

# Antioxidant Effects of Melatonin and Coenzyme Q<sub>10</sub> on Oxidative Damage Caused by Single-Dose Ochratoxin A in Rat Kidney

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## Abstract

In the study, the effects of relatively high single-dose of Ochratoxin A (OTA) and the antioxidant effects of Melatonin (Mel) and Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) on OTA-induced oxidative damages in rats were investigated. A total of 28 male Sprague-Dawley rats were divided into four groups of 7 rats each: Control, OTA, Mel+OTA and CoQ<sub>10</sub>+OTA groups. Malondialdehyde (MDA) levels in the plasma and glutathione (GSH) levels in whole blood were measured; kidneys (for histological inspection and for apoptosis detection by TUNEL method) and bone marrow samples (for chromosome aberration and mitotic index) were taken. The rats in the OTA group showed limited degeneration of tubular cells. In some tubules karyomegaly, desquamated cells and vacuolization were observed by light microscopy. Mel and CoQ<sub>10</sub> treatment significantly reduced the severity of the lesions. MDA levels of the OTA group were significantly higher than the control, OTA+Mel and OTA+CoQ<sub>10</sub> groups, while GSH levels were significantly lower than the control, OTA+Mel and OTA+CoQ<sub>10</sub> groups. Higher incidences of apoptotic bodies were observed in the kidneys of the OTA group although OTA administration did not significantly change the incidence of apoptotic bodies when compared to the control and antioxidant administrated groups. Although the percentage of the mitotic index was lowest in the OTA group, no statistical difference was found among the groups. Additionally, OTA had no numerical and structural significant effects on chromosomes. It was observed that single-dose OTA administration caused oxidative damages in rat kidney and Mel or CoQ<sub>10</sub> treatment appeared to ameliorate the OTA-induced tissue injuries.

**Key Words:** coenzyme Q<sub>10</sub>, melatonin, mitotic index, ochratoxin A, oxidative stress

## Introduction

Ochratoxin A (OTA) is a mycotoxin produced by *Aspergillus ochraceus* and other molds as a natural contaminant of moldy food and feed. OTA has been identified in blood, bile and urine of human and animals after consumption of contaminated food (16).

OTA is a nephrotoxic, hepatotoxic and teratogenic mycotoxin and a potent renal carcinogen in rodents and rats (15). Exposure to low concentrations of this toxin causes morphological and functional changes in the kidney and liver of some domestic and experimental animals (3). Furthermore, OTA seems to be involved in the pathogenesis of Balkan Endemic neph-

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ropathy, chronic interstitial nephritis and karyomegalic interstitial nephritis, and exerts teratogenic effects (15). OTA-induced oxidative stress and apoptosis may play key roles in the development of chronic tubulo interstitial nephritis connected to long term exposure to this food contaminant (24). In addition, it has been reported that OTA may induce apoptosis (35).

The exact mechanism of OTA nephrotoxicity is not completely understood. It may act as a carcinogenic activity and/or its nephrotoxicity may be due to inhibition of protein synthesis, and genotoxic effects such as DNA adduct formation and DNA single-strand breaks (7, 25). However, in recent years several studies have stressed the importance of oxidative stress (8, 33). OTA induces oxidative damage in cultured cells and in experimental animals. Moreover, antioxidant administration is able to reduce OTA-induced nephrotoxicity and its histopathologic abnormalities (1, 4, 20, 23).

Chronic effects of OTA in the kidney are usually related to primary lesion of proximal tubules followed by spontaneous damages of glomeruli and involution of the interstitial (14). Acute OTA intoxication after several consecutive daily doses produces massive acidophilic degeneration with necrosis and desquamation of epithelium in proximal tubules (13). In acute toxicity studies, OTA-induced cell death was reported *in vivo* in rat renal tubules (2). However, some investigators suggested that after short-term administration of OTA to rats, the renal proximal tubule did not appear to be the main target for nephrotoxicity although decreased capacity to eliminate the toxin might result in self-enhancing effects (9).

Melatonin (Mel) and Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) are well known antioxidants and free radical scavengers. Mel, N-acetyl-5-methoxytryptamine, is a hormonal product of the pineal gland that plays many roles within the body including control of reproductive functions, modulation of immune system activity, limitation of tumorigenesis and effective inhibition of oxidative stress (31). CoQ<sub>10</sub> is an integral component of the mitochondrial oxidative phosphorylation system and is a lipid-soluble redox carrier between particular respiratory enzyme complexes in the electron transport chain in the mitochondrial inner membrane (17).

In this study, we aimed to demonstrate morphologic and biochemical changes in the kidney of the rat administered with single relatively high doses of OTA, and to demonstrate the effects of free radical scavengers (Mel and CoQ<sub>10</sub>) on cells damaged by OTA.

## Materials and Methods

### Animals

A total of 28 Sprague-Dawley male rats weighing 230-250 g were maintained on OTA-free lab diet and water *ad libitum* at the Animal House, Medical School Research Center, Eskisehir, Turkey. Acclimatization period lasted for 2 weeks. The animals were housed in filter-top polycarbonate cages in a temperature (23 ± 1°C) and humidity (55 ± 5% humidity) controlled and artificially illuminated room (12:12 h light:dark cycle) free from any source of chemical contamination. Seven animals were housed within each cage. All rats received human care in compliance with the guidelines of the Animal Care and Use Committee of Eskisehir Osmangazi University, Medical Faculty, Turkey.

The rats were divided into 4 groups of 7 rats each: [1] Control (C), [2] OTA group (only 2.2 mg/kg bw OTA), [3] Mel+OTA group (10 mg/kg bw Mel and 2.2 mg/kg bw OTA), and [4] CoQ<sub>10</sub> + OTA group (10 mg/kg bw CoQ<sub>10</sub> and 2.2 mg/kg bw OTA).

### Reagents

Benzene-free OTA and CoQ<sub>10</sub> were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Melatonin was obtained from Merck Chemical Co. (Darmstadt Germany). Identification of apoptosis in kidney sections was performed by TdT-mediated dUTP-nick end-labeling (TUNEL) using the *In situ* Death Detection Kit, Fluorescein (Cat. No. 1-684-795; Roche Molecular Biochemical's, Mannheim, Germany).

### Experimental Design

After the acclimatization period of 2 weeks, the experimental period lasted for 3 days.

**Control group:** The control group received 0.5 M NaHCO<sub>3</sub> and ethanol (1:100, v/v) in tap water during the experimental period.

**OTA group:** Benzene-free ochratoxin A was dissolved in 0.1 M NaHCO<sub>3</sub> and administered orally at a single dose of 2.2 mg/kg body weight by gastric gavage on the first day of the experimental period.

**Mel+OTA group:** Melatonin was initially dissolved in dimethylsulfoxide (DMSO) and diluted with distilled water. The Mel+OTA group intraperitoneally received Mel, 10 mg/kg bw, three times (1st, 2nd and 3rd day, during dark periods, because of circadian rhythm), and on the first day ochratoxin A was administered by gastric gavage 30 min after the Mel treatment.

**CoQ<sub>10</sub>+OTA group:** Coenzyme Q<sub>10</sub> was initially dissolved in DMSO and diluted with distilled water. The CoQ<sub>10</sub>+OTA group intraperitoneally received CoQ<sub>10</sub>, 10 mg/kg bw, three times (1st, 2nd and

3rd day), and on the first day ochratoxin A was administered by gastric gavage 3 h after CoQ<sub>10</sub> treatment.

At the end of the experimental period, all the animals were intraperitoneally injected 4 mg/kg colchimid for the chromosome analysis. After two h of colchimid application, intracardiac blood samples were collected, under ether anesthesia, into two different heparinized tubes from all animals after being fasted for 12 h (with free access to drinking water) and the rats were then sacrificed. First tubes were centrifuged at 1,500 rpm for five min to separate the plasma. Whole blood and plasma samples were stored at -80°C until measurement of levels of malondialdehyde (MDA) and glutathione (GSH).

MDA levels in the plasma were determined by a spectrophotometric method as described by Ohkawa *et al.* (22). The principle of the method was based on spectrophotometric measurement of the color occurring at 532 nm during the reaction to thiobarbituric acid (TBA) (22).

GSH levels in the whole blood samples were measured as described by Beutler (5). The principle of the method was also based on spectrophotometric measurement of the color occurring at 412 nm during the reaction to 5-5 dithiobis-2-nitrobenzoic acid (DTNB).

#### *Chromosome Analysis and Mitotic Index (MI)*

Both femora were dissected out and bone marrow was flushed by a syringe with 5 ml solution of fetal calf serum and transferred into a test tube for chromosome analysis. Bone marrow cells were harvested by centrifugation and resuspended in a hypotonic solution (0.075 M KCl) for 20 min at 37°C and then rinsed twice in a *methanol:acetic acid* (3:1) fixative. Cells were finally spun down, dropped on to slides in residual fixative and air-dried. The preparations were stained in 5% Giemsa solution for 5 min and evaluated by light microscopy. A total of 200 metaphases were counted and chromosome abnormalities were determined for all the rats.

MI was used to determine the rate of cell division. Randomly selected views on the slides were monitored to determine the number of dividing cells (metaphase stage) and the total number of cells. MI was evaluated by counting at least 1,000 cells per treatment: the number of dividing cells was divided by the total number of cells.

#### *Histological Examination*

The kidneys of the necropsied rats were dissected and immediately fixed in neutral-buffered formalin. Kidneys were embedded in paraffin; sections of 5 µm

thickness (microtome, Leica Rm 2145) were stained in haematoxylin and eosin. The sections were examined by an Olympus PM 10 ADS microscope (Olympus America Inc. Melville NY, USA). The histopathological findings of the kidney tissues were scored according to the degree of histopathological damage: (-) showing no changes, and (+), (++) and (+++) indicating minimum, moderate and maximum changes, respectively.

For electron microscopy, randomly selected renal cortex samples (0.5-1.0 mm<sup>3</sup>) were fixed in 2.5% glutaraldehyde (pH 7.3), washed in phosphate buffer, post fixed in 1% osmium tetra oxide, dehydrated in graded alcohols and propylene oxide, and flat embedded in Araldite (CY). Semi-thin sections were stained with toluidine blue and ultra thin sections were stained with uranyl acetate and lead acetate, and examined under JEOL TEM 1220 electron microscope (Tokyo, Japan).

#### *In Situ Apoptosis Detection*

Cleavage of genomic DNA during apoptosis may yield double-stranded, low-molecular-weight DNA strand breaks that can be labeled by terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotides to free 3'-OH ends in a template-independent manner (TUNEL reaction). For counting the apoptotic cells (cells showing high-intensity fluorescence), 25 randomly selected areas for each sample were evaluated by an OLYMPUS-BX61 microscope (Olympus America Inc.). On average, 25-30 slices were collected per animal. The number of apoptotic bodies was expressed as number per 100 nuclei.

#### *Statistical Analysis*

Data were expressed as means ± SEM. SPSS version 10.0 for Windows was used to evaluate the data. Data were statistically analyzed by using ANOVA test, tukey test for post hoc multiple comparison, Kruskal-Wallis and Mann-Whitney U-test to evaluate the effects on histopathological changes. Rejection of the null hypothesis was set at  $P < 0.05$ .

## **Results**

The rats in the OTA group showed a limited degeneration of tubular cells. There were a few degenerative tubules mainly located on the inner part of the cortex. The renal lesions were seen in proximal convoluted tubules. In some tubules karyomegaly, desquamated cells and vacuolization were observed by light microscopy (Figs. 1a and 2a). No significant lesions were observed in the control (Figs. 1b and 2b)

**Table 1. Histopathological changes affected by OTA, OTA+Mel and OTA+CoQ<sub>10</sub>**

Study groups	Control (I)				OTA (II)				OTA+Mel (III)				OTA+CoQ <sub>10</sub> (IV)				Kruskal-Wallis <sup>†</sup>	Mann-Whitney U <sup>‡</sup>
	-	+	++	+++	-	+	++	+++	-	+	++	+++	-	+	++	+++		
Karyomegaly	7	0	0	0	0	4	3	0	4	3	0	0	3	4	0	0	< 0.01	I-II, I-IV, II-III, II-IV
Desquamated cells	5	2	0	0	0	3	4	0	3	4	0	0	2	5	0	0	< 0.01	I-II, II-III, II-IV
Vacuolization	6	1	0	0	0	5	2	0	4	3	0	0	4	3	0	0	< 0.05	I-II, II-III, II-IV
Tubular dilatation	6	1	0	0	2	5	0	0	4	3	0	0	3	4	0	0	> 0.05	

\*Score (-): no significant change. Score (+): mild degree. Score (++): moderate degree. Score (+++): severe degree.

<sup>†</sup>The data were statistically analyzed by the Kruskal-Wallis test.

<sup>‡</sup>Significant differences between the groups were tested by the Mann-Whitney U-test.

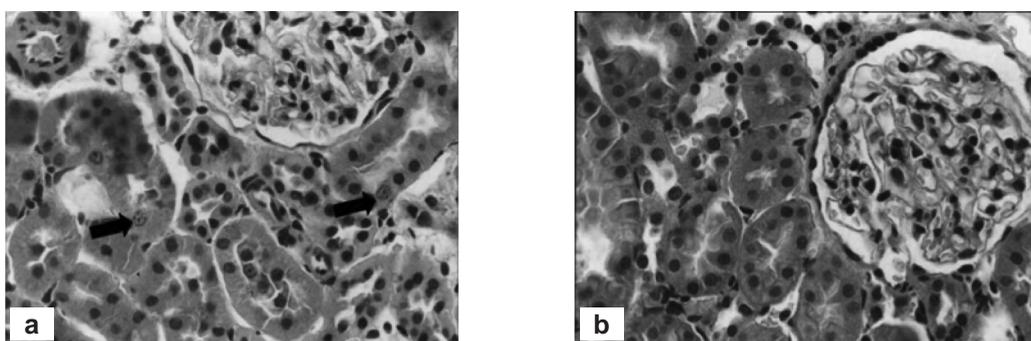


Fig. 1. Few karyomegali (arrows) in proximal tubules in the OTA group (a), and proximal tubules in the control group (b), H&E staining. Original magnification: 132 ×.

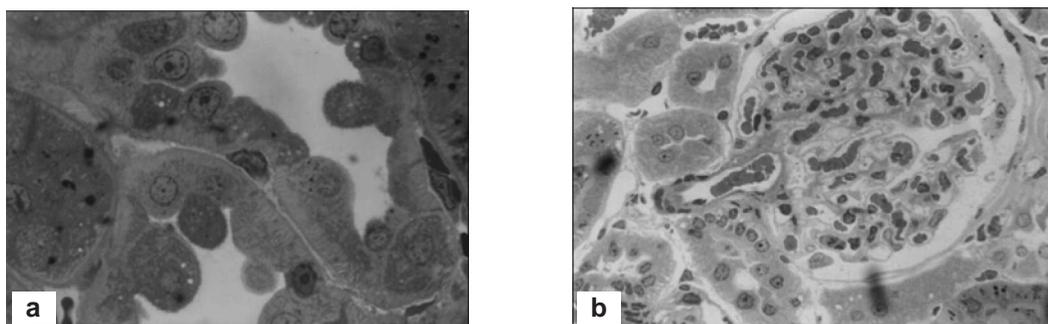


Fig. 2. Vacuolization and desquamation in proximal tubule cells in the OTA group in semi-thin section (a), and normal proximal tubules and glomerules in the control group in semi-thin section (b), Toluidine blue staining. Original magnification: 132 ×.

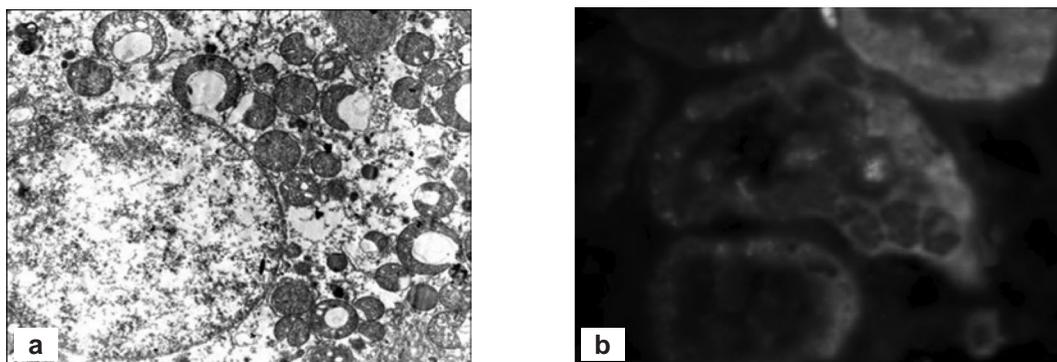


Fig. 3. Vacuolization and crista degeneration in mitochondria of a proximal tubule in the OTA group under a JEOL TEM 1220 electron microscope. Original magnification: 5,000 × (a), and TUNEL-positive cells in proximal tubules in the OTA group (b). Original magnification: 132 ×.

**Table 2. Plasma antioxidant concentrations in the study groups**

Antioxidants	Control	OTA	OTA+Mel	OTA+CoQ <sub>10</sub>
MDA (nmol/mL)	3.36 ± 0.2	5.93 ± 0.55*	4.09 ± 0.27 <sup>†</sup>	4.53 ± 0.21 <sup>‡</sup>
GSH (μM)	3,251 ± 282	1,480 ± 79*	2,966 ± 226 <sup>†</sup>	2,777 ± 92 <sup>†</sup>

\*different from the control group;  $P < 0.001$ .

<sup>†</sup>different from group OTA;  $P < 0.01$ .

<sup>‡</sup>different from group OTA;  $P < 0.05$ .

**Table 3. Ratio of TUNEL-positive cells in the study groups**

Treatment	TUNEL-positive cells (%)
Control group	0.21 ± 0.03
OTA group	0.31 ± 0.05
OTA+Mel group	0.26 ± 0.04
OTA+CoQ <sub>10</sub> group	0.28 ± 0.05

Data are shown as “means ± SEM”.

No difference was found between the groups.

**Table 4. Mitotic index (MI) and chromosome abnormalities in bone marrow cells**

Treatment	MI (%)	Gap	Chromatide type breakage
Control group	19.31 ± 1.90	1	0
OTA group	14.57 ± 0.72	2	1
OTA+Mel group	16.25 ± 1.02	1	0
OTA+CoQ <sub>10</sub> group	15.83 ± 1.77	1	2

Data are shown as “means ± SEM”.

No difference was found between the groups.

or antioxidant administrated groups. Quantification scores and statistical analysis of the data showing histopathological changes affected by OTA, OTA+Mel and OTA+CoQ<sub>10</sub> are given in Table 1. By electron microscopy, it was observed that only few tubule cells had condensed nuclei and mitochondrial degeneration from mild to moderate swelling (Fig. 3a). No glomerular lesions were seen.

MDA levels of the OTA group were significantly higher than those of the control, OTA+Mel and OTA+CoQ<sub>10</sub> groups. However, no differences were found between the the OTA+Mel and OTA+CoQ<sub>10</sub> and control groups (Table 2).

GSH levels of the OTA group were significantly lower than those of the control, OTA+Mel and OTA+CoQ<sub>10</sub> groups, and also no differences were found between the OTA+Mel, OTA+CoQ<sub>10</sub> and control groups (Table 2).

By TUNEL staining method, it was found that OTA administration (Fig. 3b) did not significantly change the incidence of apoptotic bodies when compared to the control and the antioxidant administrated groups (Table 3).

Although the percentage of the mitotic index was the lowest in the OTA group, no statistical difference was found among the groups. Additionally, OTA had no numerical and structural significant effects on chromosomes (Table 4).

## Discussion

The first aim of this study was to determine the oxidative damages caused by single relatively high doses of OTA administration and the second aim was to determine whether treatment with antioxidants such as melatonin and CoQ<sub>10</sub> has protective effects against oxidative damages induced by OTA. Our results showed that single-dose OTA administration caused oxidative damage, and melatonin or CoQ<sub>10</sub> treatment appeared to ameliorate it.

Within the complex and heterogeneous structure of the kidney, the proximal tubule is one of the main targets for nephrotoxic compounds such as OTA. Therefore, we aimed to evaluate the histopathological changes appeared in the kidney due to OTA administration.

OTA is known to affect multiple sites of the nephron: acute exposure mainly affects the post-proximal parts while chronic exposure leads predominantly to damages of the proximal tubule (10). Ochratoxin-induced non-neoplastic renal tubular epithelial changes includes cytoplasmic alteration, degeneration, karyomegaly, proliferation, hyperplasia and massive acidophilic degeneration with necrosis and desquamation of epithelium in proximal tubules (4, 6, 13).

In a study conducted by Sutken *et al.* (36), OTA was administrated for a four-week period for evaluation of subchronic effects of OTA (36). In their study, they reported mild degenerative changes in tubular epithelium with prominent dilated tubules and interstitial fibrosis and tubular atrophy. The tubular epithelial nuclei were enlarged and necrotic. Moreover, the necrotic cells had no nuclei and the remaining cytoplasm was eosinophilic. They reported that they observed reduced degeneration in the kidney in the OTA+Mel treated group. But in the OTA+CoQ<sub>10</sub> treated group, cell necrosis, karyomegaly and desquamation were observed in proximal tubules (36).

In the OTA group in our study, karyomegaly was observed in some of the proximal tubules and some of the proximal tubule lumens also had desquamated cells. These degenerative tubules were mainly in the inner part of the cortex. Only a few cells had condensed nuclei and showed mitochondrial degeneration. These findings were in an agreement with previous studies (4, 6, 13, 36). Quantification scores showed that Mel and CoQ<sub>10</sub> treatment significantly reduced the severity of the histopathological lesions caused by OTA.

Toxicological data point to a number of effects *in vivo* including inhibition of protein synthesis, induction of lipid peroxidation and inhibition of mitochondrial ATP production but consensus as to which effects represent the primary mechanisms of ochratoxicosis is still controversial. Oxidative stress contributes to the tubular toxicity of OTA. Highly destructive hydroxyl radicals attack cellular fatty acids causing a weakening in many subcellular structures and the cell membrane (8).

Malondialdehyde (MDA) is an end product of lipid peroxidation and may thus be considered as a late biomarker of oxidative stress and cellular damage. OTA has been reported to increase MDA formation *in vitro* upon incubation with rat liver microsomes in the presence of NADPH (29). GSH provides major protection in oxidative injury by participating in the cellular system of defense against oxidative damage. The levels of reactive oxygen species are controlled by antioxidant enzymes and non-enzymatic scavengers like GSH. In this study, it was observed that OTA

clearly increased MDA and decreased GSH formation, confirming previously published results (1, 20, 23, 29, 36).

Administration of antioxidant such as Mel (30) and CoQ<sub>10</sub> (28) is able to reduce OTA nephrotoxicity. Mel, a pineal secretory product, is known to function as an antioxidant (30), *i.e.* it is a scavenger of hydroxyl (27) and peroxy radicals (26). There is a substantial body of evidence for the protective effect of melatonin to DNA, lipids and proteins, which are the results of a number of endogenous and exogenous free-radical generating processes. It has been demonstrated that OTA-induced structural tissue damage in the kidney and the administration of Mel together with OTA significantly reverses the effects caused by the toxin alone (23, 30). In recent studies, it was concluded that Mel exhibits a preventive effect against OTA-induced oxidative stress through its role in the scavenging of free radicals and/or the prevention of lipid peroxidation (1) and stimulation of glutathione-S-transferase (GST) activities (20). CoQ<sub>10</sub> has several biochemical functions. The well-recognized functions are in mitochondrial energy coupling and its action as a primary regenerating antioxidant (28).

In this study, it was found that Mel and CoQ<sub>10</sub> treatment suppresses the OTA-induced increased MDA levels and decreased GSH levels in the plasma. This was similar to the results of previous studies indicating that Mel exhibits a preventive effect against OTA-induced oxidative stress in rat kidneys (1, 20, 23). Moreover, we also found that histopathological lesions were significantly reduced by Mel and CoQ<sub>10</sub> treatment. This finding was also in agreement with previous results (4). In addition, we observed condensed nuclei in some tubule cells and mitochondrial degeneration by electron microscopy in OTA-treated groups although no mitochondrial damage was detected in Mel- and CoQ<sub>10</sub>-treated groups. These data show that ROS damage in mitochondria was inhibited by antioxidants, in particular by CoQ<sub>10</sub>.

Generally, after a population of tubule cells are exposed to a nephrotoxicant, the cells respond and ultimately the nephron recovers its function, or if cell death and loss are extensive, nephron function ceases. Terminally injured cells undergo cell death through oncosis or apoptosis (3). For this reason, all degenerations in the kidney are important. However, some investigators suggested that after short-term administration of OTA to rats, the renal proximal tubule did not appear to be the main target for nephrotoxicity, although decreased capacity to eliminate the toxin might result in self-enhancing effect (9). In addition, Petrik *et al.* (24) have demonstrated that relatively low OTA concentrations activate apoptotic processes and oxidative damage in kidney cells (24). More recent results indicate that OTA is able to induce

apoptosis in MDCK-C7 cells, in cultured human proximal tubule cells and in HeLa cells (11, 34, 35). Using the TUNEL staining method in this study, higher incidences of apoptotic bodies were observed in the kidneys of the OTA group although OTA administration did not significantly increase the incidence of apoptotic bodies when compared to the control and the antioxidant administrated groups. This finding may be due to the single-dose administration of OTA.

Several studies demonstrating the genotoxic effects of OTA *in vitro* and *in vivo* in some mammalian species have confirmed the genotoxicity of OTA (18, 25, 32). The metabolic pathway of OTA leading to genotoxic compounds is not yet known. The genotoxic effects, inhibition of DNA synthesis and mitosis may be explained by OTA-inflicted DNA damage which includes DNA adduct formation and DNA single-strand breaks (21, 25). Lower OTA doses can also lead to genotoxic effects such as chromosome aberrations and oxidative DNA damages in cultured human lymphocytes (19) and in rats (12). In this study, we evaluated the genotoxic potential of a single relative high dose of OTA *in vitro* in rat bone marrow. We noted the same MI values and chromosome aberrations in the OTA group as compared to the control group. In other words, single dose of OTA did not have a genotoxic potential by means of bone marrow chromosome aberration and mitotic index in rats. Also, this finding may be due to the single-dose administration of OTA, as mentioned above.

In conclusion, single-dose OTA administration causes oxidative damage and Mel or CoQ<sub>10</sub> treatment appears to ameliorate OTA-induced kidney injury in rats. We also demonstrated that the exposure to the relatively high single-dose OTA concentration did not activate apoptotic processes and genotoxic damages in the kidney although oxidative damages and related histopathologic findings were seen.

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