

## Chromium(III)-Induced 8-Hydroxydeoxyguanosine in DNA and Its Reduction by Antioxidants: Comparative Effects of Melatonin, Ascorbate, and Vitamin E

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Chromium compounds are well documented carcinogens. Cr(III) is more reactive than Cr(VI) toward DNA under *in vitro* conditions. In the present study, we investigated the ability of Cr(III) to induce oxidative DNA damage by examining the formation of 8-hydroxydeoxyguanosine (8-OH-dG) in calf thymus DNA incubated with CrCl<sub>3</sub> plus H<sub>2</sub>O<sub>2</sub>. We measured 8-OH-dG using HPLC with electrochemical detection. In the presence of H<sub>2</sub>O<sub>2</sub>, we observed that Cr(III)-induced formation of 8-OH-dG in isolated DNA was dose and time dependent. Melatonin, ascorbate, and vitamin E (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% was 0.51, 30.4, and 36.2 μM for melatonin, ascorbate, and Trolox, respectively. The results show that melatonin is 60- and 70-fold more effective than ascorbate or vitamin E, respectively, in reducing oxidative DNA damage in this *in vitro* model. These findings also are consistent with the conclusion that the carcinogenic mechanism of Cr(III) is possibly due to Cr(III)-mediated Fenton-type reactions and that melatonin's highly protective effects against Cr(III) relate, at least in part, to its direct hydroxyl radical scavenging ability. **Key words:** ascorbate, chromium, 8-hydroxydeoxyguanosine, melatonin, vitamin E. *Environ Health Perspect* 108:399–402 (2000). [Online 16 March 2000] <http://ehpnet1.niehs.nih.gov/docs/2000/108p399-402qi/abstract.html>

Chromium is a widely used industrial chemical, with uses in steel, alloy cast irons, chrome, paints, metal finishes, and wood treatments (1). Cr causes allergic dermatitis and has other toxic and carcinogenic effects in humans and animals (2,3). Epidemiologic studies have shown that industrial workers exposed to Cr have a higher incidence of respiratory cancer than does the unexposed population (2–5). Dermal, renal, and hepatic toxicity have been reported in Cr-exposed humans (5,6). Cr can also induce tumors in experimental animals and cause genotoxicity, i.e., chromosome aberrations, sister chromatid exchanges, cell transformations, and gene mutations in mammalian cell cultures (7–10).

Cr is found in the workplace primarily in the valence forms Cr(VI) and Cr(III) (11). Cr(VI) compounds are more toxic and carcinogenic than Cr(III) (12,13) because Cr(VI), in contrast to Cr(III), can readily cross cellular membranes via nonspecific anion carriers (13–15). However, once inside cells, Cr(VI) is reduced through reactive Cr intermediates such as Cr(V) and Cr(IV) to the ultimate kinetically stable Cr(III) by cellular reductants including glutathione and vitamin C (13,16). Therefore, Cr(III) or other intermediate oxidation states probably play an important role in Cr(VI)-induced toxicity (16).

Cr(III), which was initially thought to be relatively nontoxic, recently was found to be more effective than Cr(VI) in causing

genotoxicity in cell-free systems (11). Cr(III) interacts 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) (17–21). 8-OH-dG is a key biomarker relevant to carcinogenesis because the formation of 8-OH-dG in DNA causes misincorporation during replication and subsequently leads to G→T transversions (22,23). The carcinogenic mechanisms of Cr(III) relate to its ability to generate hydroxyl radicals (\*OH) from H<sub>2</sub>O<sub>2</sub> via a Fenton-type reaction (20,24). The highly toxic \*OH then targets DNA, resulting in oxidative DNA base adducts such as 8-OH-dG.

Melatonin, an indoleamine product of the pineal gland, is an endogenous \*OH scavenger and a highly effective antioxidant (25,26). *In vitro* melatonin is as effective or more effective than either glutathione and mannitol in reducing \*OH toxicity (25) and is possibly more efficient than vitamin E in reducing the toxicity of the peroxy radical (27). Moreover, melatonin is highly lipophilic (28) as well as somewhat hydrophilic (29); therefore, it easily passes all known morpho-physiologic 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 (30). By measuring a variety of oxidative indexes (including levels of 8-OH-dG), earlier studies have shown

that melatonin effectively protects DNA from oxidative damage induced by a number of free-radical-generating agents including safrole, kainic acid, lipopolysaccharide, ferric nitrilotriacetate, ischemia/reperfusion, and ionizing radiation both *in vitro* and *in vivo* (31–36).

In the present study, we investigated the ability of melatonin to reduce Cr(III)-induced oxidative DNA damage *in vitro* and compared melatonin's efficacy to that of two well-known antioxidants, vitamins E and C. We examined the formation of 8-OH-dG in calf thymus DNA with Cr<sub>3</sub>Cl plus H<sub>2</sub>O<sub>2</sub> using HPLC with electrochemical detection.

### Materials and Methods

**Reagents.** We purchased calf thymus DNA, CrCl<sub>3</sub>•6H<sub>2</sub>O, H<sub>2</sub>O<sub>2</sub>, and ascorbate from Sigma (St. Louis, MO), and we obtained Trolox from Aldrich (Milwaukee, WI). Pure melatonin was a gift from Helssin Chemicals SA (Biasca, Switzerland). We purchased nuclease P<sub>1</sub> and alkaline phosphatase from Boehringer Mannheim (Indianapolis, IN). We used MilliQ-purified H<sub>2</sub>O to prepare all solutions. All other chemicals were of the highest quality available.

**Treatment.** We dissolved calf thymus DNA (500 μg) in 10 mM potassium phosphate buffer (pH 7.4) at a final volume of 0.45 mL. In the first study, we incubated DNA with six concentrations of CrCl<sub>3</sub> (10, 50, 100, 250, 500, or 750 μM) in the presence of 0.5 mM H<sub>2</sub>O<sub>2</sub> for 1 hr at 37°C in a water bath. This study was performed to establish the concentration of Cr(III) required to induce an appropriate amount of 8-OH-dG formation. In the second study, we selected 500 μM CrCl<sub>3</sub> for incubation with DNA in the presence of 0.5 mM H<sub>2</sub>O<sub>2</sub> for 0, 20, 40, 60, 80, or 100 min to determine the optimal incubation time. In the final study, we used several concentrations of

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melatonin (0.25, 0.5, 1, 2.5, 5, or 10  $\mu\text{M}$ ), ascorbate (1, 10, 25, 50, 100, or 250  $\mu\text{M}$ ), or Trolox (1, 10, 25, 50, 100, or 250  $\mu\text{M}$ ) in combination with 500  $\mu\text{M}$   $\text{CrCl}_3$  plus 0.5 mM  $\text{H}_2\text{O}_2$  for 60 min to test the efficacy of these antioxidants in altering oxidative DNA damage.

**Assay for 8-OH-dG.** After incubation, we added 50  $\mu\text{L}$  sodium acetate (3 M, pH 5.0) and two volumes of  $-20^\circ\text{C}$  to each sample to terminate the reaction. DNA was precipitated and washed once with 70% ethanol. The DNA sample was dried and dissolved in 200- $\mu\text{L}$  20 mM sodium acetate (pH 5.0); the samples were denatured by heating at  $95^\circ\text{C}$  for 5 min and then cooled on ice. The DNA samples were digested to nucleotides by incubation with 8 U nuclease  $\text{P}_1$  at  $37^\circ\text{C}$  for 30 min. Next, we added 20  $\mu\text{L}$  1-M Tris-HCl (pH 8.0) to the samples and they were treated with 4 U alkaline phosphatase at  $37^\circ\text{C}$  for 1 hr. We filtered the resulting deoxynucleoside mixture through a Millipore filter (0.22  $\mu\text{m}$ ; Millipore) and analyzed it using HPLC with an electrochemical detection system. We used an ESA HPLC system (ESA, Chelmsford, MA) equipped with an eight-channel CoulArray 5600 electrochemical detector: YMC-BD (4.6 mm  $\times$  250 mm, Partisil 5  $\mu$  OD53; Waters, Milford, MA) column (3  $\mu\text{m}$ , 150  $\times$  4.6 mm i.d.). The eluent was a 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetic acid, 30 mM sodium hydroxide, and 10 mM acetic acid at a flow rate of 1 mL/min. We measured the quantities of 8-OH-dG and 2-deoxyguanosine (2-dG) using different channels and two oxidative potentials (300 and 900 mV, respectively). The level of 8-OH-dG in each sample was expressed as the ratio of 8-OH-dG to  $10^5$  2-dG (37).

**Statistical analysis.** We analyzed all data by a one-way analysis of variance followed by the Tukey test.

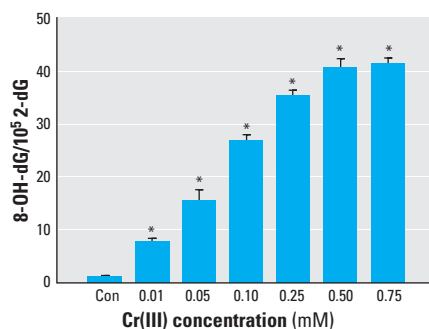
## Results

The levels of 8-OH-dG increased in a dose-dependent manner with increasing concentrations of  $\text{CrCl}_3$  (Figure 1). All concentrations of Cr(III) from 10  $\mu\text{M}$  to 0.75 mM caused significant increases in 8-OH-dG levels in DNA. We selected a concentration of 0.5 mM Cr(III) for the subsequent studies because it yielded high levels of 8-OH-dG. In the second study, 8-OH-dG levels increased essentially in a linear manner during the incubation period when 0.5 mM  $\text{CrCl}_3$  plus 0.5 mM  $\text{H}_2\text{O}_2$  were incubated with DNA (Figure 2). We selected an intermediate time of 60 min for the subsequent studies because this incubation time produced optimal levels of 8-OH-dG. Figure 3 shows that melatonin inhibited

Cr(III)-induced formation of 8-OH-dG in a dose-dependent manner. All melatonin concentrations  $>$  0.25  $\mu\text{M}$  significantly reduced 8-OH-dG formation in DNA induced by 0.5 mM Cr(III) plus 0.5 mM  $\text{H}_2\text{O}_2$  ( $p < 0.05$ ). Figure 4 shows that ascorbate inhibited Cr(III)-induced formation of 8-OH-dG in a dose-dependent manner. The effective concentrations of ascorbate against Cr(III)-induced formation of 8-OH-dG in DNA were between 1 and 250  $\mu\text{M}$ . Figure 5 shows that the formation of 8-OH-dG in DNA was also inhibited by Trolox in a dose-dependent manner. The effective concentrations of Trolox ranged from 10 to 250  $\mu\text{M}$ . To compare the efficacy of melatonin, ascorbate, and Trolox, we calculated the percentage-inhibition curves (Figure 6). The  $\text{IC}_{50}$  is the concentration of a particular agent that inhibits the formation of 8-OH-dG in DNA by 50%. The  $\text{IC}_{50}$  for melatonin was 0.51  $\mu\text{M}$ ; this value is much less than for ascorbate ( $\text{IC}_{50} = 30.4$   $\mu\text{M}$ ) or Trolox ( $\text{IC}_{50} = 36.2$   $\mu\text{M}$ ).

## Discussion

$\text{H}_2\text{O}_2$  is a normal metabolite in the cell; its steady-state concentrations range from  $10^{-9}$  to  $10^{-8}$  M (38). The concentrations of  $\text{H}_2\text{O}_2$  may markedly increase in tissues when they are subjected to ionizing radiation, during the metabolism of carcinogens, and at sites of inflammation (39–41). Although  $\text{H}_2\text{O}_2$  may not cause DNA damage under physiologic conditions, it participates in the metal ion-catalyzed Haber-Weiss reaction and generates the highly reactive  $\cdot\text{OH}$ , which can target DNA, resulting in oxidative DNA damage (42). Electron spin resonance spectroscopy studies have shown that  $\cdot\text{OH}$  are generated in a DNA-free solution containing Cr(III) and  $\text{H}_2\text{O}_2$  (43). The present study demonstrates that Cr(III) plus  $\text{H}_2\text{O}_2$  is capable of inducing oxidative DNA damage. When we incubated calf thymus DNA with  $\text{CrCl}_3 + \text{H}_2\text{O}_2$ , the levels of 8-OH-dG detected were approximately 40 times higher

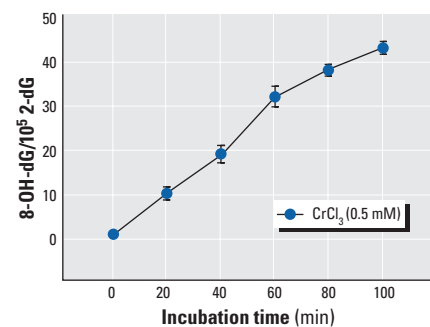


**Figure 1.** The effect of Cr(III) concentrations on the levels of 8-OH-dG in DNA incubated with 0.5 mM  $\text{H}_2\text{O}_2$ . Con, control. The incubation time was 60 min. Values are given as means  $\pm$  SE ( $n = 5$ ).  $*p < 0.05$  as compared to the Con group.

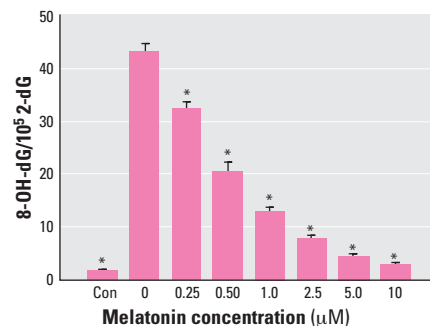
than those in the untreated controls. Furthermore, the formation of 8-OH-dG increases in a dose- and time-dependent manner in the presence of 0.5 mM  $\text{H}_2\text{O}_2$ . Melatonin, ascorbate, and vitamin E (Trolox) all function as free radical scavengers and markedly inhibited the formation of 8-OH-dG in a concentration-dependent manner but, clearly, with different efficacies.

Vitamin E, a well-known antioxidant and inhibitor of lipid peroxidation in biologic membranes, has protective effects against the carcinogenic or mutagenic activity of chemical agents and ionizing radiation (16). We found that Trolox, a water-soluble vitamin E analogue, successfully inhibited the Cr(III)-induced formation of 8-OH-dG in isolated DNA in a concentration-dependent manner. Trolox concentrations  $>$  10 mM significantly reduced 8-OH-dG levels. The  $\text{IC}_{50}$  value for Trolox was 36.2  $\mu\text{M}$ .

In this *in vitro* system, we also found that ascorbate, a water-soluble physiologic antioxidant, had a protective effect, with an  $\text{IC}_{50}$  value of 30.4  $\mu\text{M}$  calculated from its percent-inhibition curve. Vitamin C has antiviral, anticancer, and antimutagenic activity (16). However, under certain conditions, vitamin C acts as prooxidant, generating free radicals (44). In a number of studies, ascorbate



**Figure 2.** A time course of Cr(III)-induced formation of 8-OH-dG in DNA incubated with 0.5 mM  $\text{H}_2\text{O}_2$ . Results are given as means  $\pm$  SE ( $n = 5$ ). The concentration of Cr(III) was 0.5 mM.

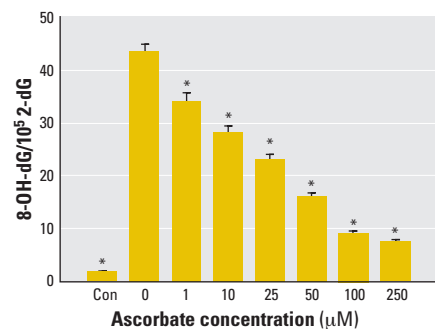


**Figure 3.** The effect of melatonin concentrations on Cr(III) (0.5 mM) plus  $\text{H}_2\text{O}_2$  (0.5 mM)-induced 8-OH-dG levels in DNA. The incubation time was 60 min. Results are given as means  $\pm$  SE ( $n = 5$ ).  $*p < 0.05$  as compared to the Cr(III) +  $\text{H}_2\text{O}_2$  treatment group.

potentiates the production of reactive oxygen species, DNA strand breaks, and 8-OH-dG formation induced by Cr(VI) plus H<sub>2</sub>O<sub>2</sub> (45–47). Thus, although ascorbate functions as a free radical scavenger in the Cr(III) plus H<sub>2</sub>O<sub>2</sub> system, the utility of ascorbate in Cr detoxification *in vivo* should be cautiously considered.

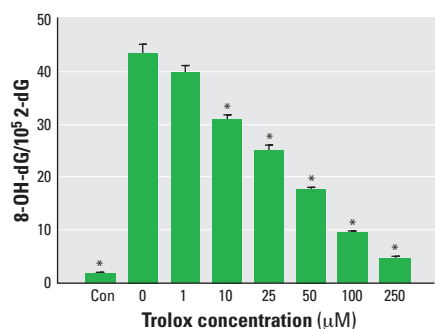
As compared to ascorbate (IC<sub>50</sub> = 30.4 μM) and vitamin E (IC<sub>50</sub> = 36.2 μM), melatonin was more effective in reducing the formation of 8-OH-dG in this system (IC<sub>50</sub> = 0.51 μM). Thus, melatonin was roughly 60- and 70-fold more effective in reducing oxidative damage to DNA than ascorbate and vitamin E, respectively. Also, the minimal concentration of melatonin required to significantly reduce 8-OH-dG formation was much less than that of either vitamin. A melatonin concentration of 0.25 μM significantly reduced the 8-OH-dG formation, and a 10-μM concentration of the indole essentially reduced 8-OH-dG levels to control levels.

In the present study, melatonin's highly effective protection against Cr(III)-induced formation of 8-OH-dG in DNA may relate to several actions of the indoleamine. First, melatonin is a direct free radical scavenger



**Figure 4.** The effect of ascorbate on Cr(III) (0.5 mM) plus H<sub>2</sub>O<sub>2</sub> (0.5 mM)-induced 8-OH-dG levels in DNA. The incubation time was 60 min. Results are given as means ± SE (n = 5).

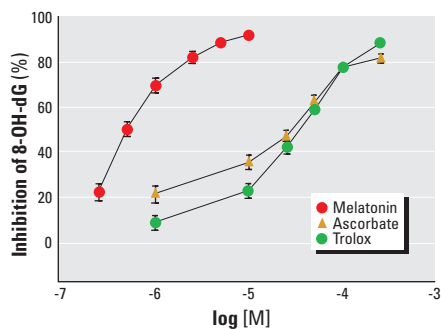
\*p < 0.05 as compared to the Cr(III) + H<sub>2</sub>O<sub>2</sub> group.



**Figure 5.** The effect of Trolox concentrations on Cr(III) (0.5 mM) plus H<sub>2</sub>O<sub>2</sub> (0.5 mM)-induced 8-OH-dG levels in DNA. The incubation time was 60 min. Results are given as means ± SE (n = 5).

\*p < 0.05 as compared to the Cr(III) plus H<sub>2</sub>O<sub>2</sub> group.

and is a particularly efficient scavenger of the highly toxic <sup>•</sup>OH (25,48,49). Melatonin neutralizes two <sup>•</sup>OH for each melatonin molecule, resulting in the formation of the product cyclic 3-hydroxymelatonin (50). In the present study, the formation of 8-OH-dG was thought to be due to a Cr(III)-mediated Fenton-type reaction that generates <sup>•</sup>OH, which in turn attacked DNA, resulting in the accumulation of the oxidative DNA base adduct 8-OH-dG (20,24). Second, melatonin not only detoxifies the highly toxic <sup>•</sup>OH, but also scavenges its precursor, H<sub>2</sub>O<sub>2</sub>. We recently uncovered a new pathway in which melatonin interacts with H<sub>2</sub>O<sub>2</sub> to yield N<sup>1</sup>-acetyl-N<sup>2</sup>-formyl-5-methoxykynuramine (51). The structure of the product was confirmed using mass spectrometry, proton nuclear magnetic resonance, and carbon nuclear magnetic resonance. By lowering the concentration of H<sub>2</sub>O<sub>2</sub>, <sup>•</sup>OH generation in this system would also be proportionally reduced. Such a dual strategy of antioxidant protection would be much more efficient than simply scavenging <sup>•</sup>OH. Third, because melatonin is highly lipophilic (28) as well as somewhat hydrophilic (29), it easily enters cells and sub-cellular compartments. Intracellularly, the highest radioimmunoassayable concentrations of melatonin are measured in the nuclei of brain cells after its peripheral administration to animals (30). Melatonin has a high affinity for the nucleus (and possibly DNA itself), which may contribute to its protective effect against Cr-induced formation of 8-OH-dG. Cr(III) accumulates in nuclei and has a high affinity for DNA (52). Melatonin may prevent the formation of 8-OH-dG by displacing Cr(III) from the Cr–DNA binding complex and thereby reduce H<sub>2</sub>O<sub>2</sub>-mediated <sup>•</sup>OH generation in the vicinity of DNA. Fourth, melatonin and its precursors reportedly have a high metal-binding affinity (53). Limson et al. (53) showed that melatonin chelated aluminum, cadmium, iron, copper, and lead,



**Figure 6.** Percent-inhibition curves of concentrations of melatonin, ascorbate, and Trolox in reducing Cr(III)-induced 8-OH-dG formation in DNA. The incubation time was 60 min with 0.5 mM H<sub>2</sub>O<sub>2</sub>. Results are given as means ± SE (n = 5).

etc. Although the authors did not investigate Cr, melatonin may also chelate this transition metal ion to prevent the formation of the <sup>•</sup>OH via the Cr-mediated Fenton-type reaction: Cr(III) + H<sub>2</sub>O<sub>2</sub> → Cr(IV) + <sup>•</sup>OH + OH<sup>-</sup>.

Susa et al. (54) used different end points and cultured primary rat hepatocytes and found that melatonin markedly reduced nuclear DNA single-strand breaks induced by K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> [Cr(VI)]. They speculated that melatonin protected cells from free radical toxicity by one of several means, including melatonin's ability to preserve intracellular levels of vitamins E and C, stimulation of catalase activity, and/or by directly scavenging the <sup>•</sup>OH. In current studies, two of the options proposed by Susa et al. (54), i.e., maintenance of Vitamin E and C levels and the stimulation of catalase activity, were clearly not involved in melatonin's protection of DNA from oxidative damage. Thus, the most likely explanation for the current findings is that melatonin's effects were a consequence of its ability to scavenge the <sup>•</sup>OH and possibly also H<sub>2</sub>O<sub>2</sub>.

In the current *in vitro* study, which used purified DNA, the curves for the inhibition of DNA damage by each of the three antioxidants, i.e., melatonin, ascorbate, and Trolox (Figure 6), were quite different. The relevance of these curves to the pharmacologic utility of these molecules in protecting nuclear DNA from oxidative damage *in vivo* remains to be investigated. However, in *in vivo* studies where other free-radical-generating agents were used, melatonin also proved highly effective in reducing DNA damage consistent with its ability to enter the nucleus with ease (55,56). There have been no *in vivo* studies where vitamin E, ascorbate, and melatonin were compared for their relative efficacies in protecting DNA from oxidative destruction.

Melatonin as an antioxidant is effective in protecting membrane lipids, nuclear DNA, and protein from oxidative damage induced by a variety of free-radical-generating agents and processes both *in vitro* as well as *in vivo* (26,55–58). Considering the apparent virtual absence of acute or chronic toxicity, melatonin's clinical application against Cr-induced genotoxicity in occupational and environmental situation where this metal is a problem should be considered.

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