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Protective Effects of New Antioxidants in OTA-Treated Chicken Kidney †

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Abstract: Ochratoxin A (OTA) is a mycotoxin which represents an emerging problem for both animal and human health, due to its high presence in feed and foods. Exposure to OTA is associated with oxidative stress-induced nephrotoxicity. Therefore, the identification of new antioxidant or adsorbent substances with protective action constitutes one of the main challenges to reduce the negative effects induced by mycotoxins. For this purpose, we investigated the effect of two innovative feed additives, a bio-organoclay (CHS) and a mixture of a tri-octahedral Na-smectite with a ligno-cellulose based material (MIX) alone or in combination with OTA in kidneys of treated chickens. Real-Time PCR analyses for NADPH oxidase 4 (NOX) and p47-phox were performed to evaluate oxidative stress. Our results demonstrated an increase in NOX and p47-phox levels in OTA-treated chickens. Moreover, CHS, more than MIX, was able to reduce OTA-induced toxicity, restoring NOX levels. Taken together, these findings highlight the potential beneficial role of CHS in reverting OTA-induced nephrotoxicity in chickens and could lead to the production of healthier foods with beneficial consequences for human and animal health.

Keywords: poultry; Ochratoxin A; mycotoxin-binders; oxidative stress; feed additives

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1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several fungi species belonging to the genera Aspergillus and Penicillium [1]. The presence of OTA in animal feed represents an emergency problem for both animal and human health [2,3]. Indeed, contaminated animal products, such as eggs, milk and meat can also be an important source of human exposure to OTA [4].

The target organ of OTA is the kidney, in fact OTA exposure has been linked to nephrotoxicity in humans and in several animal species [5], including chickens [6]. OTA is well known to increase reactive oxygen species (ROS) levels, promoting DNA damage, cell cycle arrest and apoptosis [7]. Despite the existence of several methods of detoxification from OTA [8], the presence of OTA in animal feed is still a serious problem in poul-

try farms [9]. However, organic binders and inorganic binders, such as silicates, used as additives in feed, represent innovative mycotoxin-detoxifying agents because of their adsorbent action and their ability to bind mycotoxins, thus reducing their negative effects [10].

The aim of our work was to evaluate the potential protective action of two mycotoxin binders, MIX and CHS, in reducing renal oxidative stress in broiler chickens fed an OTA-containing diet. For this purpose, since NADPH oxidase 4 (NOX), the most abundant isoform of the NADPH oxidase family in the kidney, plays an essential role in the production of ROS by reducing molecular oxygen to superoxide [11], we analyzed the transcript levels of NOX and its subunity, p47-phox.

2. Experiments

2.1. Ethical Statement

The use and the care of animals in this work was approved by the Bioethic Committee of the University of Turin (Italy) (Approval Number: 319508/2017-PR).

2.2. Animal Treatments and Experimental Plan

Thirty-six 21-day-old, female Ross broiler (ROSS 308) chickens (average body weight 860.25 ± 25.2 g) used in this study were randomly divided into six experimental groups (6 chickens for each group) and were housed in cages under the conditions laid down in Directive 2007/43/CE. Chickens were fed a basal diet (190–210 g Kg^{-1}crude proteins; 12.6–13.6 MJ Kg^{-1} Metabolizable Energy) ad libitum. After a 4-day adaptation period, chickens were treated daily orally for 10 days as follows: control group (basal diet 2 Kg/chicken/die); OTA group (0.3 mg/Kg feed); CHS group (5 g/Kg feed); MIX group (5 g/Kg feed); OTA plus CHS group (0.3 mg OTA/Kg feed +5 g CHS/Kg feed) and OTA plus MIX (0.3 mg OTA/Kg feed + 5 g MIX/Kg feed). At the end of the treatments, chickens were sacrificed and kidneys were collected. All samples were stored at −80 °C until analysis.

2.3. RNA Extraction and Complementary DNA Synthesis

Kidneys were homogenized with 1 mL of the TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in TissueLyser (MM300, Retsch, Conquer Scientific, San Diego, CA, USA) for 5 min at 20.1 Hz using Tungsten Carbide Beads (3 mm) (Qiagen, Venlo, The Netherlands). After the complete homogenization of samples, total RNA extraction was carried out as recommended in the TRizol manufacturer's protocol and DNA was eliminated by using DNase. Nano-Drop (ND-1000 UV–Vis spectrophotometer; NanoDrop Technologies, Wilmington, NC, USA) was used to evaluate RNA concentration in samples, setting the absorbance at 260 nm. Complementary DNA synthesis was performed by retro-transcription of 1000 ng of each RNA sample with the iScriptTM cDNA Synthesis Kit (BIORAD, Hercules, CA, USA), according to manufacturer's instructions using the GeneAmp PCR System 9700 (Perkin Elmer, Waltham, MA, USA).

2.4. Primer Design for Selected Genes of Interest (GOI) and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The selected GOI for our experiments were NOX andp47-phox. 18S was used to normalize the expression levels of GOI.Primer3 v. 0.4.0 (http://frodo.wi.mit.edu/primer3/) and Gene Runner software (V3.05, Hasting Software, Hastings, NY, USA) were used to design primers (Table 1) and to predict the primer's melting temperature (Tm) respectively. In addition, it was checked if the primers formed dimers and internal loops. The evaluation of the specificity of primers and RT-qPCR experiments were performed as described previously [12]. Briefly, a Via7 real-time PCR system (Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA) was used for RT-qPCR. PCR volume of each sample was 10 μL with 5 μL of Fast Start SYBR Green Master Mix (Roche, Basilea, Switzerland), 0.7

pmol/μL for each oligo, and 1 μL of the cDNA template (at a dilution of 1:10). All RT-qPCR reactions were performed in triplicate, considering three no-template negative controls (NTC) for each primer pair. Primer reaction efficiency (E) and correlation factor (R2) were determined by serial dilutions of cDNA (1:5, 1:10, 1:50, 1:100 and 1:500). Each oligonucleotide pair standard curve was plotted with the obtained dilution points by using the cycle threshold (Ct) value against the logarithm factor of each dilution and using the equation $E = 10^{-1}/slope$. Primer efficiencies (E) ranged from 93 to 100%.

Table 1. Gene names, primer forward (F) and reverse (R), amplicon size, oligo efficiencies (E) and correlation factors (R2), and GenBank accession numbers.

Gene Name	Primer F Primer R	Amplicon Size	E	R2	Acc.Number
NOX	TCGGGTGGCTTGTTGAAGTA- GTCTGTGGGAAATGAGCTTGG	224	90	0.99	NM_053524
p47-phox	TACGCTGCTGTTGAAGAGGA- GATGTCCCCTTTCCTGACCA	105	100	0.99	AY029167.1
18S	AGAAACGGCTACCACATCCA- CCCTCCAATGGATCCTCGTT	158	93	0.99	NR_046237.1

3. Results

Real-Time PCR analyses were performed to evaluate the transcript levels of NOX and p47-phox in chicken kidneys. OTA-treatment significantly increased NOX and p47-phox levels compared to the control group (Figures 1 and 2).

The treatment with CHS restored NOX levels in OTA-treated chickens (Figure 1a); but no effect was observed in p47-phox levels (Figure 1b). On the contrary, CHS alone exhibited a significant reduction in p47-phox levels compared to the control (Figure 1b).

In contrast, MIX did not show a protective effect on NOX and p47-phox levels when used in co-treatment with OTA (Figure 2a,b).

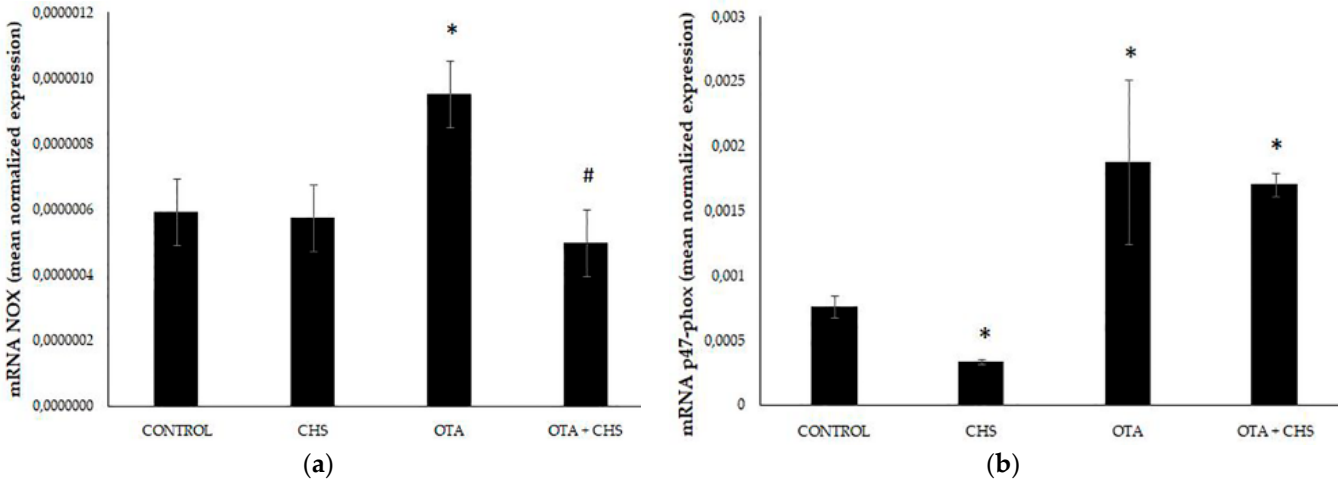

Figure 1. Effect of a bio-organoclay (CHS) on oxidative stress: (**a**) mRNA NOX level in the Control group (CONTROL), bio-organoclay group (CHS), Ochratoxin A group (OTA) and Ochratoxin A plus bio-organoclay group (OTA + CHS); (**b**) mRNA p47-phox level in Control group (CONTROL), bio-organoclay group (CHS), Ochratoxin A group (OTA) and Ochratoxin A plus bio-organoclay group (OTA + CHS). The experiments were conducted in triplicate, and values were presented as mean normalized expression (MNE) normalized towards 18S expression (mean±standard error) (* $p < 0.05$ versus control; # $p < 0.05$ versus OTA).

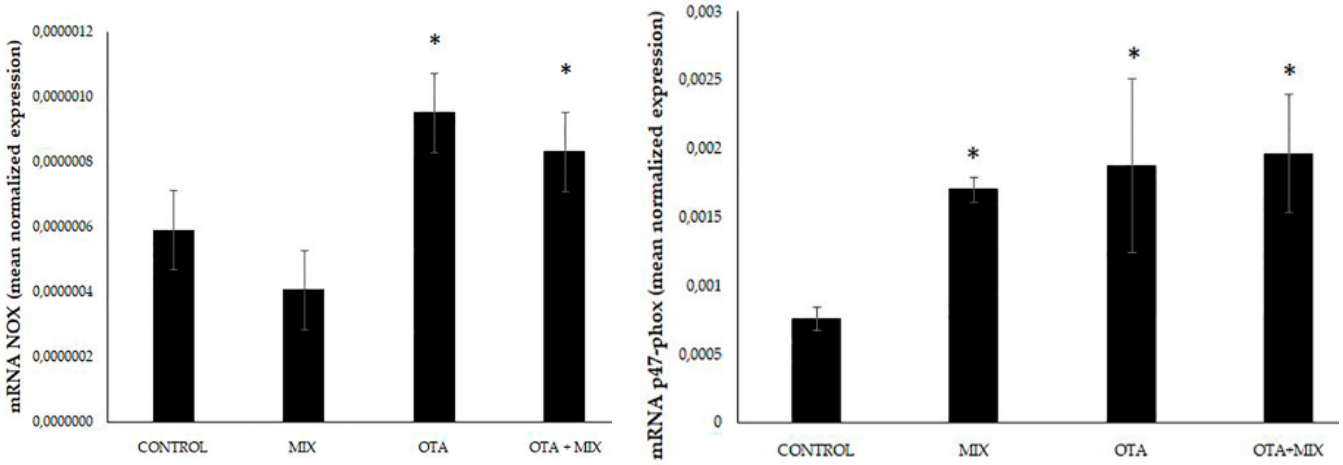

Figure 2. Effect of a mixture of a tri-octahedral Na-smectite with a ligno-cellulose-based material (MIX) on oxidative stress: (**a**) mRNA NOX level in the Control group (CONTROL), MIX group (MIX), Ochratoxin A group (OTA) and Ochratoxin A plus MIX group (OTA + MIX); (**b**) mRNA p47-phox level in Control group (CONTROL), MIX group (MIX), Ochratoxin A group (OTA) and Ochratoxin A plus MIX (OTA + MIX). The experiments were conducted in triplicate, and values were presented as mean normalized expression (MNE) normalized towards 18S expression (mean±standard error) (* $p < 0.05$ versus control).

4. Discussion

The presence of OTA in feed and food represents a widespread problem both for animal and human health. Indeed, OTA exposure is associated with nephrotoxicity in several animal species and with Balkan Endemic Nephropathy (BEN) in humans [5].

Moreover, Pozzo et al. demonstrated that the presence of OTA could be even detected in kidneys of broiler chickens fed an OTA-contaminated diet at the maximum levels recommended by the EU. In turn, thiobarbituric acid reactive substances (TBARs) levelswere increased as a consequence of OTA-exposure [13].

Several studies proved that oxidative stress was involved in OTA-induced nephrotoxicity and the use of antioxidants reduced OTA-induced renal injury [14–16]. For this purpose, in this work we investigated the effects of two antioxidants, CHS and MIX, in reducing OTA-induced oxidative stress in chicken kidneys.

NOX, together with its subunities, plays a main role in renal diseases where oxidative stress is involved [12]. From our RT-qPCR analysis, OTA increased NOX and p47-phox levels (Figures 1 and 2), confirming the relationship between OTA exposure and oxidative stress in agreement with data in the literature [17].

In addition, we found that NOX levels were completely restored after the treatment with CHS in OTA-exposed animals (Figure 1a). These results were in line with several studies showing the adsorbent and antioxidant action of CHS components [10,18].Therefore, our findings highlighted the protective action of CHS in reducing OTA-induced oxidative stress in chicken kidneys.

However, after the treatment with CHS, p47-phox levels were not decreased in OTA-exposed chickens (Figure 1b).

In our RT-qPCR analyses NOX and p47-phox transcript levels were not significantly reduced after the dietary MIX supplementation in OTA-treated chickens (Figure 2). However, the evaluation of antioxidant enzymes levels will be necessary to better understand the role of MIX on oxidative stress.

5. Conclusions

CHS, more than MIX, was capable of reducing OTA-induced toxicity in chicken kidneys. Dietary CHS supplementation could be a winning strategy to protect animal

health and reduce the economic adverse effects due to the presence of OTA in poultry farms.

Author Contributions: S.D., S.F., R.C., A.S. and G.A., conceived and designed the experiments; E.A., C.L. (Consiglia Longobardi), M.L., C.L. (Chiara Lauritano), S.D., R.C., G.A. and W.J., performed the experiments; E.A., C.L. (Consiglia Longobardi), C.L. (Chiara Lauritano), S.D. and R.C., analyzed the data; E.A., S.D. and R.C., wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The use and the care of animals in this work was approved by the Bioethic Committee of the University of Turin (Italy) (Approval Number: 319508/2017-PR).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

OTA Ochratoxin A
ROS Reactive Oxygen Species
NOX NADPH oxidase 4

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