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ORIGINAL ARTICLE

Melatonin protects against oxidative stress induced by the kidney carcinogen $KBrO_3$

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Abstract

OBJECTIVES: Free radical scavengers can protect against the genotoxicity induced by chemical carcinogens by decreasing oxidative stress. The protective effect of the antioxidant melatonin was studied in the kidney and liver of rats treated with the kidney-specific carcinogen potassium bromate ($KBrO_3$). The major endpoint of oxidative damage measured in this report was lipid peroxidation.

METHODS: Four groups of male rats (controls, melatonin-injected [10 mg/kg ×4], $KBrO_3$-injected [100 mg/kg], and melatonin+$KBrO_3$) were used in the current study. The concentrations of malondialdehyde (MDA) were assayed as an index of oxidatively damaged lipid in the kidney and liver.

RESULTS: Twenty-four hours after $KBrO_3$ administration, MDA levels were significantly increased in the kidney while the increase in the liver was not statistically significant compared to levels in control rats. The percentage increases in lipid peroxidation products were 32.8% and 12.6% for the kidney and liver, respectively. In rats given melatonin 30 minutes before $KBrO_3$, and three more times after $KBrO_3$ (i.e., every 6 hours), the increase in MDA levels was reduced in the kidney. Histopathological examination demonstrated marked changes in the structure of the kidney and slight changes in the liver. In the kidney, microscopic examination revealed atypical tubules, atypical hyperplasia, hyaline droplet degeneration, necrotic changes and stratified squamous cell metaplasia. Again, melatonin treatment inhibited the tissue damage associated with $KBrO_3$ administration.

CONCLUSION: These results show that melatonin as an antioxidant and free radical scavenger can prevent oxidative stress induced by the carcinogen $KBrO_3$.

Abbreviations

$KBrO_3$	potassium bromate
LPO	lipid peroxidation
MDA	malondialdehyde
8-oxodG	8-hydroxydeoxyguanosine

Introduction

Potassium bromate ($KBrO_3$) has been used as a food additive in the treatment of flour and of barley and as a constituent in cold-wave hair solutions [1, 2]. In 1992, the FAO/WHO Joint Committee on Food Additives (JECFA) withdrew approval for use in the treatment of flour [3]. $KBrO_3$ is also formed as a by-product of reactions such as disinfection of water by ozonation [4]. The WHO guideline limit value for $KBrO_3$ in drinking water is 25 μg/L, although the USEPA and the European Union are proposing a limit level of 10 μg/L. There is no doubt that $KBrO_3$ is potentially genotoxic having been found to give a positive response in bacterial mutation assays [5], in a chromosome aberration test [6], and in a micronucleus assay [7]. Also, it has been shown to cause renal cell tumors, mesotheliomas of the peritoneum and follicular cell tumors of the thyroid in the rat [8, 9].

A major contributor to the rodent carcinogenicity of $KBrO_3$ is via its ability to oxidize DNA. This is evidenced by the finding of Kasai et al. [10], Sai et al. [11, 12] and Cho et al. [13] wherein $KBrO_3$ increased the level of 8-oxo-deoxyguanosine (8-oxodG) in kidney DNA of male rats. One important feature of the DNA oxidation produced by $KBrO_3$ in F344 rat kidney is the apparent coincident formation of lipid peroxides. Associated with DNA oxidation was a significant increase in lipid peroxides [11]. The induction of lipid peroxidation (LPO) and 8-oxodG in the rat kidney is in accord with a possible oxidative mechanism [12]. The possibility arises therefore that the oxidation of DNA by $KBrO_3$ may be dependent on (and possible secondary to) lipid peroxidation. It is known that lipid peroxides can lead to 8-oxodG induction in DNA [14].

Melatonin, an indoleamine product of the pineal gland, was shown to be an endogenous hydroxyl radical (·OH) scavenger and effective antioxidant [15, 16]. In vitro, it has been shown that melatonin is equally or more effective than either glutathione, mannitol (against ·OH toxicity) [15] or vitamin E [against the toxicity of the peroxyl radical (LOO·)] in its scavenging ability [17]. Moreover, being highly lipophilic [18] as well as somewhat hydrophilic [19], melatonin easily passes all known morphophysiological barriers and enters all subcellular compartments. Melatonin protects cells, tissues and organs from oxidative damage induced by a variety of free radical generating agents and processes [20, 21]. Melatonin as an antioxidant is effective in protecting membrane lipids, nuclear DNA and protein from oxidative damage both in vivo and in vitro [22, 23, 24].

The aim of the current study is to investigate the protective effect of melatonin as an antioxidant in reducing the peroxidation of lipids and histopathological changes in the kidney and liver of rats administered with kidney-specific carcinogen ($KBrO_3$).

Material and Methods

Animals

Thirty adult male Sprague-Dawley rats weighing about 125 g purchased from Assiut University Joint Animal Breeding Unit were used in this study. All rats were kept under the same laboratory conditions of temperature (25±2°C) and lighting (14:10 h light:dark cycle) and were given free access to standard laboratory chow and tap water.

Chemicals

$KBrO_3$, melatonin, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane were purchased from Sigma (St. Louis, MO). Melatonin was dissolved in a small amount of ethanol before being diluted with saline. The final concentration of ethanol in the melatonin was <1%. All other chemicals were of highest quality available.

Experimental design

The rats were divided into four groups. The first group (7 rats) served as controls and received an intraperitoneal injection of physiological saline. The second group (7 rats) was given intraperitoneal melatonin only at a dose of 10 mg/kg body weight. The third group (8 rats) was given a single intraperitoneal injection (100 mg/kg body weight) of $KBrO_3$ only. The final group (8 rats) was given a similar injection of $KBrO_3$, which was preceded, by 30 min, by an intraperitoneal injection of melatonin (10 mg/kg b.w). For groups 2 and 4, melatonin administration was repeated every 6 h up to 24 h (i.e., three additional melatonin injections of 10 mg/kg each) after $KBrO_3$ administration. 24 hours after the administration of $KBrO_3$, rats were sacrificed and the liver and kidneys were removed, frozen and stored at –60°C.

Measurement of lipid peroxidation

The method was based on that of Ohkawa et al. [25]. A 10% w/v tissue homogenate was required for this assay (this homogenate contained 1% v/v dimethyl sulfoxide to prevent further oxidation). To 0.2 ml aliquots of tissue homogenate was added 0.2 ml 8.1% w/v sodium dodecyl sulfate solution, 1.5 ml 20% v/v acetic acid solution (pH 3.5) and 1.5 ml 0.8% w/v thiobarbituric acid solution. The mixture was made up to 4.0 ml with distilled water and heated to 95°C for 1 h. The samples were cooled and centrifuged at 2000 x g for 10 min and absorbance measured at 532 nm. Results were expressed as n mol malondialdehyde formation per g tissue.

Histopathological examination

For histopathological examination, specimens of the kidney and liver were removed and preserved. The tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned. The sections were stained with H&E.

Statistical analysis

Analyses were made by the ANOVA followed by the Student-Newman-Keuls t-test. The percent stimulation (S%) or inhibition (I%) in the mean values of LPO was calculated as follows:

$$S\% = \frac{\text{mean } KBrO_3 \text{ value} - \text{mean control value}}{\text{mean } KBrO_3 \text{ value}} \times 100$$

$$I\% = \frac{\text{mean } KBrO_3\text{+melatonin value} - \text{mean } KBrO_3 \text{ value}}{\text{mean } KBrO_3\text{+melatonin value}} \times 100$$

Results

The data are summarized in figures 1-3. $KBrO_3$ administration increased LPO (as indicated by the increase in MDA levels) in the kidney (Fig. 1) with no statistically significant change being observed in the liver (Fig. 2). The increase in LPO was 31.8% in the kidney of $KBrO_3$-treated rats; this increase was statistically significant ($P<0.01$). When melatonin was administered to $KBrO_3$-treated rats, it lowered LPO levels to those measured in the controls. Statistically, melatonin significantly reduced LPO levels ($P<0.01$) in the kidney (by 30.2%). Melatonin administration did not significantly change the level of LPO products in the liver of rats that were injected with $KBrO_3$. Likewise, the injection of melatonin did not change basal levels of MDA in the studied organs.

Histopathological examination revealed major changes in the kidney and only slight changes in the liver. In the kidney, figure 3a demonstrates the normal renal structure of a control rat. After $KBrO_3$ administration, the kidney exhibited substantial morphological changes. Atypical tubules, characterized as tubules lined by a single or multiple layer of basophilic or chromophobic altered cells, not exceeding three times the size of normal tubules (Fig. 3b) were observed. Also, after $KBrO_3$ administration, atypical hyperplasia of tubules with a homogenous aggregation of cells was apparent. These were solid structures (solid tubular masses) as shown in figure 3b. Hyaline droplet degen-

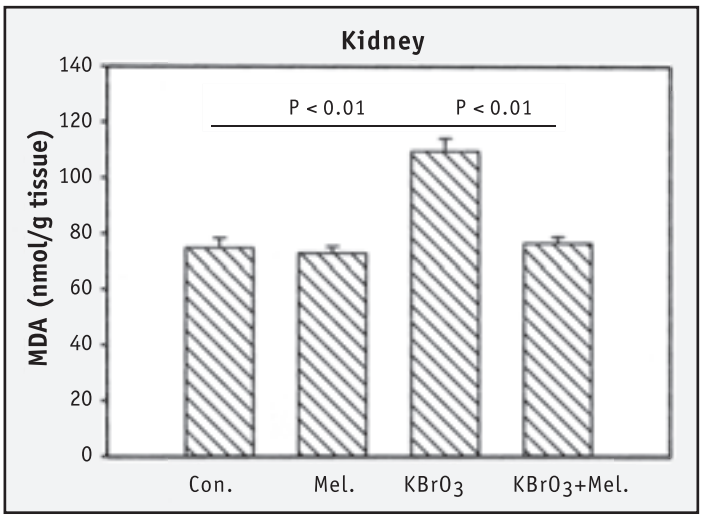

Fig.1. Inhibitory effect of melatonin on MDA level (lipid peroxidation product) in the kidney at 24 h after $KBrO_3$ administration of rats. Con, controls; Mel, melatonin (10 mg/kg × 4); $KBrO_3$, potassium bromate (100 mg/kg).

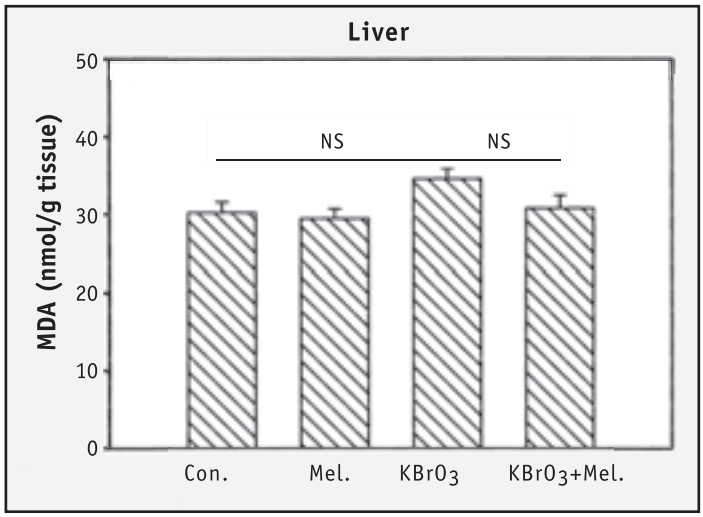

Fig.2. Effect of melatonin on MDA level (lipid peroxidation product) in the liver at 24 h after $KBrO_3$ administration. See fig.1 for legend.

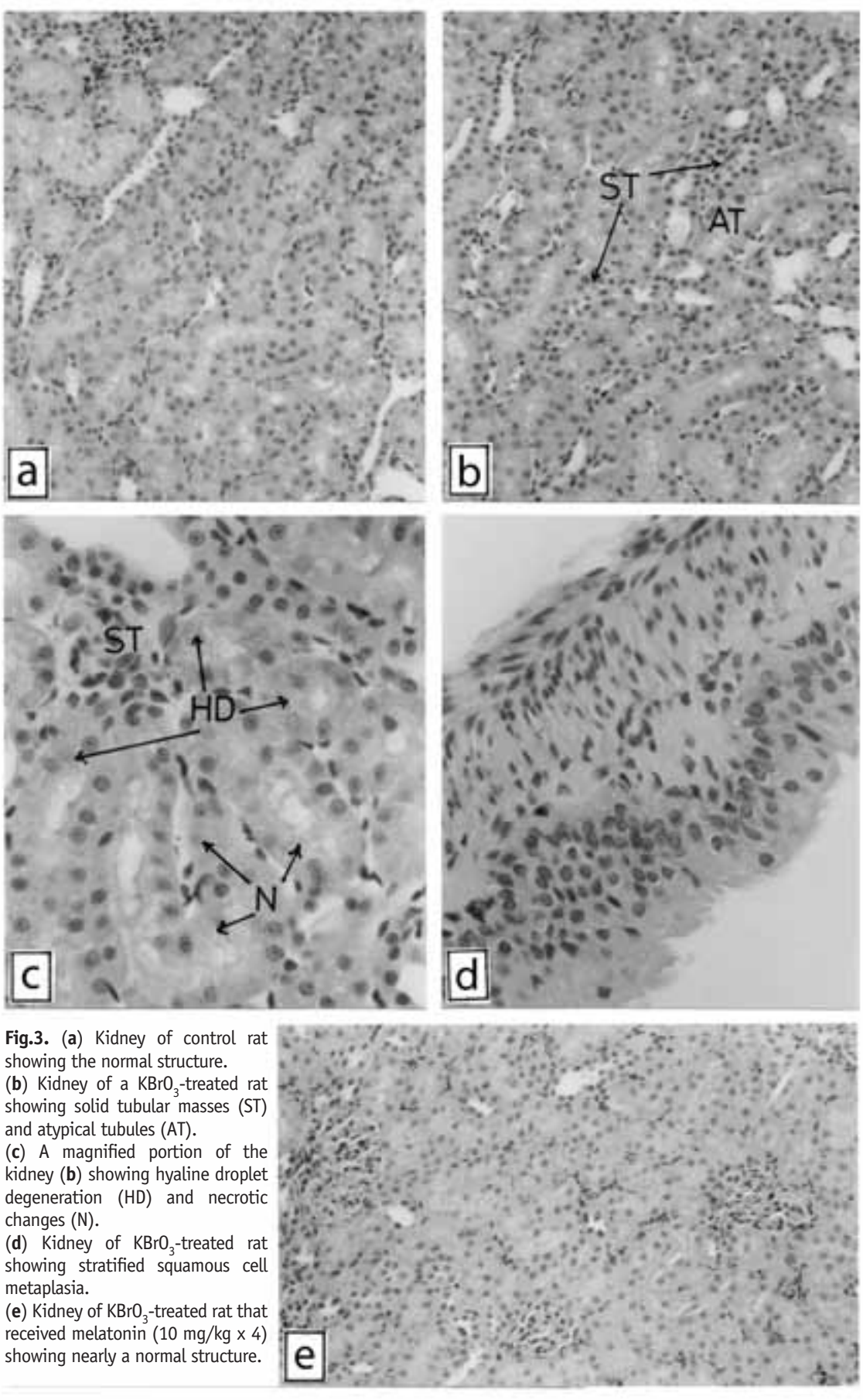

Fig.3. (**a**) Kidney of control rat showing the normal structure.
(**b**) Kidney of a $KBrO_3$-treated rat showing solid tubular masses (ST) and atypical tubules (AT).
(**c**) A magnified portion of the kidney (**b**) showing hyaline droplet degeneration (HD) and necrotic changes (N).
(**d**) Kidney of $KBrO_3$-treated rat showing stratified squamous cell metaplasia.
(**e**) Kidney of $KBrO_3$-treated rat that received melatonin (10 mg/kg x 4) showing nearly a normal structure.

eration was observed in the cortical tubules (Figs. 3b,c) and necrotic changes (karyorhyxis and karyolysis) were detected in some tubules (Fig. 3c) after $KBrO_3$ administration. In addition, stratified squamous cell metaplasia was detected in the pelvis epithelial cells (Fig. 3d). Microscopic examination of the liver of rats administered with $KBrO_3$ showed nearly no changes except some congestion and minor degenerative changes in some hepatocytes compared to controls. In general, there were no serious histopathological changes detected in the liver. When melatonin was administered to $KBrO_3$-treated rats, the kidney (Fig. 3e) as well as the liver had essentially a normal appearance on histopathological examination.

Discussion

$KBrO_3$ has been demonstrated to induce rat kidney cell tumors [26, 27, 9] and also to promote renal tumorigenesis [28]. In addition, after administration of $KBrO_3$ to rats, enhancement of the renal LPO levels has been reported [29]. Because $KBrO_3$ has oxidizing properties, the involvement of active oxygen species in its initiation/promotion of neoplasia as well as the increment of LPO has been suggested. Sai et al. [11] reported that the levels of LPO and 8-oxodG after $KBrO_3$ treatment to rats were increased in a dose-dependent manner, suggesting the involvement of LPO in the process of 8-oxodG formation.

Herein, the results of this study revealed a highly significant increase ($P<0.01$) in the levels of LPO in the kidney (a target organ) after $KBrO_3$ administration compared to control rats. In the liver (a non-target organ), there was an increase in LPO levels after $KBrO_3$ administration but this was statistically non-significant. The present results are in agreement with those of Kassi et al. [10] and Sai et al. [11], who found that 8-oxodG caused by oxygen-radical-generating agents, were detected in the kidney (a target organ) but not in the liver (a non-target organ) after treatment with $KBrO_3$. Several reports show a critical role for LPO in DNA damage and an interaction of LPO products directly with DNA [30]. It has been demonstrated that $KBrO_3$ can cause oxidative damage directly to the renal proximal tubules, the target site for renal carcinogenesis, and LPO in renal proximal tubules may be critical for oxidative DNA damage to occur [31].

It was demonstrated that kidney LPO levels increased after i.v. administration of $KBrO_3$, indicating a possible relationship between LPO and oxidative DNA damage. However, the time-course study [11] revealed that the increase of 8-oxodG levels appeared after elevation of LPO. This suggests one possible mechanism for oxidative DNA damage caused by $KBrO_3$ to be as follows. Initially, active oxygen species may be produced by $KBrO_3$ directly or as a consequence of the reaction with intracellular molecules which induce initiation of LPO via the iron-catalyzed Haber-Weiss reaction. The levels of lipid peroxide and intermediate radicals may then be amplified by a chain reaction; consequently, the reactive species probably oxidize nuclear DNA after the LPO reaction has occurred in the nuclear membrane [30, 32, 33].

In the current study, microscopic examination revealed atypical tubules, atypical hyperplasia, hyaline droplet degeneration, necrotic changes, and stratified squamous cell metaplasia in the kidney. In the liver, there were no changes except of some minor congestion. It is known that $KBrO_3$ exposure causes α_{2u}-globulin accumulation in male rats followed by sustained induction of cell proliferation in the kidney [34]. It is well known that compounds which induce α_{2u}-globulin accumulation are capable of exerting promotional actions in a two-stage rat renal carcinogenesis model [35, 36]. The observed correlation between increased cell proliferation in the kidneys and oxidation of nuclear DNA suggests a role for oxidative stress in the promotional action of $KBrO_3$ [37]. With regard to the relationship between oxidative stress and eventual promotional activity, it has been proposed that various events related to cell proliferation occur as a result of cellular oxidation, e.g., activation of early-response genes [38], increases of intracellular Ca^{++} concentration [39], activation of poly(ADP-ribose)polymerase [40] and oxidation of gap junctional protein [41]. While an in vitro study demonstrated that a single intraperitoneal administration of $KBrO_3$ at a dose of 80 mg/kg causes DNA double-strand breaks in the kidneys of rats, followed by induction of poly(ADP-ribosyl)ation [42], it remains unclear how oxidative stress due to $KBrO_3$ exposure might contribute to the process of tumor promotion. Nevertheless, it can be assumed that formation of 8-oxodG in nuclear DNA implies concurrent occurrence of oxidative damage to ubiquitous, readily oxydizable macromolecules.

Accordingly, the clear evidence which indicates that $KBrO_3$ has potential for the oxidation of nuclear DNA, induction of cell proliferation and promotion of kidney tumor development in rats [37], support the proposed hypothesis that oxidative stress is associated with tumor promotion [43, 44]. Giri et al. [45] observed an increase in renal DNA synthesis which was measured as ^{3}H-thymidine incorporation in DNA after $KBrO_3$ administration. $KBrO_3$ administration depleted the level of renal glutathione and

glutathione reductase activity in a time dependent manner. Parallel to these changes, a sharp increase in the blood urea nitrogen and serum creatinine levels was observed which is indicative of the concurrent renal damage. Umemura et al. [46] reported that oxidative damage in the kidney due to $KBrO_3$ administration is involved in its mechanisms of tumor induction. As a possible contribution of $KBrO_3$-induced oxidative damage to renal carcinogenesis, the following pathway can be considered. Peroxidized fatty acids and/or reactive derivatives, produced in nuclear membrane directly by $KBrO_3$ or through cytoplasmic oxidation, might react with nuclear DNA, resulting in 8-oxodG production [47]. The mutagenic activity of 8-oxoG in DNA finally may lead to renal carcinogenesis [48, 49].

From the results of both chromosome aberration and micronucleus tests, there is no doubt that $KBrO_3$ can act as a mutagen [9]. Likewise, some investigators have shown that induction of micronuclei by $KBrO_3$ was inhibited by co-treatment with antioxidants [50]. Accordingly, they proposed that active oxygen species may play an important role in $KBrO_3$ clastogenicity. In addition, increase of 8-oxodG levels in kidney DNA by $KBrO_3$ was found to be inhibited by treatment with antioxidants, emphasizing the involvement of active oxygen species in $KBrO_3$-induced toxicity. Results of Cadenas and Barja [51] reported that antioxidants and free radical trap, working in either water-soluble or lipid-soluble compartments, prevent the oxidative DNA damage induced in the kidney by the carcinogen $KBrO_3$.

In the current investigation, melatonin maintained the levels of lipid peroxidation produced in the kidney and liver at control values. This illustrates the protective actions of melatonin against the oxidative degradation of lipids. The protective effect of melatonin is important since the breakdown of membrane lipids due to $KBrO_3$ is devastating to the function of all cells. In the kidney, melatonin inhibited the increase of LPO levels (by 30.23%) and this inhibition was significant ($P<0.01$) while the inhibition in the liver (by 11.3%) was non-significant. Also, melatonin administration markedly reduced the histopathological changes induced by $KBrO_3$ in the kidney and liver which had essentially a normal appearance on examination. In this study, melatonin was given at individual doses of 10 mg/kg repeated at 6-h intervals throughout the experimental period. The intent was to maintain high levels of circulating melatonin throughout the $KBrO_3$ toxicity.

Melatonin's protective effect may be related to any of the following actions of the indoleamine. (1) Melatonin is a direct free radical scavenger and it is a particularly efficient scavenger of the highly toxic ·OH [15, 52, 53]. In detoxification of the ·OH, melatonin neutralizes two ·OH for each melatonin molecule resulting in the formation of the product cyclic 3-hydroxymelatonin [54]. Melatonin is also reported to neutralize several other reactive oxygen species including the peroxynitrite anion ($ONOO^-$) [55, 56], the LOO. [16], nitric oxide [57, 58] and singlet oxygen ($^1O_2$) [59, 60]. (2) In addition to its direct free radical detoxifying, melatonin also functions as an indirect antioxidant by stimulating mRNA levels and/or the activities of superoxide dismutase (SOD) [61, 62], glutathione peroxidase and glutathione reductase [63, 64]. These enzymes function to reduce ·OH generation by metabolizing its precursor to non-toxic products. Sai et al. [50] and Chipman et al. [65] reported that intracellular glutathione plays an essential protective role against renal oxidative DNA damage and nephrotoxicity caused by $KBrO_3$. (3) Being highly lipophilic [18] as well as somewhat hydrophilic [19], melatonin easily enters cells and subcellular compartments where it prevents oxidative damage to a variety of molecules [21, 24, 66]. Besides the direct scavenging properties and indirect antioxidant actions of melatonin, its ability to protect neurons probably also stems from its anti-amyloidogenic properties [67]. Also, melatonin modulates mitochondrial respiratory activity, an effect that may account for some of the protective properties of the indoleamine [68, 69]. This is a feature not shared by most antioxidants [24,70]. This combination of actions may all contribute to melatonin's ability to reduce $KBrO_3$-induced oxidative stress in the rat kidney.

This is not the first study in which melatonin has been found to reduce oxidative damage in the kidney. Thus Montilla et al. [71], Carnerio and Reiter [72] and El-Sokkary et al. [56] have shown that melatonin, in vivo, protects the kidney from the free radical damage caused by adriamycin, δ-aminolevulinic acid and zymosan, respectively. Clearly, melatonin is taken up by the kidney in sufficiently large quantities to protect it from highly damaging agents. The findings imply that the toxicity of $KBrO_3$ relates to its ability to generate free radicals while melatonin's ability to protect against this kidney carcinogen probably is due to the free radical scavenging and antioxidant activities of the

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