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Microcystin-Induced 8-Hydroxydeoxyguanosine in DNA and Its Reduction by Melatonin, Vitamin C, and Vitamin E in Mice

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Abstract—Microcystin LR (MC-LR), a liver-specific toxin synthesized by *Microcystis aeruginosa*, was investigated. MC-LR initiated reactive oxygen species formation followed by damaging DNA and some other cellular components. We investigated the ability of MC-LR to induce oxidative DNA damage by examining the formation of 8-hydroxydeoxyguanosine (8-OH-dG) using HPLC with electrochemical detection. Melatonin, vitamin C (ascorbate), and vitamin E (as Trolox), all of which are free radical scavengers, markedly inhibited the formation of 8-OH-dG in a concentration-dependent manner. The concentration that reduced DNA damage by 50% (IC_{50}) was 0.55, 31.4, and 36.8 μM for melatonin, ascorbate, and Trolox, respectively. The results show that melatonin is 60- and 70-fold more effective than vitamin C or vitamin E, respectively, in reducing oxidative DNA damage. These findings are consistent with the conclusion that melatonin's highly protective effect against microcystin toxicity relates, at least in part, to its direct hydroxyl radical scavenging ability.

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Key words: microcystin, 8-hydroxydeoxyguanosine, melatonin, vitamin C, Trolox, DNA damage

Contamination of water by toxic blooms of cyanobacteria (blue-green algae) has occurred in many regions of the world, presenting a serious public health problem [1]. Cyanobacteria are planktonic algae and some of them produce microcystins, a group of hepatotoxins with strong cytotoxic activity [2]. Toxic cyanobacterial blooms have been found in different water bodies including freshwater lakes, ponds, reservoirs, etc. [3].

Microcystins are a family of toxins produced by freshwater cyanobacteria, primarily from *Microcystis aeruginosa*, but also from other *Microcystis* species and other genera such as *Anabaena*, *Oscillatoria*, and *Nostoc* [4]. Those toxins are a family of structurally related cyclic peptide toxins. The general structure of microcystins is: cyclo[-D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha-]. Apart from two variable L-amino acids, X and Z, microcystins consist of three D-amino acids (alanine (Ala), methylaspartic acid (MeAsp), and glutamic acid (Glu)) and two unusual amino acids (*N*-methyldehydroalanine (Mdha) and 3-amino-a-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda)) (Fig. 1). The Adda amino acid is responsible for the biological activity of the toxins [5].

Microcystin is taken up into hepatocytes by multispecific bile acid transporters [6] where it induces the production of potentially harmful reactive oxygen species [7]. In mammals these free radicals interact with DNA to induce DNA strand breaks, DNA–protein cross-links, and oxidative DNA base modifications such as the formation of 8-hydroxydeoxyguanosine (8-OH-dG) [8].

8-OH-dG is a key biomarker [9] relevant to carcinogenesis because the formation of 8-OH-dG in DNA causes mis-incorporation during replication and subsequently leads to G–T transversions [10]. The carcinogenic potential of microcystin is related to its ability to generate hydroxyl radicals (˙OH) [11]. This highly toxic free radical targets DNA resulting in oxidative DNA base adducts such as 8-OH-dG [12].

Melatonin, an indoleamine product of pineal gland [13], is an endogenous ˙OH scavenger and is a highly effective antioxidant [14]. It was found that melatonin is as effective as or more effective than either glutathione or mannitol in reducing ˙OH toxicity [15], and possibly more efficient than vitamin E in reducing the toxicity of

Abbreviations: ALT) alanine transaminase; 8-OH-dG) 8-hydroxydeoxyguanosine.

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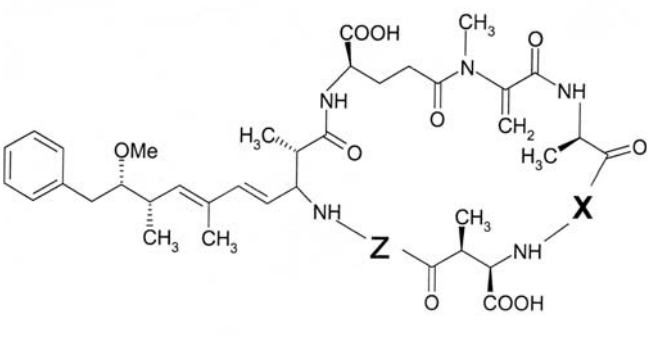

Fig. 1. Structure of microcystin LR from King Talal reservoir (Jordan).

the peroxyl radical [16]. Moreover, melatonin is highly lipophilic [17] as well as somewhat hydrophilic [18]; therefore, it easily passes all known morphophysiological barriers and enters all subcellular compartments. Melatonin has a high affinity for cell nuclei in mammalian tissues, where its concentration can be 5 times higher than levels found in blood [19].

Dietary antioxidants, such as vitamin C or vitamin E, have been identified as factors in reducing the risk of cancer [20]. Trolox, a commercial name for a water-soluble vitamin E analog, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, has been shown to protect mammalian cells from oxidative damage *in vitro* [21] as well as *in vivo* [22].

In the present study, we investigated the ability of melatonin to reduce the toxicity of microcystin-induced oxidative DNA damage in BALB/c mice, and compared melatonin efficacy to that of two well-known antioxidants, vitamins C and E.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma (Sweden) and Aldrich (USA) unless otherwise indicated. Chemicals used were of analytical grade. Preparations of *Microcystis* cells were collected from selected sites of King Talal reservoir in Jordan. *Microcystis* cells were separated from other types using a light microscope, and then they were isolated from water using a special purifying apparatus (Supelco Co., USA). They were maintained in non-axenic batch culture medium (BG 11) with micronutrients at room temperature according to [23]. *Microcystis* cells were harvested at the end of the exponential growth phase and lyophilized. Toxin was extracted from the freeze-dried cells using 70% aqueous methanol (80 µl/mg dry cell mass). The suspension was sonicated overnight (16 h) in a water bath, centrifuged to remove cell debris, and then the supernatant fluid was assayed to determine the toxin concentration using protein phosphatase according to [24].

The toxin extract was analyzed by HPLC (Knawar, Germany) on RPC-18. The samples were run in parallel to a known MC-LR standard (Entrolox, USA) under the same conditions. Forty BALB/c mice (10-week-old) were divided into eight groups (five mice in each) as follows. Control (C) group mice received neither toxin nor any supplement (only 1 ml of normal saline). Melatonin control (MC) group received melatonin supplementation (0.55 µM per mouse per day for two weeks), but no toxin, as recommended in [23]. Vitamin C control (CC) group received ascorbate supplementation (30.4 µM per mouse per day for two weeks), but no toxin, as recommended in [7]. Trolox control (TC) group received Trolox supplementation (36.2 µM mouse/day), without toxin as recommended in [7]. Toxin control (Tox) group received microcystin in normal saline at the LD_{50} dose (75 µg/kg body weight as intraperitoneal injection) without any supplement. Toxin + melatonin (ToxM) group received microcystin intraperitoneally (75 µg/kg body weight) plus 0.55 µM of melatonin per mouse according to [24]. Toxin + vitamin C (ToxC) group received microcystin intraperitoneally (75 µg/kg body weight) plus 30.4 µM of ascorbate per mouse. Toxin + Trolox (ToxT) group received microcystin intraperitoneally (75 µg/kg body weight) plus 36.2 µM of Trolox per mouse according to the method recommended in [25].

Mice were sacrificed 24 h after injection with toxin. The LD_{50} dose and the 24 h time point were established from our unpublished data. Blood was collected, serum isolated, livers were removed immediately and perfused with Hanks-buffered saline to remove excess blood. Livers of all groups were incubated with 0.5 mM H_2O_2 for 60 min at 37°C in a water bath as recommended in [7] to test the efficacy of these antioxidants in altering oxidative DNA damage.

To assay 8-OH-dG, the method in [26] was used. Alanine transaminase (ALT) level was determined in the collected serum according to the method recommended in [27].

All data were analyzed with ANOVA test.

RESULTS

The average serum ALT levels per ml were calculated as shown in Table 1. The average ALT values in group Tox, which received toxin only, showed a significant increase, which indicates a higher rate of liver damage. Our results showed a significant increase in the level of 8-OH-dG in toxin-treated mice. This increase was dose-dependent, i.e. levels of 8-OH-dG increased with increasing concentrations of MC-LR as shown in Fig. 2 after incubation with 0.5 mM H_2O_2 for 60 min at 37°C in a water bath (this incubation time produced optimal level of 8-OH-dG). Table 2 shows that melatonin inhibited MC-LR-induced formation of 8-OH-dG in a dose-

Table 1. Alanine transaminase content (units/liter) in serum of mice of different groups

C	CM	CC	CE	Tox	ToxM	ToxC	ToxE
88.28 ± 0.71	6.56 ± 0.06	6.72 ± 0.04	6.70 ± 0.11	244.23 ± 1.24	102.22 ± 1.10	138.64 ± 0.06	135.36 ± 0.77

dependent manner. All melatonin concentrations reduced the formation of 8-OH-dG in DNA induced by MC-LR plus 0.5 mM H_2O_2. Table 2 shows also that ascorbate inhibited MC-LR-induced formation of 8-OH-dG in a dose-related fashion. Similarly, the formation of 8-OH-dG in DNA also was inhibited by Trolox showing the same trend.

Comparison of the efficacy of melatonin, ascorbate, and Trolox reveals that melatonin was the strongest inhibitor for microcystin oxidative stress since it markedly decreased the level of ALT in the serum (102.22 versus 244.23 units/liter). We calculated the percentage inhibition curve (not shown). IC_{50} is the concentration of antioxidant that reduced microcystin-induced 8-OH-dG in DNA by 50%. The IC_{50} for melatonin was 0.55 μM; this value is much less than for ascorbate (33.4 μM) or Trolox (36.8 μM).

DISCUSSION

In this study, *Microcystis aeruginosa* from King Talal reservoir in Jordan was used as a source of microcystin (MC-LR). Phosphatase inhibitory activity proved the bioactivity of the toxin while HPLC analysis confirmed that the extract was MC-LR [28]. The BALB/c mouse LD_{50} dose (intraperitoneally) for this extract as determined in our laboratory was found to be well within the range reported in the literature [29].

Table 2. Effect of melatonin, ascorbate, and Trolox concentrations on MC-LR-induced 8-OH-dG formation in mouse liver after incubation for 60 min at 37°C in a water bath with 0.5 mM H_2O_2

Melatonin (μM/mouse)	8-OH-dG/10^5 2dG
0	47 ± 0.04
0.25	33 ± 0.04
0.5	24 ± 0.04
1.0	18 ± 0.04
2.5	8 ± 0.03
5.0	4 ± 0.01
10.0	2 ± 0.03
Ascorbate (μM/mouse)	**8-OH-dG/10^5 2-dG**
0	48 ± 0.4
1	38 ± 0.4
10	30 ± 0.4
25	24 ± 0.3
50	18 ± 0.3
100	10 ± 0.1
250	8 ± 0.08
Trolox (μM/mouse)	**8-OH-dG/10^5 2-dG**
0	45 ± 0.4
1	40 ± 0.4
10	32 ± 0.2
25	27 ± 0.2
50	18 ± 0.2
100	10 ± 0.2
250	4 ± 0.04

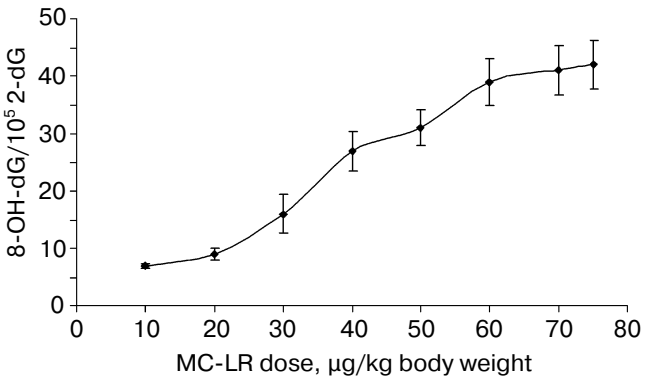

Fig. 2. Effect of concentration of MC-LR on levels of 8-OH-dG in liver incubated with 0.5 mM H_2O_2 for 60 min at 37°C in a water bath.

Previous studies [30] have shown that certain antioxidants are capable of reducing MC-LR-induced liver damage if administered *prior to* the toxin dose [31].

The generation of 8-OH-dG-adducted bases is an oxidative modification of DNA [32]. A strong correlation between higher amounts of 8-OH-dG adducts and greater degree of oxidative stress, DNA strand breaks, or DNA damage has been reported [33]. As we expected, the antioxidants melatonin, vitamin C, and Trolox (vitamin E analog) reduced the levels of 8-OH-dG. The present study confirms that those antioxidants are important antioxidants involved in protection against intracellular damage induced by free radicals (Table 1).

H_2O_2 is a normal metabolite in the cell; its steady-state concentrations range from 10^{-9}-10^{-8} M [34]. Although H_2O_2 may not cause DNA damage under physiological conditions, it participates in the metal ion-cat-

alyzed Haber–Weiss reaction and generates the highly reactive hydroxyl radical (˙OH), which can target DNA resulting in oxidative DNA damage [35]. The present study demonstrates that the concomitant microcystin and H_2O_2 treatment is capable of inducing oxidative DNA damage. Furthermore, the formation of 8-OH-dG increases in dose- and time-dependent manner in the presence of 0.5 mM H_2O_2 [36]. Melatonin, vitamin C (ascorbate), and vitamin E (Trolox) function as free radical scavengers and markedly inhibit the formation of 8-OH-dG in a concentration-dependent manner [37] but, clearly, with different efficacies [38]. It is notable that co-injection of MC-LR with an antioxidant significantly reduced the levels of 8-OH-dG. Calculations from the percent-inhibition curve demonstrate that compared to vitamin C (IC_{50} = 33.4 μM) and Trolox (IC_{50} = 36.8 μM), melatonin was more effective in reducing the formation of 8-OH-dG in this study (IC_{50} = 0.55 μM). Thus, melatonin was roughly 60- and 70-fold more effective in reducing oxidative damage to DNA than vitamin C and vitamin E, respectively. Furthermore, the minimal concentration of melatonin required to significantly reduce 8-OH-dG formation was much less than that of either vitamin.

In the present study, melatonin's highly effective protection against microcystin-induced formation of 8-OH-dG in DNA may relate to several actions of the indoleamine [39]. First, melatonin is a direct free radical scavenger and is a particularly efficient scavenger of the highly toxic ˙OH [40]. Melatonin neutralizes two ˙OH for each melatonin molecule, resulting in the formation of the product cyclic 2-hydroxymelatonin [41]. Our finding, the formation of 8-OH-dG resulting from the ˙OH generated by microcystin, supports previous reports indicating accumulation of the oxidative DNA base adduct 8-OH-dG [42]. Second, melatonin not only detoxifies the highly toxic ˙OH, but also scavenges its precursor, H_2O_2. It was found in [43] that melatonin is highly lipophilic as well as somewhat hydrophilic, and therefore it easily enters cells and subcellular compartments. In [44] it was found that melatonin has a high affinity for the nucleus (and possibly DNA itself), thus, it may contribute to its protective effect against formation of 8-OH-dG induced by microcystin. Other studies using other free radical-generating agents showed that melatonin was highly effective in reducing DNA damage consistent with its ability to enter the nucleus with ease. In fact, there have been no *in vivo* studies where melatonin, vitamin C, and vitamin E were compared for their relative efficacies in protecting DNA from oxidative destruction.

In conclusion, one practical application of the present data is the possible clinical use of melatonin against microcystin-induced cytotoxicity and genotoxicity in occupational and environmental situations where microcystin is a health problem. However, additional studies are also needed to investigate the implications of interaction between MC-LR and antioxidants in several animal models using different types of tissues.

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