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Impact of Copper II Albumin Complex on Kidney Impairment Induced By Aflatoxin B1 in Rats

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Abstract

This study aimed to assess the renal protective efficacy of the Copper II albumin Complex (Cu-II-Album Complex) compound against aflatoxin-B1 (AFB1) in rats. Forty adult male albino rats were divided into four groups (10 rats/group): group-1 served as a negative control, the remaining groups received an oral dose of (50µg/kg) of the AFB1 daily for 3-weeks according to the following protocol: one group did not receive any treatment (group-2), while the remaining two groups received 0.05 g/kg of Cu-II-Album Complex either every other day (group-3) or intoxicated with AFB1 for 3-weeks then treated daily with Cu-II-Album Complex for another 3-weeks. Serum urea and creatinine, renal histopathology, indicators of kidney repair, nuclear factor-κB (NF-κB), antioxidant gene inducer (nuclear factor erythroid-2; Nrf2), and metabolic homeostasis indicator (peroxisome proliferator-activated receptor-gamma; PPARγ) were evaluated. The Cu-II-Album Complex significantly reduced serum urea, creatinine levels, and renal NF-κB and significantly increased the renal Nrf2 and PPARγ expression. The AFB1 induced renal degenerative changes were significantly reduced in the alternative treatment with Cu-II-Album Complex. In conclusion, the Cu-II-Album Complex is an effective renal protective agent against AFB1 as indicated by maintaining the renal functions and histology and upregulating the expression of the kidney antioxidant genes and renal metabolic homeostasis indicator.

Keywords: Kidney; Aflatoxin B1; Copper II complex; Albumin; PPARγ; Nrf2; Rats

Introduction

Mycotoxins are biologically active, poisonous, secondary metabolites generated by toxigenic fungi, primarily Aspergillus, Fusarium, and Penicillium species, which infect and develop on crops during storage under favorable temperature and humidity [1, 2]. These secondary fungal metabolites pose a human health risk and can cause hepatotoxicity, teratogenicity, and immunotoxicity [3, 4].

Even though 18 aflatoxins have been identified, only four are well-known and have been thoroughly researched from a toxicological standpoint. Because they are highly luminous in ultraviolet light, the four are abbreviated as B1, B2, G1, and G2 that denote their blue and green fluorescence. Aflatoxins B1, B2, and G1 are prevalent in food; however, the aflatoxin B1 (AFB1) predominates (60-80% of total aflatoxin content [5, 6]. Acute dietary exposure to AFB1 has been linked to epidemics of acute hepatic injury. However, the chronic aflatoxicosis resulting from the ingestion of low to moderate levels of aflatoxins cause subclinical effects, including teratogenic and congenital abnormalities, as well as mutagenic or carcinogenic effects [7].

The kidney is also susceptible to many toxic agents, including mycotoxins [8]. Aflatoxins,

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particularly AFB1 and its metabolites, affects different regions of the nephron, causing nephrotoxicity before it is eliminated in the urine and the aflatoxin-induced decrease in protein content is attributed to increased renal necrosis [9, 10]. In experimental animals, AFB1 has been found to produce kidney tumors, while a combination of AFB and AFG has been found to cause renal and hepatic tumors in 80% of intoxicated hamsters [11]. In rats, aflatoxins and their metabolites caused a decrease in glomerular filtration rate, glucose reabsorption, tubular transport of electrolytes and organic anions, decreased activity of renal alanine amino transferase and aspartate aminotransferase, as well as alkaline phosphatase [11, 12].

Various natural compounds prevent aflatoxin contamination in food through different ways, including inhibition of the growth of aflatoxigenic fungi, blocking aflatoxin biosynthesis, and removal or degradation of aflatoxin [13]. Recently, a promising approach using nanoparticles adsorbents (such as hydrated sodium aluminum silicates, copper oxide, and zinc oxide nanoparticles) was evaluated as multi-mycotoxin binders [14, 15]. These inhibitors are highly promising for developing new approaches to control aflatoxin contamination in food and can replace or complement conventional strategies [14].

Copper (Cu) is an essential trace element that cannot be synthesized in-vivo and has a vital role in many physiological functions in the nervous, hematological, cardiovascular, and immune systems. Most of the Cu biological functions is believed to be associated with the Cu role as a ligand in the active site of metalloenzymes [16].

Copper complexes usually exhibit more potent activity than their parent compounds without copper [17]. The copper nicotinate complex efficiently prevented the AFB1-induced nephrotoxicity by promoting phase II detoxification glutathione-S-transferase activity [18]. Additionally, scientists utilized the affinity of Copper (II) complexes to the mammalian albumin to generate Copper (II) albumin complex compounds [19]. With the additional affinity of the mammalian albumin to mycotoxins [20], it was suggested that the Copper (II) albumin would be more potent anti-mycotoxins compounds.

The current study aimed to evaluate the efficacy of the Copper (II) albumin Complex (Cu-II-Album Complex) compound as renal protective agents against AFB1 in rats.

Materials and Methods

Experimental Animals

Forty male albino rats (8-weeks old, 50- 200 gm body weight) were received from the Laboratory Animal House at Assiut University, Egypt. The experimental protocol was approved by the research ethics committee at Faculty of Medicine, Assiut University, Assiut, Egypt (approval number IRB-17300660). A standard commercial pellet feed and water were provided ad libitum.

Chemicals

The aflatoxin was kindly provided in a corn oil preparation by Dr. Ahmed Shatat (Chemistry Department, Faculty of Science, Al-Azhar University). The provided aflatoxin content was verified by thin-layer chromatography (TLC). Briefly, the TLC was done on plats of aluminum sheet silica gel 60 pre-coated 20×20 cm, layer thickness 0.2mm (E-Merck, Darmstadt). The solvent was a mixture of chloroform-methanol (98:2 v/v); 100 µL of the sample was spotted as a streak on the TLC plate in a very tightly closed jar for two hours. The chromatogram zones were demonstrated in a UV cabinet room at a wavelength of 365 nm. The AFB1 appeared as a faint blue-colored zone. The AFB1 was confirmed to be the major component in the mixture (data not shown).

The Copper II albumin Complex (Cu-II-Album Complex) was kindly provided by Dr. Ahmed Nasar (Biochemistry Department, Faculty of Medicine, Assiut University, Egypt) (patented in the international Bureau of world intellectual property Organization (WIPO), Geneva, Switzerland "World Organization, 2008 028497"). The complex is mixed in dairy milk as 0.05 g/kg body weight.

Experimental Design

Experimental animals were allocated into 4 groups (10 rats each) as shown in Table 1. The aflatoxicosis was induced as previously described by Williams (1996). Briefly, a daily 50µg/kg of the AFB1 was administered orally for 3 weeks. The Cu-II-Album Complex was prepared as 0.05g/kg dissolved in milk and administered orally alternatively every other day of the aflatoxin treatment in the first treatment group or for additional 3 weeks following the aflatoxin treatment in the second treatment group.

Sampling

At the end of the experimental period, individual blood samples were collected into 2 tubes. One containing EDTA as an anticoagulant and the second tube was plain. Both were kept at room temperature for 15 minutes, then sera and plasma were collected by centrifugation at 4000 rpm. for 10 minutes. Collected sera and plasma were kept at -20°C until analyzed. Kidney tissue samples were washed by shield saline, dried by filter paper, and collected in 4% neutral buffered formalin for histological and immunohistochemical evaluation.

Determination of serum urea and creatinine (Cr)

Serum urea and serum creatinine levels were determined using the Spinreact Urea-B and Creatinina-J commercial kits (Spinreact, S.A., Spain) according to the method described by [21].

Histopathology and immunohistochemistry

Kidney tissue samples were fixed in 10% neutral-buffered formalin, then embedded in paraffin, sectioned at 4μm, stained by hematoxylin and eosin (H&E), and examined by light microscopy [22].

For immunohistochemistry, glass slides from the fixed tissues were prepared. Xylene was used to deparaffinize the tissues. Three percent hydrogen peroxide was used for blocking endogenous peroxidase. The slides were treated with pepsin at 42°C for 5 min to obtain an adequate signal. Afterthought, the slides were incubated overnight at 4°C with mouse anti-nuclear factor-κB (NF-κB) antibody (AbCam)[23], 1: 100 dilution of anti-nuclear factor erythroid-2 (Nrf2), and anti-peroxisome proliferator-activated receptor-γ (PPARγ) (Santa Cruz Biotechnologies) [24, 25]. Sections were then incubated with a Biotin-conjugated secondary antibody and Streptavidin-Enzyme Conjugate (LSAB System HRP, BIOCARE). The immune reaction resulted in the oxidation of the 3,3′-diaminobenzidine by peroxidase (Liquid DAB, DAKO Carpinteria, CA) into an insoluble brown precipitate. The reaction sites were visualized as brown staining. Counterstaining with hematoxylin was performed after immunostaining [26].

Statistical analysis

Data were presented as mean ± SD. One-way analysis of variance (ANOVA) followed by post hoc least significant difference analysis (LSD) was performed using the statistical package for social science (SPSS) version 19 to compare all groups. The value of $p \leq 0.05$ was considered statistically significant. The % of difference representing the percent of variation with respect to the negative and positive control groups was also calculated [27].

Table 1. Experimental design

Group	Aflatoxicosis induction	Treatment with Copper II albumin Complex	End of experiment and sample collections time
Negative Control (NC)	None (received only the vehicle oil)	None (received only the vehicle oil)	3 weeks
Aflatoxicosis (AF)	Daily for three weeks	None	3 weeks
Aflatoxicosis and Cu-Complex preventive (AF/CUC-P)	Every other day for 3 weeks	Cu_ Complex alternating with aflatoxin for 3 weeks	3 weeks
Aflatoxicosis then Cu-II-Album Complex treated (AF/CUC-T)	Daily for 3 weeks	Daily for another 3 weeks	6 weeks

Results

Kidney weight, serum urea and creatinine levels

In all groups, there was no significant change in the kidney weight. However, the serum urea and creatinine (Cr) levels were significantly increased in control group ($P < 0.05$). In either the alternative or end treatment groups, the copper complex compound significantly decreased serum urea levels at $P < 0.05$. the aflatoxicosis-induced group compared to the However, serum Cr was significantly reduced in the alternative copper complex treatment group only (Figure 1).

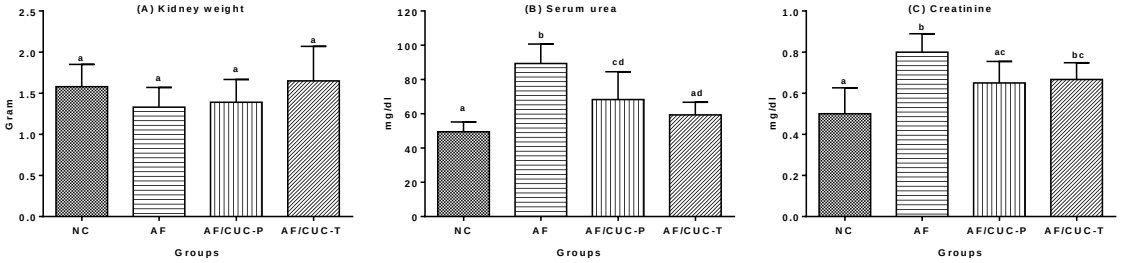

Figure 1. Effect of Copper II albumin complex (Cu-II-Album Complex) treatments on kidney function markers. (A) kidney weight, (B) serum urea, and (C) Creatinine. Each value represents the mean ± SD. Statistical significance is indicated by different small letters (a, b and c) (P <0.05). Abbreviations: NC; negative control, AF, Aflatoxicosis; AF/CUC-P, Aflatoxicosis Cu-II-Album Complex alternatively; AF/CUC-T, Aflatoxicosis then Cu-II-Album Complex treated. Similar characters denote insignificance between groups.

Histopathology in aflatoxicosis induced group versus different treatment groups

The histopathological changes in the kidney of aflatoxicosis induced groups were characterized by necrosis of the glomerular capillary tuft (Figure 2 A and B), widening the Bowman's space, and resulting in accumulation of hyaline casts in the tubular lumen and Bowman's space (Figure 2 B). The renal tubules showed severe degenerative and necrotic changes which their cells had pyknotic or karyolitic nuclei. Shortening and flattening of the renal tubular epithelium were also noticed (Figure 3 A) and severe vacuolation of their cytoplasm. Moderate to severe infiltration of lymphoid cells were seen in the renal cortex and medulla (Figure 2 C).

However, these renal changes were markedly reduced when the Cu-II-Album Complex was administered alternatively with aflatoxin or administered for another 3 weeks following the aflatoxicosis induction. Renal sections from the alternatively treated group showed mild degeneration and necrosis of the renal tubules, including shortened or flattened epithelium. Very few cells had vacuolated cytoplasm. Hyaline casts were seen only in the tubular lumen. The medulla was mildly infiltrated with lymphoid cells (Figures 3). On the other hand, histopathological examination of the kidney samples from the group treated with Cu-II-Album Complex after aflatoxicosis induction revealed that some renal tubules had shortened or flattened epithelium with minimal hyaline casts in their lumen (Figure 3 F and G).

Kidney immunohistochemistry in different treatment groups

Negative brown immunostain for NF-kB was observed in the kidneys of control rats (Figure 4 A). While, in aflatoxicosed rats resulted in strong positive brown immunostain in the kidneys (Figure 4 B). In contrast, both treatments of Cu-II-Album Complex have obviously reduced brown staining (Figure 4 C and D), and the brown immunostained areas in the rat's kidney were significantly reduced ($P<0.05$) (Figure 4).

The immunohistochemistry to identify the Nrf2 in rats kidneys revealed slight immunostaining for Nrf2 in kidney sections of control and aflatoxicosed groups (Figure 5 A and B). However, compared to the controls, increased Nrf2 immunostaining was detected in the basement membrane, and proximal tubular cells of kidney in both Cu-II-Album Complex treated groups ((Figure 5 C and D), with a significant increase in the immunostained areas ($P < 0.05$) (Figure 5).

The intensity of PPARγ immunoreactivity in the glomeruli and renal tubules was assessed. The aflatoxicosed group showed a very low immunoreactivity (Figure 6 B); however, both Cu-II-Album Complex treated rat kidneys, PPARγ immunoreactivity was higher than that of the negative control and aflatoxicosed groups (Figure 6 Cand D). meanwhile, the copper complex treatment for 3 weeks following the aflatoxicosis showed the most significant PPARγ expression in terms of intensity (Figure 6) and positive area of immunostaining ($P < 0.05$).

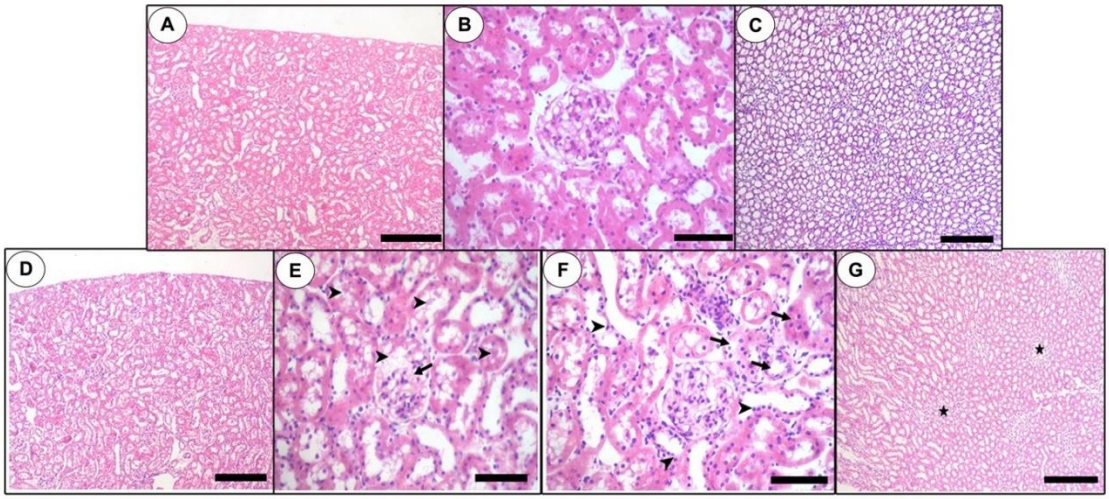

Figure 2. Kidneys of the negative control and induced aflatoxicosis groups. (A-B) kidney showing normal renal cortical glomerulus and renal tubules and medulla (C) in the control group. Hematoxylin and eosin stain. Bars A, C = 200 µM, B = 50 µM. The kidney in the induced aflatoxicosis group showing glomerular necrosis (D), accumulation of hyaline casts in the Bowman's space, and tubular lumen (arrowheads) (E). The renal tubules showed degeneration and necrosis (arrows) and shortening or flattening of the epithelium (arrowheads) (F). Lymphoid cell aggregation in the renal medulla (star) (G). Hematoxylin and eosin stain. Bars D and G = 200 µM. Bars E and F = 50 µM.

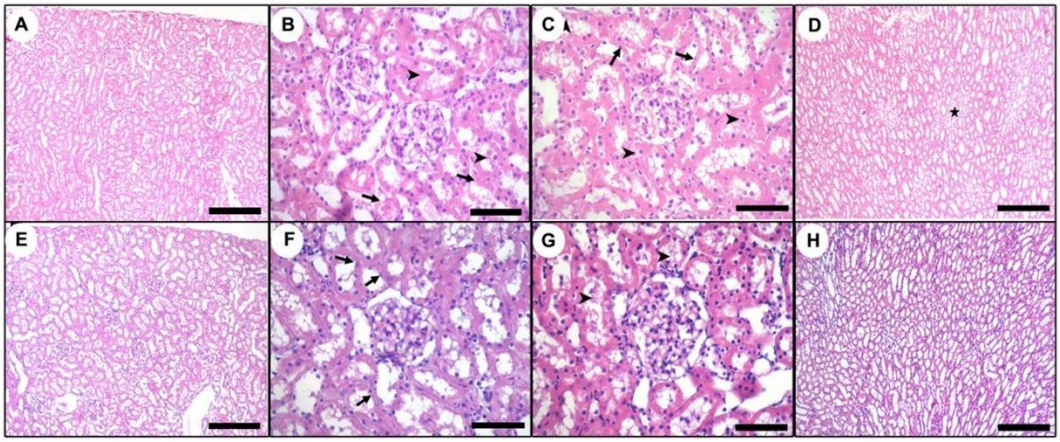

Figure 3. Histopathological changes of kidneys in the aflatoxin with copper complex alternative or end treatments groups. Kidneys showing mild necrotic renal tubules (arrows) (A and B), shortening and flattening of the tubular epithelium (arrows), hyaline casts in the tubular lumen (arrowheads), few vacuolated cells (B and C), and mild infiltration of lymphoid cells in the medulla (D). Hematoxylin and eosin stain. Bars A and D = 200 µM. Bars B and C = 50 µM. Kidney in the copper complex treatment group showing shortening or flattening of few renal tubular epithelia (arrows) (F) with mild accumulation of hyaline cast in the tubular lumen (arrowheads) (G) with normal structure of the medulla (H). Hematoxylin and eosin stain. Bars E and H = 200 µM. Bars F and G = 50 µM.

Discussion

Kidneys are one of the main target organs of AFB1 that caused renal edema and cytomorphosis, severe inflammatory cell infiltration, and hemorrhage [9]. copper is an important trace element in several physiological cellular functions, however, in its free form is highly toxic [28]. Hence, Copper-based complexes were investigated as promising anti-cancer, anti-inflammatory and anti-mycotoxin [14, 29]. The Copper (II) albumin complexes were suggested to be potent anti-mycotoxins considering their dual affinity to mycotoxins [19]. In the current study, the Copper II albumin Complex (Cu-II-Album Complex) was evaluated as ani-mycotoxin

(specifically AFB1) in aflatoxicosed rats. Two treatment schedules were examined, including an alternative treatment (every other day) and an end treatment after 3 weeks of aflatoxicosis. Results revealed that AFB1 induced significant elevations in serum urea and creatinine which indicates renal damage that involves inflammation, cell necrosis, and toxicosis [30]. However, Cu-II-Album Complex, either as an alternative or end treatment, significantly reduced the serum urea and Cr levels.

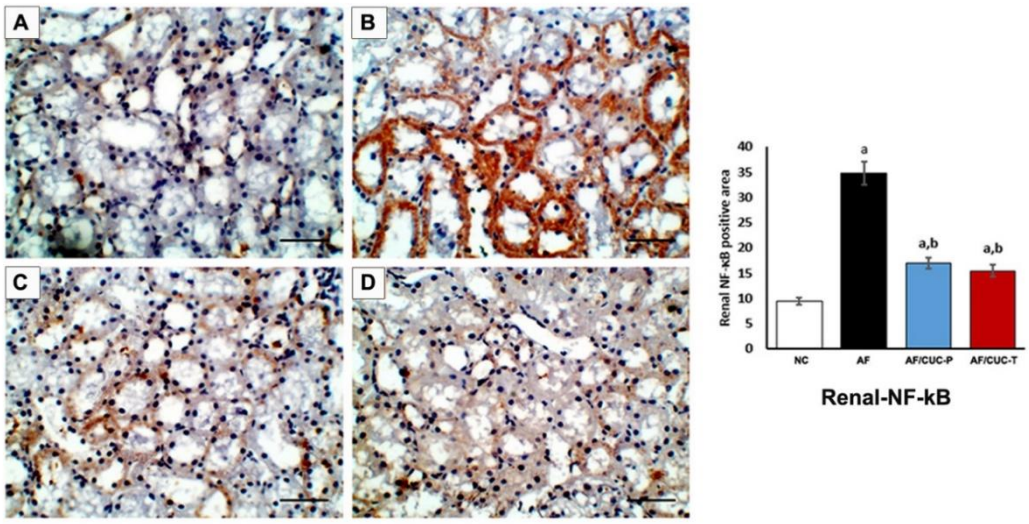

Figure 4. Immunohistochemical staining of NF-κB in kidney tissue sections of treated and control rats. Statistical significance is indicated by different small letters (a, b and c) (P <0.05). NC; negative control, AF, Aflatoxicosis; AF/CUC-P, Aflatoxicosis Cu-II-Album Complex alternatively; AF/CUC-T, Aflatoxicosis then Cu-II-Album Complex treated. Bar = 50 µM. Similar characters denote insignificance between groups.

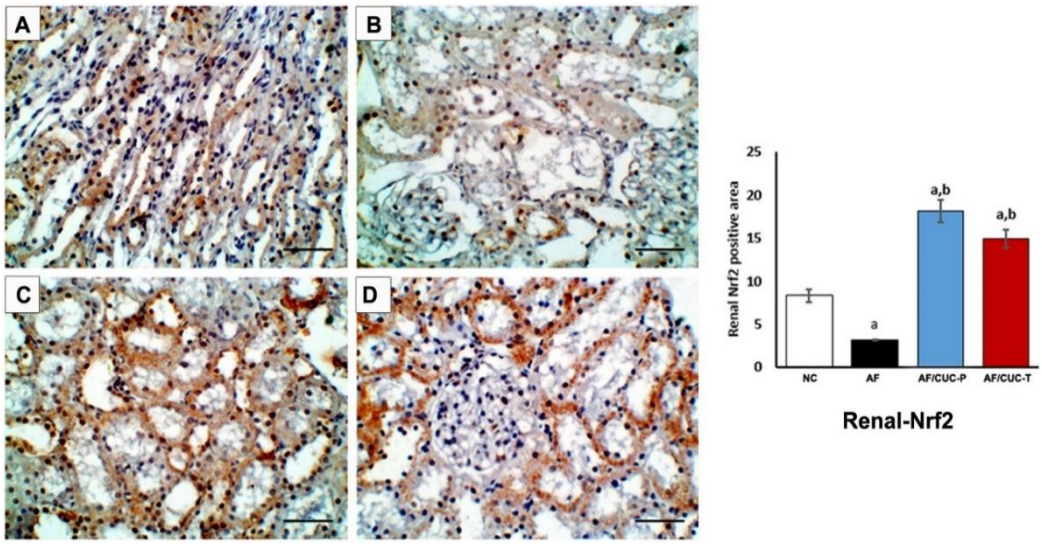

Figure 5. Immunohistochemical staining of nuclear factor erythroid-2 in kidney tissue sections of treated and control rats. Statistical significance is indicated by different small letters (a, b and c) (P <0.05). NC; negative control, AF, Aflatoxicosis; AF/CUC-P, Aflatoxicosis Cu-II-Album Complex alternatively; AF/CUC-T, Aflatoxicosis then Cu-II-Album Complex treated. Bar = 50 µM. Similar characters denote insignificance between groups.

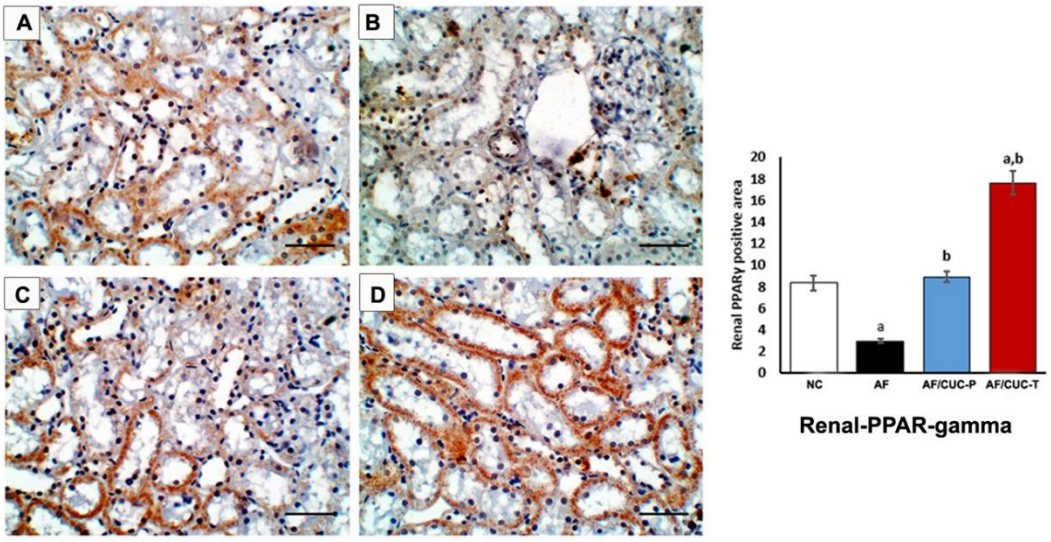

Figure 6. Immunohistochemical staining of peroxisome proliferator-activated receptor gamma; PPARγ in kidney tissue sections of treated and control rats. Statistical significance is indicated by different small letters (a, b and c) (P <0.05). NC; negative control, AF, Aflatoxicosis; AF/CUC-P, Aflatoxicosis Cu-II-Album Complex alternatively; AF/CUC-T, Aflatoxicosis then Cu-II-Album Complex treated. Bar = 50 µM. Similar characters denote insignificance between groups.

The immediate effect of the alternative Cu-II-Album Complex treatment protocol may explain the relatively lower levels of serum urea and creatinine compared to the end treatment, where some renal changes may be irreversible. The histopathology of kidneys further confirms this observation Cu-II-Album Complex treatments markedly alleviated the renal changes induced by AFB1 toxicity. However, rats treated after 3 weeks of intoxication showed mild degeneration and necrosis of the renal tubules that occasionally had shortened or flattened epithelium with minimal hyaline casts.

Several studies revealed that induction of acute kidney injury is associated with increased expression of NF-κB members [31, 32]. The decrease in NF-κB activation protects mice from ischemic acute kidney injury through reduced apoptosis and chemokine expression [33]. In this study, the Cu-II-Album Complex treatment significantly modulates the NF-κB that may be reflected as reduced cell apoptosis in the treated groups that remarkably maintained its normal structures compared to the aflatoxicosis group. A previous study reported an artificial copper complex as an NF-κB inhibiting Cu(2+) complex with improved cell-penetrating activity than free copper [34]. Meanwhile, the free copper and copper complexes were found to react with reactive oxygen species (ROS) that regulate the NF-kB activation signaling pathway [34, 35]

The Nrf2 is a redox-regulated transcription factor that plays a crucial role in protecting against oxidative damage and nitrosative stress by inducing cytoprotective and antioxidant genes (such as superoxide dismutase-2) [36]. Additionally, Nrf2 has been identified as an inhibitor of apoptosis in several studies, including Fas-induced apoptosis [37]. It was reported that the knockdown of Nrf2 expression aggravated the NF-κB pathway and increased the levels of reactive oxygen species, inducible nitric oxide synthase "iNOS", transforming growth factor beta 1"TGFβ1", and fibronectin [38]. AFB1 was found to reduce the gene and protein expression levels of Nrf2 and its downstream genes especially Heme oxygenase-1 (HO-1) as one of the most important anti-oxidative stress pathways [39].

In this study, a strong immunostaining of Nrf2 was observed in rat's kidneys treated with Cu-II-Album Complex that more likely neutralized the reactive oxygen species through downregulating the NFκB. Similar to our finding, the *Bacillus amyloliquefaciens* B10 probiotics alleviated the AFB1-induced kidney oxidative stress and apoptosis in mice via upregulation of the Nrf2 protein expression [40]. In another study, the curcumin (300 mg/kg) was found to alleviate AFB1-induced liver damage in broiler chickens by suppressing the oxidative stress via the same mechanism of Nrf2/HO-1 pathway activation [39].

In the kidney, PPARγ is expressed in different regions of the renal collecting system, renal medullary interstitial cells, and the juxtaglomerular apparatus, and the glomeruli [41]. Its activation in the kidney plays a critical role in renal function

regulation and involves the renin-angiotensin-aldosterone system that controls systemic blood pressure [42]. In this study, the PPARγ was strongly expressed in treated rats' renal tubules, indicating a maintained kidney metabolic homeostasis and function [43]. Furthermore, several studies suggested that PPARγ agonists have beneficial actions to induce PPARγ that modulate the renal fibrotic, inflammatory, immune, proliferative, reactive oxygen, and mitochondrial injury pathways [44-47]. Hence the increased expression in kidneys of treated rats indicates the restoration of podocytes after injury, especially in the end treatment of the Cu-II-Album Complex treated group.

In conclusion, the current study demonstrates the beneficial effect of Copper II albumin Complex (Cu-II-Album Complex) as a renal protective agent against aflatoxicosis by maintaining the renal functions and normal histological structure. Furthermore, the downregulation of NF-kB and the upregulated expression of renal Nrf2 and PPARγ in treated rats indicate the restoration of the renal podocytes, renal functions, and renal metabolic homeostasis.

Conflicts of interest

There are no conflicts to declare.

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