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Curcumin mitigates ochratoxin A-induced oxidative stress and alters gene expression in broiler chicken liver and kidney

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RESEARCH ARTICLE



ABSTRACT

The study aimed to evaluate the effect of curcumin (CURC) supplementation on broiler chickens exposed to ochratoxin A (OTA), by examining biochemical parameters and the expression of glutathione redox system genes and their regulation. OTA reduced glutathione content in the liver while increasing glutathione peroxidase activity. CURC showed no significant effects. Kidney parameters remained mostly unaffected. Gene expression analysis revealed OTA-induced upregulation of *KEAP1*, *NRF2*, *AHR*, *GPx4* and *GSR* genes in the liver. CURC supplementation led to the upregulation of *GPx4* and *AHR* genes with OTA+CURC treatment, resulting in the downregulation of *GPx4*, *KEAP1*, *NRF2* and *AHR* genes compared to OTA treatment alone. In the kidney, *GPx4* was downregulated, and *NRF2* and *AHR* were upregulated as an effect of OTA, while CURC upregulated the *NRF2* gene only. OTA+CURC treatment led to the downregulation of *GPx4*, *GSS* and *AHR* genes compared to the control and downregulation of *NRF2* and *AHR* genes compared to OTA. The results suggested that CURC is partly effective against OTA-induced oxidative stress and that the effect of OTA and CURC on the antioxidant response is regulated through the KEAP1-NRF2-ARE and AHR pathways.

KEYWORDS

ochratoxin A, curcumin, glutathione-peroxidase, aryl hydrocarbon receptor, oxidative stress, gene expression

1. INTRODUCTION

Oxidative stress and the generation of oxygen-free radicals have been widely implicated as one of the potential causes of mycotoxin toxicity, attracting significant attention both in animal and human studies (da Silva et al., 2018). Oxidative stress arises from an imbalance between the excessive production of oxygen and nitrogen-free radicals and the compromised functioning of the antioxidant defence system, leading to damage in cellular biomolecules, including nucleic acids, lipids, and proteins across diverse tissues. This damage can result in reversible or irreversible harm to cells and tissues (Valko et al., 2007). However, in the context of mycotoxin toxicity, the specific mechanisms through which mycotoxins promote lipid peroxidation remain unclear. It is uncertain, whether mycotoxins directly enhance the formation of reactive oxygen species (ROS) or increase tissue sensitivity to lipid peroxidation by compromising antioxidant defence. Previous studies suggest that these processes often occur concurrently (Yilmaz et al., 2017). Due to their chemical structure, mycotoxins and their bioactive metabolites directly activate the oxygen-free radical formation, thus activating

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the redox-sensitive regulatory pathways. Still, oxidative stress impairs the integrity of proteins, such as the activity of the enzymes responsible for antioxidant defence, such as the glutathione redox system.

Ochratoxin A (OTA), one of the most commonly occurring mycotoxins, is primarily produced by various fungal species belonging to the genera *Aspergillus* and *Penicillium* (Van der Merwe et al., 1965). OTA contamination can affect a wide range of agricultural products, including cereals, cereal-based products (Sun et al., 2017), grapes (Freire et al., 2017), herbs, coffee, cocoa and tea, and indirectly, through the feeds of fish, pork, milk and its products, poultry meat and eggs (Iqbal et al., 2014; Huong et al., 2016). Chemically, OTA is a pentaketide with a stable dihydrocoumarin linked to an L- β -phenylalanine residue (Longobardi et al., 2022). Its extraordinary stability during food and feed processing makes its complete elimination in the food chain nearly impossible (EFSA, 2020).

According to the 2022 DSM World Mycotoxin Survey (DSM, 2022), OTA contamination in Europe was 10% in corn and 6% in cereals, with the average positive samples containing $31 \mu\text{g kg}^{-1}$ in corn and $20 \mu\text{g kg}^{-1}$ in cereals, respectively. In poultry species, ochratoxin toxicity is often associated with decreased feed intake, feed refusal, lower weight gain, and reduced egg production (Duarte et al., 2011). Chickens are particularly sensitive to OTA, with an oral LD_{50} value of $3.6 \pm 0.6 \text{ mg kg}^{-1}$ body weight (b.w.) at 21 days of age (Huff et al., 1974). OTA is primarily known for its nephrotoxic effects. Still, it also exhibits hepatotoxic, carcinogenic, genotoxic, immunotoxic and potentially neurotoxic effects in humans and various animal species, including chickens (Kőszegi and Poór, 2016; Damiano et al., 2021). However, it is hypothesized that overproduction of ROS, hence OTA-induced oxidative stress, is the main reason behind these effects (Damiano et al., 2020).

The pivotal regulator of the oxidative stress response is the redox-sensitive transcription factor, nuclear factor-erythroid 2 p45-related factor 2 (NRF2) (Kensler et al., 2007) in connection with Kelch-like ECH-associated protein 1 (KEAP1) (Suzuki and Yamamoto, 2015). OTA-induced oxidative stress affects non-enzymatic and enzymatic antioxidant defenses, mainly decreasing the reduced glutathione (GSH) content (Cavin et al., 2007; Kőszegi and Poór, 2016). GSH is important for maintaining cellular redox balance (Schafer and Buettner, 2001). OTA can also impair the NRF2 mRNA and protein expression, its translocation into the cell nucleus and its binding to DNA (Limonciel and Jennings, 2014) as a transcription factor that regulates the expression of the Antioxidant Response Element (ARE) gene cluster (Boesch-Saadatmandi et al., 2009), therefore inducing the expression of detoxification and antioxidant enzyme genes, and consequently adequate oxidative stress response. Shin et al. (2019) showed that OTA-induced oxidative stress *in vitro* in human hepatocytes is associated with aryl hydrocarbon receptor (AHR) and NRF2; additionally, the increased expression and nuclear localization of NRF2 were observed. Ayed-Boussema et al. (2012) similarly found *in vitro* in primary cultured human hepatocytes that OTA treatment induced overexpression of genes encoding the

xenobiotic metabolizing CYP1A1 and CYP1A2 enzymes accompanied by an increase in AHR mRNA expression.

Phytochemicals, i.e. naturally occurring plant-derived compounds, have emerged as a promising approach to mitigate the toxicity of environmental contaminants such as mycotoxins. Curcumin (CURC), the primary bioactive component of the rhizome of *Curcuma longa* (turmeric), exhibits a wide range of pharmacological effects, including antioxidant, anti-inflammatory (Jin et al., 2021), antibacterial (Zheng et al., 2020), antifungal (Chen et al., 2018) and antitumor activities (Walker and Mittal, 2020; Kabir et al., 2021). Its antioxidant effects manifest in reducing the levels of different reactive oxygen and nitrogen species, e.g., superoxide (O_2^-), peroxynitrite (ONOO^-), nitric oxide (NO), hydroperoxyl (HO_2) and hydroxyl radicals ($\text{OH}^\cdot$) (Halliwell and Gutteridge, 2015). Furthermore, CURC can indirectly induce the expression of the genes encoding antioxidant proteins, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR), glutathione-S-transferase (GST) and γ -glutamyl cysteine ligase (gGCL), required for the GSH synthesis (Trujillo et al., 2013). Furthermore, CURC induces the upregulation of the antioxidant genes through the induction of NRF2 (Shahcheraghi et al., 2021). CURC was also reported to be an activator of the aryl-hydrocarbon receptor (AHR) transcription factor (Lin et al., 2022), which is primarily responsible for the expression of genes encoding the phase I and II xenobiotic metabolizing enzymes (Dietrich, 2016). Interestingly, there is an overlap between NRF2 and AHR target genes, which means that both can regulate the antioxidant gene cluster. The functional basis of this overlap is that the promoters of these target genes contain functional Xenobiotic Response Elements (XRE) and AREs; consequently, induction of these genes requires activation of AHR and/or NRF2 (Ma et al., 2004). The effect of CURC was proven in heat-induced oxidative stress *in vitro* (Wu et al., 2019) and against oxidative stress-induced liver damage in AFB1 toxicosis in chickens (Li et al., 2019; Damiano et al., 2022). It was also reported that CURC affects OTA-induced oxidative stress in rats (Damiano et al., 2021) and ducks (Ruan et al., 2019; Jin et al., 2021). However, there is no data about this effect in broiler chickens.

Therefore, this study aimed to evaluate the potential of CURC in mitigating OTA-induced toxicity by examining lipid peroxidation processes, markers of the glutathione redox system and the expression of genes related to redox-sensitive transcription pathways. Specifically, we aimed to elucidate the role of the KEAP1/NRF2/ARE and AHR pathways in regulating genes linked to the glutathione redox system in broiler chickens following 21 days of treatment.

2. MATERIALS AND METHODS

2.1. Production and determination of ochratoxin A

Ochratoxin A was produced by artificial infection of sterile ground corn substrate with *Aspergillus albertensis* strain



(SZMC 22107) deposited in the Microbiological Collection of the University of Szeged, Hungary. The mold strain was grown at 25 °C on a petri dish in PDA media for eight days. After that, the PDA surface was washed with DI water to get the inoculum (strain suspension). For infecting the corn matrix with the inoculum, 1 kg of corn matrix was moistened with 400 mL of DI water, sterilized and cooled down. After that, 2 mL of strain inoculum, with an OD of 2.0, was added and mixed into the matrix. The prepared matrix was incubated at 25 °C for 30 days. After 30 days, the OTA content was measured on the matrix. Ochratoxin A content was determined by the HPLC method with fluorescence detection according to [Stroka et al. \(2009\)](#) after immune-affinity clean-up with OchraStar R IAC column (Romer-Labs, Tulln) in triplicates.

An appropriate amount of OTA-containing ground corn was mixed with the basal diet (BD) to reach the predicted OTA concentration of 1,000 µg kg⁻¹. The average amount of OTA in the artificially contaminated diet (5 samples) was 1,030 µg kg⁻¹, while the amount of OTA in the BD used for the control group was 1.53 µg kg⁻¹. The concentration of some other mycotoxins, aflatoxin B₁ (0.1 µg kg⁻¹), T-2 toxin (10 µg kg⁻¹), deoxynivalenol (20 µg kg⁻¹) and zearalenone (3 µg kg⁻¹) was lower than the detection limit.

2.2. Animals, treatments, sample preparations, and determination of lipid peroxidation and antioxidant parameters

A total of 24 one-day-old broiler cockerels were used for the experiment with an initial body weight of 38.28 ± 1.94 g that were randomly divided into four experimental groups (*n* = 6 in each) to investigate the effect of both OTA and CURC: control (Ctr) group (basal diet (BD) without OTA, or CURC); CURC group (BD + 400 mg kg⁻¹ feed CURC); OTA group (BD + 1 mg kg⁻¹ feed OTA); OTA + CURC group (BD + 1 mg kg⁻¹ feed OTA +400 mg kg⁻¹ feed CURC). Curcumin obtained from the Advance Nutraceutics Ltd (Budapest, Hungary). The product is a turmeric extract with 95% curcumin content. The OTA concentration was ten times the EU recommended limit for poultry feed (2006/576/EC) to provoke toxic effects, and the concentration of CURC was chosen according to literature data ([Ruan et al., 2019](#); [Zhai et al., 2020](#); [Damiano et al., 2022](#)). Animals were kept in deep litter under a natural light regimen (15L/9D). Feed and water were provided *ad libitum* during the trial.

The calculated nutrient content of the broiler chicken diet was 13.4 MJ AME/kg, 20% crude protein, 10% ether extract and 3.5% crude fiber. The experimental period lasted for 21 days. Mortality, body weight and body weight gain were measured weekly.

At the end of the experiment, birds were euthanized by cervical dislocation. To determine biochemical parameters, liver and kidney samples were removed and collected on ice. In contrast, for gene expression studies, portions of the liver and the kidney were frozen in liquid nitrogen immediately after sampling and stored at -70 °C until analysis, preventing RNA degradation. The markers of lipid peroxidation conjugated dienes (CD), conjugated trienes (CT) and malondialdehyde (MDA) were determined from 1:9 homogenates of the liver and kidney in physiological saline. The GSH concentration and the activity of GPx were determined from the 10,000 g supernatant fraction of the 1:9 homogenates of the liver and kidney. Methods of analysis of the abovementioned biochemical parameters were described in our previous study ([Kövesi et al., 2019](#)). GSH concentration and GPx activity were normalized to the protein content of the 10,000 g supernatant fraction using the Folin-Ciocalteu phenol reagent ([Lowry et al., 1951](#)).

2.3. Quantitative Real-Time PCR

Total RNA was extracted from each group’s liver and kidney of six chickens. The RNA sample preparation, qPCR procedure and relative RNA abundance qualification were conducted without modifications as previously described ([Kövesi et al., 2019](#)). Primers for the glutathione peroxidase 3, 4 (GPX3, GPX4), glutathione synthetase (GSS), glutathione reductase (GSR), nuclear factor-erythroid 2 p45-related factor 2 (NRF2), kelch-like ECH-Associated protein 1 (KEAP1), aryl-hydrocarbon receptor (AHR) and reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed by Primer Express 3.0.1 (Thermo Fisher Scientific, San Jose) and are shown in [Table 1](#). The StepOne-Plus™ Real-Time PCR System from Applied Biosystems™ was used for the qPCR analysis. The threshold cycle (Ct) of the target and the endogenous control gene was determined by StepOne™/StepOnePlus™ Software v2.2 (Thermo Fisher Scientific, San Jose) by applying the comparative Ct method. The delta Ct (ΔCt), delta-delta Ct (ΔΔCt) and relative quantification (RQ = 2^{-ΔΔCt}) values were calculated ([Livak and Schmittgen, 2001](#)).

Table 1. Primers of target and endogenous control genes

Gene	Forward (5'-3')	Reverse (5'-3')	GenBank accession No.
GAPDH	TGACCTGCCGTCTGGAGAAA	TGTGTATCCTAGGATGCCCTTCAG	NM_204305.1
KEAP1	CATCGGCATCGCCAACTT	TGAAGAACTCCTCCTGCTTGGA	KU321503.1
NRF2	TTTTCGCAGAGCACAGATAC	GGAGAAGCCTCATTGTCATC	NM_205117.1
GPX3	ATCCCCCTTCCGAAAGTACGC	GACGACAAGTCCATAGGGCC	NM_001163232.2
GPX4	AGTGCCATCAAGTGGAACCTTCAC	TTCAAGGCAGGCCGTCAT	NM_001346448.1
GSS	GTACTCACTGGATGTGGGTGAAGA	CGGCTCGATCTTGTCATCAG	XM_425692.6
GSR	CCACCAGAAAGGGGATCTACG	ACAGAGATGGCTTCATCTTCAGTG	XM_015276627.2
AHR	GAAGACGGGTGAGAGTGGAA	CGCTTCCGTAGATGTTCTGC	NM_204118.3



2.4. Statistical analysis

All data are presented as mean $\pm$ standard deviation (SD). The Shapiro–Wilk test confirmed the normality of distribution, and homogeneity of variance was tested with Bartlett and Browne–Forsythe tests. Data with these conditions were analyzed by one-way ANOVA followed by Tukey’s post hoc test. Statistical analysis was performed using GraphPad PRISM version 7 software (GraphPad, San Diego), and $P \leq 0.05$ was considered significant.

2.5. Ethics

The guidelines set by the European Communities Council Directive (86/609 EEC) were followed during the experiment. The Food Chain Safety Food Chain Safety, Land Use, Plant and Soil Protection and Forestry Directorate of the Pest County Governmental Office (PE/EA/1964-7/2017) approved the experimental protocol with the lowest number of animals possible for an accurate statistical analysis. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Animal Ethics Committee of Szent István University (MKK-TAKT-003/2019, 27 February 2019).

3. RESULTS

3.1. Clinical findings

Clinical signs of toxicity were not observed during the trial in the experimental groups. Most of the mortality occurred in the first few days after hatching. Based on necropsy data, mortality during the first three weeks was not related to ochratoxin treatment, as no typical signs of ochratoxin contamination were observed (Table 2). Body weight of birds in the different treatment groups was only significantly different on the 1st week of the experiment, while relative

Table 2. The effect of ochratoxin A and Curcumin on mortality

Mortality	Week 1 (%)	Week 2 (%)	Week 3 (%)
Ctr	8	2	0
CURC	8	0	0
OTA	5	0	5
OTA+CURC	3	7	0

Ctr, control group; CURC, Curcumin group (400 mg kg⁻¹ feed); OTA, Ochratoxin A group (1 mg kg⁻¹ feed); OTA + CURC, Ochratoxin A (1 mg kg⁻¹ feed) and Curcumin (400 mg kg⁻¹ feed).

liver weight did not change significantly between the experimental groups (Table 3). Feed consumption could not be measured individually; therefore, statistical analysis was not possible. However, feed intake was reduced by 21 days of age in each OTA-supplemented diet group (Table 4). Overall feed efficiency was the best in the control group for the 3-week experimental period. Ochratoxin does not seem to have a drastic effect on the performance of the birds when it is loaded in the diet at a concentration of 1 mg kg⁻¹ (Table 5).

3.2. Effects of CURC and OTA on hepatic and renal lipid peroxidation

Markers of the initial phase of lipid peroxidation, CD and CT, showed a significant reduction in broilers’ liver treated with OTA alone, compared with the combined OTA and CURC treatment. Still, the difference was not significant in the control or CURC groups. On the other hand, there were no significant changes in the kidneys as an effect of the treatments at the end of the 21-day trial (Fig. 1). In addition, the level of one of the main termination markers of lipid peroxidation processes, thiobarbituric reactive substances

Table 4. Daily body weight gain (g/bird/day) in the different treatments throughout the experiment

Daily body weight gain (g/bird/day)			
g/bird/day	Week 1	Week 2	Week 3
Ctr	16.2	27.7	52.1
CURC	15.0	29.9	41.0
OTA	12.4	26.1	44.8
OTA+CURC	15.2	25.4	43.0

Table 5. Daily and total feed consumption

g/day	Week 1	Week 2	Week 3	Total (g/bird)
Ctr	40.4	46.7	75.8	1140.3
CURC	40.5	46.1	71.9	1109.5
OTA	38.1	42.7	75.1	1091.3
OTA+CURC	40.7	41.2	70.4	1066.1

Table 5. Average feed conversion ratio for the experimental period

FCR	Total
Ctr	1.70
CURC	1.85
OTA	1.87
OTA+CURC	1.82

Table 3. The effect of ochratoxin A and Curcumin on body weight

	Ctr	CURC	OTA	OTA+CURC
Week 1	152.3 $\pm$ 11.7a	142.0 $\pm$ 7.0ab	127.0 $\pm$ 27.6b	143.5 $\pm$ 11.9ab
Week 2	346.6 $\pm$ 60.6	351.0 $\pm$ 45.0	309.8 $\pm$ 64.0	321.2 $\pm$ 29.7
Week 3	711.4 $\pm$ 71.7	638.3 $\pm$ 86.9	623.6 $\pm$ 101.1	622.3 $\pm$ 107.9

Data are expressed as mean $\pm$ standard deviation (SD), $n = 6$; Ctr, control group; CURC, Curcumin group (400 mg kg⁻¹ feed); OTA, Ochratoxin A group (1 mg kg⁻¹ feed); OTA + CURC, Ochratoxin A (1 mg kg⁻¹ feed) and Curcumin (400 mg kg⁻¹ feed). Data with different letters in the same row are significantly different – $P < 0.05$.



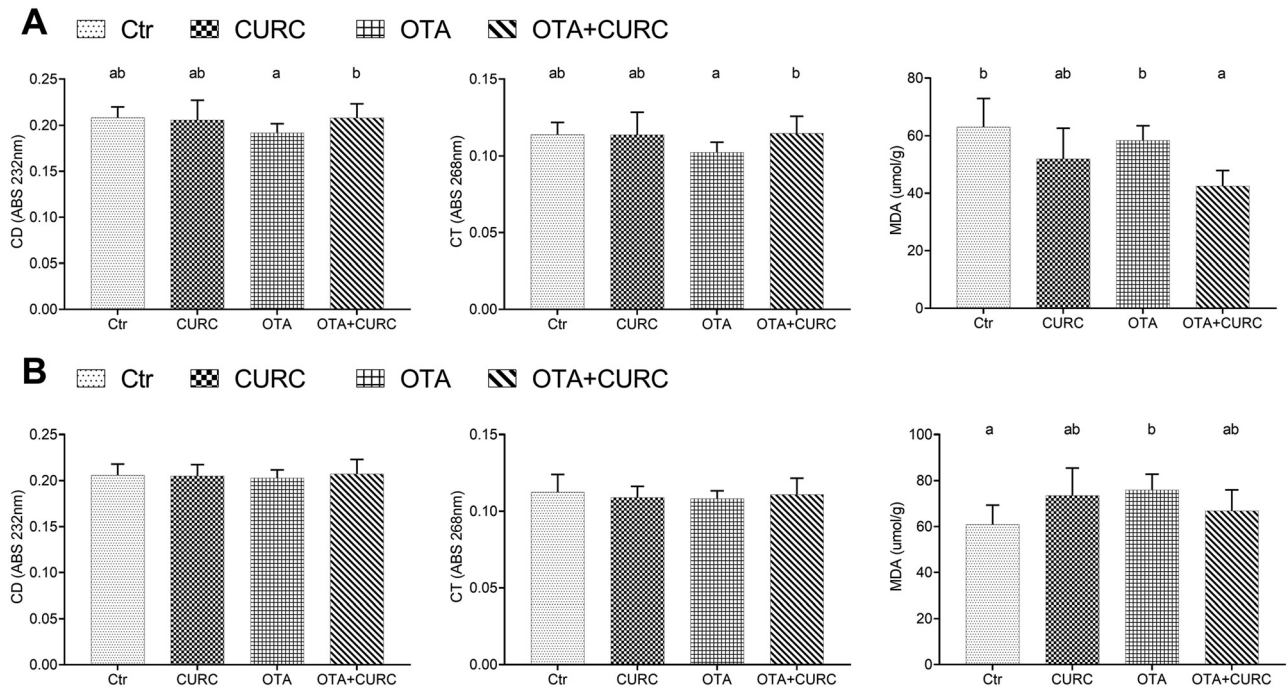


Fig. 1. Effect of 21 days OTA and CURC treatment on conjugated diene, -triene, and malondialdehyde content in the liver (A) and kidney (B) homogenates. Data are expressed as mean $\pm$ standard deviation (SD), $n = 6$; Ctr, control group; CURC, Curcumin group (400 mg kg^{-1} feed); OTA, Ochratoxin A group (1 mg kg^{-1} feed); OTA + CURC, Ochratoxin A (1 mg kg^{-1} feed) and Curcumin (400 mg kg^{-1} feed). Different superscripts mean significant differences at $P < 0.05$, while the same letter indicates no significant difference between groups

(TBARS), expressed as malondialdehyde (MDA), were significantly lower in the liver in the OTA+CURC group when compared to the OTA treatment and then to control. Still, OTA alone did not cause a significant difference from the control. However, in the case of the kidney, OTA treatment caused a significant increase in MDA levels compared to the control group, but it was the same as the control in the OTA+CURC group at the end of the trial.

3.3. Effects of CURC and OTA on hepatic and renal GSH content and GPx enzymatic activity

Figure 2 shows that GSH content in the 10,000 g supernatant fraction of liver homogenates in the OTA group was

significantly lower than in the control and OTA+CURC groups at the end of the 21-day trial. In contrast, GPx activity in the 10,000 g supernatant fraction of liver homogenates showed a significantly higher value in the OTA and OTA+CURC than in the control and CURC groups (Fig. 2). On the other hand, there were no significant alterations in GSH content and GPx activity of kidney homogenates.

3.4. Effects of CURC and OTA on hepatic and renal gene expression

OTA exposure induced the relative expression of the *KEAP1*, *NRF2*, *AHR*, *GPX4* and *GSR* genes in the liver as an effect of 21 days of OTA exposure compared to the control.

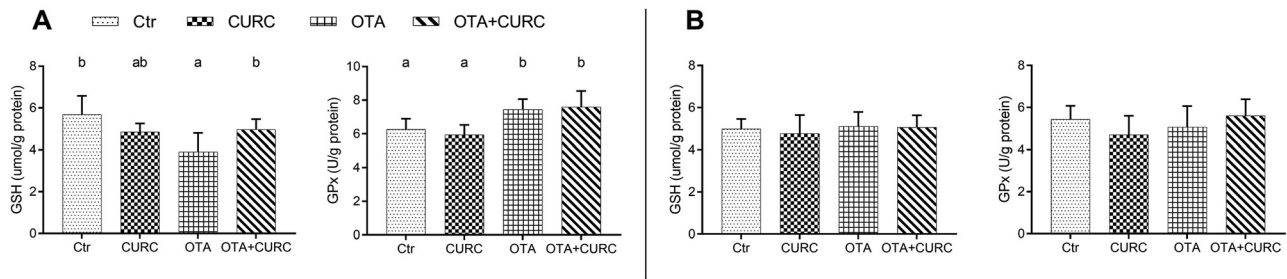


Fig. 2. Effect of 21 days OTA and CURC treatment on GSH content and GPx activity in the 10,000 g supernatant fraction of liver (A) and kidney (B) homogenates. Data are expressed as mean $\pm$ standard deviation (SD), $n = 6$; Ctr, control group; CURC, Curcumin group (400 mg kg^{-1} feed); OTA, Ochratoxin A group (1 mg kg^{-1} feed); OTA + CURC, Ochratoxin A (1 mg kg^{-1} feed) and Curcumin (400 mg kg^{-1} feed) group. Different superscripts in the same column mean a significant difference at $P < 0.05$, while the same letter indicates no significant difference between groups



Otherwise, curcumin induced the relative expression of *GPX4* and *AHR* genes only. CURC supplementation together with OTA exposure (OTA+CURC) induced a significant reduction in the relative expression of *GPX4*, *KEAP1*, *NRF2* and *AHR* genes compared to the OTA group (Fig. 3). Relative expression of *GPX4* was lower, but *NRF2* and *AHR* genes were higher than control in the OTA treated group in the kidney at the end of 21 days exposure. Curcumin supplementation of the diet caused significantly higher relative gene expression in the kidney only in the case of *NRF2*. However, curcumin supplementation together with OTA exposure (OTA+CURC) resulted in significantly lower values than the control in the case of the relative expression of *GPX4*, *GSS* and *AHR* genes. In addition, curcumin supplementation in combination with OTA exposure (OTA+CURC) revealed significantly lower relative gene expression than OTA alone in the case of *NRF2* and *AHR* (Fig. 3).

4. DISCUSSION

Ochratoxin A (OTA) is a well-known mycotoxin commonly found in poultry feeds, capable of inducing a range of detrimental or even lethal effects (Tahir et al., 2022). The kidney is a primary target organ for OTA toxicity, due to its slow elimination and the susceptibility of kidney tubular cells to OTA-induced oxidative damage (Lee et al., 2018). Nevertheless, OTA can also exert hepatotoxic effects (Li et al., 2020). Therefore, it is essential to enhance the antioxidant status of birds by supplementing their feeds with natural antioxidants with oxygen-free-radical scavenging capacities, e.g., curcumin, to counteract the adverse effects of OTA-induced oxidative stress.

Our investigation revealed that markers of the initial phase of lipid peroxidation, specifically CD and CT, decreased due to OTA exposure and MDA, as an effect of CURC alone or combined with OTA in the liver. However, CD and CT levels remained unchanged, but MDA increased as an effect of OTA in the kidney. These results suggested that lipid peroxidation in both organs was probably in a later stage, when most of the unsaturated double bonds of the fatty acids were saturated. Consequently, no further diene or triene formation occurred by the end of the 21-day trial. However, in the kidney, lipid peroxidation reached its terminal phase, as evidenced by the higher malondialdehyde (MDA) content following OTA treatment.

Intriguingly, the glutathione redox system did not show activation in the kidney, in response to lipid peroxidation, indicating an inadequate antioxidant response. This hypothesis is supported by the downregulation of genes encoding components of the glutathione redox system in the kidney. In contrast, the liver exhibited a different trend, with decreased lipid peroxidation following OTA+CURC treatment, possibly due to forming termination markers other than malondialdehyde, e.g., hydroxyalkenals. The activated antioxidant response did not prove this effect, because gene expression of the master regulator, *NRF2*, did not increase as an effect of CURC in OTA-induced oxidative stress.

The observed alterations in lipid peroxidation parameters appear to be closely linked to changes in glutathione redox parameters. The GSH content and the activity of GPx exhibited consistent increases during OTA exposure. However, the GSH content in the liver decreased due to OTA exposure, indicating an oxidative stress response, which was subsequently restored with CURC supplementation. The increased GPx activity occurred due to OTA exposure, and CURC supplementation showed the same effect, meaning that CURC did not modify the OTA-induced glutathione

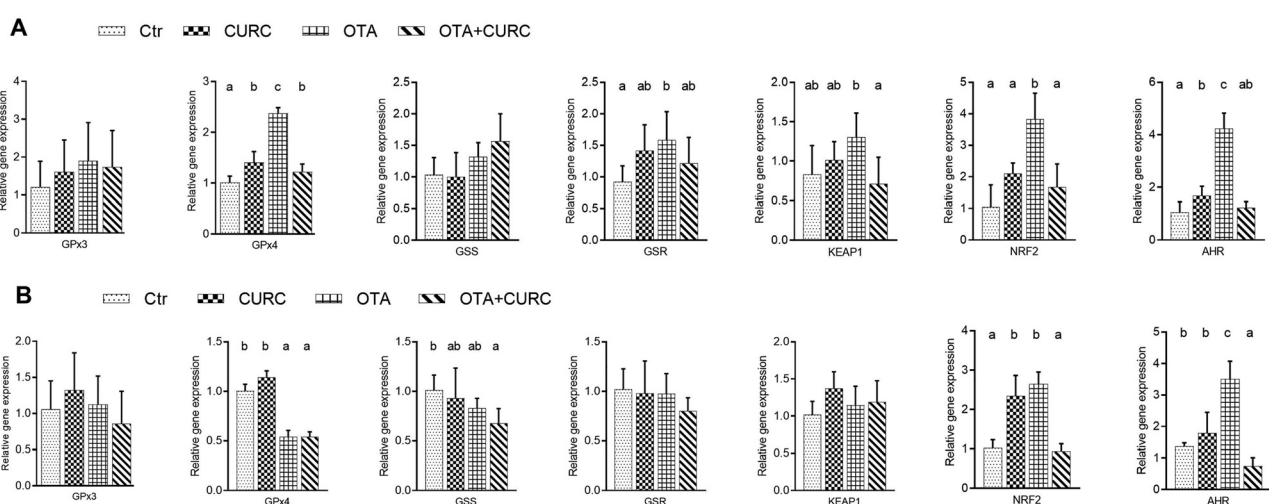


Fig. 3. Effect of 21 days OTA and CURC treatment on the relative expression of *GPX3*, *GPX4*, *GSS*, *GSR*, *KEAP1*, *NRF2*, and *AHR* genes in liver (A) and kidneys (B) of broiler chickens. Values are presented as mean normalized expression toward GAPDH expression. Ctr, control group; CURC, Curcumin group (400 mg kg⁻¹ feed); OTA, Ochratoxin A group (1 mg kg⁻¹ feed); OTA + CURC, Ochratoxin A (1 mg kg⁻¹ feed) and Curcumin (400 mg kg⁻¹ feed). Data are expressed as mean $\pm$ standard deviation (SD), $n = 6$; a,b Different superscripts mean significant difference at $P < 0.05$, while the same letter indicates no significant difference between groups

redox response. Therefore, the lower GSH content in the liver of the OTA group can be explained by the higher GPx activity, because GSH is the co-substrate of GPx. In the case of its higher activity, GSH pools will be exhausted if the reduction of glutathione disulfide to GSH and/or GSH synthesis is inadequate.

The redox-sensitive KEAP1-NRF2 pathway is a primary inducible defense against oxygen and nitrogen-free radical-initiated oxidative and electrophilic stress by regulating the expression of several genes of the antioxidant gene cluster in the ARE, such as the glutathione metabolism enzymes, such as GSS and GSR and glutathione peroxidases (GPX3 and GPX4) (Baird and Yamamoto, 2020).

CURC has been reported to induce the NRF2 pathway, contributing to cellular protection against oxidative stress and its consequences, such as apoptosis (Ashrafizadeh et al., 2020). This effect may be mediated through PKC δ -mediated p62 phosphorylation at Ser351 (Park et al., 2021), disrupting the binding of NRF2 to KEAP1 and decreasing NRF2 degradation.

The mRNA expression of *NRF2* showed upregulation as an effect of OTA in the liver and kidney. The previous findings supported that OTA induces oxidative stress, activating the stress response and its master regulator, NRF2 (Kensler et al., 2007). However, the literature does not agree on whether OTA induces or inhibits *NRF2* mRNA expression. Ferenczi et al. (2020) reported that OTA exposition significantly increased the *NRF2* mRNA levels in an *in vivo* experiment with mice using 1 and 10 mg kg⁻¹ b.w. in single (24 h) and repeated (72 h) and 0.5 mg kg⁻¹ b.w. in repeated 21-days exposure. Our previous *in vivo* study with broiler chickens also demonstrated increased *NRF2* mRNA levels as an effect of 1 mg OTA/kg feed oral exposure for 21 days (Kövesi et al., 2019). Also, Shin et al. (2019) in an *in vitro* model (50–200 nmol for 72 h) showed that OTA-induced oxidative stress in human hepatocytes was associated with increased expression of the *NRF2* gene and nuclear localization of Nrf2. In the study of Boesch-Saadatmandi et al. (2009), OTA (2, 5, and 10 μ mol for 24 h) decreased the expression of *NRF2* *in vitro* in cells derived from pigs' kidneys. A similar effect was observed *in vitro* in porcine renal proximal tubular cells (Stachurska et al., 2013). This discrepancy likely arises from variations in OTA doses, species, and differences between *in vitro* and *in vivo* models. The effect of CURC was different in the kidney and liver because *NRF2* gene expression increased in the kidney but not in the liver.

The effect of CURC on the expression of *NRF2* expression was described in different *in vitro* and *in vivo* models (Ashrafizadeh et al., 2020). Still, organ differences found in the present study require further research. However, the treatment with OTA+CURC showed a marked decrease in the *NRF2* expression in the liver and kidney, as compared to the individual effect of OTA in the liver and the individual effect of OTA and CURC in the kidney. These results suggested that CURC supplementation did not mitigate the OTA-induced Nrf2 response in the liver and kidney.

Among the GPx isoenzymes, glutathione peroxidase 3 (GPX3), which originates from kidney tubular cells (Avissar

et al., 1994), showed minor changes but significant down-regulation of *GPX4*, as an effect of OTA during the experimental period, which is partly contradictory to our previous findings where 21 days oral exposure of the same dose (1 mg OTA/kg feed) caused significant upregulation of *GPX3* and significant downregulation of *GPX4* in the kidney of broiler chickens (Kövesi et al., 2019). Explaining the discrepancy in the case of the expression of *GPX3* requires further research. Still, it is probably caused by the different ages of the birds because the previous study started at 21 days, but the recent one was at one day of age.

The effect of CURC on OTA toxicity was not demonstrated in the present trial on the expression profile of the *GPX3* gene, which probably means that the applied dose of CURC did not activate the antioxidant response, in this case, GPx3 protein expression, which requires the elimination of the effect of OTA. The other possible explanation is that the effect of OTA and CURC overlap. On the other hand, the gene expression of the other glutathione peroxidase enzyme, *GPX4*, increased as an effect of CURC and OTA treatment in the liver, but decreased significantly due to CURC supplementation (CURC+OTA group). This result supports our hypothesis about the overlap between the effects of OTA and CURC when added together. In contrast, OTA exposure caused downregulation of *GPX4* gene expression in the kidney, and the CURC supplementation did not modify its effect. This result suggested an impairment of the activation of the antioxidant gene cluster in the kidney. Still, gene expression of *NRF2* and *AHR* did not support it, probably because of other factors, such as miRNAs (Yang et al., 2011), that may modify transcription factors' effect at the DNA level.

Furthermore, GSS and GSR gene expression did not increase with OTA exposure or CURC supplementation, consistent with previous studies where OTA exposure downregulated the genes involved in glutathione (GSH) metabolism (Marin-Kuan et al., 2006). However, CURC at the applied dose did not modify the effect of OTA.

OTA exposure significantly increased the aryl hydrocarbon receptor (AHR) expression in the liver and kidney. This increase was also observed in the liver following individual CURC treatment but not in the kidney. The combined OTA+CURC treatment restored AHR levels in both organs. These findings align with existing literature, as both CURC and OTA have been reported to activate the gene expression of the AHR transcription factor (Lee et al., 2018; Shin et al., 2019). However, in the case of OTA exposure, CURC supplementation eliminates this effect. However, according to our knowledge, this is the first experimental evidence of the upregulation of *AHR* in OTA and CURC treatment and the mitigating effect of CURC in OTA exposure in broiler chicken.

5. CONCLUSIONS

In summary, our study has shed light on the impact of OTA on the lipid peroxidation process in broiler chickens, revealing that this process had reached its terminal phase by



the end of the 21-day trial. While CURC did not demonstrate a significant antioxidant effect in mitigating lipid peroxidation in the kidney, it did exhibit some antioxidant activity in the liver. Notably, both OTA and CURC treatments, individually and in combination (OTA+CURC), resulted in the upregulation of mRNA expression of *NRF2* and *AHR* transcription factors along with their corresponding genes in the liver and kidney of chickens after 21 days of treatment. This activation appeared to offer partial protection against OTA-induced oxidative stress, with CURC showing a modest modifying effect, possibly due to overlapping effects between OTA and CURC. However, it's important to note that the gene expression of the glutathione redox system did not consistently reflect the changes in the transcription factors induced by OTA or CURC, whether applied individually or in combination.

These findings suggest a complex interplay between OTA and CURC, influencing the antioxidant response and potentially impacting pathways such as KEAP1-NRF2-ARE and AHR. The complex dynamics unveiled by this research underscore the need for further investigation into the interactions between mycotoxins and natural antioxidants in poultry health.

Future research should delve deeper into the mechanisms underlying these interactions and explore their practical applications in poultry farming and food safety. While CURC's mitigating effect on OTA-induced oxidative stress may be modest, it opens the door to a broader exploration of dietary interventions to enhance avian health and productivity.

Data availability statement: The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

Conflicts of interest: The authors do not declare a conflict of interest.

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