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Effects of curcumin on performance, antioxidation, intestinal barrier and mitochondrial function in ducks fed corn contaminated with ochratoxin A

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Curcumin has been attributed with antioxidant, anti-inflammatory, antibacterial activities, and has shown highly protective effects against enteropathogenic bacteria and mycotoxins. Ochratoxin A (OTA) is one of the major intestinal pathogenic mycotoxins. The possible effect of curcumin on the alleviation of enterotoxicity induced by OTA is unknown. The effects of dietary curcumin supplementation on OTA-induced oxidative stress, intestinal barrier and mitochondrial dysfunctions were examined in young ducks. A total of 540 mixed-sex 1-day-old White Pekin ducklings with initial BW (43.4 ± 0.1 g) were randomly assigned into controls (fed only the basal diet), a group fed an OTA-contaminated diet (2 mg/kg feed), and a group fed the same OTA-contaminated feed plus 400 mg/kg of curcumin. Each treatment consisted of six replicates, each containing 30 ducklings and treatment lasted for 21 days. There was a significant decrease in average daily gain (ADG) and increased feed : gain caused by OTA ($P < 0.05$); curcumin co-treatment prevented the decrease in BW and ADG compared with the OTA group ($P < 0.05$). Histopathological and ultrastructural examination showed clear signs of enterotoxicity caused by OTA, but these changes were largely prevented by curcumin supplementation. Curcumin decreased the concentrations of interleukin-1β, tumor necrosis factor-α and malondialdehyde, and increased the activity of glutathione peroxidase induced by OTA in the jejunal mucosa of ducks ($P < 0.05$). Additionally, curcumin increased jejunal mucosa occludin and tight junction protein 1 mRNA and protein levels, and decreased those of ρ-associated protein kinase 1 ($P < 0.05$). Notably, curcumin inhibited the increased expression of apoptosis-related genes, and downregulated mitochondrial transcription factors A, B1 and B2 caused by OTA without any effects on RNA polymerase mitochondrial ($P < 0.05$). These results indicated that curcumin could protect ducks from OTA-induced impairment of intestinal barrier function and mitochondrial integrity.

Keywords: curcumin, ochratoxin A, enterotoxicity, intestinal function, ducks

Implications

Ochratoxin A (OTA) is one of the most common mycotoxins detected in poultry feedstuff, and one of the major intestinal pathogenic mycotoxins. Curcumin alleviates the negative effects caused by enteropathogenic bacteria and mycotoxins. However, the possible effects of curcumin as well as the mechanism involved in alleviating the enterotoxicity induced by OTA are unknown. The results obtained in this experiment resolved these two questions, thereby helping to understand the molecular mechanism that allows curcumin to alleviate the destruction of the intestinal barrier by mycotoxins, and providing a natural additive to prevent poor performance and reduce the economic losses associated with mycotoxins.

Introduction

Mycotoxins, the secondary metabolites of fungal species, are the most frequently occurring natural food contaminants in human and animal feeds. Ochratoxin A is a well-known and widely spread mycotoxin that is mainly produced by *Aspergillus* and *Penicillum* molds and found in cereals, feeds, meat,

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vegetables, fruits, wine, beer, coffee, etc. (Kőszegi and Poór, 2016). Ochratoxin A inhibits protein synthesis, and promotes membrane peroxidation, and disrupts calcium homeostasis, as well as mitochondrial dysfunctions and DNA adducts (Ringot *et al*., 2006). The main target organ for OTA toxicity in poultry and other species is identified as the kidney, while its well-known inhibition of protein synthesis and production of reactive oxygen (ROS) and nitrogen species reveal that liver and gastrointestinal tract may be a possible target for OTA as well (Grenier and Applegate, 2013). High concentrations of OTA have been found to damage the intestinal mucosa in chickens (Solcan *et al*., 2015), resulting in a rapid inflammatory effect associated with mucosal necrosis and sloughing. Furthermore, OTA also reduces local immunity, which leads to higher susceptibility to colibacillosis and salmonellosis (Śliżewska and Pitotrowska, 2014). In Caco-2 cells, OTA exhibited an increase in permeability of tight junctions due to altered distribution of specific claudin isoforms, which were integral membrane components of the tight junctions (Maresca *et al*., 2001; McLaughlin *et al*., 2004). Also, Ranaldi *et al*. (2007) found that OTA caused faster and irreversible increase in tight junctions permeability, intracellular delocalization of claudin-4 and extensive apoptosis. These studies indicate that OTA is able to alter the intestinal barrier function.

Curcumin is a polyphenolic compound isolated from the rhizome of *Curcuma longa* Linn which has a variety of pharmacological activities, including anti-oxidative, anti-inflammatory, antibacterial and antifungal properties (Moghadamtousi *et al*., 2014). *In vivo* studies showed that curcumin could increase the expression of various detoxifying enzymes in mouse liver, small intestine and kidney tissues of mice (Shen *et al*., 2006). Wang *et al*. (2012) concluded that curcumin could protect human intestinal epithelial cells against H_2O_2-induced disruption of tight junctions via the heme oxygenase 1 pathway. Supplementation of curcumin to broiler feed increased the villus absorptive area of the small intestine (Rajput *et al*., 2013). Muhammad *et al*. (2017) reported that curcumin could prevent the harmful effects of aflatoxin B_1 in broilers.

The young animals and ducks or turkeys are usually the most sensitive species with regard to their response to mycotoxin-contaminated feed (Stoev, 2016). Because OTA was shown to affect organization and permeability of tight junctions in the intestine, but the related mechanisms were not clear, it was considered important to investigate whether an adequate intake of curcumin might alleviate the enterotoxicity of OTA. The main objective of this study was to determine the protective effects of added curcumin in an OTA-contaminated diet on performance, antioxidant status, intestinal barrier and mitochondrial function in young ducks.

Material and methods

Preparation of moldy corn

Aspergillus ochraceus AS3.3876 isolate was purchased from Guangdong Institute of Microbiology (Guangzhou, Guangdong, China). The fungus was grown on Czapek Dox Medium (CDM) at 28°C for 7 days, mycelia were then picked with an inoculation loop and inoculated into six Erlenmeyer flasks (1 l), each containing 500 ml CDM liquid medium and incubated at 28°C for 7 days to prepare the conidial suspension. Conidial concentration was 8×10^5/ml. Ground corn (200 kg) was sterilized in a dryer at 105°C for 48 h, and was uniformly spread on the floor of a sterilization room, then inoculated with the suspension of *A. ochraceus* conidia and incubated for 7 days at 29°C to 30°C. Twice daily (at 0800 and 2000 h), 3 l of autoclaved CDM medium was added to the inoculated corn meal and thoroughly mixed. All operations were carried out using protective clothing in a sterile environment except for the *A. ochraceus*. The incubation was terminated on day 7 of incubation, when the contaminated corn was collected and autoclaved at 121°C for 15 min. Ochratoxin A was extracted with a mobile phase of acetonitrile : water : acetic acid (99 : 99 : 2, v/v/v) and quantified by HPLC (Rao *et al*., 2013). The moldy ground corn used here was shown to contain 12.7 mg/kg OTA.

Experimental design, animals, housing and diets

The experimental protocol was approved by the Animal Care and Use Committee of South China Agricultural University. All animal care procedures throughout the study followed Experimental Animal Care and Use Guidelines of China. A total of 540 mixed-sex 1-day-old White Pekin ducklings (from Guiliu Poultry Co., Ltd, Foshan, China) with initial BW of 43.4 ± 0.1 g were randomly assigned to three dietary treatments, using six replicate plastic wire-floor pens (length 300 cm × width 200 cm) each with 30 birds and they were studied for 21 days. The birds were provided with *ad libitum* access to water and pelleted diets. Daylight was eliminated but 18-h/day lighting was provided from incandescent bulbs. The temperature of the room was maintained at 32°C to 34°C for the first 3 days and then reduced by 2°C to 3°C per week to a final temperature of 26°C. Control birds, not exposed to OTA, were fed a corn–soybean meal basal diet formulated to meet the nutritional requirements for starter ducks (National Research Council, 1994), as shown in Table 1. Birds exposed to OTA were fed the same diet with 16% moldy corn (described above) replacing fresh corn providing a total content of 2 mg of OTA/kg. The third group (CUR + OTA) was fed this diet with 2 mg/kg OTA, further supplemented with 400 mg/kg curcumin. Natural curcumin (>98%, by HPLC analysis) used in the experiment was provided by Nanjing Nutri-herb Biotech Co., Ltd (Nanjing, Jiangsu, China).

Performance variables

Feed intake was recorded daily on a per replicate basis. Birds were weighed at the start and the end of 3-week test period to determine the average daily gain (ADG). The feed consumed from day 1 to day 21 was measured on a pen basis to calculate average daily feed intake (ADFI) and feed : gain (F/G). Mortality was checked daily and dead birds were weighed in order to adjust F/G.

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Table 1 *Ingredient composition and nutrient content of the basal diet (%, as-fed basis)*

Items	Content
Ingredients (%)	
Corn (CP 8.7%)	63.80
Soybean meal (CP 46%)	32.00
L-Lysine·HCl	0.10
DL-Methionine	0.14
Limestone	1.06
Calcium hydrogen phosphate	1.60
Salt	0.30
Premix[1]	1.00
Total	100
Calculated nutrient[2]	
Metabolizable energy (kcal/kg)	2819
CP (%)	20.00
Crude fat (%)	2.87
Calcium (%)	0.90
Total phosphorus (%)	0.66
Non-phytate phosphorus (%)	0.42
Lysine (%)	1.10
Methionine (%)	0.45
Methionine + cystine (%)	0.80
Threonine (%)	0.80
Tryptophane (%)	0.24
Analyzed values[3]	
Dry matter (%)	88.57
CP (%)	20.05
Ether extract (%)	2.96

[1]The premix provided per kilogram diet: vitamin A 10 000 IU, vitamin D_3 3000 IU, vitamin E 20 mg, vitamin K_3 2.0 mg, vitamin B_1 2.0 mg, vitamin B_2 8 mg, vitamin B_6 4.0 mg, vitamin B_{12} 0.02 mg, choline chloride 500 mg, nicotinic acid 50 mg, D-pantothenic acid 20 mg, folic acid 1 mg, biotin 0.2 mg, Fe 60 mg, Cu 10 mg, Mn 80 mg, Zn 60 mg, I 0.2 mg, Se 0.3 mg, Co 0.25 mg.
[2]Values were calculated based on the data provided by Feed Database in China (2004).
[3]CP was analyzed by Kjeldahl method; ether extract was analyzed by Soxhlet extraction.

Tissue sampling and storage

After an overnight (12 h) feed withdrawal at day 21, 12 ducks per treatment (two per replicate) with BW close to the replicate average were selected. Individual blood samples were collected from the wing vein; plasma samples were separated by centrifugation of blood at 1200 × **g** for 10 min at 4°C and stored at −20°C for analysis. These birds were slaughtered quickly after blood collection; the entire liver was then collected and weighed, and the midpoints of jejunum of each bird were harvested and rinsed quickly with cold PBS. Portions were fixed in 10% formalin or 2.5% glutaraldehyde plus 2% paraformaldehyde and mucosa was scraped from the remainder, snap frozen in liquid nitrogen, and stored at −80°C.

Biochemical variables and measurement of jejunal mucosa cytokines

The levels of plasma malondialdehyde (MDA), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) were assayed using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' instructions. The activity of diamine oxidase (DAO) in plasma was determined according to the method of previous assay (Li *et al.*, 1996).

Jejunal mucosa samples (1 g) were homogenized on ice in 9 ml ice-cold PBS (pH 7.4) using an Ultra-Turrax T8 homogenizer (IKA Labortechnik, Staufen, Germany) and centrifuged at 3000 × **g** for 20 min at 4°C to collect the supernatants. The levels of interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) were determined using an ELISA kits (Beijing Fangcheng Biotechnology Co., Ltd, Beijing, China). The mucosal content of MDA, and activities of SOD, and GSH-Px were measured with kits, as described above. All samples were measured in duplicate, at appropriate dilutions, to give activities of the enzymes in the linear range of standard curves constructed with pure enzymes. The protein content of supernatants was determined using the Coomassie Brilliant Blue G250 from Sigma Chemical Co. (St Louis, MO, USA) with bovine serum albumin as the standard.

Histopathological analysis of jejunum

Jejunal segments were carefully oriented with regard to the crypt to villus axis during embedding in paraffin. Sections of 5-μm thickness were dewaxed, rehydrated and stained with hematoxylin–eosin. Tissue sections were scanned with the Pannoramic MIDI (3D Histech, Budapest, Hungary). Thereafter, they were analyzed, quantified and photographed with the virtual microscopy software (Pannoramic Viewer version 1.15.2, 3D Histech) to determine histomorphometric variables: villus height and crypt depth. The morphometric analysis of the sections was performed on 10 randomly selected, well-oriented villi and crypts per duck.

Ultrastructure of tight junctions and mitochondria in jejunal enterocytes using transmission electron microscopy

Jejunal samples were processed for transmission electron microscopy (TEM) following standard procedures. Ultra-thin sections were mounted on copper grids, contrasted with standard uranyl acetate and lead citrate double-staining, and examined with a TEM (Hitachi HT7700, Tokyo, Japan) at 80 kV with an Ultrascan CCD camera. To evaluate changes in the ultrastructure of tight junctions, only sections with longitudinally oriented microvilli were examined in each sample. Ultrastructure morphometric assessments of mitochondria were conducted on every sixth epithelial cell CCD micrographs using NIH ImageJ software. Examinations were performed independently in a blinded manner per duck, using non-overlapping fields.

RNA isolation and real-time PCR

Total RNA was isolated from the frozen jejunal mucosa samples using Trizol reagent (Invitrogen Biotechnology, Carlsbad, CA, USA), and dissolved in RNase-free water. The concentration of extracted RNA was qualified by the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the integrity of RNA was verified

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using denatured RNA electrophoresis. The complementary DNA (cDNA) was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology, Dalian, China), and was stored at −20°C. The cDNA was diluted with nuclease-free water (1 : 9) directly before quantitative real-time PCR analysis. The primers in the study, as shown in Table 2, were designed based on duck gene sequences using Primer Premier 5.0 and prepared from Shanghai Shenggong Biological Company (Shanghai, China).

Quantitative real-time PCR was performed using a iTaq™ Universal SYBER® Green Supermix (Takara Biotechnology, Dalian, China) in CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The protocol of PCR was as follows: denaturation at 95°C for 3 min followed by 39 cycles at 95°C for 15 s, 30 s at the annealing temperature (T_A) and 72°C for 30 s. Specificity of PCR products was evaluated by the analysis of melting curve. All analyses were performed in triplicate, each in a volume of 20 μl, and the average values were obtained. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative mRNA expression of intestinal genes. The expression of β-actin was selected as an internal control to normalize the expression of targeted genes.

Western blotting analysis

Western blotting for detection of occludin (OCLN), tight junction protein 1 (TJP1), ρ-associated protein kinase 1 (ROCK1) and myosin light-chain kinase (MLCK) proteins was performed followed previously described methods with minor modifications (Bradford, 1976). In brief, 1 g of Jejunal mucosa tissue was sampled from each duck (one duck per replicate, six ducks per treatment), 0.5 g of tissue was homogenized on ice in lysis buffer (3 ml/g) with a protease inhibitor enzyme for 5 min. The homogenized mixture was centrifuged at 12 000 × **g** for 20 min at 4°C, and 20 μl of supernatant was boiled for 5 min before being loaded into the gels. The protein concentrations were determined using the BCA protein assay kit (ThermoFisher Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Protein samples (50 μg) were subjected to 12% SDS-PAGE (25 min at 60 V and after that 60 min at 120 V). Then, the separated

Table 2 *Primers used for quantitative real-time PCR*

Gene	GenBank accession	Primer sequence (5′ to 3′)	Products size (bp)
OCLN	XM_013109403.1	GCTGGGCTACAACTACGGGT ACGATGGAGGCGATGAGC	240
CLDN1	XM_013108556.1	GGCATCATATTCAGCACCTTC GCCTTACGCACTACATCTTGG	134
TJP1	XM_013104936.1	TCAGCGAGATGAACGAGCC TCTGAAGGCTCTGACCTCTGG	189
ROCK1	XM_005009390.2	TCCCTAAGAACCCGCCATC TCAGACCGCATCCCAGGA	241
MLCK	XM_005017538.2	AGGTAATGGATGGAAGTCAAGTG TGTCAATGTAGCCTGCGTCT	156
Calmodulin	D83350.1	AGGTAATGGATGGAAGTCAAGTG TGTCAATGTAGCCTGCGTCT	167
BCL2	XM_005028719.1	ACCTGGTTCTGAATAAGTGGGAT GGTTGTCTTCTCAGTGTTGCCT	187
BAK1	XM_005026830.2	CAGGAGAGAGAAGAGAGCG TTGATGAAGTAATCGTAGGC	206
CASP3	XM_005030494.1	TGTTGAGGCAGACAGTGGACC GGAGTAATAGCCTGGAGCAGTAGA	100
CASP9	XM_013095294.1	TGCTTCAACTTCCTCCGTAA ATTCCTTCAGATGCCCCTTC	267
POLRMT	XM_021279073.1	CCACCAGAAGATCATGCAGC CGATGGTCAGCAGCTTTCTC	134
TFAM	XM_013107580.1	AGCATCACAGAAGCAGGTTTA TGCCAGTCGTTTCCTCCT	139
TFB1M	XM_013098238.1	GTTCCAAAACCAGAGGTGGA TCTGCTGCCATCATCAACTC	203
TFB2M	XM_005023464.1	TAGTCCTCCATACTCCAACCTG TTATTCACTCGGCCTCCC	141
β-Actin	EF667345.1	GCTATGTCGCCCTGGATTT GGATGCCACAGGACTCCATAC	160

OCLN = occludin; *CLDN1* = claudin1; *TJP1* = tight junction protein 1; *ROCK1* = ρ-associated protein kinase 1; *MLCK* = myosin light-chain kinase; *BCL2* = B-cell CLL/lymphoma 2; *BAK1* = BCL2-antagonist/killer 1; *CASP3* = apoptosis-related cysteine peptidase 3; *CASP9* = apoptosis-related cysteine peptidase 9; *POLRMT* = RNA polymerase mitochondrial; *TFAM* = mitochondrial transcription factor A; *TFB1M* = mitochondrial transcription factor B1; *TFB2M* = mitochondrial transcription factor B2.

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proteins were transferred on a polyvinylidene fluoride (PVDF) membrane. PVDF membranes were blocked using tris buffered saline with tween (TBST) containing 5% (w/v) non-fat milk powder for 1 h at room temperature, followed by overnight incubation at 4°C with the following primary antibodies at the indicated dilutions: anti-OCLN (rabbit monoclonal antibody, 1 : 10000 dilution, ab167161, Abcam, MA, USA); anti-TJP1 (rabbit IgG antibody, 1 : 1000 dilution, 13663, CST, MA, USA); anti-ROCK1 (rabbit monoclonal antibody, 1 : 1000 dilution, ab45171, Abcam); anti-MLCK (rabbit monoclonal antibody, 1 : 3000 dilution, ab76092, Abcam); anti-GAPDH (rabbit polyclonal antibody, 1 : 1500 dilution, AB-P-R, Hangzhou Xianzhi, Hangzhou, China). Next, the membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibody (1 : 2500 dilution, Boster, Wuhan, China) for 1 h at room temperature. Analysis of blot images was performed by ImageJ program (National Institutes of Health, MD, USA) to measure the densitometry value. The relative densitometry value, *D*, was calculated as $D = Dg/Da$, where Dg is the densitometry value of target protein and Da the densitometry value of GAPDH.

Statistical analysis

Replicate ($n = 6$) was taken as the experimental unit. Except where otherwise noted, two sampled birds per replicate were used. Statistical analysis was performed using the one-way ANOVA procedure of SAS 9.3 (SAS Institute, Cary, NC, USA). Where appropriate, differences among treatments were tested using Duncan's multiple-range test at a significance level of $P < 0.05$.

Results

Growth performance

The results for performance of the ducks are summarized in Table 3. Compared with controls, ducks fed OTA diet had significantly lower 21-day BW, ADFI and ADG, and higher absolute and relative liver weight ($P < 0.05$). Addition of curcumin to the OTA diet (CUR + OTA) significantly increased 21-day BW, ADFI and ADG to the values of the controls, while partially ameliorated the increase in liver weight ($P < 0.05$).

Table 3 *Effect of ochratoxin A and curcumin on growth performance of ducks from day 1 to day 21*

Variables	Control	OTA	CUR + OTA	SEM	*P*-value
1-day BW (g)	43.39	43.44	43.38	0.039	0.478
21-day BW (g)	1094.2^{a}	970.2^{b}	1064.4^{a}	14.9	<0.001
LW (g)	27.67^{b}	31.84^{a}	28.83^{b}	1.912	0.006
RLW (g/kg)	25.29^{c}	32.61^{a}	27.58^{b}	2.152	0.003
ADFI (g/day)	85.19^{a}	77.30^{b}	83.87^{a}	1.389	0.008
ADG (g/day)	47.76^{a}	42.06^{b}	46.41^{a}	0.567	0.002
F/G	1.79	1.86	1.81	0.042	0.083

OTA = Ochratoxin A (2 mg/kg); CUR + OTA = curcumin (400 mg/kg) + ochratoxin A (2 mg/kg); LW = liver weight; RLW = relative liver weight; ADFI = average daily feed intake; ADG = average daily gain; F/G = feed : gain.
[a,b,c]Values within a row with different superscript letters differ significantly at $P < 0.05$.

Antioxidant parameters and jejunal cytokines

Table 4 shows the effect of the OTA-contaminated diet on antioxidant indices in plasma and jejunal mucosa cytokines in the ducks at 21 days. The plasma activities of SOD and T-AOC in ducks fed the OTA-contaminated diet decreased and DAO increased along with levels of MDA ($P < 0.05$). In jejunal mucosa, contents of inflammatory cytokines like IL-1β and TNF-α increased and IL-10 decreased, compared with the controls ($P < 0.05$); mucosal activities of GSH-Px and SOD decreased whereas the content of MDA was increased to 483%. Additional supplementation with curcumin significantly improved antioxidant status in jejunum in terms of GSH-Px and SOD ($P < 0.05$), decreased jejunal IL-1β and TNF-α levels ($P < 0.05$), and further decreased both plasma and jejunal content of MDA ($P < 0.01$). There were no differences in jejunal levels of IL-8 among dietary treatments ($P > 0.05$).

Histomorphological changes in jejunum

Representative photomicrographs of jejunal villi in are depicted in Figure 1. Compared with the controls, ducks fed the diet containing OTA showed villous blunting and epithelial denudation along with a related decrease in villous length and increased the depth of crypts. Supplementation of the OTA diet with curcumin partly restored villus length in jejunum.

Transcript abundance of tight junction-related proteins in jejunal mucosa

Jejunal mucosa expression of genes related to tight junctions were quantified at day 21. As shown in Table 5, transcripts of *OCLN* and *TJP1* in ducks fed the diet with OTA

Table 4 *Effect of ochratoxin A and curcumin on biochemical indices and jejunal immunity of 21-day ducks*

Variables	Control	OTA	CUR + OTA	SEM	*P*-value
Plasma					
GSH-Px (U/ml)	835.2	700.7	792.1	44.82	0.089
SOD (U/ml)	133.3^{a}	110.4^{b}	120.5^{a}	5.120	0.037
T-AOC (U/ml)	10.10^{a}	7.40^{b}	8.29^{b}	0.531	0.042
MDA (nmol/ml)	2.05^{b}	4.86^{a}	2.74^{b}	0.143	0.004
DAO (U/l)	16.77^{b}	23.64^{a}	16.55^{b}	2.215	0.045
Jejunum					
GSH-Px (U/mg prot)	58.08^{b}	47.89^{a}	55.51^{b}	2.424	0.032
SOD (U/mg prot)	148.3^{a}	77.4^{c}	123.8^{b}	3.672	0.022
IL-1β (ng/g prot)	7.29^{b}	10.53^{a}	8.48^{b}	0.516	0.017
IL-8 (ng/g prot)	45.85	45.67	45.12	1.496	0.938
IL-10 (ng/g prot)	2.31^{a}	1.73^{b}	2.20^{ab}	0.126	0.037
TNF-α (ng/g prot)	18.75^{b}	23.55^{a}	18.59^{b}	0.543	<0.001
MDA (nmol/mg prot)	0.35^{c}	2.04^{a}	0.65^{b}	0.142	<0.001

OTA = Ochratoxin A (2 mg/kg); CUR + OTA = curcumin (400 mg/kg) + ochratoxin A (2 mg/kg); GSH-Px = glutathione peroxidase; SOD = superoxide dismutase; T-AOC = total antioxidant capacity; MDA = malondialdehyde; DAO = diamine oxidase; IL-1β = interleukin-1β; IL-8 = interleukin-8; IL-10 = interleukin-10; TNF-α = tumor necrosis factor-α.
[a,b,c]Values within a row with different superscript letters differ significantly at $P < 0.05$.

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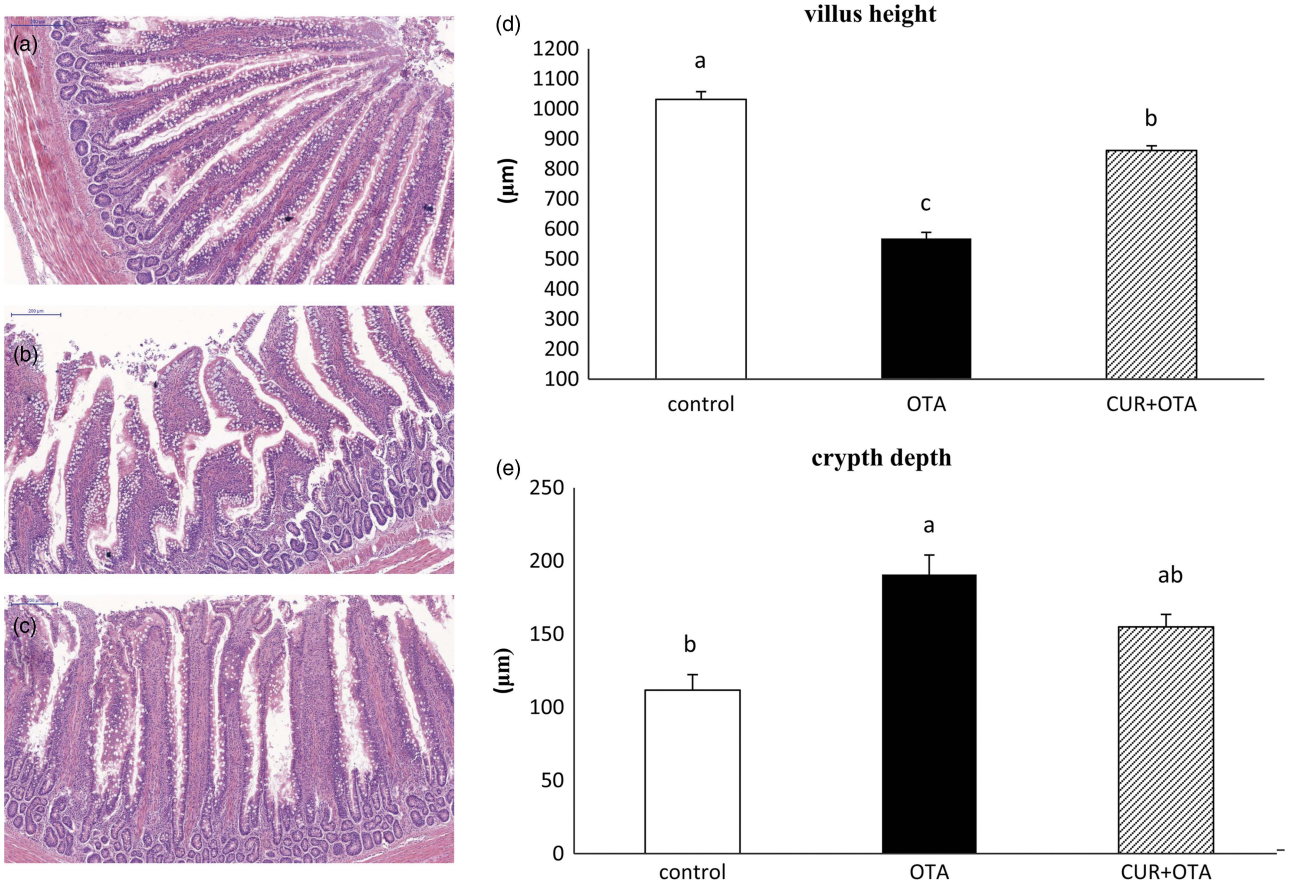


Figure 1 Representative photomicrographs of hematoxylin–eosin stained of villi in jejunum (magnification, 200×; scale bars, 200 μm). (a) The jejunal mucosa of the controls showed normal villous structure. (b) Ducks fed the diet with 2 mg/kg ochratoxin A (OTA) showed villous blunting and epithelial denudation in the jejunum, compared with the controls. (c) Supplementation of the 2 mg/kg OTA diet with 400 mg/kg curcumin (CUR) improved villous height and width in jejunum. (d) The villous height was significantly decreased in the jejunum of ducks exposed to 2 mg/kg OTA compared with the controls while supplementation of the OTA diet with 400 mg/kg CUR increased villus height. (e) Crypt elongation was significantly increased in the jejunum of ducks fed the diet with 2 mg/kg OTA compared with the controls and supplementation of the OTA diet with 400 mg/kg CUR decreased crypt elongation. Results are expressed as means ± SEM; $n = 6$ animal/group. ^{a,b,c}Means with different letters were significantly different ($P < 0.05$).

were significantly decreased than those in controls ($P < 0.01$); and those of *ROCK1* and *MLCK* were increased ($P < 0.05$). The expression of *OCLN* and *TJP1* in ducks fed curcumin in addition to OTA were significantly higher than with the toxic diet alone ($P < 0.01$); simultaneously, *ROCK1* and *MLCK* transcripts were reduced ($P < 0.05$). There were no differences in the jejunal relative expression of *claudin 1* or *Calmodulin* among dietary treatments ($P > 0.05$).

Relative expression of genes related to apoptosis and mitochondrial transcription factors in jejunal mucosa

Table 5 also shows the relative abundance of apoptosis and mitochondrial transcription factors transcripts in jejunal mucosa. The transcript abundance of BCL2-antagonist/killer 1 (*BAK1*) and apoptosis-related cysteine peptidase 3 (*CASP3*) increased over controls in jejunal mucosa of ducks fed OTA while there were no changes in these transcripts between CUR + OTA ducks and controls ($P > 0.05$). The expression of B-cell CLL/lymphoma 2 (*BCL2*) mRNA in ducks fed the diet with OTA was significantly lower ($P < 0.01$) than in the controls and was intermediate when curcumin was given, in addition to OTA. Ducks fed the diet containing OTA showed increases ($P < 0.01$) in mitochondrial transcription factor A (*TFAM*), mitochondrial transcription factor B1 and B2 (*TFB1M*, *TFB2M*). In all cases, the addition of curcumin to the OTA diet attenuated the increased expression of these transcription factors.

Protein expression of tight junction-related proteins in jejunal mucosa

The full gels with molecular weight ladder and the appropriate controls are presented in Supplementary Figure S1. Immunoblotting (Figure 2a) and subsequent densitometry (Figure 2b) demonstrated that OCLN and TJP1 protein expression were significantly decreased in ducks fed the diet with OTA compared with the controls ($P < 0.05$); and those of ROCK1 was increased ($P < 0.05$). Occludin and TJP1 expression were decreased by 1.6-fold and 1.5-fold, respectively, whereas ROCK1 expression was increased by 1.2-fold. The protein expression of OCLN and TJP1 in ducks fed curcumin in addition to OTA were significantly higher than with the toxic diet alone ($P < 0.01$); simultaneously, ROCK1 protein expression was reduced ($P < 0.05$).

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Table 5 *Effects of ochratoxin A and curcumin on relative jejunal mucosa expression of genes related to tight junctions and mitochondrial function in day 21 ducks*

Gene	Control	OTA	CUR + OTA	SEM	*P*-value
OCLN	1.01[a]	0.27[b]	0.92[a]	0.105	<0.001
CLDN1	1.04	0.90	1.15	0.089	0.535
TJP1	1.02[a]	0.21[c]	0.68[b]	0.109	0.008
ROCK1	1.07[b]	1.95[a]	1.17[b]	0.186	0.014
MLCK	1.01[c]	2.77[a]	1.36[b]	0.192	<0.001
Calmodulin	1.08	0.98	1.25	0.123	0.214
BCL2	1.00[a]	0.66[b]	0.87[b]	0.092	0.004
BAK1	1.01[b]	1.48[a]	1.15[b]	0.104	0.008
CASP3	1.00[b]	1.26[a]	1.08[b]	0.114	0.019
CASP9	1.02[c]	1.32[a]	1.11[b]	0.155	0.022
POLRMT	1.00	1.22	1.01	0.153	0.788
TFAM	0.98[b]	2.09[a]	1.36[b]	0.181	0.006
TFB1M	0.99[b]	1.93[a]	1.11[b]	0.121	0.011
TFB2M	1.02[b]	1.70[a]	1.25[ab]	0.125	0.031

OTA = Ochratoxin A (2 mg/kg); CUR + OTA = curcumin (400 mg/kg) + ochratoxin A (2 mg/kg); *OCLN* = occludin; *CLDN1* = claudin1; *TJP1* = tight junction protein 1; *ROCK1* = ρ-associated protein kinase 1; *MLCK* = myosin light-chain kinase; *BCL2* = B-cell CLL/lymphoma 2; *BAK1* = BCL2-antagonist/killer 1; *CASP3* = apoptosis-related cysteine peptidase 3; *CASP9* = apoptosis-related cysteine peptidase 9; *POLRMT* = RNA polymerase mitochondrial; *TFAM* = mitochondrial transcription factor A; *TFB1M* = mitochondrial transcription factor B1; *TFB2M* = mitochondrial transcription factor B2.

[a,b,c]Values within a row with different superscript letters differ significantly at $P < 0.05$.

Ultrastructure of tight junctions and mitochondria in jejunal mucosa

The ultrastructure of tight junctions and mitochondria in jejunal mucosa, visualized by TEM (magnification 5000 ×), is shown in Figure 3. Enterocytes of control ducks demonstrated unaffected tight junctions and normal mitochondria with clear cristae. In contrast, enterocytes from ducks exposed to OTA had obviously damaged microvilli, widened intercellular spaces, condensed cytoskeleton and mitochondria appeared to be swollen and suffering from the disappearance of the inner and outer membrane and there were no visible cristae. These diverse morphological alterations of tight junctions and mitochondria suggest intestinal barrier and mitochondrial dysfunctions in the enterocytes. These adverse effects were alleviated in ducks fed curcumin along with the OTA-contaminated corn.

Discussion

Ochratoxin A, as a secondary metabolite of *Penicillium* and *Aspergillus* microfungi, is a well-known and widely spread mycotoxin all over the world, which exerts hazardous effects on human and animal health (Kőszegi and Poór, 2016). This study used an *in vivo* exposure model to study the harmful effects of OTA in White Pekin ducklings. Feed containing 2 mg/kg OTA significantly decreased ADFI, ADG, and increased F/G between day 1 and day 21, all of which agrees with earlier reports of performance-depressing effects of OTA (Prior *et al*., 1980; Hoehler *et al*., 1996; Zhong, 2008). The additional supplementation with 400 mg/kg curcumin improved the performance, or offset the toxicity of OTA, of the ducks studied here. No information appears to be available in the literature regarding the effect of curcumin on ameliorating the decrease in BW of ducks fed OTA-contaminated feed. There have been such effects in broilers fed aflatoxin B_1-contaminated rations, where the co-administration of curcumin, in a dose-dependent manner partially ameliorated the decrease in BW (Gowda *et al*., 2009; Muhammad *et al*., 2017). Additional evidence was obtained here for the improved performance of ducks fed the diet containing 400 mg/kg curcumin with the OTA being attributable to the high antioxidant properties claimed of curcumin.

Numerous mycotoxins are able to alter intestinal barrier function and the intestinal mucosa represents the first site of interaction between noxious agents of dietary origin and the organism. In the present experiment, the enterotoxicity of OTA was observed in terms of deleterious modifications in villus architecture, grossly evident in the shortened villi and elongated crypts. These changes were largely reversed by co-exposure to curcumin (Figure 1). Mucosal architecture injury is initiated by increasing number of intraepithelial lymphocytes. Crypt elongation is a compensatory feature occurring in response to villous shortening, which may be caused by releasing various growth factors and cytokines from inflammatory cells due to the stromal–epithelial interactions. Then villous atrophy associated with crypt hyperplasia occurred (Solcan *et al*., 2015). Therefore, it can be speculated that curcumin might control the number of intraepithelial lymphocytes, and inhibit secretion of inflammatory cytokines to protect intestinal integrity. Contact between intestinal epithelial cells includes three ultrastructural components: desmosomes, adherens junctions and tight junctions. The latter is the most important structures in intestinal barrier function, and play a decisive role in intestinal mucosa permeability. Proteins of tight junctions include transmembrane proteins (OCLN, CLDN, tricellulin, etc.) and intracellular proteins (zonula occludens-1, -2, -3, cingulin, etc.). These tight junction proteins interact with F-actin and MLC and anchor them to the cytoskeleton structure. The kinase ROCK plays an important role in maintaining the structure of tight junctions by regulating the phosphorylation status of downstream MLC to influence overall cytoskeletal structure (Du *et al*., 2016). In the present study, ducks fed the OTA-contaminated diet had significantly reduced *OCLN* and *TJP1* transcripts in the jejunal mucosa, but increased expression of *ROCK1* and *MLCK* mRNA. Western Blot analyses also showed a significant decrease in OCLN and TJP1 protein mass, and increased protein expression of ROCK1 (Figure 2) without any changes in MLCK in OTA diet, the addition of curcumin to the OTA diet attenuated the modifications of those proteins. Furthermore, histomorphological and ultrastructural examination of the jejunum showed villous blunting and epithelial denudation along with damaged microvilli and widened intercellular spaces in ducks fed the diet containing OTA. These findings indicate that OTA

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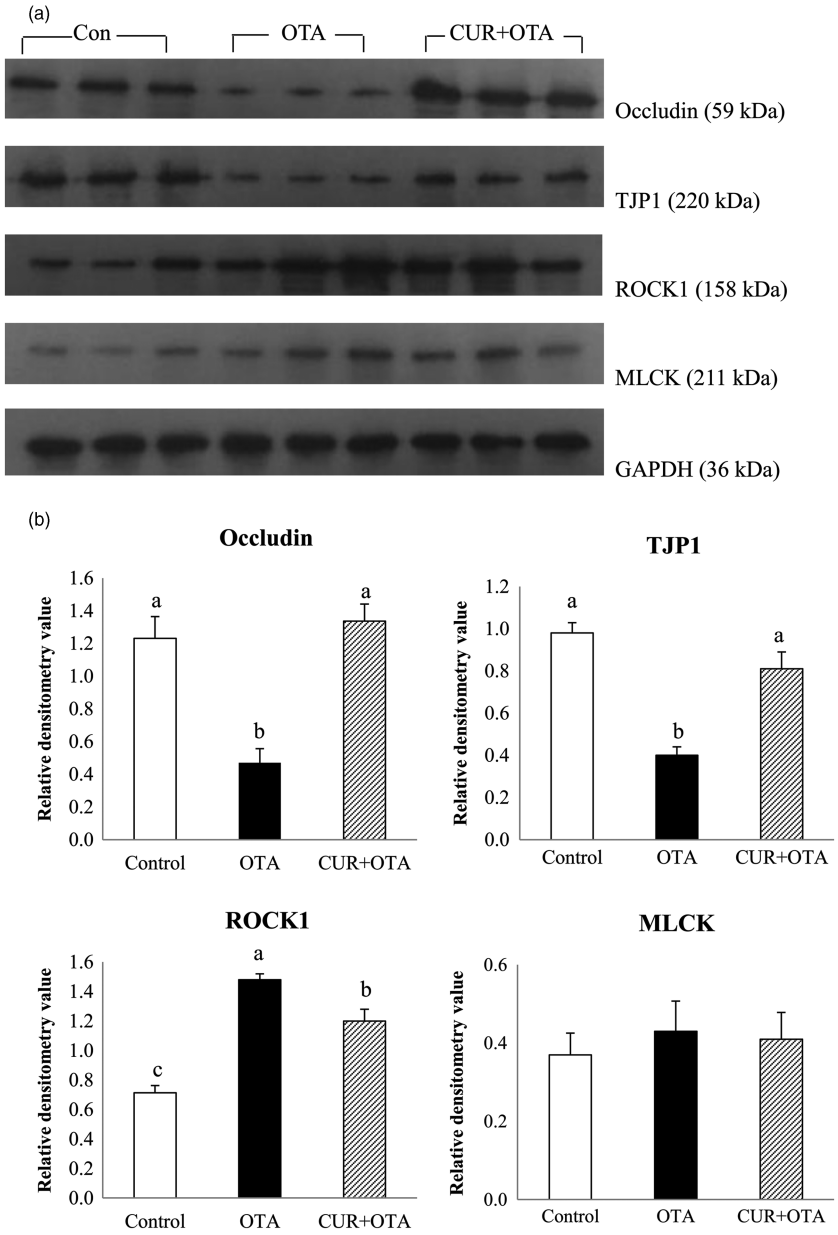


Figure 2 Western blotting analysis of jejunal mucosa tight junction and cytoskeleton-related protein expression in the control, 2 mg/kg ochratoxin A (OTA) group and co-administration of 400 mg/kg curcumin (CUR) group of ducks after 21 days of feeding. (a) A representative immunoblot of three replicates is shown. (b) Densitometry value of protein expression levels is shown. Data are expressed as means ± SEM of all determinations. TJP1 = tight junction protein 1; ROCK1 = ρ-associated protein kinase 1; MLCK = myosin light-chain kinase protein. [a,b,c]Columns with different letters are considered significant ($P < 0.05$).

may have affected the phosphorylation of MLC through the ROCK pathway, thereby destroying the cytoskeletal structure, and leading to delocalization of tight junction proteins. Other studies have reported that human enterocytes exposed to OTA reduced the barrier function, by removal of specifical claudin isoforms and affecting of lipid rafts in biological membranes (McLaughlin *et al*., 2004). Mainly involved in protein synthesis inhibition and oxidative stress-related cytotoxicity could also participate in the effect of OTA on intestinal permeability (Ranaldi *et al*., 2007; Maresca *et al*., 2001; Grenier and Applegate, 2013). In addition, the intestinal barrier dysfunction is associated with intestinal inflammation, which is based on the destruction of tight junctions (Turner, 2009). Tumor necrosis factor-α which is secreted by activated T cells increases intestinal permeability, and may directly or indirectly inhibit the expression of tight junction proteins (Chung *et al*., 2002). Al-Anati *et al*. (2010) reported that only macrophages and macrophage-derived cells respond to OTA and were considered to be sources of the TNF-α, released by exposure to OTA. In the present study, the contents of IL-1β, and TNF-α in jejunal mucosa were increased in ducks given OTA-contaminated feed but were decreased by co-supplementation with curcumin. These results showed that curcumin largely protected T cells from OTA-induced impairment. The protective role of curcumin is mainly due to its antioxidant and anti-inflammatory properties.

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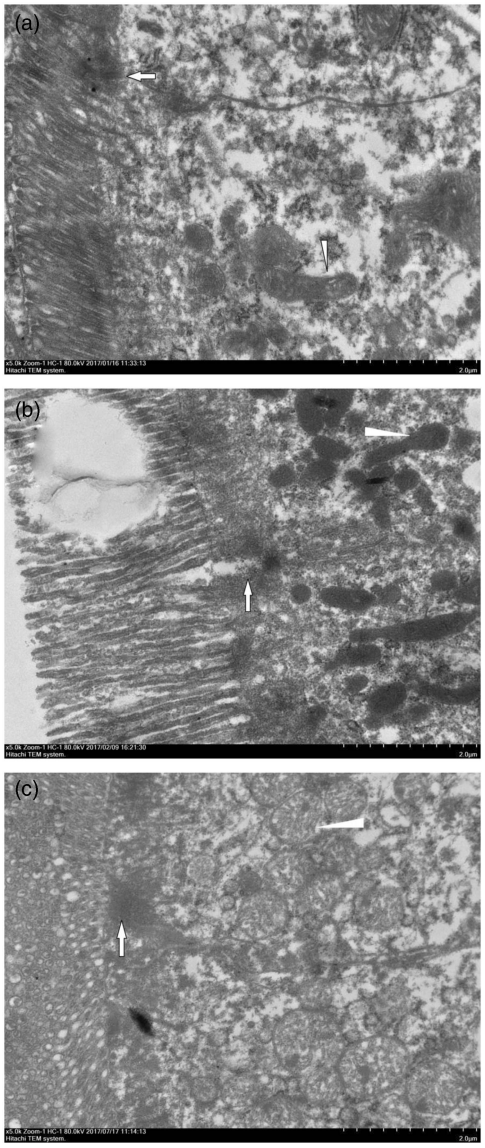


Figure 3 Ultrastructure of tight junctional complex and mitochondria in jejunal mucosa. These panels show representative micrographs of the jejunal epithelium (original magnification 5000×; scale bars, 2.0 μm). (a) Enterocyte of the control exposed to vehicle with unaffected tight junction region (arrow) and normal mitochondria (arrowhead). (b) Widening of the intercellular space (arrow), absence of mitochondria membrane with fuzziness and derangement of the cristae (arrowhead) after being fed the diet with 2 mg/kg ochratoxin A (OTA). (c) Normal intercellular space (arrow), visible mitochondria membrane and regular cristae (arrowhead) after feeding with the diet containing 2 mg/kg OTA plus 400 mg/kg curcumin (CUR).

The mitochondrion is the predominant site for intracellular production of ROS production and a prime target for oxidative damage, especially of the exposed mitochondrial DNA and membrane unsaturated fatty acids (Venditti *et al.*, 2013). Several studies indicate that both *in vivo* and *in vitro* exposure to OTA results in the overproduction of ROS. In the present experiment, OTA-induced lipid peroxidation in jejunum epithelial cells, and was offset by dietary curcumin. As a liposoluble substance, it may use lipophilic integration into the epithelial cell's membrane and mitochondrial membrane phospholipids, thereby directly neutralizing ROS in its role of an antioxidant, thus significantly alleviating OTA-induced oxidative damage. Previous study pointed out that the natural flavonoids could be specifically enriched in mitochondria, thereby playing a role in protecting the body (Dorta *et al.*, 2005). Curcumin, as a plant-derived polyphenolic substance, may achieve its antioxidant activity by similar subcellular localization like natural flavonoids, but this remains to be demonstrated. The mitochondrial genome is transcribed by specialized machinery that includes RNA polymerase mitochondrial (*POLRMT*), *TFAM*, *TFB1M* and *TFB2M* paralogues (Asin-Cayuela and Gustafsson, 2007). The *TFAM*, which was cloned as a transcription factor for mitochondrial DNA (mtDNA), may play a crucial role in maintaining mtDNA as a main component of the nucleoid (Kang *et al.*, 2007). A strong negative effect of OTA on cellular energy (ATP) production (Poór *et al.*, 2014) is consistent with OTA penetrating into mitochondria and most probably binding to proteins participated in maintenance of membrane potential and oxidative phosphorylation via interfering with phosphate transport along with suppression of electron transport (Wei *et al.*, 1985). This may resulted in depletion of mtDNA and mutation-mediated mitochondrial toxicity. Increased expression of *TFAM*, *TFB1M* and *TFB2M* was observed here in ducks given OTA-contaminated feed with no marked difference in *POLRMT*. We hypothesized that the enhanced expression of genes for these factors may represent a compensatory mechanism in response to oxidative stress-related mitochondrial dysfunctions, including deficient electron transport system, incomplete mitochondrial biogenesis and excessive cell apoptosis. Taha *et al.* (2010) reported that iron–ascorbate-mediated lipid peroxidation raised the gene expression and protein mass of *TFAM*, *TFB1M* and *TFB2M* in Caco-2/15 cells. Accordingly, *TFAM* was found to be upregulated in response to lipopolysaccharide-induced oxidative damage to mitochondria, presumably to enhance mtDNA levels and oxidative phosphorylation activity (Suliman *et al.*, 2003). Ikeda *et al.* (2015) found that overexpression of *TFAM* increased mtDNA copy number, suppressed mitochondrial ROS, and facilitated cardioprotection associated with limited mitochondrial oxidative stress. It was demonstrated that a low level of curcumin was able to elicit mitochondrial biogenesis in cells and tissues through induction of peroxisome proliferator-activated receptor γ co-activator 1-α, modulating mitochondrial transcription factors (Rainey *et al.*, 2017). This suggests the existence of a retrograde signaling pathway from mitochondria to the nucleus, to precisely regulate the related factors expression. The mechanism involved needs further investigation. The ultrastructure of jejunum epithelial cells of ducks oxidative stressed by the OTA-contaminated feed showed that inner and outer mitochondrial lipid bilayer and cristae were obscure whereas nuclei did not seem to be affected (Figure 3); this is again consistent with mtDNA being more vulnerable than nuclear DNA to oxidative damage (Kanki *et al.*, 2004).

In conclusion, ducklings fed OTA had depressed performance, exhibited intestinal lipid peroxidation, disrupted tight junctions, cell apoptosis and caused mitochondrial dysfunction,

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likely related to oxidative stress. Addition of dietary curcumin effectively prevented OTA-induced toxicity: caused less lipid peroxidation, improved intestinal barrier and mitochondrial function, mainly by affecting tight junctions, cytoskeleton proteins, apoptotic proteins and mitochondrial transcription factors. These results may be helpful in revealing the molecular mechanism of curcumin in alleviating the destruction of the intestinal barrier caused by mycotoxins.

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Declaration of interest

The authors declare that there are no conflicts of interest.

Ethics statement

The experimental animal protocol for this study was approved by the Animal Care and Use Committee of South China Agricultural University. All animal care procedures throughout the study followed Experimental Animal Care and Use Guidelines of China.

Software and data repository resources

The authors declare that the data of this research are not deposited in any official repository.

Supplementary material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731118000678

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