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Cytotoxicity and apoptosis induced by mixed mycotoxins (T-2 and HT-2 toxin) on primary hepatocytes of broilers in vitro

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ABSTRACT

T-2 and HT-2 (T-2/HT-2) induced cytotoxicity and apoptosis in hepatocytes from broilers. In this study, hepatocytes treated with T-2/HT-2 were analyzed for cytotoxic effects and apoptosis and for the associated mechanisms. To assay cytotoxicity, we used the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) viability assay, hematoxylin-eosin staining and aspartase transaminase and alanine transaminase (ALT/AST) activities. We evaluated apoptosis by fluorescence microscopy using the Terminal transferase nick-end labeling (TUNEL) assay. The apoptotic ratio and the apoptotic stage of the hepatocytes were next assessed with fluorescently labeled (FITC) Annexin V and propidium iodide (PI) staining. Finally, expression levels of apoptosis-related mRNAs were assessed by real-time PCR and those of apoptosis-related proteins by western blotting. We found that cells treated with T-2/HT-2 showed, in a dose dependent manner, significantly lower cell viabilities ($P < 0.05$) and markedly increased intercellular spaces, dead cells and ALT/AST activities. T-2/HT-2 treatment also significantly increased the number of apoptotic cells and the apoptotic ratio ($P < 0.05$). T-2/HT-2 induced early stage apoptosis of the hepatocytes and levels of apoptosis-related mRNAs and proteins changed in a manner implicating them in the apoptotic process. These changes occurred from 0 to 24 h of T-2/HT-2 exposure. Expression of bax and caspase-7 mRNAs was significantly upregulated, in a time-dependent manner, during this period ($P < 0.05$). Levels of mRNAs for caspase-3 and caspase-9 were increased from 0 to 12 h ($P < 0.05$) and then decreased after 12 h ($P < 0.05$). There were no significant effects on expression of bcl-2 mRNA ($P > 0.05$). Expression of all apoptosis-related proteins examined, except for bcl-2, was significantly increased from 0 to 24 h in a time-dependent manner ($P < 0.05$). Overall, T-2/HT-2 induced cytotoxicity and apoptosis in hepatocytes. The resulting changes in mRNA and protein expression were shown that several apoptosis-related proteins were involved in the liver toxicity of these agents.

1. Introduction

T-2 toxin (T-2) and HT-2 toxin (HT-2), both metabolites of fusarium, are health hazards to humans and animals (Kalantari and Moosavi, 2010; Li et al., 2011). T-2 and HT-2 are Type A trichothecenes, and T-2 has received much attention because it is the most toxic of the trichothecenes (Sudakin, 2003). T-2 and HT-2 were reported to coexist in contaminated grains (Li et al., 2011) and HT-2 was identified as the major metabolite of the T-2 both *in vivo* and in vitro (Mark et al., 2011). The toxic effects of T-2 and HT-2 are quite similar because of their closely related epoxy sesquiterpenoids (JECFA) (2001). In a previous report (Richard et al., 1978), T-2 was rapidly metabolized to the HT-2 toxin *in vivo* and in vitro, indicating that the toxicity of T-2 might be partly attributable to HT-2 (Königs et al., 2009).

To study toxic, including apoptosis-inducing, effects of mycotoxins, cell culture experiments have been a useful tool. Among

Abbreviations: T-2, T-2 toxin; HT-2, HT-2 toxin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 Associated X protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, Terminal transferase nick-end labeling; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; D-HBSS, D-Hank's balanced salt solution; DMSO, dimethyl sulfoxide; FITC, Fluorescein Isothiocyanate; PI, Propidium Iodide; T-TBS, Tris Buffered Saline–Tween; S.E., standard error of measurement; PVDF, polyvinylidene fluoride; T-TBS, Tris Buffered Saline–Tween.

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fusarial toxins, T-2 was the most cytotoxic compound for various cell lines (Bouaziz et al., 2012; Königs et al., 2009; Yuan et al., 2016). T-2 is known to inhibit protein synthesis by binding peptidyl-transferase (Shifrin and Anderson, 1999) and was also reported to induce DNA lesions, potentially causing DNA fragmentation (Bouaziz et al., 2006). T-2 was described as an inhibitor of eukaryotic protein biosynthesis and as pro-apoptotic (Bouaziz et al., 2008). T-2 caused apoptosis in various cell types, including HL-60 cells, human B and T lymphoid cells, mammalian kidney cells and Vero cells (Nagase et al., 2001).

T-2 and HT-2 increased expression of mRNAs and proteins related to apoptosis in cells (Sehata et al., 2005). Both compounds, at similar concentrations, induced activation of caspase-3, an apoptosis indicator, in primary cells (Weidner et al., 2012). The caspase protein family was reported to play a central role in apoptosis and caspase activation can be regulated by many factors including the B-cell lymphoma 2 (Bcl-2) protein family (Wyllie, 2010). The anti-apoptotic protein, Bcl-2, and the pro-apoptotic protein, Bcl-2 Associated X protein (Bax), are well-known members of the Bcl-2 family, acting in opposition to regulate apoptosis. The P53 protein is a key tumor suppressor protein involved in cellular oxidative stress, a condition that can also lead to apoptosis (Chen et al., 2008).

The liver is the main target of the toxicity and the metabolite of the T-2 toxin *in vivo* (Lesson et al., 1995; Medina et al., 2010). In the previous research (Yang et al., 2016), the authors had explored oxidative stress induced by mixture of T-2 and HT-2 *in vivo* and in vitro on broilers, but there have been no reports on pro-apoptotic effects of T-2 or HT-2 in primary liver cells on broilers.

The aim of this study was to provide references for study on apoptosis mechanism induced by T-2 and HT-2 via detecting cytotoxic and pro-apoptotic effects of mixture of T-2 and HT-2 in primary hepatocytes on broilers. Cytotoxicity was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and hematoxylin-eosin (HE) staining. The apoptosis rate was determined by terminal transferase nick-end labeling (TUNEL) assay and annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining, using flow cytometry and fluorescence microscopy. The mechanism of apoptosis induced by mixture of T-2 and HT-2 was stated by monitoring expression of mRNAs and proteins related to apoptosis using real-time PCR and western blotting, respectively.

2. Material and methods

2.1. Reagents and kits

T-2 toxin (T-2), HT-2 toxin (HT-2) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), fetal calf serum, MTT, penicillin–streptomycin solution and insulin were from Life Technologies Co., Ltd (Carlsbad, CA, USA). The TUNEL assay kit (Cat.No.11684795910) was from Roche (Basel, Switzerland) and the Annexin V-FITC/PI Apoptosis Detection kit was from Miltenyi Biotec (Shanghai, China). Alanine aminotransferase (ALT) and asparaminotransferase (AST) assay kits were from the Jiancheng Bioengineering Institute (Nanjing, China). PrimeScript™ RT reagent kit (Perfect Real Time) and SYBR Premix Ex Taq kits were from Takara Biotechnology (Dalian, China).

2.2. Cell culture and chemical treatment

Arbor Acres broilers (age 42 d) were purchased from Institute of Animal Science, Hunan Academy of Agricultural Sciences (Changsha, China). All animals in this experiment were approved by the Animal Ethics Committee of Hunan Agricultural University, China. As previously described (Yang et al., 2016), primary hepatocytes were isolated from broiler livers. The chicken liver cells were plated and cultured (96-well, 24-well or 6-well culture plates) at approximately 5×10^5 viable cells/mL and incubated for 24 h at 37 °C in DMEM with 10% fetal calf serum, 1% penicillin–streptomycin solution and 0.5 mg/L insulin in a humidified incubator equilibrated with 5% CO_2 in air. T-2 and HT-2 solutions (100 μg/mL) were diluted in serum-free DMEM and cells were treated with T-2 and HT-2 (administered together, T-2 and HT-2) at 0, 10, 20 or 50 nM each. For these treatments, the medium was then changed to complete culture medium with the indicated concentrations of compounds and cells were incubated for either 24 or 48 h prior to analysis.

2.3. HE staining and MTT assay

For HE staining, cells (2×10^5) were left untreated or were treated with T-2 and HT-2 (10, 20 or 50 nM, respectively) for 24 h. Hepatocytes were subsequently washed in D-Hank's balanced salt solution (D-HBSS), fixed with 4% neutral formalin for 30 min and stained with hematoxylin and eosin (HE) for 5 min. After HE staining, cells were washed with D-HBSS and observed by light microscopy (Nikon TS100, Tokyo, Japan).

Hepatocytes (1×10^5), after treatment with various concentrations of T-2 and HT-2, were incubated in 96-well culture plates for 24 h. As described of Mosmann (1983), MTT (100 ng) was then added to each well and cells were incubated at 37 °C for 4 h. The medium was then removed and cells were washed with D-HBSS and 150 μL DMSO was added to each well to dissolve the formazan crystals. After agitation of the plates for 10 min, absorbances at 570 nm were detected with a microplate reader (Bio-Rad Laboratories, CA, USA). Cell viabilities = (OD_{570} (treatment)/OD_{570} (control)) × 100%. All of these procedures were performed under gnotobasis.

2.4. ALT and AST assays

After incubation in 24-well culture plates for 24 h, cells were left untreated or treated with various concentrations of T-2 and HT-2 (control or 10, 20 or 50 nM, respectively) for 24 h and the medium was tested for ALT or AST activity using the corresponding assay kits. The reaction solutions were transferred to a 96-well microplate and the absorbances were measured at 510 nm to determine ALT and AST activities in the medium.

2.5. TUNEL assay

Hepatocytes (2×10^5) were incubated on glass slides in 24-well culture plates and treated with various concentrations of T-2 and HT-2 (10, 20 or 50 nM, respectively) for 24 h. The cells were then washed with D-HBSS and fixed in 4% neutral formalin for 30 min. After washing three times with D-HBSS, cells were stained according to the manufacturer's instructions for the TUNEL cell death detection kit following the protocol previously described (Barroso et al., 2000).

2.6. Apoptosis cell detection by double staining with annexin V–FITC and PI

2.6.1. Flow cytometry

The flow cytometry assay was performed according to the manufacturer's instructions for the Annexin V–FITC/PI kit (Henry et al., 2013). Hepatocytes were left untreated or treated for 24 h with T-2/HT-2 at various concentrations (10, 20 or 50 nM,