem et al. 2015). Similar findings were also

confirmed in mouse Leydig cells, showing that treatment with ZEN caused apparent dose-dependent upregulation in the protein levels of GRP78, CHOP, and caspase-12 (Lin et al. 2015). These observations suggest that activation of ER stress may be a common feature of mycotoxin toxicity.

Se, an effective antioxidant agent, is known to improve antioxidant capacity and provide protection from ROS-induced cell damage (Tapiero et al. 2003). Indeed, our results showed that co-treatment with Se and ZEN effectively attenuated the ROS generation, lipid peroxidation formation, and antioxidant components depletion, suggesting Se could restrain the ZEN-induced oxidative stress and reduce ZEN toxicity by scavenging free radical in chicken spleen lymphocytes. Interestingly, the phenomenon was supported in previous studies, where Se could enhance the decrease of SOD, CAT, GSH, and GSH-Px levels, and suppress MDA formation as induced by lead and cadmium in chicken (Jiao et al. 2017; Liu et al. 2015b). Moreover, in ZEN-induced toxicity, Se had protective effects on reproductive system damage in mice that

might be associated with recovering activity of antioxidant enzymes and decreasing free radical-mediated lipid peroxidation (Long et al. 2016).

Furthermore, Se appears to be mediated through its anti-apoptosis effect to protect against tissue or cell damage. Previous studies have demonstrated that Se significantly ameliorated apoptosis and the changes of apoptosis-related genes or proteins via decreasing the expression of the ER stress-related genes or proteins GRP78, GRP94, ATF4, ATF6, and IRE in cadmium or lead-treated chicken kidney and ovary (Liu et al. 2015b; Wan et al. 2018; Wang et al. 2018). Also, our results showed that Se clearly inhibited ER stress and apoptosis induced by ZEN exposure. An important observation in this study is that Se restrained the expression of ER stress-related genes and proteins (GRP78, ATF6, ATF4, and IRE), upregulated the Bcl-2 gene and protein, and attenuated the caspase-12 and -3 activities and apoptosis ratio. These findings, taken together, indicated that ER stress signal pathway played important role in protection of Se against ZEN-induced apoptosis.

Conclusion

This study provided new insight into the molecular mechanisms of ZEN toxicity in chicken spleen lymphocytes, and a way to reduce the toxicity of ZEN. We demonstrated that ZEN increased ROS generation, induced ER stress and triggered apoptosis in chicken spleen lymphocytes. Further, we also observed that Se protect cells from ZEN toxicity by inhibiting ER stress. We speculate that antioxidant treatment might be helpful to prevent ZEN-related apoptosis through ER stress signaling pathway in chicken spleen lymphocytes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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