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ARTICLES

# Relative Efficacies of Indole Antioxidants in Reducing Autoxidation and Iron-Induced Lipid Peroxidation in Hamster Testes

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**Abstract** Increased iron stores are associated with free radical generation and carcinogenesis. Lipid peroxidation is involved in DNA damage, thus indirectly participating in the early steps of tumor initiation. Melatonin and structurally related indoles are effective in protecting against oxidative stress. The aim of the study was to compare the relative efficacies of melatonin, *N*-acetylserotonin (NAS), indole-3-propionic acid (IPA), and 5-hydroxy-indole-3-acetic acid (5HIAA) in altering basal and iron-induced lipid peroxidation in homogenates of hamster testes. To determine the effect of the indoles on the autoxidation of lipids, homogenates were incubated in the presence of each agent in concentrations of 0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM. To study their effects on induced lipid peroxidation, homogenates were incubated with $FeSO_4$ (30 μM + $H_2O_2$ (0.1 mM) + each of the indoles in the same concentrations as above. The degree of lipid peroxidation was expressed as concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) per mg protein. The indoles decreased both basal and iron-related lipid peroxidation in a concentration-dependent manner. Melatonin reduced basal MDA + 4-HDA levels when used at the concentrations of 0.25 mM or higher, and prevented iron-induced lipid peroxidation at concentrations of 1.0, 2.0, 2.5, or 5.0 mM. The lowest effective concentrations of NAS required to lower basal and iron-related lipid peroxidation were 0.05 mM and 0.25 mM, respectively. IPA, only when used in the highest concentrations of 2.5 mM or 5 mM inhibited basal lipid peroxidation levels and it was ineffective on the levels of MDA + 4-HDA due to iron damage. 5HIAA reduced basal lipid peroxidation when used at concentrations of 0.25 mM or higher, and it prevented iron-induced lipid peroxidation only at the highest applied concentration (5 mM). In conclusion, melatonin and related indoles at pharmacological concentrations protect against both the autoxidation of lipids as well as induced peroxidation of lipids in testes. In doing so, these agents would be expected to reduce testicular cancer that is initiated by products of lipid peroxidation. J. Cell. Biochem. 81:693–699, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** melatonin; indoles; testes; iron; lipid peroxidation; cancer

Increased body iron stores are associated with an increased risk of cancer [Stevens et al., 1988]. The contribution of iron to the process of carcinogenesis is a consequence of several processes. Iron-related generation of free radicals, a process directly associated with carcinogenesis [Halliwell and Gutteridge, 1986], and the fact that iron may be a limiting nutrient for the growth and development of cancer [Weinberg, 1996], are of particular importance. Bivalent iron (ferrous, $Fe^{2+}$), which initiates the Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$), is frequently used to experimentally induce oxidative damage including that caused by lipid peroxidation [Chen et al., 1997; Karbownik et al., 2000c].

Although testes do not especially serve as a major reservoir for iron, testicular iron accumulation accompanied by oxidative damage due to acute iron intoxication is observed [Lucesoli et al., 1999]. Testicular cell membranes are rich in polyunsaturated fatty acids which easily undergo peroxidative changes, leading to a

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chain reaction involving various radicals and reactive oxygen species (ROS).

*N*-acetyl-5-methoxytryptamine (melatonin) (Fig. 1), the chief indoleamine produced by the pineal gland, is well known to protect against oxidative damage and carcinogenesis [Reiter, 1997, 1998, 1999, 2000a,b; Blask et al., 1999; Reiter et al., 2000; Karbownik and Reiter, 2000; Karbownik et al., 2000a–c]. Recently, other melatonin-related molecules have attracted interest due to their protective actions against oxidative stress. *N*-acetyl-5-hydroxytryptamine (*N*-acetylserotonin, NAS) (Fig. 1), the immediate precursor of melatonin, protects against oxidative damage under a variety of experimental conditions [Barsacchi et al., 1998; Qi et al., 2000]. Indole-3-propionic acid (IPA) (Fig. 1), a deamination product of tryptophan, possesses a structure similar to that of melatonin and is a newly discovered free radical scavenger effective in preventing oxidative damage [Chyan et al., 1999; Poeggeler et al., 1999; Qi et al., 2000]. 5-hydroxy-indole-3-acetic acid (5HIAA), the main metabolite of serotonin which is excreted in urine, has been sparingly examined in relation to oxidative stress. It is of interest, however, since elevated urinary concentrations of this derivative are found in patients suffering from neuroendocrine tumors [Torney and FitzGerald, 1995].

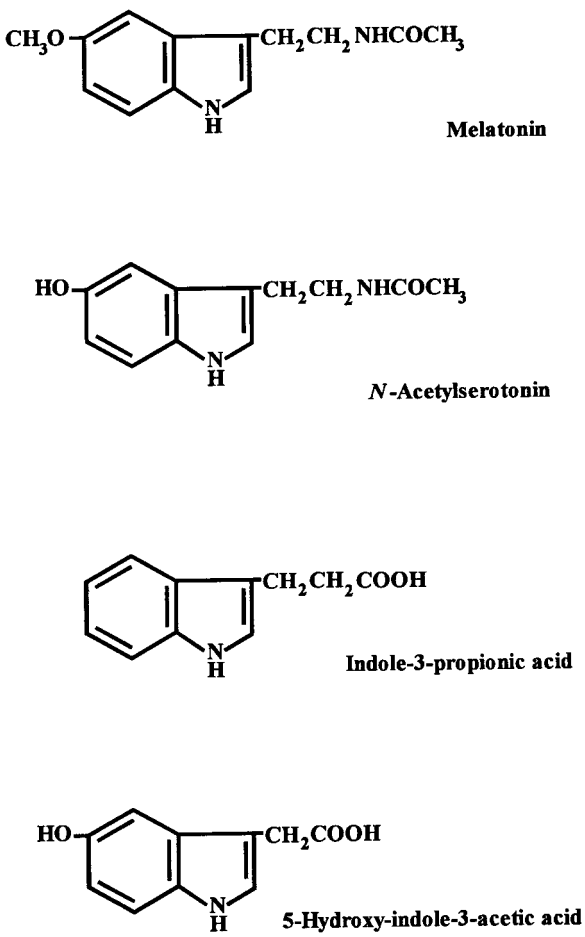


**Fig. 1.** Chemical structures of the indoles tested in the current study.

The aim of the current study was to evaluate the potential protective effects of melatonin, NAS, IPA, and 5HIAA against both basal as well as $Fe^{2+}$-induced lipid peroxidation in homogenates of hamster testes.

## MATERIALS AND METHODS

### Chemicals

Pure melatonin was a gift from Helsinn Chemicals SA (Biasca, Switzerland). NAS, IPA, 5HIAA, ferrous sulfate ($FeSO_4$) and hydrogen peroxide ($H_2O_2$) were purchased from Sigma (St. Louis, MO). The LPO-586 kit for lipid peroxidation was obtained from Calbiochem (La Jolla, CA). Other chemicals used were of analytical grade and came from commercial sources.

### Animals

The procedures used in the study were approved by the Institutional Animal Care and Use Committee. Male Syrian hamsters (*Mesocricetus auratus*) (~120 g) were purchased from Harlan (Houston, TX) and housed in plexiglas cages (three animals per cage) in a windowless room with automatically regulated temperature ($22 \pm 2^{\circ}C$) and lighting (14 h light/10 h dark, with light on from 06.00 to 20.00 h). The animals received standard chow (Ralston Purina Co., Inc., St. Louis, MO) and water ad libitum. After one week of acclimatization, the hamsters were killed by decapitation and testes were collected, frozen on solid $CO_2$ and stored at $-80^{\circ}C$ until assay. The testes of 32 hamsters were used in these studies.

### Incubation of Testicular Homogenates

Individual testes were homogenized (Euro Turrax T20B homogenizer) in ice cold 50 mM Tris-HCl buffer (pH = 7.4) (10%, w/v), and then incubated for 60 min at $37^{\circ}C$ in the presence of either melatonin, NAS, IPA, or 5HIAA in the following concentrations: 0.0, 0.01, 0.05, 0.1,

0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM. For each concentration of indole, duplicate samples of homogenate were run; each experiment was performed in triplicate. In another set of experiments, testicular homogenates were incubated in the presence of $FeSO_4$ (30 μM) + $H_2O_2$ (0.1 mM) to induce lipid peroxidation, and, additionally, in the presence of the same indoleamines used at the same concentrations as in the first study. The reactions were stopped by cooling the samples on ice.

### Measurement of Products of Lipid Peroxidation

The concentrations of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA), as an index of lipid peroxidation, were measured in testicular homogenates. Homogenates were centrifuged at 3,000*g* for 10 min at 4°C. The supernatant was mixed 650 μl of methanol:acetonitrile (1:3, v/v) solution containing a chromogenic reagent, N-methyl-2-phenylindole, and vortexed. After adding 150 μl 15.4 M methanesulfonic acid, incubation was carried out at 45°C for 40 min. The reaction between MDA + 4-HDA and N-methyl-2-phenylindole yields a chromophore, which is measured spectrophotometrically at the absorbance of 586 nm using a solution of 10 mM 4-hydroxynonenal as the standard. The level of lipid peroxidation is expressed as the amount of MDA + 4-HDA (nmol) per mg protein.

### Measurement of Protein

Protein was measured using the method of Bradford [1976], with bovine albumin as the standard.

### Statistical Analyses

Results are expressed as means ± SE. The data were statistically analyzed using a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test. Statistical significance was determined at a level of $<0.05$.

## RESULTS

### Effect of Melatonin on Basal (Autoxidation) and Iron-Induced Lipid Peroxidation in Testicular Homogenates

The incubation of testicular homogenates in the presence of melatonin only resulted in the concentration-dependent decrease in the level of lipid peroxidation products with the lowest effective concentration being 0.25 mM (Fig. 2A).

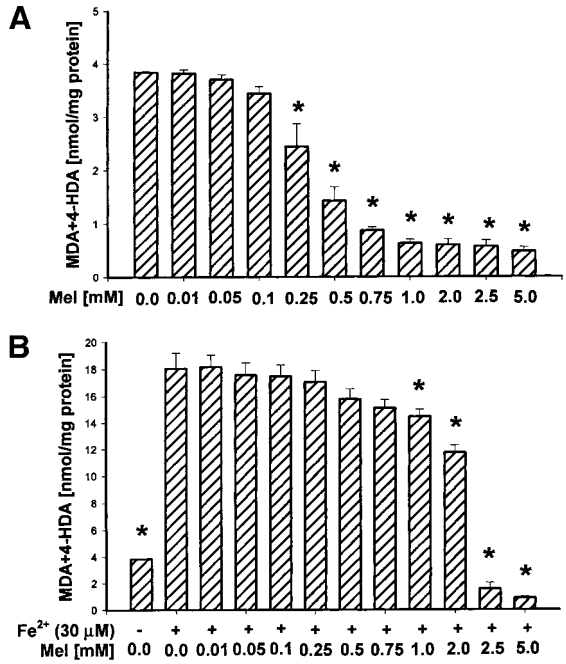


**Fig. 2.** The concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) in hamster testicular homogenates incubated for 1 h in the presence of melatonin (0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(A)**, or in the presence of $FeSO_4$ (30 μM) + $H_2O_2$ (0.1 mM), used to induce lipid peroxidation, and melatonin (0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(B)** Bars represent the mean ± SE of three independent experiments. $^*P < 0.05$ vs. Control [in the absence of indole (A) or in the presence of $Fe^{2+}$ and in the absence of indole (B)].

The increased lipid peroxidation, induced by iron, was also significantly reduced by melatonin in the concentrations of 1.0, 2.0, 2.5, and 5.0 mM (Fig. 2B).

### Effect of NAS on Basal and Iron-Induced Lipid Peroxidation in Testicular Homogenates

NAS, added to the incubation medium at concentrations of 0.05 mM or higher, decreased basal levels of MDA + 4-HDA in testicular homogenates (Fig. 3A). Likewise, NAS was effective in protecting against iron-induced lipid peroxidation at concentrations of 0.25 mM and higher (Fig. 3B).

### Effect of IPA on Basal and Iron-Induced Lipid Peroxidation in Testicular Homogenates

Unexpectedly, IPA was shown to be weakly effective in protecting against the autoxidation of lipids in homogenates of hamster testes. Only in concentrations of either 2.5 or 5 mM IPA did it reduce basal levels in testicular membranes (Fig. 4A); additionally, it was totally ineffective

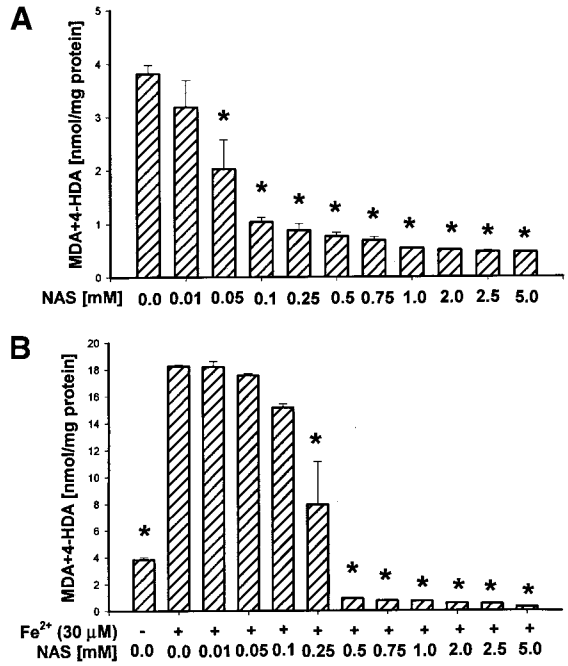


**Fig. 3.** The concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) in hamster testicular homogenates incubated for 1 h in the presence of *N*-acetylserotonin (NAS, 0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(A)**, or in the presence of $FeSO_4$ (30 μM) + $H_2O_2$ (0.1 mM), used to induce lipid peroxidation, and NAS (0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(B)** Bars represent the mean ± SE of three independent experiments. $^*P < 0.05$ vs. Control (in the absence of indole (A) or in the presence of $Fe^{2+}$ and in the absence of indole (B)).

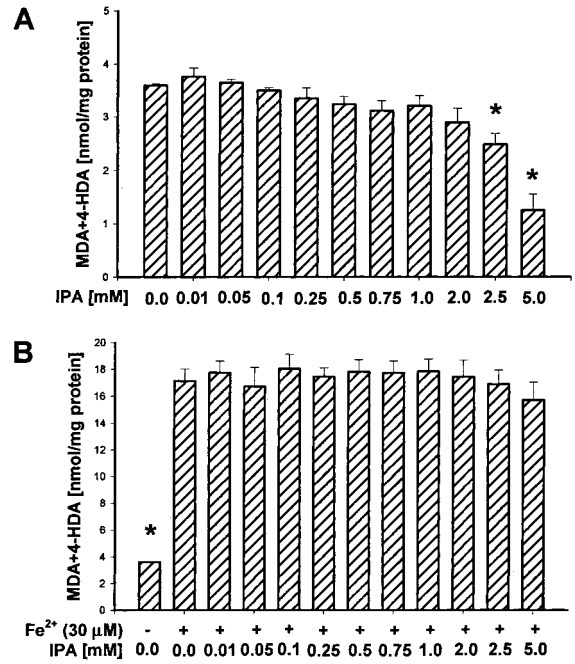


**Fig. 4.** The concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) in hamster testicular homogenates incubated for 1 h in the presence of indole-3-propionic acid (IPA, 0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(A)**, or in the presence of $FeSO_4$ (30 μM) + $H_2O_2$ (0.1 mM), used to induce lipid peroxidation, and IPA (0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(B)** Bars represent the mean ± SE of three independent experiments. $^*P < 0.05$ vs. Control (in the absence of indole (A) or in the presence of $Fe^{2+}$ and in the absence of indole (B)).

in altering lipid peroxidation due to iron (Fig. 4B).

### Effect of 5HIAA on Basal and Iron-Induced Lipid Peroxidation in Testicular Homogenates

5HIAA was effective in reducing autoxidative processes when used in concentrations of 0.25 mM or higher (Fig. 5A). 5HIAA prevented iron-induced lipid peroxidation only when the indole was applied at a concentration of 5 mM (Fig. 5B).

## DISCUSSION

The contribution of iron to testicular malignancy may involve two primary mechanisms. The first, which is not specific for testes, consists of iron-related free radical generation followed by damage to macromolecules. The second mechanism involves the association of iron and ROS and their induction on sperm abnormalities and a reduction of their fertilizing ability [Chen et al., 1997; Aitken et al., 1998]. Infertility is strong a risk factor for testicular cancer [Möller and Skakkebaek, 1999]. It was shown recently that acute iron overload leads to its accumulation in rat testes followed by increased lipid peroxidation, oxidation of DNA bases, and depletion of lipid-soluble antioxidants [Lucesoli and Fraga, 1995; Lucesoli et al., 1999]. In these studies, iron accumulation was positively correlated with the level of lipid peroxidation products and with antioxidant depletion, but not with DNA damage. Therefore, the authors suggested that oxidation of DNA bases in vivo due to iron overload is an indirect one and is a consequence of the iron-related increase in lipid peroxidation rather than to a direct action of iron in the nucleus [Lucesoli and Fraga, 1995]. Indeed, the importance of the contribution of lipid peroxidation products to DNA damage, genotoxicity, mutagenicity and, consequently, carcinogenesis is well documented [Burcham, 1998; Marnett, 1999].

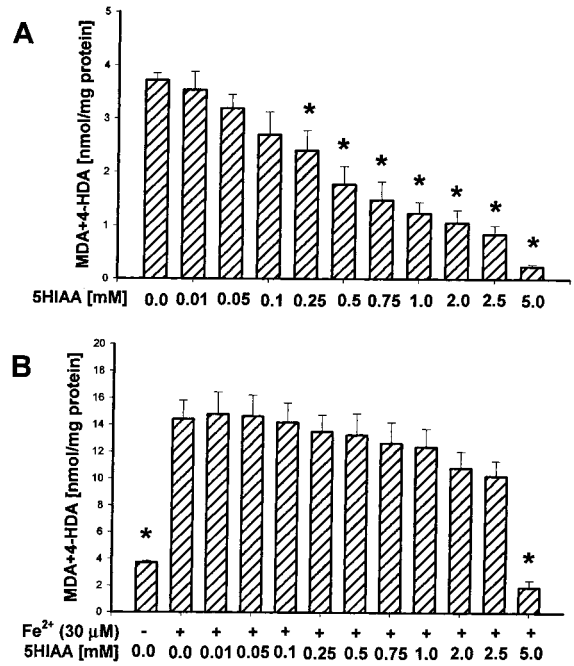


**Fig. 5.** The concentration of malondialdehyde + 4-hydroxyalkenals (MDA + 4-HDA) in hamster testicular homogenates incubated for 1 h in the presence of 5-hydroxy-indole-3-acetic acid (5HIAA, 0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(A)**, or in the presence of $FeSO_4$ (30 μM) + $H_2O_2$ (0.1 mM), used to induce lipid peroxidation, and 5HIAA (0.0, 0.01, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.0, 2.5, or 5.0 mM) **(B)** Bars represent the mean ± SE of three independent experiments. $^*P < 0.05$ vs. Control (in the absence of indole (A) or in the presence of $Fe^{2+}$ and in the absence of indole (B)).

Studies using other agents have also shown melatonin to be effective in protecting testicular lipids from lipid peroxidative processes. Thus, co-treatment with melatonin prevented ethanol-induced lipid peroxidation in rat testes in vivo [El-Sokkary et al., 1999], and likewise melatonin applied in vitro suppressed experimentally-induced lipid peroxidation in sperm membranes in samples from infertile men [Gavella and Lipovac, 2000].

The protective effects of melatonin and related molecules against oxidative damage to cellular membranes has been observed previously [Reiter, 1998; Calvo et al., 2000; Garcia et al., 2000; Karbownik et al., 2000a, b, 2001a]. The mechanisms by which these agents reduce lipid peroxidation likely involve their indirect antioxidant and direct free radical scavenging activities. According to the current view [Reiter, 2000a], the protective effect of melatonin against lipid peroxidation relies primarily on its ability to scavenge several toxic reactants, e.g., $^{\bullet}OH$ and $ONOO^-$, which initiate the peroxidative process. Both, melatonin and IPA scavenge the highly toxic $^{\bullet}OH$ with a rate constant roughly on the order of $10^{10}$/M/s [Tan et al., 1993; Chyan et al., 1999; Poeggeler et al., 1999; Brömme et al., 2000]. Conversely, NAS was shown ineffective in scavenging $^{\bullet}OH$ which were generated by the ultraviolet photolysis of $H_2O_2$ [Tan et al., 1993].

As mentioned, melatonin also has been shown to detoxify the $ONOO^-$, the highly destructive product of the interaction between the $O_2^{-\bullet}$ and $NO^{\bullet}$ [Blanchard et al., 2000], and to directly neutralize $NO^{\bullet}$ [Noda et al., 1999]. These actions of melatonin would contribute to melatonin's ability to reduce both autoxidation and iron-induced lipid peroxidation since the $ONOO^-$ is sufficiently reactive to initiate the breakdown of lipids. Melatonin may indirectly detoxify the superoxide anion ($O_2^{-\bullet}$) [Reiter, 1998] and IPA quenches $O_2^{-\bullet}$ [Hardeland et al., 1999]. Recently, melatonin was found to directly detoxify $H_2O_2$ [Tan et al., 2000]. Also, IPA was reported to protect primary neurons, neuroblastoma cells, and rat brain against oxidative damage due to $H_2O_2$ [Chyan et al., 1999; Poeggeler et al., 1999]. Furthermore, melatonin quenches singlet oxygen ($^1O_2$), a high energy form of $O_2$, which exhibits high toxicity at the molecular level [Reiter et al., 2000]. While melatonin highly effectively reduces lipid peroxidation, whether it does so by directly detoxifying the peroxyl radical remains controversial [Pieri et al., 1994; Antunes et al., 1999].

Melatonin's protective effects against lipid peroxidation in vivo may also involve its ability to stimulate antioxidative enzymes which remove oxygen and nitrogen-based reactants. Thus, melatonin increases intracellular glutathione (GSH) concentration by stimulating the rate limiting enzyme in its synthesis, γ-glutamylcysteine synthetase [Urata et al., 1999]. Additionally, melatonin functions in the recycling of GSH by promoting the activities of several enzymes including glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) [Pablos et al., 1998] and glucose-6-phosphate dehydrogenase (G6PD) [Pierrefiche and Laborit, 1995]. In reference to IPA, Poeggeler et al. [1999] showed that it acts synergistically with GSH to scavenge the $^{\bullet}OH$. Melatonin is also known to increase tissue mRNA levels for superoxide dismutase (SOD), the enzyme which dismutases $O_2^{-\bullet}$ to $H_2O_2$, and of catalase (CAT), which metabolically removes $H_2O_2$ from the

intracellular environment, thereby further reducing $H_2O_2$ levels and $^{\bullet}OH$ generation [Reiter, 1998; Reiter et al., 2000]. The latter property is important since ferrous iron induced lipid peroxidation in testes is reduced by catalase [Melin et al., 1997]. Melatonin also inhibits the activity of a pro-oxidative enzyme, i.e., nitric oxide synthase (NOS), which catalyzes the formation of $NO^{\bullet}$ [Reiter, 1998]. Finally, melatonin increases the efficiency of the electron transport chain in the inner mitochondrial membrane with a consequent reduction of free radical generation [Absi et al., 2000; Martin et al., 2000; Acuña-Castroviejo et al., 2001].

Melatonin which is highly lipid soluble, is believed to be widely distributed in cellular membranes [Reiter et al., 2000] where it may intercalate between the polar heads of fatty acids [Ceraulo et al., 1999]. This action has been proposed as another means by which melatonin may reduce peroxidative damage to lipids.

Clearly, the efficacies of the indoles used in this study varied in terms of reducing lipid peroxidation. These differences likely relate to their particular structures (Fig. 1). Antioxidants which possess a reactive hydroxyl group are often hydrogen donors, thereby reducing free radicals that promote radical chain reactions and effectively reduce lipid peroxidation [Moosmann et al., 1997; Barsacchi et al., 1998]. At the same time, however, they may autoxidize in the presence of transition metals and increase the formation of primary radicals. Two indoles (NAS and 5HIAA) applied in the current study, possess a hydroxyl group and reduced both basal and iron-induced lipid peroxidation in vitro. In vivo, however, they could pose a potential risk by enhancing oxidative damage when they encounter a transition metal. Conversely, the two other indoles (melatonin and IPA) do not possess a hydroxyl group. They act as endogenous electron donors, primarily detoxifying reactive radicals, but, at the same time they do not undergo autoxidation in the process of redox recycling or in the presence of transition metals [Chyan et al., 1999]. Thus, they are more promising agents in terms of protecting against oxidative damage in vivo. In support of this, we have recently found that melatonin and IPA, but not NAS, prevented in vivo DNA damage in hamster kidney following treatment with estradiol [Karbownik et al., 2001b].

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