ABSTRACT The purpose of this study was to investigate the immunopathological effects of combinations of ochratoxin A (OTA) and T-2 toxin on broilers. Four hundred eighty 1-d-old broilers were randomly assigned to 4 groups, each group consisting of 4 duplicates each with 30 broilers. The 4 groups were fed the following diets for 4 wk: group 1 = basal diet (control, mycotoxin-free); group 2 = basal diet + 2,000 mg/kg of Mycofix Plus; group 3 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2; and group 4 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 + 2,000 mg/kg of Mycofix Plus. The feeding of OTA-T-2 toxin diets reduced (P < 0.05) the level of anti-Newcastle disease virus antibody titers by 10.4%. When broilers were administered lipopolysaccharide, the results of real-time PCR showed that broilers fed OTA-T-2 toxin reduced the cytokine mRNA expression levels of interleukin-2 and interferon-γ to some extent but not significantly (P > 0.05). The concentrations of interleukin-2 and interferon-γ in serum were significantly decreased (P < 0.05) by OTA-T-2 toxin combination. Histopathological studies demonstrated that OTA-T-2 toxin combination caused abnormalities in the thymus, bursa of Fabricius, spleen, and liver. Ochratoxin A-T-2 toxicity could be counteracted by Mycofix Plus partially but not significantly (P > 0.05). The concentrations of OTA and T-2 toxin used in this study are under the maximum tolerated levels recommended by Canadian Food Inspection Agency. Our study clearly put the standard and detoxification method for these toxins into question. We suggest that it may be time to reduce the maximum allowable limits of OTA and T-2 mycotoxins in feeds to improve animal health and the safety of the food chain.

Key words: broiler, immunopathological effect, ochratoxin A, T-2 toxin

INTRODUCTION

Ochratoxin A (OTA) is an important mycotoxin due to its toxicity and occurrence in poultry feedstuffs. Studies have shown that OTA causes detrimental effects both on growth (Huff et al., 1988) and immune functions of birds (Al-Anati and Petzinger, 2006). A significant feature of OTA is its presence in a wide variety of commodities in varying amounts often at very high levels (CAST, 2003).

The T-2 toxin is predominant in tropical and sub-tropical regions (Bamburg et al., 1970). It has been shown to cause reductions in feed intake and weight gain (Wyatt et al., 1973; Chi et al., 1977) and has immunomodulatory activity (Pestka et al., 2004).

The additive effects of co-contamination of OTA and T-2 toxin have been reported in broilers. The effects of OTA (2 mg/kg) and T-2 toxin (4 mg/kg) were additive for reduced BW gains, serum levels of total protein, and lactate dehydrogenase activity (Kubena et al., 1988). Broilers fed 0.567 mg/kg of OTA or 0.927 mg/kg of T-2 toxin had a lower BW and feed intake reduction, and broilers fed both toxins showed toxic additive effects (García et al., 2003). In our previous study, we investigated the effects of different combinations of OTA and T-2 toxin in yellow-feathered broilers and found that the combination of OTA and T-2 toxin impaired chicken immune function in terms of immune organs and lymphocytes even at combined concentrations as low as 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin (Wang et al., 2009).

The Canadian Food Inspection Agency recommended that the maximum tolerated levels of OTA and T-2 toxin for poultry are 2 mg/kg and 1 mg/kg, respectively. However, OTA and T-2 toxin caused detrimental effects in poultry at lower values than the published ones. Therefore, the aim of this study was to further investigate effects of the combination of OTA and T-2 toxin.
Gene Expression by Real-Time PCR

Detection of Proinflammatory Cytokines

Immune Function Evaluations

Mycotoxins and MPL

the adverse effects of the mycotoxins in China.

which is a widely recommended feed additive against MPL; Biomin Corporation, Shanghai, China), plus (growing broilers and to evaluate the effect of Mycofix toxin at low levels on immune organs and molecules of broilers selected randomly from each treatment to determine the Newcastle disease virus (NDV) antibody titers by hemagglutination inhibition assay (Alexander, 1988). Two broilers were picked up randomly and killed from every duplicate at 42 d of age to collect blood. After centrifugation at 3,000 × g for 10 min, the sera were collected for determination of IL-2 by IL-2 ELISA kit (96T, QRCT-310123EIA\UTL, ADL, Guangzhou, China; Yuan et al., 2009) and IFN-γ by IFN-γ ELISA kit (96T, QRCT-313313EIA\UTL, ADL). Two broilers were picked up randomly and killed from every duplicate at 42 d of age. Specimens of thymus, bursa of Fabricius, spleen, and liver were removed and fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined microscopically.

Statistical Analysis

All data from this study were analyzed by ANOVA according to the GLM procedure of SAS (SAS Institute, 1994). Differences between individual means were determined by Duncan’s new multiple range test. All statements of differences were based on significance at P < 0.05 unless otherwise stated. Data are expressed as the mean ± SE.

MATERIALS AND METHODS

Birds and Treatments

Four hundred eighty 1-d-old Guangdong yellow-feather broilers were allotted randomly into 4 groups, each group consisting of 4 duplicates each with 30 broilers. All broilers were fed with the normal starter diet for 2 wk to become environmentally acclimated and then were fed the following experimental diets for 4 wk: group 1 = basal diet; group 2 = basal diet + 2,000 mg/kg of MPL; group 3 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2; and group 4 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 + 2,000 mg/kg of MPL. The basal diet was primarily based on corn-soybean meal and formulated according to Nutrient Requirements of Guangdong Yellow-Feather Broiler (NY/T 33-2004, China; Wang et al., 2009). Broilers were maintained on a 24-h constant light schedule and allowed ad libitum access to feed and water. The ethical guidelines for animal protection rights in China were observed.

Mycotoxins and MPL

The OTA and T-2 toxin were kindly provided by GTI GmbH (IFA-Tulln, Austria), and MPL was kindly provided by Bionin Corporation (Shanghai, China). Crystalline OTA and T-2 toxin were dissolved in 95% ethanol. The dissolved toxins or MPL, or both, were first mixed with 1 kg of basal diet, and then the mixture was dried and blended with proportional basal diet to produce the experimental diets. The content of mycotoxins added in diets was confirmed by an ELISA.

Immune Function Evaluations

Broilers were vaccinated subcutaneously with 1 dose of Newcastle disease vaccine at 7 d of age and received a booster 2 wk later. At 21 and 42 d of age, blood samples were collected via cardiac puncture from 24 broilers selected randomly from each treatment to determine the Newcastle disease virus (NDV) antibody titers by hemagglutination inhibition assay (Alexander, 1988).

Detection of Cytokine in Serum

Detection of Proinflammatory Cytokine Gene Expression by Real-Time PCR

Lipopolysaccharide Challenge. At 42 d of age, 2 broilers were picked up randomly from every duplicate. The broilers were challenged with lipopolysaccharide (Salmonella Typhimurium, Sigma, St. Louis, MO) at 1 mg/kg of BW via wing vein injection and were killed after 2 h. The mRNA expressions of interleukin-2 (IL-2), interferon-γ (IFN-γ), and β-actin were monitored using the real-time PCR method.

Total RNA Extraction and Real-Time PCR. The total RNA from spleen tissue (0.1 g) was extracted by the Trizol reagent method (Invitrogen, Carlsbad, CA) and reverse transcription was performed. The cytokine nucleotide sequences of PCR primers and hybridization oligonucleotides are as follows: IL-2: primer-forward, 5′-TGA TGT GCA AAG TAC TGA TCT TTG G-3′, probe, 5′-TCT TGC ATT CAC TTC CGG TGT-3′; IFN-γ: primer-forward, 5′-ACT TGT TTG TTC TTT CTA TCA TG-3′, probe, 5′-CTG GCC AAG CTC CCG ATG AAC G-3′, and primer-reverse, 5′-AGA CTG CCT TTT CCT TTT G-3′; β-actin: primer-forward, 5′-TTC CGG TGT-3′, probe, 5′-TGA TGT GCA AAG TAC TGA TCT TTG-3′, and primer-reverse, 5′-TGG AAG GTG AGC GAG G-3′.

Histology

The real-time PCR was performed in an ABI 7500 Fluorescence Quantitative PCR Cycler (Applied Biosystems, Foster City, CA) starting with a 2-min incubation step at 50°C and a 10-min activation step at 95°C, followed by 40 cycles of denaturing at 95°C for 30 s and annealing-Extending for 30 s at 60°C. The transcription abundance of each cytokine was normalized by the abundance of β-actin mRNA.

Statistical Analysis

All data from this study were analyzed by ANOVA according to the GLM procedure of SAS (SAS Institute, 1994). Differences between individual means were determined by Duncan’s new multiple range test. All statements of differences were based on significance at P < 0.05 unless otherwise stated. Data are expressed as the mean ± SE.

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Histology

Two broilers were picked up randomly and killed from every duplicate at 42 d of age. Specimens of thymus, bursa of Fabricius, spleen, and liver were removed and fixed in neutral-buffered 10% formalin, embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin, and examined microscopically.

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RESULTS

Antibody Titers

At 21 d of age, there was no significant difference of anti-NDV antibody titer among the groups. At 42 d of age, when compared with the control, the OTA-T-2 toxin combination decreased anti-NDV antibody titer 10.4% ($P < 0.05$) that was not affected significantly by 2,000 mg/kg of MPL ($P > 0.05$) (Table 1), which indicated that OTA-T-2 toxin combination impaired the production of anti-NDV antibodies in the serum and MPL was unable to reduce the toxicity.

Gene Expression of Cytokines

The real-time PCR results of mRNA expressions of IL-2 and IFN-γ in the spleens of broilers were expressed as relative mRNA expression of β-actin. The cytokine mRNA expression levels of IL-2 and IFN-γ were decreased to some extent but not significantly ($P > 0.05$) by OTA-T-2 toxin combination (data not shown).

Cytokine in Serum

The content of IL-2 and IFN-γ in serum was illustrated in Figure 1. The OTA-T-2 toxin combination caused the content of IL-2 and IFN-γ in serum to decrease ($P < 0.05$) by 12.99 and 13.16%, respectively. The detrimental effects of OTA-T-2 toxin combination on the content of the 2 cytokines in serum were alleviated to some extent but not significantly ($P > 0.05$) by 2,000 mg/kg of MPL.

Histopathological Examination

The histopathological changes were caused by OTA-T-2 toxin combination. The pathological changes had multi-organ toxic characteristics including thymus, bursa of Fabricius, spleen, and liver. In broilers fed OTA-T-2 toxin, thymus, bursa of Fabricius, and spleen showed lymphoid depletion, lymphocytosis, and congestion. Hemorrhage in the spleen and degeneration in the thymus were seen. Liver showed degeneration and necrosis. With the addition of 2,000 mg/kg of MPL, the severity of histopathological lesions caused by OTA-T-2 toxin combination was somewhat reduced. No histopathological alterations were observed in the control group, and there was no toxin effect of MPL by itself.

DISCUSSION

Both OTA and T-2 toxin are important mycotoxins due to their toxicity and their occurrence in the contaminated diets in southern China. The Canadian Food Inspection Agency recommended that the maximum tolerated levels of OTA and T-2 toxin for poultry are 2 and 1 mg/kg, respectively. But from our previous study, we found that the combination of OTA and T-2 toxin impaired chicken immune function in immune organs and lymphocytes even at combined concentrations as low as 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin (Wang et al., 2009). In this study, we attempted to further investigate the wide-ranging effects of the OTA-T-2 toxin combination on immune functions of broilers and evaluate the detoxification ability of MPL.

Determination of serum antibody titers to the corresponding viruses after regular vaccination was used to evaluate the effects of feeding mycotoxin-contaminated diets on immune system competence (Danicke et al., 2003; Chen et al., 2008). In this study, OTA-T-2 toxin combination had significant ($P < 0.05$) effects on the level of anti-NDV serum antibody titer, indicating that mycotoxins significantly impaired humoral-mediated immunity. Verma et al. (2004) demonstrated that a significant decrease in hemagglutination titer against SRBC was recorded in broilers given 4 mg/kg of OTA or a combination of aflatoxin (1 or 2 mg/kg) and OTA (2 or 4 mg/kg). In terms of mean skin thickness sensitive to dinitrochlorobenzene, cell-mediated immunity was significantly reduced for broilers given the combination of 2 mg/kg of aflatoxin and 4 mg/kg of OTA. Although the previous study focused on different toxin effects on different immune responses, it is commonly accepted that mycotoxins could impair immune function greatly. In contrast, no effects of T-2 toxin (1 mg/kg) were observed on antibody production to antigens administered by enteral or parenteral routes (Sklan et al., 2001).

As important regulators in the network of immune responses, cytokines are small secreted proteins that account for communication, activation, maturation, and differentiation among immune cells. Interleukin-2 stimulates proliferation of antigen-activated T and B cells, and IFN-γ activates macrophages and stimulates proliferation of T helper 2 cells. The decline of IFN-γ expression may impair the antivirus ability of the host and the cell-mediated and humoral immune response networks are interfered by a decrease of IL-2 level (Cheng et al., 2006). Our findings showed that the

Table 1. Effects of the ochratoxin A (OTA)-T-2 toxin combination on antibody titers against Newcastle disease virus in broilers (n = 4)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>21 d (Log2 titer)</th>
<th>42 d (Log2 titer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2.96 ± 0.03a</td>
<td>3.44 ± 0.02a</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.92 ± 0.05a</td>
<td>3.42 ± 0.03a</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.93 ± 0.06b</td>
<td>3.07 ± 0.02b</td>
</tr>
<tr>
<td>Group 4</td>
<td>2.94 ± 0.05b</td>
<td>3.04 ± 0.05b</td>
</tr>
</tbody>
</table>

Within a column, means without a common superscript are significantly different ($P < 0.05$).

1Group 1 = basal diet (control, mycotoxin-free); group 2 = basal diet + 2,000 mg/kg of Mycofix Plus (MPL; Biomin Corporation, Shanghai, China); group 3 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2; group 4 = basal diet + 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 + 2,000 mg/kg of MPL.

2Antibody titers were subjected to log2 transformation.

As important regulators in the network of immune responses, cytokines are small secreted proteins that account for communication, activation, maturation, and differentiation among immune cells. Interleukin-2 stimulates proliferation of antigen-activated T and B cells, and IFN-γ activates macrophages and stimulates proliferation of T helper 2 cells. The decline of IFN-γ expression may impair the antivirus ability of the host and the cell-mediated and humoral immune response networks are interfered by a decrease of IL-2 level (Cheng et al., 2006). Our findings showed that the
combination of 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin has an immunosuppressive effect by decreasing the content expression of cytokines (IFN-γ and IL-2) in serum of Guangdong yellow-feather broilers and not by inhibiting the gene expression of cytokines, suggesting that the combination may affect cytokine production at the level of expression. Li et al. (2006) observed that T-2 toxin (1.75 mg/kg in feed) suppressed IFN-γ responses in Peyer’s patch to reovirus at 3 and 7 d as compared with infected controls, whereas IL-2 mRNA concentrations were unaffected. The differences between the result and our findings may be due to different species, toxic dose, and methodology of immune measurements.

The histopathological changes in the OTA and T-2 toxin-challenged group showed the characteristics of multi-organ toxicity including thymus, bursa of Fabricius, spleen, and liver pathology. The T-2 toxin can induce necrosis and depletion of lymphoid cells in the thymus, spleen, and lymph nodes of chicken, and the main toxic effects of T-2 toxin in vivo are in the liver (Sokolović et al., 2008). Hanif et al. (2008) demonstrated that as low as 0.5 mg/kg of OTA could cause the marked histopathological changes in kidney, liver, bursa, and spleen of broilers. Although the liver has the remarkable potential regeneration and clearance of apoptotic cells in vivo, the toxic effect of mycotoxins still exists in the liver.

Due to health problems in flocks and potential economic losses caused by the toxic effects of mycotoxins, it is important to test a potential mycotoxin adsorbent. In this study, the effect of MPL that are widely recommended in China was evaluated. Mycofix Plus alleviated the detrimental effects of OTA-T-2 toxin combination to some extent but not significantly. García et al. (2003) demonstrated that chickens fed 0.567 mg/kg of OTA and 0.927 mg/kg of T-2 toxin showed toxin additive effects, and no protection of Zeotek (Nutek S.A. de C.V., Tehuacan, Mexico) or Mycofix adsorbents was observed.

Overall, our study showed that the combination of 0.25 mg/kg of OTA and 0.5 mg/kg of T-2 toxin impaired the immune functions in Guangdong yellow-feather broilers, confirming the additive effects of OTA and T-2 toxin on immune functions. We suggest that with the increasing availability of cellular and molecular technologies, it may be time to reduce the maximum allowable limits of OTA and T-2 mycotoxins in feeds to improve animal health and the safety of the food chain.

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