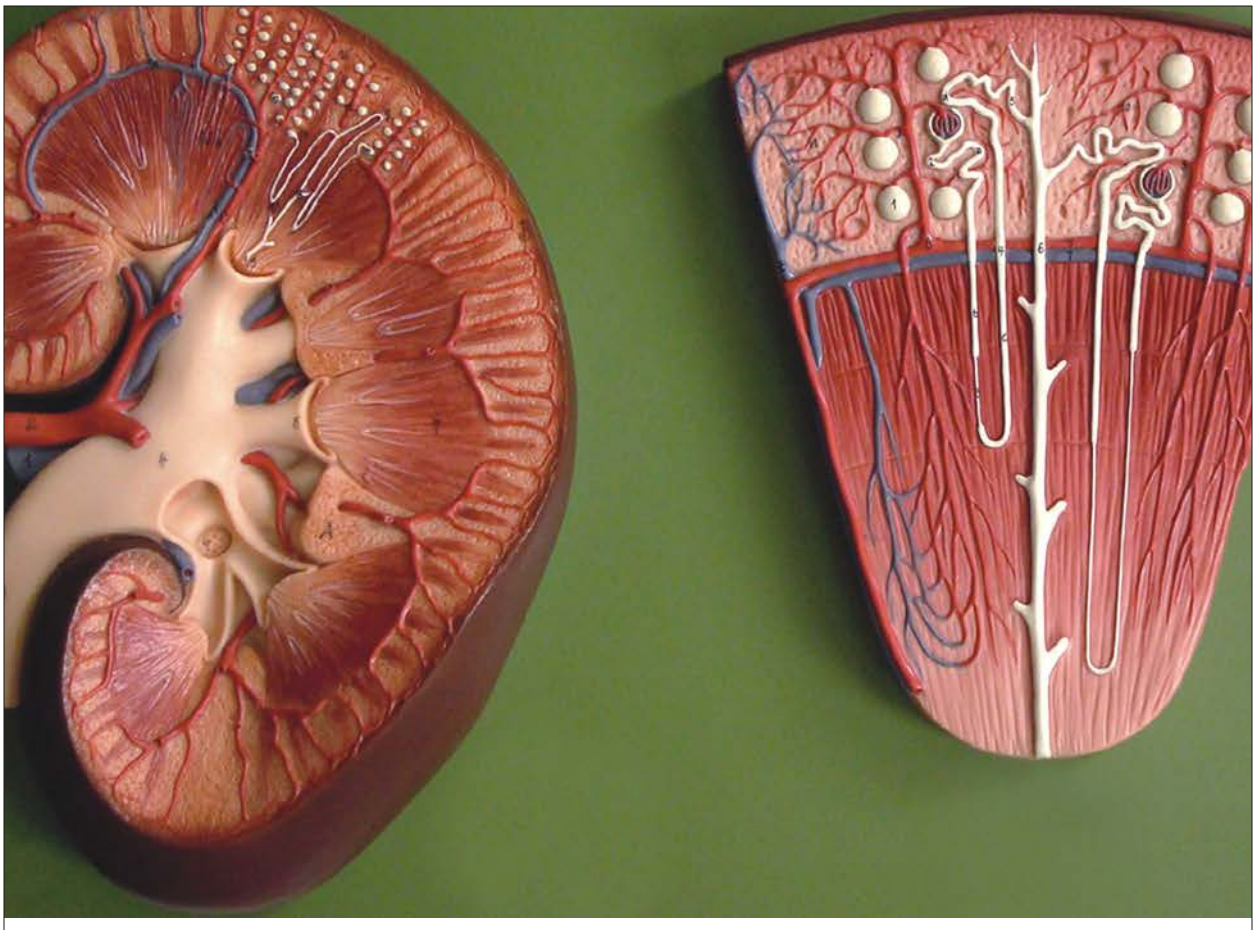

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Renoprotective Effect of Date Fruit Extract on Ochratoxin (A) Induced-oxidative Stress in Distal Tubules of Rat: A Light and Electron Microscopic Study

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ABSTRACT

Nowadays, people's exposure to mycotoxin such was continuously on the rise more and more. Theses compounds had induced an excessive production of free radicals which were responsible for several cell alterations in the organism. Recent investigations had proved the crucial role of nutritional antioxidants to prevent the damage caused by toxic compounds. In this study, we investigated the role of date fruit extract in protection against oxidative damage and distal tubules toxicity induced by chronic exposure to ochratoxin (A) (OTA). The animals were assigned into four groups (n = 8) including control and test groups. Control group received sodium bicarbonate and the animals in the test groups received (289 μg OTA kg^{-1} b.wt. day^{-1}), (1 mg Ajwa kg^{-1} b.wt. day^{-1}) and (289 μg OTA kg^{-1} b.wt. day^{-1}+1 mg Ajwa kg^{-1} b.wt. day^{-1}), respectively during 28 consecutive days. Oral administration of OTA caused nepherotoxicity as monitored by histological alterations marked by appearance of numerous apoptotic cells with swollen mitochondria and disintegrated membranes. Surprisingly, histopathological examinations showed that date, exerted a protective effect on OTA-induced damages, as revealed by improvement of histopathology changes whereas, in Ajwa date extract+OTA group the severity of the lesions was significantly reduced. In conclusion, this data indicated that OTA, at least partly by interfering in oxidative stress system, exerted its toxic effects on distal tubules whereas *in vivo* date with antioxidant properties could fairly protected rats against OTA toxic effects and be useful for the prevention of oxidative stress induced nepherotoxicity.

Key words: 1-Ochratoxin (A)2- light microscope 3-date 4- morphometry 5- distal tubules 6-electron microscope

INTRODUCTION

Contamination of cereals and grains with *Aspergillus* and *Penicillium* fungi had resulted in the production of the mycotoxin Ochratoxin A (OTA). This contamination with OTA had been associated with the induction of Balkan nephropathy in humans and porcine nephropathy in domestic swine, as well as carcinogenesis (Tapia and Seawright, 1984) where OTA was a potent renal carcinogen (Adler *et al*., 2009) and carcinoma arised from male rat renal parenchyma was an aspect of the nephrotoxicity of OTA and was a factor in considering application of animal data to human health risk assessment (Mantle and Kulinskaya, 2010). Importantly, many of the genes found to be deregulated in response to OTA had been linked to chromosomal instability and malignant transformation, supporting the hypothesis that aberrant mitosis, resulting in blocked

or asymmetric cell division, accompanied by an increased risk of aneuploidy acquisition, might play a critical role in OTA carcinogenicity (Adler *et al*., 2009).

OTA-induced oxidative stress (Malekinejad *et al*., 2010) where Reactive Oxygen Species (ROS) might lead to cellular damage when the rat of this generation suppressed the rate of its decomposition by antioxidant defense system (Datta *et al*., 2000). Lipid peroxidation was one of the main manifestations of oxidative damage and it had been found to have important roles in the toxicity and carcinogenicity of many xenobiotics (Anane and Creppy, 2001). Antioxidants in *Syzygium aromaticum* L (SA) (Aisha *et al*., 2011) and dates extracts (*Phoenix dactylifera* L.) (Hasan *et al*., 2010) were known to reduce oxidative radical-induced reactions (El-Demerdash, 2004). The fruits of Date were consumed throughout the world and were an important part of the diet in the Middle East. Dates at the rutab and tamar maturity and ripening stages contained a wide array of phenolic antioxidants (Hong *et al*., 2006) where they possess antioxidative properties *in vitro* (Rock *et al*., 2009). Saafi *et al*. (2010) indicated that *in vivo* date palm fruit might be useful for the prevention of oxidative stress induced hepatotoxicity. There was scarce information on sub-cellular changes as a result of interaction of date in OTA_ treated rats. In view of these findings, the present investigation was undertaken to assess the renal ultrastructural alterations in rat fed a diet containing OTA and Date, either alone or in combination to investigate possible protective effects of chronic administration of antioxidant date on oxidative stress after OTA-treatment in renal distal tubules of rats. Also, this research tested the hypothesis that OTA-induced nepherotoxicity might be the result of distal tubular injury rather than classic proximal tubular injury.

MATERIALS AND METHODS

Chemicals: Ochratoxin A: Ochratoxin A (Cat. No. 01877) were purchased from Sigma-Aldrich chemical company (USA). It was dissolved in 0.5 M HNaCO3 PH 7.4.

Preparation of date fruit extract: Fruit flesh was extracted according to Saafi *et al*. (2010).

Animals: Twenty eight weaning male albino rats (*Rutus narvegigus*) weighing 40±5 g. were used in this study and obtained from the Experimental Animal House Center, King Abdul-Aziz University Jeddah- Saudi Arabia at 2008 . They were fed daily tap water and pellet foods including 21% pure protein under optimum laboratory conditions (temperature, 21°C; humidity, 40-60%; light/dark period: 12h/12h, optimum air condition system) and housed in metal cages free from any source of chemical contamination. The use of animals was approved by the ethical requirements approved by the Animals Research Ethic Committee of KAU.

Experimental design: After acclimatization period of one week, the animals were distributed equally into four groups and were treated for four weeks (5 days/week) by gavage:

- **First group:** Control animals received sodium bicarbonate buffer: 0.1 M, pH 7.4
- **Second group:** Animals received (289 μg OTA kg^{-1} b.wt. day^{-1})
- **Third group:** Animals received (1 mg Ajwa dates kg^{-1} b.wt. day^{-1})
- **Fourth group:** Animals received (1 mg Ajwa dates+289 μg OTA kg^{-1} b.wt. day^{-1})

Histopathological procedures: At the end of the experiments, the animals of each group were killed by decapitation. The kidney specimens of the all groups were obtained and its volume

measured (Paltiel *et al.*, 2002) then processed for light and electron microscopical examination. For light microscopical observation, kidney specimens were embedded in the paraffin blocks after they had been fixed in 10% buffered formalin solution . Five micrometer (μm) sections were obtained and stained with hematoxylin+eosin and periodic acid-Schiff (Bancroft and Gamble, 2002). Other biopsies were immediately collected into electron microscopy processing.

Morphometric analysis: Five micrometer paraffin sections were stained with periodic acid-Schiff for morphometry. By using photo-analysis program under a light microscope (Olympus Cx 31): 1-Ten distal tubules area from each rat (at a magnification of 40x) (i.e., 80 tubules for each group) were chosen randomly. 2-The kidney capsule thickness of four locations from each rat(at a magnification of 100x) were chosen randomly. Four samples (renal cortex) from each animal of each group were evaluated to judge the severity of lesions in distal tubules. The lesions were classified as mild (+), moderate (++), intense (+++) and severe (++++) on the basis of changes in distal tubules.

Statistical analysis: Kidney volume, tubular area and capsule thickness were analyzed by using the program SPSS version 15 where One way ANOVA (Mould, 1989) was used to assess the significance of changes between control and treated rats. The data were expressed as Mean± Standard Errors (SE) and A p-value<0.05 was considered statistically significance.

RESULTS

No changes were noticed on the morphology and behavior of the animals. In addition, no macroscopic lesions were observed on the kidney. In this study, the kidney volume was slightly decreased in the OTA and Ajwa Dates+OTA-group and slightly increased in the Ajwa date-group compared to the control. No significant changes were noticed in the distal tubules areas in all groups but there was significant increase in the capsule thickness of OTA-group (Table 1). It was noticed from (Table 1)that ,the maximum value for distal tubule area (1784.28±107.11 μm^2) and kidney capsule thickness (2.68±0.18 μm) obtained by OTA-group and the minimum value obtained by Ajwa date-group respectively (1617.82±92.35 μm^2), (1.54±0.07 μm) but the maximum value for kidney volume (0.74±0.07 cm^3) obtained by Ajwa date-group and minimum value obtained by OTA-group (0.60±0.08 cm^3).

In rats, the intensity of tubule lesions varied between the treatments, whereas within the group the lesions were similar in nature i.e., moderate to intense in OTA-group, mild to moderate to in OTA+date-group (Table 2) i.e., the maximum amount of lesions noticed in OTA-group and the minimum one noticed in Ajwa date-group .

Histopathological alterations induced by OTA in rat tubules included dilatation of the tubule with extruded nuclei, hypertrophied cell, necrotic cell or single cell death (Fig. 1a), dilated

Table 1: Morphometric analysis for distal tubules area, kidney capsule thickness and kidney volume

	Experimental groups			
Variable	Control	OTA	Ajwa date	Ajwa date +OTA
Distal tubules area (μm^2)	1624.98±61.46	1784.28±107.11	1617.82±92.35	1625.54±47.33
Kidney capsule (μm) thickness	1.83±0.15	2.68±0.18*	1.54±0.07	1.72±0.14
Kidney volume (cm^3)	0.72±0.06	0.60±0.08	0.74±0.07	0.66±0.01

*Significantly different from the control at p<0.05. Values are expressed as Mean±SE

Table 2: The characteristics of tuble lesions observed at 28 days post-treatment

	Ggroups			
Parameters	Control	OTA	Ajwa date	Ajwa date + OTA
Tubular cell degeneration	+	+ + +	-	+ +
Tubular cell necrosis	-	+ +	-	+
Dilation of tubular lumen	-	+ +	-	+
Vessels congestion	+	+ + +	-	+ +
Mitochondrial changes	-	+ ++	-	++

Lesions described: -: No lesion, +: Mild, ++: Moderate, +++: Intense; ++++: Severe

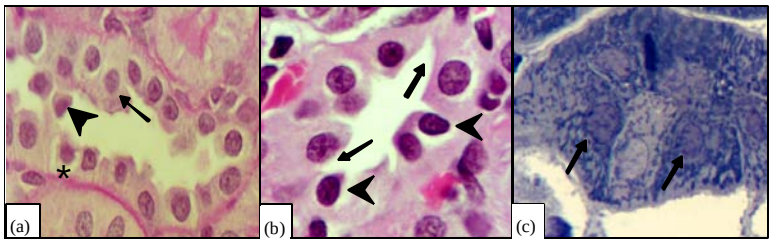

Fig. 1: Light photograph for section of OTA-group distal tubules showing: (a)Dilated tubule with extruded nuclei (head arrow), hypertrophied cell (arrow) and necrotic cells (*) (P.A.S. x600). (b) Tubule with dilated intercellular spaces (arrows), pyknotic nuclei (head arrows) (H And E x1000) and (c) Hypertrophied tubules with no lumen and extruded nuclei (arrows) (TB x1000)

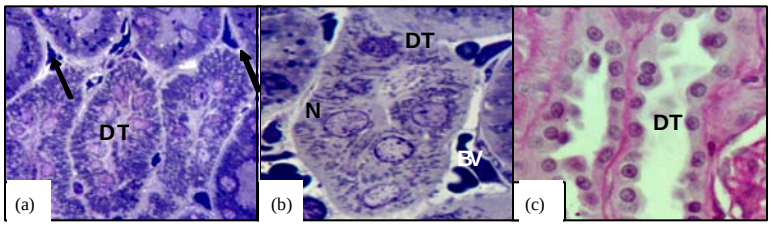

Fig. 2: Light photograph for section of Date-group showing: (a) Cortical mass contain normal distal tubules (DT) separated by thin interstitial tissue (arrows) (TB x400). (b) Tubule (DT) with cuboidal cell have oval nuclei (N) .Not, blood vessel (BV) (TB x1000) and (c) Tubules (DT) with wide lumen and low height cells (PAS x400)

intercellular spaces, pyknotic nuclei(Fig. 1b) and hypertrophied tubules with no lumen and extruded nuclei as indication for stimulation of cell proliferation (Fig. 1c).

In date-treated group animal,there were no significant histological abnormalities seen in comparison with the control group and majority of Distal Convoluted Tubule (DCT) cells revealed almost normal appearance where cortical mass contain normal distal tubules separated by thin interstitial tissue (Fig. 2a). The tubule lined with cuboidal cell have oval nuclei (Fig. 2b) and had wide lumen surrounded by these low height cells (Fig. 2c).

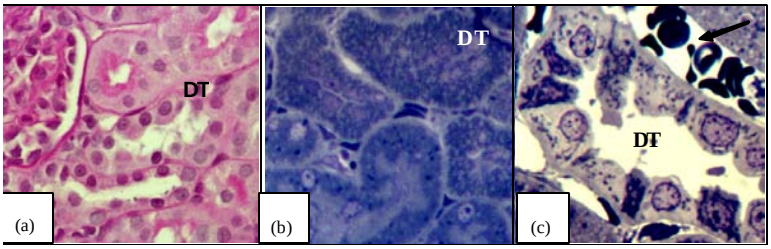

Fig. 3: Light photograph for section of OTA+date-group showing: (a) Distal tubule (DT) with cytoplasmic basophilia (PAS x400). (b) Nearly normal tubules (TB x400) and (c) Distinct nuclei in distal tubule (DT) with visible lumen and blood vessel in interstitial tissue (arrow) (TB x1000)

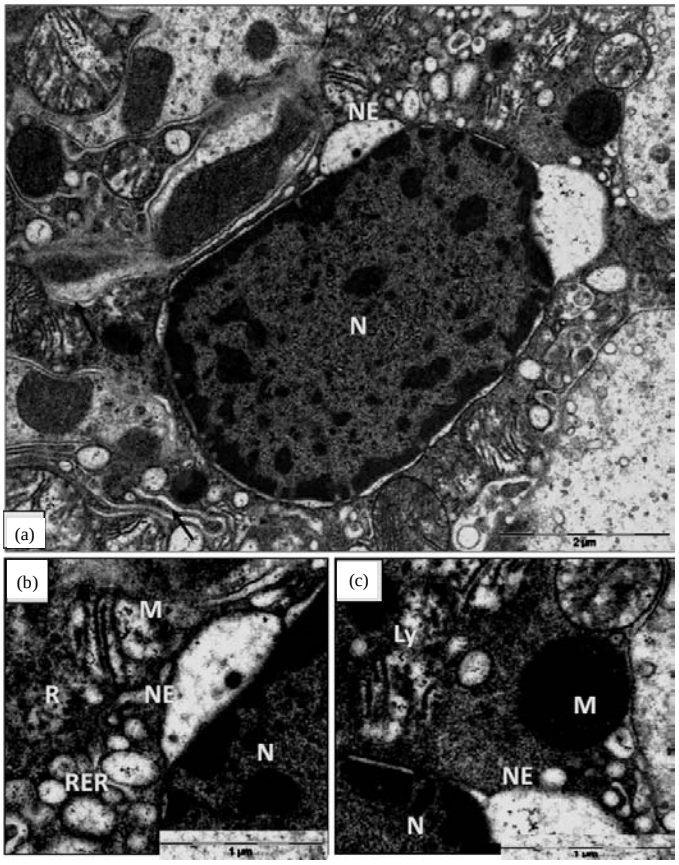

Fig. 4: Transmission electron micrograph of ultrathin OTA sections: (a) apoptotic distal tubule cell with high dens electron cytoplasm, hyperchromatic nucleus (N) and disrupted basal infolding (arrows). (b) detail swollen mitochondria (M) with few cristae, nuclear envelope blabbing (NE), vesiculated Rough Endoplasmic Reticulum(RER) and free ribosome (R) and (c) detail highly dense mitochondria (M) and lysosome (Ly)

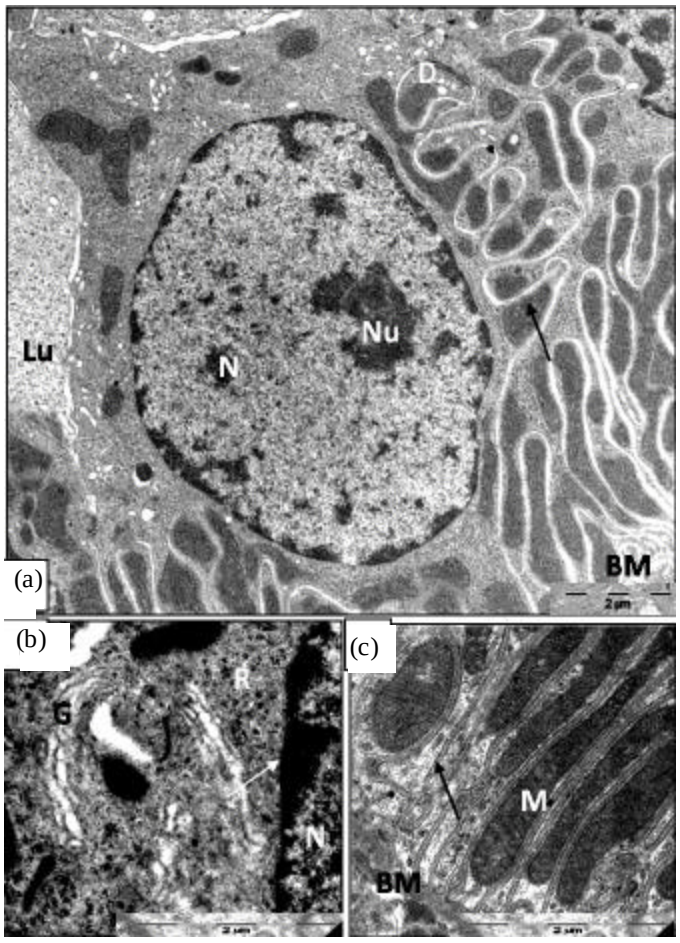

Fig. 5: Transmission electron micrograph of ultrathin of Ajwa date-sections: (a) distal tubule cell with wide lumen (Lu), normal active central round nucleus (N) have nucleolus (Nu), lateral infolding (arrow)and desmosome (D), (b) detail nucleus (N) with heterochromatin marginalization, free ribosome (R) and Golgi complex (G) and (c) detail elaborate basal infoldings (arrows) from basement membrane (BM) inbetween parrall elongated mitochondria (M) with distinct cristae

Treatment of rats with Date ameliorated the pathological changes induces by OTA where the the morphmetric measurements were almost like the controls (Table 1). Additionally, the extent of the lesions to the tubules was also reduced (Table 2). The tubular organization and the cytoplasmic basophilia were also similar to the control group (Fig. 3a). The nearly normal tubules (Fig. 3b) had low height cells with distinct nuclei and clearly lumen visible in most of the tubules (Fig. 3c).

The decrease in antioxidant status in rat kidney treated with OTA was clear by ultrastructural alterations in OTA-group primarily involving DCT epithelium were mainly associated with an increased number of cells undergoing apoptosis (Fig. 4). The apoptotic cells were visualized through dilation of the nuclear envelope forming membrane blabbing, uneven distribution of nuclear chromatin and disappearance of nucleoli. Condensed cytoplasmic organelles were also seen

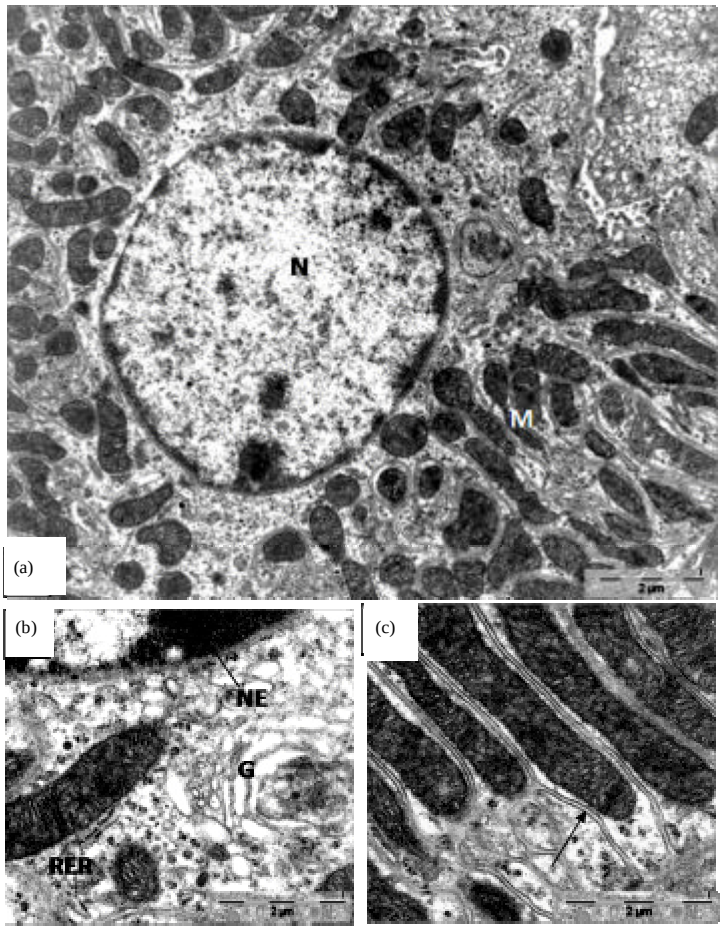

Fig. 6: Transmission electron micrograph of ultrathin of Ajwa dates+OTA sections: (a) distal tubular cell has euchromatic nucleus (N) and many mitochondria (M), (b)detail nucleus surrounded with double nuclear envelop (NE), short Rough Endoplasmic Reticulum (RER) and Golgi complex with parallel cisternae (G) and (c) detail elongated mitochondria with distinct cristae and numerous normal basal Infolding

deteriorated, loss of membrane integrity was detected in most organelles in high dens electron cytoplasm (Fig. 4a). Degeneration and distortion of swollen mitochondria with complete loss of cristae, nuclear envelope blabbing, vesiculated rough endoplasmic reticulum, free ribosome and formation of empty spaces in the cytoplasm were the consistent findings (Fig. 4b). In other area highly dense mitochondria and lysosome were recorded (Fig. 4c).

An ultrastructural examination of the distal tubular cells in the date-group revealed the presence of well formed nuclei centrally located and normal shape, size and number of cytoplasmic organelles especially lateral infolding, desmosome (Fig. 5a), free ribosome and Golgi complex (Fig. 5b). Also, densely packed parallel elongated mitochondria with distinct cristae arranged in the form of tubular rays arising from their membrane inbetween elaborated basal infoldings (Fig. 5c).

Clearly, the ultrastructural changes were lessened by administration of Ajwa date, the cell nuclei were slightly more heterochromatic. Although the mitochondria were again randomly scattered throughout the cytoplasm, they were more homogenous in terms of their shapes and sizes (Fig. 6a). Euchromatic nucleus surrounded with double nuclear envelop, short rough endoplasmic reticulum, Golgi complex with parallel cisternae (Fig. 6b) and elongated mitochondria with cristae structure appeared almost normal, with no observable damage (Fig. 6c) were observed.

DISCUSSION

Microinfusion of OTA into superficial nephrons showed that it was reabsorbed in proximal as well as distal parts of the nephron. About two-thirds of total reabsorption (which amounts to 60-70% of the tubular load) occurred before OTA reached the distal tubule, i.e., in the proximal convoluted tubule and/or in the short loops of Henle. The remaining reabsorption took place in the distal tubule and/or the collecting duct. In this case OTA would be trapped within tubular cells, might lead to accumulation of the toxin in renal tissue, thus enhancing its toxicity (Zingerle *et al*., 1997).

In the present study, the intensity of tubule lesions varied between the treatments, whereas within the group the lesions were similar in nature i.e., moderate to intense in OTA-group, mild to moderate to in OTA+date-group. Also, Kumar *et al*. (2007) observed mild to moderate lesions in distal convoluted tubules in OTA-treated rabbits.

Histopathological alterations induced by OTA in rat tubules hypertrophied tubules with no lumen and extruded nuclei as indication for stimulation of cell proliferation. Based on these observations, it had been suggested that disruption of mitosis by OTA might be the principal cause of cell death and subsequent triggered for cell proliferation to compensate for cell loss (Adler *et al*., 2009).

In date-treated group animal, there were no significant histological abnormalities seen in comparison with the control group and majority of Distal Convoluted Tubule (DCT) cells revealed almost normal appearance as described before (Junqueira and Carneiro, 2003).

Treatment of rats with date ameliorated the pathological changes induces by OTA where the morphmetric measurements were almost like the controls. Additionally, the extent of the lesions to the tubules was also reduced. The tubular organization and the cytoplasmic basophilia were also similar to the control group, with the lumen clearly visible in most of the tubules as, date flesh demonstrated a strong free radical scavenging activity (Chaira *et al*., 2007).

The decrease in antioxidant status in rat kidney treated with OTA might be ultimately interlinked in the pathogenic network of the OTA toxicity (Meki and Hussein, 2001) and this was clear by ultrastructural alterations in OTA-group primarily involving DCT epithelium were mainly associated with an increased number of cells undergoing apoptosis. Nuclear changes noticed in the toxin treated groups might be due to free radical formation as a result of toxic injury to cells (Hoehler *et al*., 1997). Oxidative damage including lipid peroxidation might be one of the mechanisms of cellular damage in the toxicity due to OTA (Petrik *et al*., 2003). The nuclear condensation with cytoplasmic bleb formation (apoptosis) observed in the present study was a characteristic feature recorded in OTA- group. OTA induced apoptosis has been earlier reported *in vivo* in rat kidney and *in vitro* in human kidney epithelial cells (Rached *et al*., 2006). The tubular cells apoptosis played an important role in the development of the kidney insult (Balachandran *et al*., 2005). Degeneration and distortion of mitochondria with complete loss of cristae and formation of empty spaces in the cytoplasm were the consistent findings in this study,

the mitochondrial swelling and lysis of cristae may reflect the disturbances in oxy-reduction processes taking place in the organelle (Thevenod, 2003). The mitochondrial alterations observed in the present study were similar to those described in chicks, pigs and rats (Satheesh *et al*., 2004) where mitochondrial swelling and misshapen appearance might be due to the accumulation of intracellular water as a result of toxic stress. OTA inhibits mitochondrial oxidative phosphorylation by acting as a competitive inhibitor of carrier proteins in the inner mitochondrial membrane (Meisner and Chan, 1974).

An ultrastructural examination of the distal tubular cells in the Date- group revealed the presence of well formed nuclei and normal shape, size and number of cytoplasmic organelles i.e.normal structure as illustrated before (Junqueira and Carneiro, 2003).

Clearly, the ultrastructural changes were lessened by administration of Ajwa date, the cell nuclei were slightly more heterochromatic. Although the mitochondria were again randomly scattered throughout the cytoplasm, they were more homogenous in terms of their shapes and sizes, since mitochondrial electron transport has been negatively affected (Ezeji *et al*., 2009). Wang and Zhang (2008) provided *in vitro* evidence that recombinant human erythropoietin mediated renoprotective effect against aristolochic acid injury in renal tubular cells by ameliorating the damage of cytoskeleton, reducing the number of apoptotic cells and promoting cell regeneration and this might be the same renoprotective effect for date in this study.

CONCLUSION AND RECOMMENDATIONS

It may be concluded that on simultaneous exposure-date potentiated the toxic effects of OTA on renal structure and chronic administration of date would be effective in protecting against OTA-induced tissue damage in rat distal tubules. The toxic effect of OTA is somehow minimized by a compensatory mechanism involving date via the induction of antioxidant enzyme activity and that is why further studies should be conducted to better understand the protective mechanisms of concomitant treatment of date.

To our knowledge, no study has been conducted on the co-effect of date on OTA accumulation and on the histology of distal tubules. The results highlight the need to reduce exposure to myotoxines, with particular attention being paid to the known sources of myotoxines. At the same time, the maintenance of a diet that is rich in date should be beneficial in the alleviation of myotoxines toxicity.

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