

# Effects of zearalenone on calcium homeostasis of splenic lymphocytes of chickens in vitro

Y. C. Wang,\* J. L. Deng,\*<sup>1</sup> S. W. Xu,†<sup>1</sup> X. Peng,‡ Z. C. Zuo,§ H. M. Cui,‡ Y. Wang,§ and Z. H. Ren§

\*College of Veterinary Medicine, Sichuan Agricultural University, Yaan, Sichuan 625014, P.R. China;

†Department of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, P.R. China;

‡Sichuan Key Laboratory of Animal Disease and Human Health, Yaan, Sichuan 625014, P.R. China;

§Sichuan Key Laboratory of Environmental Hazards Disease, Yaan, Sichuan 625014, P.R. China

**ABSTRACT** Zearalenone (ZEA) is an estrogenic mycotoxin. It is produced by several *Fusarium* species and can contaminate food and feed. To investigate the role of calcium homeostasis in ZEA-induced toxicity of poultry and elucidate its cytotoxic mechanism, splenic lymphocytes isolated from chickens were exposed to ZEA (0–25 µg/mL) for 48 h. The intracellular calcium concentration ( $[Ca^{2+}]_i$ ), pH, calmodulin (*CaM*) mRNA levels, and  $Na^+/K^+$ -ATPase activities and  $Ca^{2+}$ -ATPase activities were detected by the fluorescent dyes Fluo-3/AM and BCECF/AM, quantitative real-time PCR, and chromatometry. Supernatant CaM concen-

trations were simultaneously detected by ELISA. As the ZEA exposure concentration increased, the  $[Ca^{2+}]_i$  and *CaM* mRNA levels gradually increased, while intracellular pH, CaM concentrations of supernatants, and intracellular  $Na^+/K^+$ -ATPase and  $Ca^{2+}$ -ATPase activities gradually decreased in a dose-dependent manner. There were significant differences ( $P < 0.05$  or  $P < 0.01$ ) between the treatment groups and the control group. These results indicate that ZEA cytotoxicity arises by causing an imbalance in calcium homeostasis and intracellular acidification in lymphocytes.

**Key words:** zearalenone, calcium homeostasis, ATPase, calmodulin, mycotoxin

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## INTRODUCTION

Mycotoxins are toxic metabolites of fungi that are commonly found in foods and animal feeds, and they cause serious health problems in both animals and humans. Zearalenone (ZEA), also known as F-2 mycotoxin, is a main mycotoxin produced by fungi in foods and feeds. It is also one of the most common contaminants of cereal grains worldwide (Collins et al., 2006). The molecular mechanism of ZEA toxicity is poorly understood, but because the chemical structure of ZEA is similar to that of 17β-estradiol, ZEA can bind to estrogen receptors and is involved in estrogen-mediated events (Martin et al., 1978; Miksicek, 1994). It has been frequently implicated in hyperestrogenism and other reproductive disorders in laboratory and farm animals (Gutleb et al., 2002; Abid-Essefi et al., 2004; Cetin and Bullerman, 2005; Kouadio et al., 2005; Hassen et al., 2007; Bouaziz et al., 2008). In humans, ZEA has been associated with precocious pubertal changes

and endometrial adenocarcinoma and hyperplasia in women (Abid-Essefi et al., 2004; Hassen et al., 2007). Moreover, ZEA was found to be hepatotoxic, disruptive toward hematological parameters, and associated with several alterations of immunological parameters in humans and rodents (Abid-Essefi et al., 2004; Hassen et al., 2007). Zearalenone exerts several in vivo and in vitro genotoxic effects (Abid-Essefi et al., 2004; Cetin and Bullerman, 2005; Kouadio et al., 2005; Hassen et al., 2007; Bouaziz et al., 2008). It also induces lipid peroxidation and cell death and inhibits protein and DNA syntheses (Abid-Essefi et al., 2004; Kouadio et al., 2005; Hassen et al., 2007; Bouaziz et al., 2008). Recently, it was demonstrated that ZEA reduced cell viability in correlation with increases in generation of reactive oxygen species and formation of malondialdehyde in concentration- and time-dependent manners. This suggests that cytotoxicity and generation of reactive oxygen species are mechanisms of mycotoxin-mediated toxicity (Ferrer et al., 2009).

However, very few studies have shown the toxicity of ZEA in cell homeostasis. Notably, the exact mechanism of ZEA toxicity has not been completely elucidated (Abdellah et al., 2007). It is well known that cell

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<sup>1</sup>Corresponding authors: dengjl213@126.com and shiwenxu@neau.edu.cn

homeostasis is a major factor in maintenance of normal cell function. Intracellular dyshomeostasis, as occurs during oxidative stress, results in pH and ion concentration disorders, especially cellular calcium homeostasis disruptions, which may cause serious damage or death. Therefore, in the present study, we investigated the *in vitro* effects of different doses of ZEA on intracellular pH, calcium concentration, and ATPase activity in chicken spleen lymphocytes to reveal the signaling pathway underlying the cytotoxicity of ZEA.

## MATERIALS AND METHODS

### Reagents

Zearalenone, RPMI 1640 medium, Histopaque 1077, chicken calmodulin (CaM) ELISA Kit, Fluo-3/AM, and 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein, acetoxymethyl ester (BCECF/AM) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum was purchased from Sijiqing Biological Engineering Materials Co. Ltd. (Hangzhou, China). The Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase analysis kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Trizol reagent was purchased from Invitrogen Biotechnology Co. Ltd. (Shanghai, China). The SYBR PremixScript real-time (RT)-PCR Kit II was purchased from TaKaRa (Shiga, Japan).

### Cell Culture

All procedures in this experiment were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. The spleens of 2-month-old Isa Brown chickens were ground at low temperature and teased through a 200-mesh cell strainer into a Petri dish containing phosphate-buffered saline. The cell suspension was overlaid onto Histopaque 1077 and centrifuged at 400 × *g* for 15 min at room temperature. The lymphocytes at the interface were collected, washed twice with PBS at 250 × *g* for 5 min at room temperature, and suspended in RPMI-1640 medium (without phenol red, a weak estrogen mimic) supplemented with 10% fetal calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. More than 95% of cells were viable based on trypan blue dye exclusion. The spleen cells were cultured in 6-well tissue culture plates (6 × 10<sup>6</sup> cells/mL) in triplicate and stimulated with a range of ZEA concentrations (0, 0.1, 0.4, 1.6, 6.25, and 25 μg/mL) at 41.5°C in a humidified 5% CO<sub>2</sub> environment for 48 h (Okamura et al., 2004). The mycotoxin concentrations were selected based on preliminary dose-response experiments (data not shown). After 48 h of incubation, supernatants were collected for analysis of CaM concentration and frozen at -80°C, while cells were collected for RNA isolation, intracellular pH, intracellular calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and Ca<sup>2+</sup>-ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase activities. All tests were performed in 3 independent experiments.

### Measurement of Intracellular pH with BCECF

Intracellular pH was measured by loading cells with the membrane-impermeant dye BCECF. This detailed procedure was performed according to Hirpara et al. (2001). Briefly, harvested cells were incubated with BCECF/AM (2 μM final concentration) in 1 mL of serum-free culture medium. After 30 min of incubation in the CO<sub>2</sub> incubator at 37°C, cells were pelleted, rinsed twice with PBS buffer, and resuspended at an appropriate density for fluorescence measurements. A minimum of 10,000 cells was analyzed, and the ratio of BCECF fluorescence at 525 and 610 nm was used to obtain intracellular pH from a calibration curve.

### Analysis of [Ca<sup>2+</sup>]<sub>i</sub>

Fluo-3/AM was chosen for use as an intracellular free calcium fluorescent probe for analysis of [Ca<sup>2+</sup>]<sub>i</sub> in ZEA-exposed cells (Harrison et al., 1993; Guthrie et al., 2011). The harvested cells were loaded with Fluo-3/AM (1 μM final concentration) for 30 min in the dark at 37°C and then washed with D-Hank's solution. Intracellular [Ca<sup>2+</sup>]<sub>i</sub> levels were represented by the fluorescence intensity (FL1, 530 nm) of 10,000 cells on a flow cytometer. Fluorescence intensity of cells and the background were measured and recorded. The relative fluorescence intensity represented the [Ca<sup>2+</sup>]<sub>i</sub>.

### Activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase

A minimum of 6 × 10<sup>7</sup> cells was analyzed, and the harvested cells were homogenized in ice-cold physiological saline in an ultrasonic disintegrator. The cell homogenates were centrifuged at 1,000 × *g* for 10 min. Supernatants were obtained and their protein concentration was determined by the Folin-phenol method, using bovine serum albumin (BSA) as a standard (the protein concentration in the assay was 3–5 mg/mL; Lowry et al., 1951). Activities of Na<sup>+</sup>/K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase from supernatants were determined as described by Bonting (1970) and Hjertén et al. (1983), respectively. The activities were indirectly measured by estimating the phosphorous liberated after the incubation of supernatants in a reaction mixture containing the substrate ATP with the cosubstrate elements at 37°C for 15 min. The reactions were arrested by adding 1.0 mL of 10% trichloroacetic acid (TCA). The phosphorus content from the TCA supernatants was then determined as described by Fiske and Subbarow (1925). The ATPase activity was expressed as micromoles of phosphorus liberated in 1 min per milligram of protein at 37°C.

### Quantification of CaM mRNA

Total RNA was isolated from cells using Trizol reagent according to the manufacturer's instructions. The

**Table 1.** Real-time PCR primer sequences and products

Gene	Primer	Sequence (5'→3')	Product size (bp)
β-actin	β-Actin forward	CACCACAGCCGAGAGAGAAAT	135
	β-Actin reverse	TGACCATCAGGGAGTTCATAGC	
CaM	CaM forward	GATGGAGTTGGTAAAATGAGGGAA	166
	CaM reverse	ACGCACTGGAAAACACTAGGGTCA	

RNA concentrations were determined using the GeneQuant 1300.

The reverse-transcription reaction (40 μL) consisted of the following: 10 μg of total RNA, 1 μL of M-MLV reverse transcription, 1 μL of RNase inhibitor, 4 μL of dNTP, 2 μL of Oligo dT, 4 μL of dithiothreitol, and 8 μL of 5× buffer. The reverse transcription was performed according to the manufacturer's instructions (Invitrogen), and the reverse-transcription products (cDNA) were then stored at −20°C for PCR.

To design primers, we used the chicken *CaM* mRNA GenBank sequence with the accession number NM\_205005.1. Chicken β-actin (GenBank accession number L08165.1), a housekeeping gene, was used as the internal reference. The primers (Table 1) were designed using Prime 5 Software (Molecular Biology Insights, Inc., Cascade, CO) and were synthesized by Invitrogen Biotechnology Co. Ltd.

Real-time PCR was performed to detect the expression of *CaM* and β-actin genes in different cDNA samples using SYBR Premix Ex *Taq* (TaKaRa). Each sample was assayed 3 times. The reaction mixtures were incubated in an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The program included 1 cycle at 95°C for 30 s and 40 cycles at 95°C for 5 s and 60°C for 34 s. The dissociation curves were analyzed using Dissociation Curves 1.0 Software (Applied Biosystems) for each PCR reaction to detect and eliminate possible primer-dimer and nonspecific amplification. Results (fold changes) are expressed using the Pfaffl method (Chini et al., 2007; Cikos et al., 2007) with the following formula:

$$\text{Ratio} = \frac{[(E_{\text{target}})^{\Delta\text{CT},\text{target}(\text{calibrator-test})}]}{[(E_{\text{ref}})^{\Delta\text{CT},\text{ref}(\text{calibrator-test})}]}$$

where  $\Delta\text{CT},\text{target}(\text{calibrator-test})$  equals  $(\text{CT}_{\text{target}})_{\text{control group}} - (\text{CT}_{\text{target}})_{\text{treatment group}}$ ;  $\Delta\text{CT},\text{ref}(\text{calibrator-test})$  equals  $(\text{CT}_{\text{ref}})_{\text{control group}} - (\text{CT}_{\text{ref}})_{\text{treatment group}}$ ;  $E_{\text{target}}$  is the amplification efficiency of target genes; and  $E_{\text{ref}}$  is the amplification efficiency of the housekeeping gene.

### CaM Concentration of Culture Supernatants: ELISA

The concentration of CaM in supernatants was measured by ELISA using ELISA development kits for CaM. ELISA plates (96-well) were coated in quadruplicate with 100 μL of culture supernatant per well in

carbonate-bicarbonate buffer, as previously described (Döll et al., 2009), and the plates were incubated for 20 min at room temperature and at 4°C overnight. After washing 6 times with PBS containing 0.05% Tween (**PBS-T**), 100 μL per well of 1% BSA-PBS was added, the plates were incubated for 1 h at room temperature, the liquid was decanted, and 50 μL of a predetermined dose of monoclonal antibody against chicken CaM was added to each well. The plates were incubated for 1 h at room temperature and washed 5 times with PBS-T. Peroxidase-conjugated goat anti-mouse IgG antibody (100 μL per well, 1:2,000; Sigma) in 0.1% BSA-PBS was added, and the plates were incubated for 1 h at room temperature and washed. Peroxidase substrate (100 μL per well; Sigma) was then added. After 5 min of incubation, substrate reaction was stopped with 50 μL per well of 2 N H<sub>2</sub>SO<sub>4</sub>, and the optical density values were measured at 450 nm (Bio-Rad, Hercules, CA). Each assay was performed in triplicate.

### Statistical Analysis

Results were expressed as mean ± SD from at least 3 independent experiments with different batches of cells, each one performed in duplicate or triplicate. Statistical comparisons were made using one-way ANOVA and Tukey's post-test. Differences between groups were considered significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ).

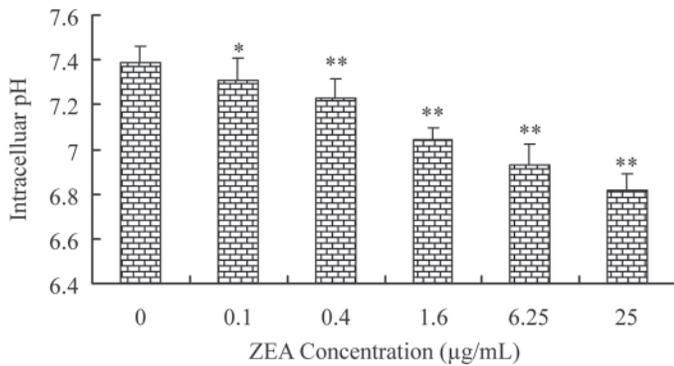
## RESULTS

### Intracellular Acidification Due to the Induction of ZEA

As shown in Figure 1, treatment of splenic lymphocytes with ZEA at concentrations ranging from 0 to 25 μg/mL consistently decreased the intracellular pH. These results were statistically significant ( $P < 0.05$ ) at a toxin concentration of 0.1 μg/mL and very significant ( $P < 0.01$ ) at toxin concentrations of 0.4, 1.6, 6.25, and 25 μg/mL.

### Effect of ZEA on [Ca<sup>2+</sup>]<sub>i</sub>

As shown in Figure 2, treatment of splenic lymphocytes with ZEA resulted in abnormal manifestations in [Ca<sup>2+</sup>]<sub>i</sub>, showing significantly ( $P < 0.01$ ) stronger [Ca<sup>2+</sup>]<sub>i</sub> fluorescence intensities at 48 h in a dose-dependent manner.



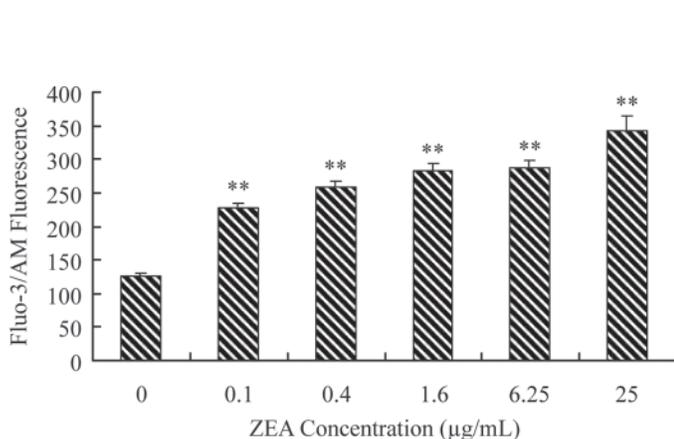
**Figure 1.** Effect of zearalenone (ZEA) on intracellular pH of splenic lymphocytes. Each bar represents mean  $\pm$  SD ( $n = 5$ ). \* $P < 0.05$  and \*\* $P < 0.01$  compared with controls using one-way ANOVA.

### Relative Quantification of CaM Expression Levels

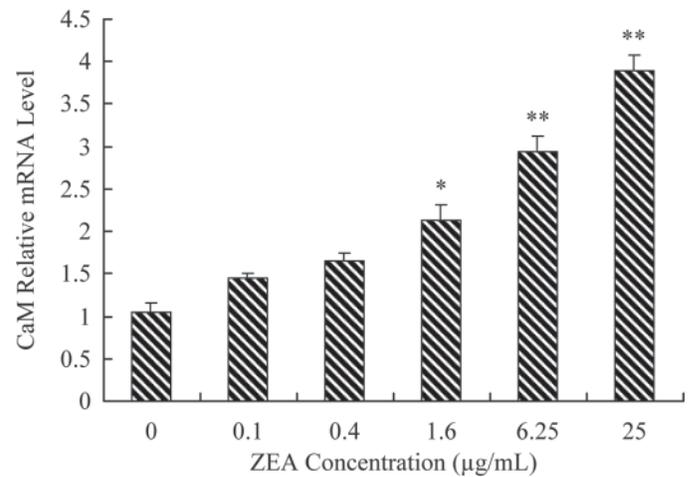
As shown in Figure 3, treatment of splenic lymphocytes with ZEA at concentrations ranging from 0 to 25  $\mu\text{g/mL}$  consistently increased the *CaM* mRNA expression levels. These results were statistically significant ( $P < 0.05$ ) at a toxin concentration of 1.6  $\mu\text{g/mL}$  and very significant ( $P < 0.01$ ) at toxin concentrations of 6.25 and 25  $\mu\text{g/mL}$ .

### CaM Concentrations of Supernatants

As shown in Figure 4, concentrations of CaM were analyzed in supernatants of splenic lymphocytes incubated for 48 h with increasing concentrations of ZEA (0–25  $\mu\text{g/mL}$ ). Supernatant concentrations of CaM in splenic lymphocytes incubated with 0.1 and 0.4  $\mu\text{g/mL}$  ZEA were slightly lower than those in the control groups. However, these decreases were statistically significant ( $P < 0.01$ ) at toxin concentrations of 1.6, 6.25, and 25  $\mu\text{g/mL}$ .



**Figure 2.** Effects of zearalenone (ZEA) on the intracellular calcium concentration of splenic lymphocytes as measured by flow cytometry. Each bar represents mean  $\pm$  SD ( $n = 5$ ). \*\* $P < 0.01$  compared with controls using one-way ANOVA.



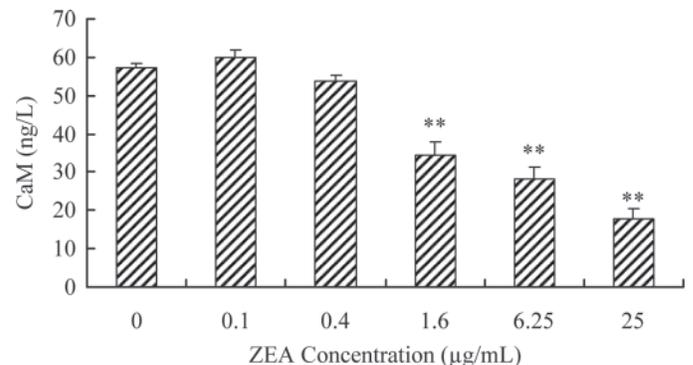
**Figure 3.** The graph represents the relative transcription levels of the gene calmodulin (*CaM*) in splenic lymphocytes of chicken exposed to zearalenone (ZEA) for 48 h. The transcription levels were quantified relative to the control group. Data represent mean  $\pm$  SD from 3 independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$  compared with controls using one-way ANOVA.

### Inhibition of $\text{Na}^+/\text{K}^+$ -ATPase and $\text{Ca}^{2+}$ -ATPase Activities by ZEA

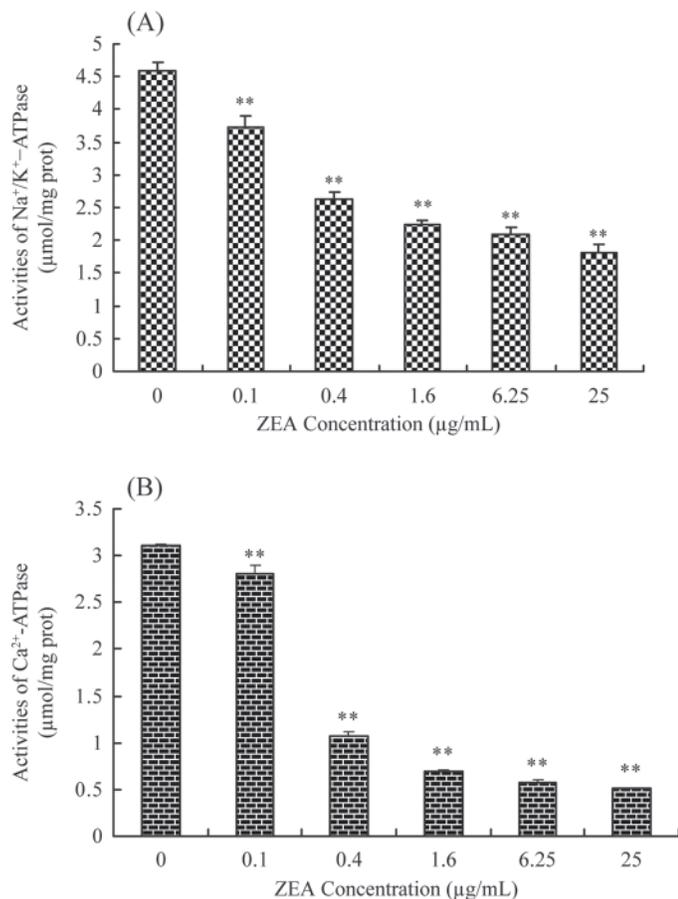
To understand whether there is a link between calcium overload and other factors, we studied the effect of ZEA on the activities of ATPases. As shown in Figure 5A,B, treatment of splenic lymphocytes with ZEA resulted in a significant loss in activities of ATPases ( $P < 0.01$ ) in a dose-dependent manner. When exposed to 1.6, 6.25, and 25  $\mu\text{g/mL}$  ZEA for 48 h, the  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Ca}^{2+}$ -ATPase activities decreased to 50% and 30% of the control value, respectively.

## DISCUSSION

Calcium homeostasis is a major factor in maintenance of cell integrity and function, and calcium had



**Figure 4.** Concentrations of calmodulin (CaM) in supernatants of splenic lymphocytes incubated for 48 h with increasing concentrations of zearalenone (ZEA; 0, 0.1, 0.4, 1.6, 6.25, and 25  $\mu\text{g/mL}$ ). Values are presented as relative concentrations, and data are mean  $\pm$  SD of 3 experiments from supernatants of different cultures, each one performed in triplicate. \*\* $P < 0.01$  compared with controls using one-way ANOVA.



**Figure 5.** Effects of zearalenone (ZEA) on ATPase activities in splenic lymphocytes. The cells were treated for 48 h with the indicated ZEA concentrations to measure the assays. Data are mean  $\pm$  SD of 3 experiments from cells of different cultures, each one performed in triplicate. \*\* $P < 0.01$  compared with controls using one-way ANOVA.

been implicated as an important second messenger and regulator of cell homeostasis (Plank et al., 2006). The  $[Ca^{2+}]_i$  is maintained at  $10^{-7}$  to  $10^{-6}$  M, enabling a simple ion to activate diverse signaling cascades affecting a range of cellular processes. Calmodulin is a highly conserved calcium-binding protein that transduces calcium signals into downstream effects, influencing a range of cellular processes, including calcium homeostasis. However, calcium homeostasis pathways involved in mycotoxin-mediated cytotoxicity differ according to the origins of the cells examined and the kinds of toxins exposed; few studies have reported on this topic (Ana-Marija and Andrey, 2011). In the present study, the use of cell cultures offered several advantages over other methods, particularly in terms of the quantification of toxic effects and definition of immune organ specificity related to a preferential action of a particular cell type. The spleen is the largest peripheral lymphoid organ in the body and contains a large number of immune cells, including B lymphocytes, T lymphocytes, macrophages, dendritic-like cells, and so on. The splenic lymphocytes of chickens have been a very useful model for many studies (Okamura et al., 2004; Li et al., 2010; Villanueva et al., 2011). Therefore, we addressed the issue

by evaluating the effects of ZEA cytotoxicity through in vitro assessment of its calcium homeostasis activity on the splenic lymphocytes of chickens.

Calmodulin plays a pivotal role in cellular calcium homeostasis through its regulation of many proteins directly involved in calcium handling, including the calcium pumps (plasma membrane  $Ca^{2+}$ -ATPase and sarco/endoplasmic reticulum  $Ca^{2+}$ -ATPase) and channels (Cheung, 1980; Means and Dedman, 1980; Arnon et al., 1997; Van Eldik and Watterson, 1998; Lee et al., 1999; Zoccola et al., 1999; Zühlke et al., 1999; Ashby and Tepikin, 2002; Saimi and Kung, 2002; Mori et al., 2004). The resulting reduction in cellular ATP levels can lead to a disruption in ionic homeostasis, which can cause an increase in  $[Ca^{2+}]_i$  and subsequent cellular apoptosis and necrosis (Grammatopoulos et al., 2004). Evidence suggests that prolonged elevation of the intracellular free calcium concentration (calcium overload) can induce apoptosis by stimulating  $Ca^{2+}$ / $Mg^{2+}$ -dependent endonucleases and modulating  $Ca^{2+}$ /CaM-dependent enzymatic activities (Orrenius et al., 1992; McConkey and Orrenius, 1997). In addition to energy production, mitochondria have the capacity to store calcium, playing a critical role in the maintenance of cellular calcium homeostasis (Foster et al., 2006). At the same time, pathological calcium accumulation (calcium overload) could lead to uncoupling of mitochondrial oxidative phosphorylation and opening of the mitochondria permeability transition pores, which produce the inhibition of oxidative phosphorylation, collapse of the proton motive force, mitochondrial swelling and release of mitochondrial calcium into the cytoplasm and contributes to cell death (Orrenius et al., 1992; McConkey and Orrenius, 1997; Foster et al., 2006). The increased  $Ca^{2+}$  binds to CaM, and this binding induces a conformational change in CaM, exposing hydrophobic patches that interact with and activate target enzymes (Lu and Means, 1993). Calmodulin is known to be expressed in all eukaryotic cells and is especially rich in the brain, testes, and ovaries (Moriya et al., 1993). In the present study, we found that ZEA could increase the total concentration of CaM in the splenic lymphocytes of chickens. This suggests that abnormal calcium homeostasis due to ZEA exposure may be another important mechanism of the development of apoptosis in these cells.

Moreover, intracellular calcium levels are maintained by other factors. Among them,  $Na^+$ / $K^+$ -ATPase and  $Ca^{2+}$ -ATPase play critical roles in intracellular calcium homeostasis by removing calcium from the cytosol across the plasma membrane; thus, blockage of these enzymes can induce cytosolic calcium overload (Fujita et al., 1998). Inhibition of  $Na^+$ / $K^+$ -ATPase and  $Ca^{2+}$ -ATPase activities by ZEA (Figure 4) may demonstrate this. In addition,  $Na^+$ / $K^+$ -ATPase and  $Ca^{2+}$ -ATPase are ATP-dependent, meaning that sufficient ATP is necessary to maintain their function. The resultant deficiency in cellular ATP levels due to disruption of mitochondrial respiration leads to inhibition of  $Na^+$ /

$K^+$ -ATPase and  $Ca^{2+}$ -ATPase (Wang et al., 2003; Qin et al., 2008). Simultaneously,  $Na^+/K^+$ -ATPase and  $Ca^{2+}$ -ATPase are highly vulnerable to the effects of oxidative damage and free radical attack (Qin et al., 2008). Therefore, it can be concluded that mitochondrial dysfunction induced by ZEA leads to the disruption of energy metabolism and results in the decrease of ATPase activities, which are involved in intracellular calcium overload.

It is reported that mycotoxins act as ionophores by forming dimeric structures that transport monovalent ions across cellular membranes, particularly the mitochondrial membrane; disturb the physiological ionic balance and the pH; challenge cellular metabolism; and cause ATP depletion (Ivanov et al., 1973; Kouri et al., 2003, 2005; Kamyar et al., 2004, 2006). In particular, pH changes have been reported to be involved in mitochondria-mediated apoptosis, with intracellular acidosis promoting cytochrome c-mediated activation of caspases (Matsuyama et al., 2000). Furthermore, interactions between  $H^+$  and  $Ca^{2+}$  ions occur at almost every level of the regulatory pathways involved in intracellular  $Ca^{2+}$  homeostasis (Austin and Wray, 2000). Previous evidence demonstrated that the inhibitory effect of arachidonic acid on mitogen-induced lymphocyte proliferation is primarily caused by blockade of transmembrane  $[Ca^{2+}]_i$  and pHi signals associated with sustained cytosolic acidification (Astashkin et al., 1993). Kume et al. (1990) also reported that in tracheal smooth muscle cells, acidosis inhibits  $Ca^{2+}$ -activated  $K^+$  channels, resulting in membrane depolarization. Schubert et al. (2001) provided a detailed description showing that in rat vascular smooth muscle cells a decrease in intracellular pH inhibits  $BK_{Ca}$  channels in inside-out patches and depresses whole-cell currents in isolated cells. Changes in  $H^+$  concentration may modify several cell membrane  $Ca^{2+}$  transport pathways (Smith et al., 1998a,b; Eto et al., 2003),  $K^+$  channel activity, mobilization of  $Ca^{2+}$  from intracellular pools,  $Ca^{2+}$  sensitivity of contractile proteins (Smith et al., 1998b; Raingo et al., 2005), and the activity of exchangers, that is,  $Na/H$  and  $Na/HCO_3$  (Neylon et al., 1990). Thus, the start of the fall in pHi and external  $Ca^{2+}$  markedly attenuated increases in  $[Ca^{2+}]_i$  (Diarra et al., 1999). As shown in Figures 1 and 5, intracellular acidification was accompanied by exposure of ZEA to splenic lymphocytes, while CaM concentrations of supernatants decreased; this may have been caused by the differences in the internal and external environments. Moreover, some evidence has demonstrated that acidification of the intracellular milieu is due to mitochondrial production of radical oxygen species, which could create an intracellular environment efficient for caspase activation to promote apoptosis (Clément et al., 1998; Hirpara et al., 2001). Therefore, it is suggested that intracellular acidification in splenic lymphocytes interrupts calcium homeostasis, which could create an intracellular environment efficient to promote apoptosis.

In conclusion, calcium homeostasis plays a critical role in ZEA-induced chicken splenic lymphocyte apoptosis or death. Apoptosis triggered by calcium dyshomeostasis was the main mechanism of death in these cells. This mechanism was likely complex, involving disturbances in the equilibrium of intracellular milieu (intracellular acidification, calcium overload, and ATPase activity level disruptions). These deregulations of calcium signaling strongly implicate mitochondrial dysfunction in the mechanism of ZEA cytotoxicity, which promotes the development of apoptosis.

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