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Prevalence of Ochratoxigenic Fungi and Ochratoxin A Residues in Animal Feeds and Modulation of Their Toxic Effects by Glutathione

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ABSTRACT

The present study was undertaken to investigate the prevalence of ochratoxigenic fungi and ochratoxin A in animal feeds. The efficacy of an antioxidant material glutathione (GSH) on ochratoxicated rats was evaluated. Our investigation revealed that, the most predominant isolated molds from the examined animal feed samples were *A. ochraceous* and *A. niger* that recovered from (30% and 25%) of dree (hay) samples, respectively. The most ochratoxigenic strains was *A. niger* that recovered from dree (hay) and yellow corn was produced ochratoxins at the mean level of (20±1.75 mg/kg) and (13.34±6.5 mg/kg), respectively. The maximum level of ochratoxin residues detected in yellow corn at a mean level of (9.84±5.33 mg/kg) and the minimum level was detected in rice straw at a mean level of (1.88±0.44 mg/kg). Significant increase in serum ALT and AST activities and urea level, decrease in serum total protein, albumin, globulin levels and corresponding reduction of A:G ratio were observed in ochratoxicated rats, in addition to an increase in MDA level. The measurement of tumor marker of liver (alfa-feto-protein) (AFP) showed significant increase among ochratoxicated group and it could be concluded that OTA had a hepatic genotoxic effect (that correlated with activation of p53 transcription). Ochratoxin A decreased serum iron, TIBC and transferrin percentage. GSH increased the normal tissue defense capability and suppressing toxicity invasion, leading to an improvement in biochemical parameter. At cellular level; cytochrome-c dependent, capase-3 apoptotic pathway was incriminated in ochratoxicosis. Glutathione can restore the DNA integrity (in ochratoxicated rats) as indicated by expression activities of p53 and mmp2 and significantly block cytochrome-c dependent- caspase-3 apoptotic pathway. Supplementation of glutathione preserved the cellular pool of glutathione and diminishes the oxidative stress involved in ochratoxicosis so indicate to the economic importance of addition of glutathione to animal healthy feeds to overcome the side dangerous effects of ochratoxicosis.

Keywords

Fungi, Ochratoxin (OTA), Animal feeds, Antioxidants, Glutathione (GSH), Alpha Feto Protein (AFP)

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Introduction

Recently, the Food and Agriculture Organization (FAO, 2011) reported that in areas such as Asia and Africa, 8–18% of cereals commodities, seeds, fruits and

vegetables are lost during post-harvest handling, processing and storage and about 13.5% of the total value of grain production was lost. The majority of these losses can be attributed to fungal growth and contamination with mycotoxins.

Ochratoxin A (OTA) is a mycotoxin detected in a variety of animal feeds from countries with a moderate temperate climate at temperatures below 30 °C and at water activity above 0.8 (Duarte *et al.*, 2010). Ochratoxin A (OTA) is produced by fungi of the genera *Aspergillus* and *Penicillium* the major species implicated in OTA production includes *Aspergillus ochraceus*, *A. niger*, *Aspergillus carbonarius*, *Aspergillus melleus*, *Aspergillus sclerotiorum*, *Aspergillus sulphureus*, *P. verrucosum* and *Pichia verrucosum* (Benford *et al.*, 2001 and El-Hamaky *et al.*, 2016).

On the other hand, Van Egmond (1991) reported that the acceptable levels of OTA ranged from 0.01 to 0.05 mg/kg for food and from 0.1 to 1.0 mg/kg for animal feed. The permissible limits OTA in different Cereal grains and their products reported recently by the European Commission Regulation (2006) must be not more than 0.005 mg/kg (5.0 µg/kg) and 0.01 mg/kg (10.0 µg/kg) in other feedstuffs.

OTA is absorbed from the gastrointestinal tract, mainly in the small intestine; it is distributed via the blood, mainly to the kidneys (the known target organ of OTA toxicity), where it accumulates, with lower concentrations found in liver, muscle and fat. Transfer to milk in mice, rats, rabbits and humans, but little OTA is transferred to the milk of ruminants (Ringot *et al.*, 2006).

The periodic annual reports of FAO/WHO (2007) noted that the dietary exposure to OTA, which causes various toxic effects, such as nephrotoxic, hepatotoxic, teratogenic, genotoxic, neurotoxicity, suppress immune function and reproductive deficiency in humans and animals (JECFA, 2007).

In general, the widespread threat of OTA to human and animal health gained urgent major

interest of researchers for detoxification of this toxin. Therefore, various physical, chemical and biological methods had been developed to reduce and/or eliminate the toxin in contaminated food and feed products to improve food safety and minimize economic losses in human and animal health (Gholampour *et al.*, 2012).

Hence, the addition of nutrients or additives with protective properties to contaminated foodstuffs is one approach that reduces the toxicity of mycotoxins. One of natural effective compounds is Glutathione (GSH), a biological intracellular antioxidant which synthesized mainly in the liver. Orally supplemented GSH is directly absorbed through the intestinal mucosa and acts as the functional component against oxidative stress (Hunjan and Evered, 1985).

Glutathione (GSH) considered the master antioxidant has the ability to regenerate other antioxidants, such as vitamins C and E and supports the function of liver, kidneys and GI tract. In addition, GSH plays an essential role in body's major detoxification pathways where, it helps eliminate toxins and ingested chemicals that the body has already absorbed; neutralizes toxins in the GI tract before they are even absorbed and stimulates the natural killing ability of immune cells (Therapeutic Research Faculty, 2009).

GSH is a most abundant intra-cellular non protein thiol that compartmented in nucleus (linked to DNA histone) (Go and Jones, 2010)), cytosol (reduced form GSH) and mitochondria (GSH/ GSSG cycle). So, it had a master role in regulating DNA repair (Chatterjee, 2013), protein post-translational and/or structural modification and oxidative status of the mitochondria (Circu and Aw, 2012). These facts indicate the basic role of glutathione in regulation and/or end cell survival decision and integrity. Mitochondria could not synthesize its glutathione (mtGSH)

by itself, so depend on the cytosolic component of de novo synthesized glutathione (Chen and Lash, 1998), and also provided by plasma glutathione pool (Ballatori *et al.*, 2005). The impairment of mtGSH homeostasis will create state of oxidative stress (Han *et al.*, 2016). The nuclear pool of glutathione also depends on the cytosolic one, with passive transportation from the cytoplasm to the nucleus; that help in preservation of nuclear protein integrity (Go and Jones, 2010) as redox alteration will led to inability of DNA to bind to the transcription factors (Green *et al.*, 2006 and Han *et al.*, 2016).

Necrosis due to oxidative stress occurs after a certain critical redox threshold is passed and below this threshold redox alterations cells sensitize to TNF-induced apoptosis by inhibiting NF- κ B activation and/or transactivation; so Chatterjee (2013) defined GSH as a single molecule with multi-function; in DNA damage and repair, redox regulation and cell signaling. Ochratoxin A had been incriminated to hinder intra-cellular glutathione production (Guilford and Hope, 2014) through blocking effect on glutathione oxidase enzyme (Schaaf *et al.*, 2002); absence of adequate GSH concentrations cause numerous oxidative and nitrosative reactive intermediates persist, ROS (O, OH, H₂O₂, and NO radicals), with possible induction of DNA adducts formation, and subsequent genotoxic and oxidative damage effects (Dickinson and Forman, 2002). So glutathione deficiency due to inhibition of its synthesis could be overcome by administration of glutathione esters which hydrolyzed intra-cellular to provide functional glutathione (Meister, 1991).

Therefore, the present study was undertaken to investigate the effect of OTA on some hepatic glutathione functions at the protective, therapeutic and competitive levels. This study included the detection of the status of

cytochrome-c (as indicator of mitochondrial integrity) and the possible associated caspase-3 activities, p53 (as a sensor of genome stability) and the subsequent release of mmp2, as an indicator for transformed behavior and evaluate the benefit effect of adding glutathione to animal feeds.

Materials and Methods

Materials

Feed samples

A total of 80 different samples of animal feeds (20 samples of each of drees (hay), yellow corn, rice straw, processed feeds (50 g of each sample) were collected from some farms and markets at Giza Governorate. In these farms, the animals were sustained from the signs of toxicosis as vomiting, diarrhea and sudden death of affected animals may be occurred.

Glutathione

The reduced glutathione was obtained from S.D. Fine-CHEM Ltd Company. It the following biochemical and physical properties (C₁₀H₁₇N₃O₆S, M.W. 307.33, B.No. K002/1396/2112/62, Pkg. 5gm and its minimum assay: 98.0%). The dose of 50 mg/Kg (b.w.)/ daily was given to experimental rats for two weeks.

Experimental animals

A total of 25 male albino rats, aged 4-6 weeks and weighing approximately 100-150 gm were obtained from faculty of veterinary medicine, Cairo University.

They were provided with feed and water *adlibitum* and maintained under laboratory conditions. Such animals were divided into five groups (5 rats in each) and caged separately.

Methods

Isolation and identification of fungi from animal feeds

Ten grams of finely ground sample was transferred aseptically into sterile blender jar, to which 90 ml of 1% peptone water were added and homogenized in a sterile warring blender for 2 minutes and the homogenate were prepared (APHA, 2003).

One milliliter quantities of the previously prepared dilutions were inoculated separately into Petri dish plates and mixed with Sabouraud's dextrose agar (SDA) medium containing 0.05 mg of Chloramphenicol/ml. The plates were then left to solidify after mixing, and incubated at 25°C for 3-5 days. The counts of mould and yeast colonies were recorded.

Individual suspected colonies were selected depending upon their morphological characters. Stock culture were made from each isolate and monitored on Czapek-Dox, malt extract and potato dextrose (PDA) agar slopes for further identification. The identification of different species was carried out by observation of their macroscopic and microscopic characteristics of mold and yeast colonies according to, Pitt and Hocking (2009).

Detection of ochratoxin A residues in the animal feed samples by thin layer chromatography (TLC) (A.O.A.C., 2000)

Animal feed samples were analyzed for detection of OTA measured qualitatively by TLC. Finely ground 25 gm of each sample was subjected for extraction, clean up and purification of OTA residues. The toxin residues were examined under long wave ultraviolet light.

The production and estimation of ochratoxin on yellow corn by the isolated *A. ochraceus* and *A. niger* from the present samples (Trenk *et al.*, 1971 and Pestka, 1996)

The *A. ochraceus* and *A. niger* that were recovered from feed samples were screened for OTA production on yellow corn. In a flask containing 100 gm of finely ground yellow corn and 40-50 ml of distilled water were mixed and autoclaved at 121°C for one hour. The flask was shaken to prevent cooking of yellow corn. It was inoculated with spore suspension of 2 slants of *A. ochraceus* or *A. niger* and incubated for 4 weeks at 25-28°C. After end of incubation period, the corn was removed from flasks, dried; finely ground and 50 g of each were subjected for estimation of OTA. The estimation of prepared OTA was measured qualitatively by TLC according to the recommended method of AOAC (2000).

Experimental design

As illustrated in (Table 1), a total of 25 albino rats were divided into 5 groups (5 rats in each). The animals in group 1 was given a healthy basal diet (B.D.) and water for 5 weeks and kept as a (negative control). While, rats of group 2 given basal diet (B.D.) for first 2 weeks, followed by administration of OTA by stomach tube (50 µg/kg b.w. dissolved in corn oil/daily) (Vettorazzi *et al.*, 2011) for next 3 weeks (positive control). On the other hand, animals of group 3 was given OTA by stomach tube (50 µg/kg b.w. dissolved in corn oil/ daily) for first 3 weeks and then, given glutathione by stomach tube (50 mg/Kg b.w./daily) for next two weeks.

Whereas, in group 4, animals were given glutathione by stomach tube (50 mg/Kg b.w./daily) for first two weeks, followed by administration of OTA by stomach tube (50 µg/kg b.w./daily) dissolved in corn oil for next

3 weeks. While, rats of group 5 fed on healthy basal diet (B.D) for first 2 weeks and then were given glutathione by stomach tube (50 mg/Kg b.w./daily), at the same time were given OTA by stomach tube (50 µg/kg b.w./daily dissolved in corn oil/ daily) for next 3 weeks.

The biochemical analysis

Blood samples

At the end of each experimental period and after overnight fasting, serum samples were collected by ocular vein puncture in dry, clean, and screw capped tubes and serum was separated by centrifugation at 3000 r.p.m for 15 minutes. The clean, clear serum was separated and received in dry sterile samples tube, then kept in a deep freeze at -20°C until used for subsequent biochemical analysis.

Biochemical analysis parameters:

The activities of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of Reitman and Frankel (1957).

Serum urea according to Wybenga *et al.*, (1971).

The estimation of serum total protein was measured as the method as SonnenWirth and Jareet (1980).

Albumin was evaluated according to the method of Webster (1977).

Globulin was determined by subtraction of albumin from total protein according to Reinhold (1953).

The lipid peroxidation as malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) were estimated as Montgomery and Dymock (1961), Aebi (1974) and Okhawa *et al.*, (1979).

For estimation of Alpha fetoprotein (AFP) the method of Chan and Miao (1986) was applied.

The measurement of serum iron, total iron-binding capacity (TIBC) and transferrin % were determined by a modification of the method of Young and Hicks (1965).

Histopathological examination

At the end of experimental period (5 weeks), animals were anesthetized by chloroform and ethically scarified, livers were dissected and preserved in formalin. Formalin fixed paraffin embedded sections were processed routinely for H&E staining (Suvana *et al.*, 2012).

Further sections on positively charged coated slides were used for IHC technique using Abs marking the target elements.

Cytochrome-c primary Ab as was used for estimation of mitochondrial membrane integrity, caspase-3 primary Ab as an indicator for apoptosis induction, p53 primary Ab to estimate DNA integrity, and matrix metalloprotease-2 (MMP2) primary Ab to explore the protease activity.

Primary antibodies and peroxidase / IHC kits were purchased from Dako. IHC protocol was applied according to the kit's manufactures' instructions.

Statistical analysis

The statistical interpretation of the results was performed with One-Way ANOVA test. The results were given as mean ± standard error using SPSS 14 (2006). The value of $p < 0.05$ was considered significant.

Results and Discussion

In the present study, the obtained results in (Table 2) showed that the most predominant isolated molds from the examined animal feed

samples were *Aspergillus* spp. (100%) from drees (hay), (70%) from straw rice and (25%) from yellow corn followed by *Fusarium* spp. and *Rhizopus* spp. (25% for both). Other mould and yeast species were isolated with low percentages from examined samples. While, *A. ochraceus* and *A.niger* were recovered from (30% and 25%) of drees samples (hay), respectively.

Several studies as (Hassan *et al.*, 2012) who recovered members of *Aspergillus* species as the most common isolated mold from animal feed (100%), followed by *Penicillium* spp. (52%), *Alternaria* and *Mucor* spp. (28% for each), *Fusarium* spp. (24%) and *Cladosporium* spp. (8%), while isolated yeasts were *C. albicans* (80%), *C.tropicalis* (40%) and *Rhodotorula rubra* (32%).

While, the genus *Aspergillus* represents a large group of fungi that occupies very diverse ecological niches (Hassan *et al.*, 2008) who reported that the isolated *A. flavus* and *A. ochraceus* from feedstuffs produced significant levels of Afs and OA and these feeds were collected from store houses under bad condition of storage at high temperatures and moisture. However, the associated mycotoxins with *Aspergillus* species include AFs, ochratoxins, versicolorins, sterigmatocystin, gliotoxin, citrinin, CPA, patulin, citreoviridin, cyclopiazonic acid, penicillic acid and tremorgenicmycotoxins (CAST, 2003 and Frisvad *et al.*, 2006 a& b).

Currently, Hassan and Omran (1996) isolated various molds including *A. flavus* and *A. ochraceus* from yellow corn and mixed feed during summer season, where high moisture content and high temperature existed and they detected significant levels of OTA in samples. While, Nooh *et al.*, (2014) recovered *Aspergillus* spp. and *Penicillium* spp. from Egyptian maize at field and earlier storage with a variety climate of temperature, wind

and rainfall during the same season, this diversity impact on type and number of toxigenic fungi that grown on maize that followed by mycotoxins type and amount influences.

While, El-Hamaky *et al.*, (2016) isolated 106 fungal isolates from feeds and the most predominant isolates were *A. niger* (30-50 %) and *A. ochraceus* (15-20%). Twenty two of 44 tested isolates of *A. niger* produced OTA with average levels of (100-550 ppb), whereas, 12 of 15 *A. ochraceus* isolates produced OTA at average levels of (300-700 ppb). However, Hassan *et al.*, (2016) recovered *Aspergillus* spp. at the top of fungi isolated from feeds (68%), followed by *Mucor* spp. (35%), *Penicillium* spp. (31%), *Fusarium* spp. (23%), *Rhizopus* spp. (21%), *Cladosporium* spp. (6%) and *Scopulariopsis* spp. (4%). Whereas, Hassan *et al.*, (2017), examined 30 of the consumed animals' feeds were collected from a private buffalo farm at Giza governorate. The most common isolated molds from samples were *Aspergillus* spp. (100%).

In our study, when the isolated *A. niger* and *A. ochraceus* were screened for OTA production, (Table 3), the most ochratoxigenic strains was *A. niger* that isolated from drees (hay) and yellow corn which produced OTA at a mean levels of (20 ± 1.75 ppm) and (13.34 ± 6.5 ppm), respectively followed by *A. ochraceus* strain that recovered from rice straw and drees (hay) with a mean levels of (8.34 ± 3.6 ppm) and (7.3 ± 1.91 ppm), respectively.

Currently, the results in (Table, 4) showed that the higher levels of ochratoxin residues were detected in examined animal feed samples, in yellow corn with a mean levels of (9.84 ± 5.33 ppm), in processed feeds with a mean levels of (7.84 ± 3.49 ppm), in drees (hay) with a mean levels of (2.65 ± 0.53 ppm) and in straw rice with a mean levels of (1.88 ± 0.44 ppm), respectively.

In our study, all the detected levels of OTA in feeds or that produced by isolated *A. niger* and *A. ochraceus* were over the permissible limits that detected by European Commission Regulation (EC) No 1881/2006 (2006) who stated that the permissible limits for ochratoxin A in different Cereal grains and their products must be not more than not more than 0.005 mg/kg (5.0 µg/kg) and 0.01 mg/kg (10.0 µg/kg) in other feedstuffs.

On the other hand, significant losses to the animal industry occur due to effects of ochratoxin A on performance and health. It causes a reduction in growth rate and feed consumption, poorer feed conversion and increased mortality (Nedeljković *et al.*, 2015). Ochratoxicosis has been implicated in a diverse range of toxicological effects, including renal toxicity, mutagenicity, teratogenicity, neurotoxicity and immunotoxicity in both animals and human (O'Brien and Dietrich, 2005). In human, OTA is implicated in Balkan endemic nephropathy (BEN) a disease characterized by tubulonephritis (Agag, 2004).

The most important factors that influence growth and mycotoxin production are environmental temperature, substrate water activity (a_w), relative humidity, gas composition, substrate composition, inoculum's concentrations, microbial interactions and mechanical or insect damage (Guynot *et al.*, 2003 and Giorni *et al.*, 2007). In particular, it is the interaction between some or all of these factors that determines whether contamination increases and mycotoxins are produced. Interactions between available water and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to germinate, grow and actively compete for the allocation of the available resources (Marín *et al.*, 2012 and Nooh *et al.*, 2014). While, Abdou *et al.*, (2017) detected incidence rates of OTA in feeds at winter season were

comparatively lower than that detected in summer season, Where, the OTA residues were detected in all examined samples of (white corn, concentrated feeds and silage) (100%), during summer season. Whereas, 83.3%, 50%, 33.3% and 72% of examined yellow corn, wheat bran, barseem hay and poultry feed samples were contaminated with OTA, respectively.

In other study, animal feed samples were examined for fungal contamination and detection of mycotoxins. They were subjected for detection of mycotoxins, the results revealed that the amounts of OTA were detected in (40%) with the mean levels of (45.00±0.30 ppb), (Hassan *et al.*, 2012).

Regarding the biochemical study on the rats' sera after the end of experimental period, the results revealed that OTA caused oxidative stress and production of free radicals in rat hepatocytes and proximal tubules of the kidneys. Where, OTA enhanced increase in the levels of reactive oxygen species (ROS) and decreased NF-E2-related factor-2 (Nrf2), which mediate GST gene transcription, this may be lead to induced nephrotoxicity (Boesch-Saadatmandi *et al.*, 2008). Other authors have reported that the OTA hydroquinone/quinone couple was generated from the oxidation of OTA (phenol oxidation) by electrochemical, photochemical, and chemical processes (Faucet-Marquis *et al.*, 2006).

OTA intoxicated rats showed a significant increase in serum ALT and AST activities and urea level when compared with normal control group table 5. These results were considered to be a more sensitive measure in evaluating liver function and damage in the sera of OTA administration (Prati *et al.*, 2016). The more severe the liver damage was the higher release of the liver enzymes (Dreisbach and Lertora, 2008).

The increase in the blood urea nitrogen (BUN) could be attributed to the renal damage. OTA acts essentially in the proximal renal tubules, inhibiting the enzyme phosphoenol-pyruvate carboxylase, which is a lipid peroxidant, and it alters the structural and functional renal ability to metabolize calcium (Betina, 1989).

Biochemical alterations in rats treated with ochratoxin A were decreased in serum total protein, albumin and globulin levels and corresponding reduction of A: G ratio (Table 5). These decreased in values might be due to OTA-induced damage to liver (Qi *et al.*, 2014). Proteinuria especially albuminuria resulting from toxic damage to nephrons could have resulted in decreased albumin fraction observed in the study by Gupta *et al.*, (2009).

OTA is a protein synthesis inhibitor that has an effect on mitochondrial oxidative enzyme activity (Vettorazzi *et al.*, 2013). Ochratoxin A can cause immunosuppression and immunotoxicity in animals. Immunosuppressant activity of OTA is characterized by size reduction of immune organs, like thymus, spleen and lymph nodes, depression of antibody responses, alterations in the number and functions of immune cells, and modulation of cytokine production. OTA has immunotoxic activity results from degenerative changes and cell death affected immune cells, due to inhibition of protein synthesis (Al-Anati and Petzinger, 2006).

In this study, an increase was observed in MDA level in all groups excluding in ochratoxicosis group (5), as compared to the control group this results agreement with Domijan *et al.*, (2005). This is an indication of cellular damage caused by OTA. Several studies had reported that OTA induces oxidative stress. The generation of ROS may be mediated via the formation of Fe_3^+ -OTA complex (Gautier *et al.*, 2001).

OTA are known to cause damage in the digestive system (Kabak *et al.*, 2009). This may altered the absorption of vitamins in the digestive tract. Thus, it may decrease vitamin absorption and reduce their levels in the body, and hence inhibit the antioxidant defense mechanism.

It is also obvious that vitamin A, stored particularly in the liver and known to play a role in the inhibition of lipid peroxidation, is among the vitamins mostly affected by OTA (Bald *et al.*, 2004). This may be one of the reasons for the increase in MDA level.

Our study shown that ochratoxin A treated group elicit GSH depletion (Table 6). GSH is the main nonprotein thiol responsible for cellular homeostasis and maintenance of the cellular redox balance Forman *et al.*, (2009). Exposure to OTA can down-regulate the formation of GCL enzyme which is responsible for GSH synthesis, this lead to a reduction in the intracellular level of GSH Guilford and Hope (2014).

Nitric oxide was naturally formed in activated macrophages and endothelial cells and was considered as an active agent in several pathologies based on inflammation, organ reperfusion and increasing level of the NO observed in this study (Table 6). This may be due to OTA inducible nitric oxide synthase (iNOS) (Mally *et al.*, 2005 and Ferrante *et al.*, 2008).

The use of AFP as biomarker is used in the detection of liver cancer in human and animals, (Masi *et al.*, 2005). The result of this study showed increase in serum AFP among ochratoxin A group (Table 6). From the results of the current study, it can be concluded that OTA induce severe hepatic toxicity. AFP is widely used as a serological marker in the diagnosis of hepatocellular carcinoma and non-seminomatous germ-cell tumors (Chua *et al.*, 2013).

OTA acts as a carcinogen in the liver by epigenetic mechanisms, which modifying the hepatocyte gap junction apparatus and influencing the expression of specific markers such as alpha-fetoprotein (AFP) (Gagliano *et al.*, 2006).

Ochratoxin A in present study decreased serum iron, TIBC and transferrin % (Table 6). The drop of serum iron and TIBC levels in lambs fed mycotoxin and probably related to inflammation of liver and protein metabolism (Edrington *et al.*, 1994 and Salem *et al.*, 2007). Ochratoxin A produces iron deficiency anemia, the most common type of hypochromic microcytic anemia (Malir *et al.*, 2016).

In our experiment, glutathion had a protective effect being able to significantly reduce the level of ROS and also to increase cell viability under the same experimental conditions. GSH can inhibit the toxicity of ochratoxin by increasing the normal tissue defense capability and suppressing toxicity invasion, leading to an improvement in biochemical parameter as in Tables 5 and 6.

GSH can protect macromolecules (i.e., proteins, lipids, DNA) either by the formation of adducts directly with reactive electrophiles (glutathiolation) or by acting as proton donor in the presence of ROS or organic free radicals, yielding GSSG (Asada, 1994).

Glutathione play a role in maintaining normal cells permeability and active cation transport by protecting SH group in the cell membrane from oxidation. Oxidation of SH groups on the surface of the cell membrane results in increased permeability, and oxidation of important SH groups of Na⁺/K⁺ ATPase impedes active transport.

Although some studies have shown that alveolar type cells can supplement the endogenous synthesis of GSH by the uptake of exogenous GSH. The antioxidative effect of exogenously administered GSH is attenuated by its instability when crossing cell membranes and its rapid hydrolysis while in circulation (Kim *et al.*, 2010).

From our results we found that the group treated with glutathione before or at the same time with OTA administration showed marked improvement in the biochemical parameters.

Histopathological investigation

Macroscopic investigation, revealed evidence of emaciation in groups that given OTA, OTA/GSH and OTA+GSH that ranged from marked to moderate and mild degrees respectively. At post mortem examination, no gross changes could be detected in the internal organs of the different investigated groups.

One case of mortality was recorded in OTA control group at the end of the 2nd week.

Table.4 Detection of ochratoxin residues in animal feed samples by TLC

Types of examined samples	Levels of ochratoxin (ppm)(mg/kg)				
	No. of +ve.	%	Min.	Max.	Mean ± S.E.
Drees (hay) (5).	3	60	1.0	4.0	2.65±0.53
Yellow corn (5).	2	66.66	2.0	20.0	9.84±5.33
Straw rice (5).	2	66.66	0.5	2.0	1.88±0.44
Processed feeds (5).	2	66.66	1.0	12.5	7.84±3.49

Permissible limits for ochratoxin A in different cereal grains and their products set by European Commission Regulation (EC) No 1881/2006 (2006): not more than 0.005 mg/kg (5.0 µg/kg) and 0.01 mg/kg (10.0 µg/kg) in other feedstuffs.

Table.1 Experimental design for induction of ochratoxicosis in rats with or without glutathione treatment (5 weeks)

Groups of rats	Ochratoxin A	Glutathione	Times of treatment
G1 (-ve control)	-	-	5 weeks
G2 ochratoxicated (+ve control)	-	-	First 2 weeks
	50µg/kg b.w./d.	-	The next 3 weeks
G3 (OTA/GSH)	50 µg/kg b.w. /d.	-	First 3 weeks
	-	50 mg/Kg b.w./d.	The next 2 weeks
G4 (GSH/OTA)	-	50 mg/Kgb.w./d.	First 2 weeks
	50µg/kg (b.w.)/d.	-	The next 3 weeks
G5 (OTA+GSH)	-	-	First 2 weeks
	50 µg/kg b.w./d.	50mg/Kg b.w./d.	The next 3 weeks

All groups were fed on basal diet with or without treatments during the period of treatment (5 weeks).

Table.2 Prevalence of fungal species in different animal feed samples

Fungal isolates.	Drees (hay) (20)		Yellow corn (20)		Straw rice (20)		Processed feeds (20)	
	No. of +ve.	%	No. of +ve.	%	No. of +ve	%	No. of +ve.	%
<i>Aspergillus</i> spp.:	20	100	5	25	14	70	4	20
1. <i>A. candidus</i> .	1	5	0	0	1	5	0	0
2. <i>A. flavus</i> .	4	20	2	10	4	20	3	15
3. <i>A. niger</i> .	5	25	3	15	3	15	1	5
4. <i>A. ochraceous</i> .	6	30	0	0	3	15	0	0
5. <i>A. parasiticus</i> .	2	10	0	0	2	10	0	0
6. <i>A. terreus</i> .	2	10	0	0	1	5	0	0
<i>Alternaria</i> spp.	3	15	1	5	2	10	1	5
<i>Cladosporium</i> spp.	2	10	0	0	2	10	0	0
<i>Fusarium</i> spp.	5	25	3	15	4	20	2	10
<i>Geotrichum</i> spp.	2	10	0	0	1	5	0	0
<i>Mucor</i> spp.	3	15	1	5	2	10	1	5
<i>Rhizopus</i> spp.	5	25	1	5	2	10	1	5
<i>Paecilomyces</i> spp.	1	5	0	0	2	10	0	0
<i>Penicillium</i> spp.	2	10	1	5	4	20	1	5
<i>Scopulariopsis</i> spp.	3	15	1	5	2	10	1	5

% was calculated according to the No. of examined samples.

Table.3 Detection of ochratoxigenicity of the isolated *Aspergillus ochraceus* and *Aspergillus niger* strains from examined animal feed samples by TLC

Source of <i>A.ochraceus</i> and <i>A.niger</i>	Levels of ochratoxin (mg/kg) (ppm) produced by toxigenic strains of											
	<i>A. ochraceus</i>						<i>A.niger</i>					
	No. of tested isolates	No. of +ve	%	Min.	Max.	Mean \pm S.E.	No. of tested isolates	No. of +ve	%	Min.	Max.	Mean \pm S.E.
Drees (hay).	6	3	50	3	15	7.3 \pm 1.91	5	3	60	15	25	20 \pm 1.75
Yellow corn.	0	0	0	0	0	0	3	2	66.66	3	25	13.34 \pm 6.5
Straw rice.	3	2	66.66	3	15	8.34 \pm 3.6	3	0	0	0	0	0
Processed feeds.	0	0	0	0	0	0	1	0	0	0	0	0

-No. = number, -S.E. = standard error. -ppm = mg/kg. -ppm= part per million.

Permissible limits for ochratoxin A in different cereal grains and their products set by European Commission Regulation (EC) No 1881/2006 (2006): not more than 0.005 mg/kg (5.0 μ g/kg) and 0.01 mg/kg (10.0 μ g/kg) in other feedstuffs.

Table.5 Effect of oral treatment with ochratoxin A and glutathione on biochemical parameters in rats (Mean \pm S.E)

Parameters Group	ALT u/L	AST u/L	Urea mg/dl	T. protein g/dl	Albumin g/dl	Globulin g/dl	A/G
G1(-ve control)	28.65 ^d \pm 0.87	53.80 ^e \pm 0.86	29.83 ^e \pm 0.65	6.74 ^a \pm 0.10	2.06 ^a \pm 0.02	4.68 ^a \pm 0.10	0.44 ^a \pm 0.01
G2(+ve control)	55.98 ^a \pm 1.64	93.02 ^a \pm 1.08	47.51 ^a \pm 1.28	4.92 ^c \pm 0.09	1.11 ^d \pm 0.03	3.81 ^b \pm 0.12	0.29 ^c \pm 0.02
G3(OTA /GSH)	40.80 ^b \pm 0.58	83.98 ^b \pm 0.94	44.02 ^b \pm 0.43	5.69 ^b \pm 0.12	1.62 ^b \pm 0.02	4.07 ^b \pm 0.13	0.40 ^{ab} \pm 0.02
G4(GSH /OTA)	34.60 ^c \pm 0.93	74.02 ^d \pm 1.19	37.30 ^d \pm 0.43	5.72 ^b \pm 0.09	1.68 ^b \pm 0.08	4.04 ^b \pm 0.06	0.42 ^{ab} \pm 0.02
G5(OTA +GSH)	41.00 ^b \pm 0.55	79.35 ^c \pm 0.88	41.74 ^c \pm 0.46	5.17 ^c \pm 0.14	1.39 ^c \pm 0.02	3.78 ^b \pm 0.13	0.37 ^b \pm 0.01

Means within each column bearing common superscript do not differ significantly (P<0.05).

Table.6 Effect of oral treatment with ochratoxin A and glutathione on oxidative and biochemical parameters in rats (Mean \pm S.E)

parameters Group	MDA nmol/ml	GSH U/ml	Nitric oxide μ mol/L	AFP IU/ml	Iron	TIBC	Transferrin %
G1(-ve control)	1.02 ^e \pm 0.05	14.48 ^a \pm 0.12	20.65 ^d \pm 0.65	2.40 ^e \pm 0.02	117.80 ^a \pm 1.39	156.80 ^a \pm 2.15	83.00 ^a \pm 0.71
G2(+ve control)	2.47 ^a \pm 0.07	11.32 ^d \pm 0.03	29.27 ^a \pm 0.35	3.76 ^a \pm 0.09	84.40 ^a \pm 1.81	127.80 ^d \pm 1.16	55.40 ^d \pm 1.21
G3(OTA/GSH)	1.81 ^b \pm 0.07	13.42 ^{ab} \pm 0.64	26.22 ^b \pm 0.21	3.21 ^b \pm 0.09	91.40 ^c \pm 2.32	139.99 ^c \pm 1.92	57.60 ^d \pm 1.57
G4(GSH/OTA)	1.21 ^d \pm 0.03	13.56 ^{ab} \pm 0.20	21.84 ^d \pm 0.34	2.81 ^d \pm 0.02	105.20 ^b \pm 1.83	147.21 ^b \pm 2.35	71.40 ^b \pm 3.34
G5(OTA+GSH)	1.43 ^c \pm 0.05	13.05 ^b \pm 0.37	24.69 ^c \pm 0.58	2.98 ^c \pm 0.04	93.40 ^c \pm 1.89	139.15 ^c \pm 1.78	64.10 ^c \pm 2.16

Means within each column bearing common superscript do not differ significantly (P<0.05).

Plate (A)

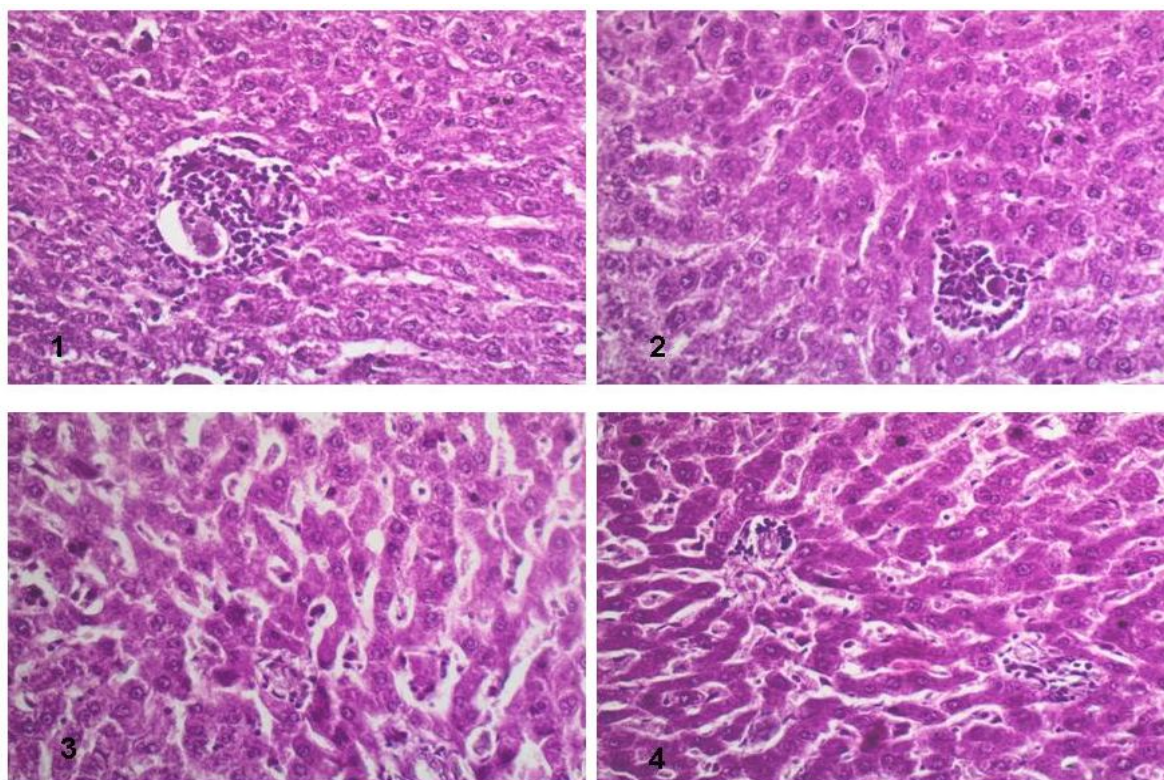


Plate A

Figure (1): Ochratoxin control group reveal large size necrotic foci. H&E X400

Figure (2): Ochratoxin/ glutathione group reveal moderate sized necrotic foci. H&E X400

Figure (3): Glutathione/ochratoxin group reveal minute necrotic foci. H&E X400

Figure (4): Ochratoxin+glutathione group reveal minute but multiple necrotic foci. H&E X400

Plate (B)

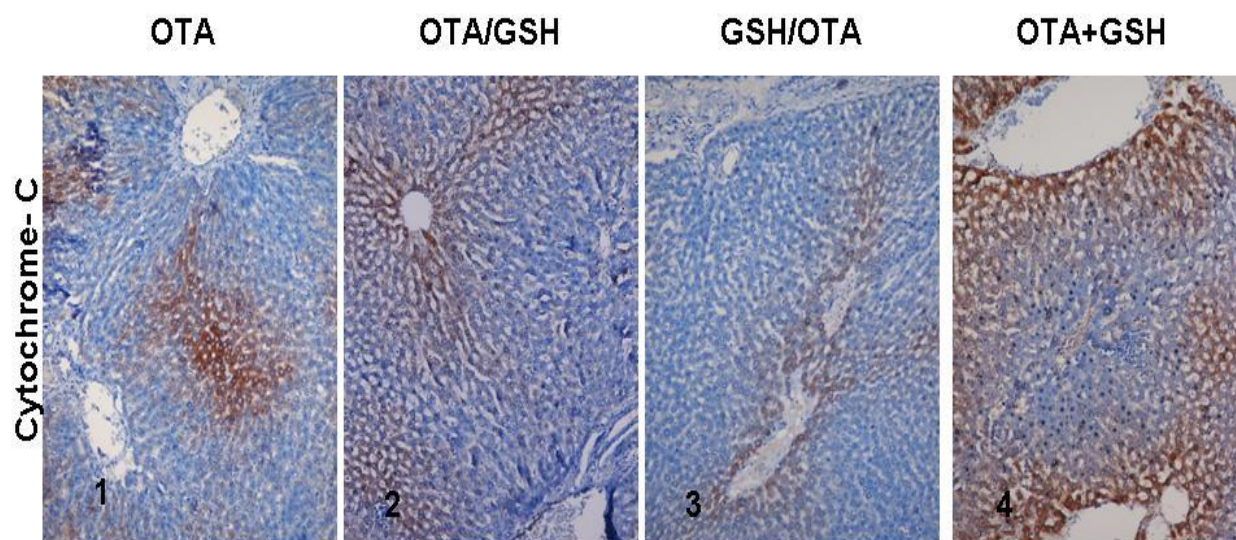


Plate B

Figure (1): OTA control group demonstrate marked release of cytochrome-c group with characteristic peri-central vein detection. IHC X200

Figure (2): OTA/GSH group demonstrate moderate reduction of cytochrome-c release around the central veins. IHC X200

Figure (3): GSH/OTA group demonstrate marked reduction in cytochrome-c release around central vein. IHC X200

Figure (4): OTA+GSH group demonstrate marked release of cytochrome-c with characteristic distribution around central vein. IHCX200

Plate (C)

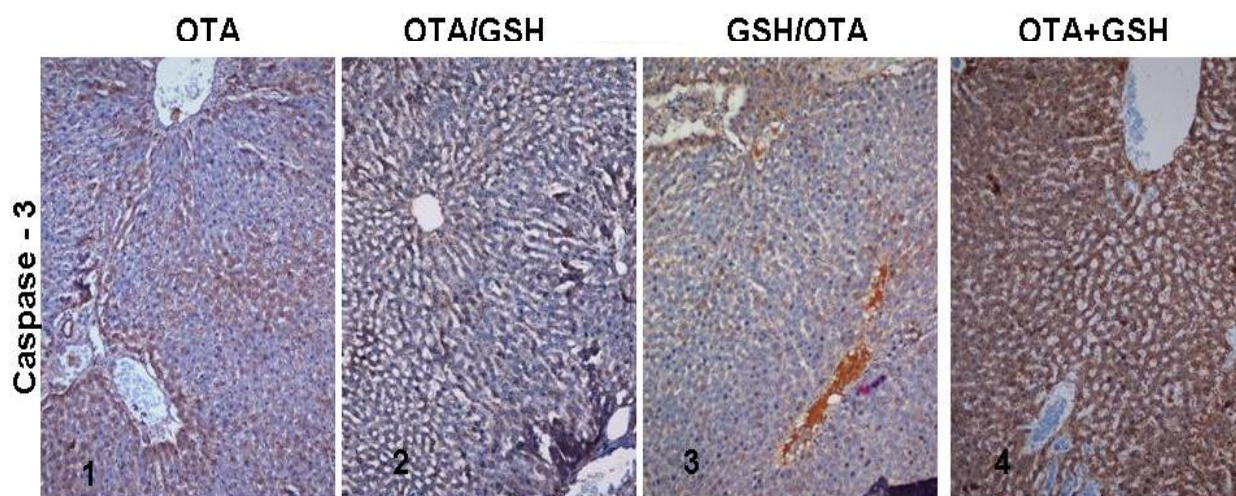


Plate C

Figure (1): OTA control group exhibit marked expression of caspase-3 with characteristic peri – central vein and along the sinusoids' at the inter-lobular borders location. IHC X200

Figure (2): OTA/GSH group showed diminishing in the level of caspase-3 expression. IHC X200

Figure (3): GSH/OTA group indicate to marked reduction in caspase-3 expression. IHC X200

Figure (4): OTA+GSH group clarify the diffuse pattern of caspase-3 expression all of the lobular area. IHC X200

Plate (D)

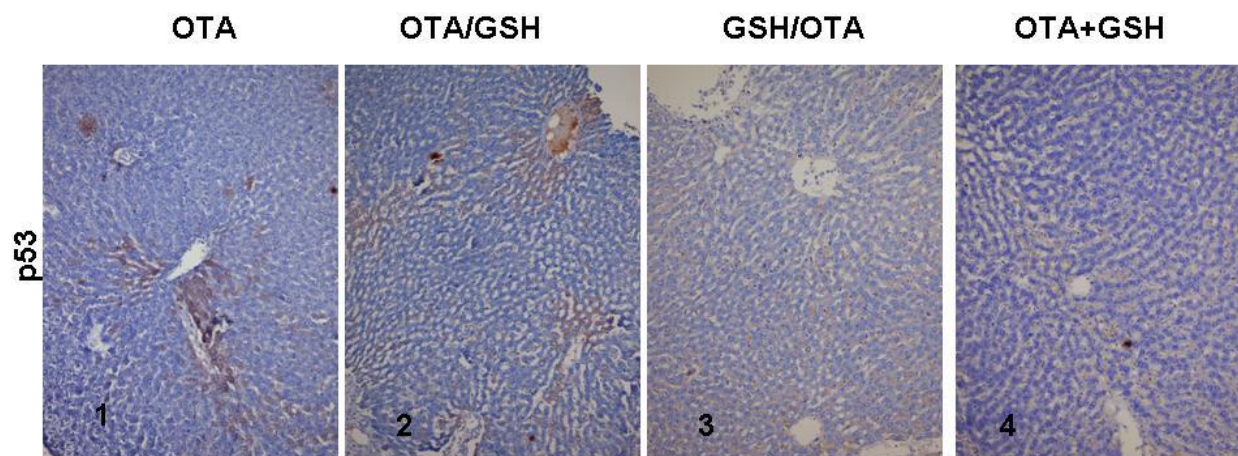


Plate D

Figure (1): OTA control group show up-regulation of p53. IHC X200

Figure (2): OTA/GSH group show moderate inhibition of p53 expression. IHC X200

Figure (3): GSH/OTA group show complete inhibition of p53 expression. IHC X200

Figure (4): OTA+GSH group show marked inhibition of p53 expression. IHC X200

Plate (E)

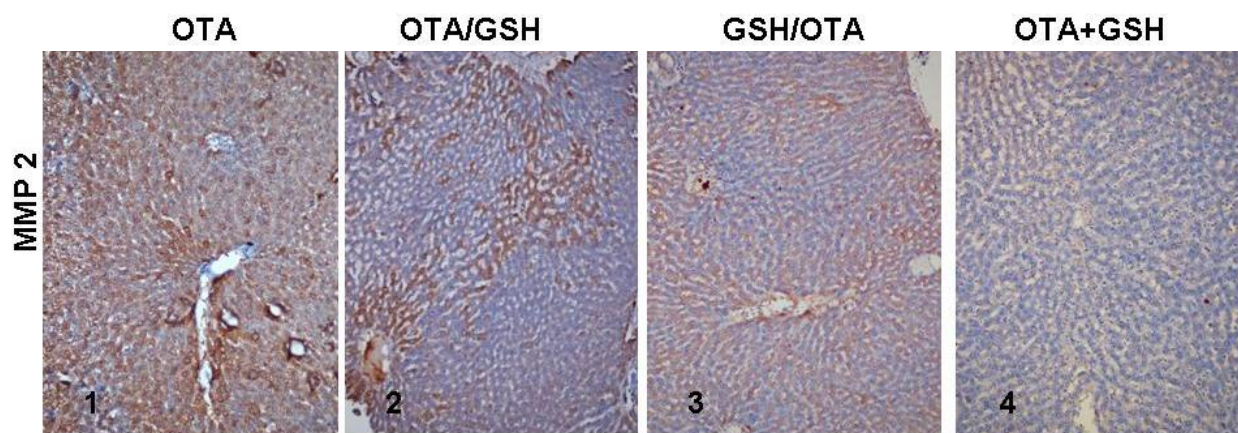


Plate E

Figure (1): OTA control group refer to expression of mmp2 in the same pattern of p53 and other un-associated areas. IHC X200

Figure (2): OTA/GSH group indicate to predominant mmp2 expression in associated and un-associated pattern with p53. IHC X200

Figure (3): GSH/OTA group indicate to scarce level of the expressed mmp2. IHC X200

Figure (4): OTA+GSH group indicate to noticeable inhibition of mmp2. IHC X200

Microscopically, hepatic investigation demonstrated necrotic foci in different treated groups, which decreased in the size in the following descending order; OTA, OTA/GSH, GSH/OTA and finally OTA+GSH (Plate A; Fig. 1, 2, 3, 4) respectively.

Attention to liver, at molecular level, was directed to estimate the expression of cytochrome-c (cyto-c), in relation to caspase-3 expression in addition to p53 expression in relation to matrix metalloprotease-2 (mmp-2), in different groups.

Cytochrome-c is one of the mitochondrial components that localized in between inner and outer mitochondrial membrane space (Galluzzi *et al.*, 2007), decrease in mitochondrial membrane potential lead to release of cytochrome-c from mitochondrial membrane and initiation of caspase-dependent apoptosis (Ow *et al.*, 2008). In our study OTA group revealed characteristic pattern of cytochrome-c release, that follow the direction from the central vein toward the periphery of the hepatic lobules (Plate B; Fig. 1 & 4); so follow the blood concentration of OTA that in consistent with El Golli Bennour *et al.*, (2009) and Chen *et al.*, (2015) who found and indicated the concentration dependent toxic effect of OTA.

In the present study marked release of cytochrome-c was detected in OTA (Plate B; Fig. 1) and OTA+GSH (Plate B; Fig. 4) groups, meanwhile noticeable decrease in cytochrome-c release was detected in OTA/GSH (Plate B; Fig. 2) and GSH/OTA (Plate B; Fig. 3) groups. The release of cytochrome-c into the cytosol is the point of no return in cell death pathway (Solary *et al.*, 2008) either through deviation of energy (impairment of ATP) and/or induction of apoptosis (Ow *et al.*, 2008).

Thus our findings refer to incorporation of mitochondrial in ochratoxicosis and the protective and therapeutic but not competitive effect of GSH in hindering ochratoxicosis.

Induction of mitochondrial oxidative stress within OTA toxicity was detected by Wang *et al.*, (2017) which was attributed to the oxidative effect of OTA on mitochondrial membrane ingredients with subsequent lipid peroxidation, decrease in mitochondrial membrane potential, leakage of cytochrome-c into the cytosol with reduction in ATP production (Chopra *et al.*, 2010) with impairment of electron transport in

mitochondrial respiratory chain (Wei *et al.*, 1985). On the other side El Golli Bennour *et al.*, (2009) detected the insignificant effect of oxidative stress in OTA induced toxicity, and in the same conclusion Bouaziz *et al.*, (2011) suggested that ROS was created due to mitochondrial impairment rather than a mechanism of OTA induced toxicities.

Regarding to caspase-3; in our study there was marked association between detection of cytochrome-c and caspase-3 (Plates B&C; Fig. 1, 2, 3 and 4) as caspase-3 followed the same pattern of cytochrome -c distribution in addition to unassociated location that follows the direction of sinusoids along the inter-lobular borders and around portal areas in OTA control group (Plate C; Fig. 1).

OTA/GSH group showed diminishing in the level of cytochrome-c release (Plate B; Fig. 2) and that effect was similarly demonstrated for caspase-3 (Plate C; Fig. 2).

In GSH/OTA group there was a marked reduction in the cytochrome-c tissue level (Plate B; Fig. 3) that in accordance with findings of caspase-3 (Plate C; Fig. 3) that could be attributed to maintenance of plasma glutathione pool and subsequently cellular pool; so blocking of caspase-3 activities due to glutathionylation of cysteine residue in caspase molecules, with hindering its activation so block the apoptosis- dependent caspase-3 cascade (Huang *et al.*, 2008). Caspase-3 inhibition led to a significant but not complete reduction of OTA-induced apoptosis, which in agreement with Chopra *et al.*, (2010) and give a chance for incorporation of other factor, but keep the prominent role of caspase-3 cascade.

GSH+OTA group revealed the loss of the blocking effect on caspase-3 release with diffuse pattern of caspase-3 expression all of the lobular area (Plate C, Fig. 4).

The present study indicated to the effect of OTA on cytochrome-c release into the cytoplasm that firmly associated with initiation of caspase-3 activity, so indicate to incorporation of caspase-3 mediated-apoptosis in the mechanism of ochratoxicosis (Gupta *et al.*, 2009). Our findings in inconsistent with Bouaziz *et al.*, (2011) who referred to induction of mitochondrial-Bcl-2 mediated pathway of apoptosis as a result of Bax-induced mitochondrial membrane potential dysfunction and release of cytochrome-c rather than caspase-3.

Our results clarified the marked reduction in cytochrome-c and the associated caspase-3 activities in glutathione protective (GSH/OTA). While GSH at therapeutic (OTA/GSH) and competitive (GSH+OTA) levels, showed prominent effect on cytochrome-c and to less degree on caspase-3; as exposure to oxidative stress that lead to rapid exhaustion of GSH (Jones, *et al.*, 1986). While fast disturbance in GSH and marked increase in GSSG level, so re-establishment of GSH/GSSG redox balance post oxidant exposure did not protect the cells from initiated apoptotic cascade (Circu and Aw, 2012), as initiation of apoptosis cascade start with early loss of GSH/GSSG redox balance.

The initiation of caspase-3 in un-associated pattern with cytochrome-c may indicate to cytochrome-c-independent- activation of caspase-3 apoptotic pathway.

Regarding to p53, which act as a sensor of genome integrity, and its activation is associated with mutant insults (Fei *et al.*, 2002), so DNA damage lead to up regulation of p53 which arrest cell cycle and hence protect from mutagen accumulation and allow DNA repair (Kim *et al.*, 2016). P53 also act as a transcription factor that regulates cell cycle, DNA repair and even induction of apoptosis (Meyer *et al.*, 2005).

P53 showed up-regulation in OTA group (Plate D, Fig. 1), that in accordance with Chopra *et al.*, (2010); while inhibition was monitored in OTA/GSH (Plate D, Fig. 2), GSH+OTA (Plate D, Fig. 4) and GSH/OTA (Plate D, Fig., 3) treated groups in descending order, that reflect the ability of Glutathione to keep DNA stability (at protective level) in concentration-dependent manner and even its ability to repair genome damage. Similar result was reported by Bouaziz *et al.*, (2008) and El Golli Bennour *et al.*, (2009).

Erster *et al.*, (2004) refer to dual effect of p53; first one represent a fast response of sensitive organs (thymus and spleen) to oxidative stress through induction of mitochondria-mediated caspase-3 activation (that could explain the un-associated release of caspase-3 independent of cytochrome-c, in the present study), the other one is delayed response (less sensitive organs; liver and kidneys); in which P53 act as a transcriptional factors for apoptogenic proteins.

Our findings refer partial but not complete association between p53 up regulation and caspase-3 activity, so we think both of fast and delayed response of p53 (Erster *et al.*, 2004) is incorporated OTA induced hepatic toxicity.

Matrix metalloproteinases (mmps) are a family of proteinases degrade fibrous matrix (Wang *et al.*, 2000), mmp2 (as a member of mmps) and its expression is associated with tissue remodeling (Bennett *et al.*, 2000) and even with ras-mediated cellular transformation; as an indication of disturbance in cellular regulatory program with capacity for malignant progression (Baruch *et al.*, 2001) through Extra-cellular matrix degradation and remodeling (Taniwaki *et al.*, 2007). Our results showed that OTA moderately enhanced p53 expression (Plate D; Fig. 1), and although mmp2 showed the

same pattern of distribution, but its expression in other areas was detected (Plate E; Fig. 1). In OTA/GSH group; the p53 had significant level of tissue expression mainly around central veins (Plate D; Fig. 2) with more predominant mmp2 expression in associated and un-associated pattern (Plate E; Fig. 2). In GSH/OTA group; despite the scarce cellular expression of p53 (Plate D; Fig. 3) there were significant level of the expressed mmp2 (Plate E; Fig. 3). In GSH + OTA group; noticeable inhibition of p53 (Plate D; Fig. 4) and mmp2 (Plate E; Fig. 4) had been observed.

According to Chatterjee *et al.*, (2013) and Wang *et al.*, (2000) mmp2 showed correlation with p53 up regulation depending on cell type. P53 mediated transcription activity of mmp2 had been documented by Bian and Sun (1997) and Meyer *et al.*, (2005). Induction of P53 expression affect on mmp2 expression, as a target gene, that is associated with cellular transformation (Bian and Sun, 1997).

Although the pattern of mmp2 expression in most our investigated cases was associated with expression of P53; the un-associated area of mmp2 expression could be attributed to ability of transformed cell-associated fibronectin to stimulate surrounding normal stromal tissue to secrete mmp2 (Saad *et al.*, 2002).

In our study, reverse association between P53 expression and suspected glutathione content of cell had been previously recorded in variable forms of hepatotoxicity (Ali *et al.*, 2014 and Sun *et al.*, 2014). Moreover it gives suggestion that the genotoxic effect of ochratoxin is reversible and mediated by oxidative stress.

Our investigation refer to protective role of GSH on OTA induced hepatotoxicity rather than competitive and to a less degree therapeutic effect (limited to p53 expression),

so we could suggested that supplementation of glutathione preserve the cellular pool of glutathione and hence diminish the oxidative stress adverse effects.

From previous findings we could hypothesis that, glutathione is a target involved in Ochratoxicosis, either as primary agent through impairment of endogenous glutathione synthesis or secondary due to glutathione utilization as ROS scavenger to overcome oxidative stress from dysfunctional mitochondria, and as OTA was incriminated to hinder the intra-cellular glutathione production (Guilford and Hope, 2014) through blocking effect on glutathione oxidase enzyme (Schaaf *et al.*, 2002). So our findings came in agreement with Novi *et al.*, (1982) in that; administration of glutathione intact molecules is suitable to restore the cellular glutathione pool and preservation of its vital functions. This investigation revealed that glutathione supplementation had a marked protective role in competing ochratoxicosis, rather than therapeutic ones.

In this study, molds were recorded to produce OTA which caused some degree of acute toxicity when consumed in high amounts. OTA demonstrate the direct correlation between ochratoxin intake and the incidence of hepatotoxicity. The results indicate that GSH have a potential protective agent for preventing the negative oxidative parameter changes caused by OTA. In addition, the glutathione supplementation had a marked protective role in amelioration the toxic effects of ochratoxicosis where GSH enhance the immune response and nutrition of host species through the production of supplemental digestive enzymes. Dietary glutathione-enhancer (GSH) was effective to eliminate these toxicological effects of OTA, especially with the high level. It is evident that GSH has a potential antioxidant activity and a protective effect against OTA toxicity.

Therefore, frequent testing program of the animal feeds and other environmental factors for fungi and mycotoxins contamination and use of feed additives as GSH; are a critical demand today to safe the animal and human health.

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