Exp Toxic Pathol 2004; **55**: 379–384
http://www.elsevier-deutschland.de/etp

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Protective effects of melatonin on the ionizing radiation induced DNA damage in the rat brain

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With 2 figures

Received: June 2, 2003; Revised: September 1, 2003; Accepted: September 24, 2003

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Key words: Ionizing radiation; Melatonin; DNA damage; Lipid peroxidation; Brain; Rat.

Summary

Melatonin is an endogenously produced antioxidant with radioprotective actions while ionizing radiation is a well-known cytotoxic and mutagenic agent of which the biological results are attributable to its free radical producing effects. The effect of melatonin on the DNA strand breakage and lipid peroxidation induced by ionizing radiation in the rat brain were investigated in order to clarify its radioprotective ability. The DNA strand breakage in rat brain exposed to 1000 cGy ionizing radiation was assessed by alkaline single cell gel electrophoresis and the lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) concentrations. A significant increase in DNA damage ($p < 0.05$) and TBARS concentrations ($p < 0.01$) was found in the radiation treated rat brain. Pre-treatment of rats with intraperitoneal doses of 100 mg/kg melatonin provided a significant decrease in the DNA strand breakage and lipid peroxidation. Our results indicate that melatonin can protect brain cells from oxidative damage induced by ionizing radiation.

Introduction

Ionizing radiation is a well-known cytotoxic and mutagenic agent that causes a variety of changes depending on exposed and absorbed dose, duration of exposure, interval after exposure, and susceptibility of tissues. DNA is the most often reported molecule to be damaged by ionizing radiation. Ionizing radiation induces predominantly single-strand breaks but also double-strand breaks, alkali labile sites, and oxidized purines and pyrimidines (Frankenberg-Schwanger 1990; Halliwell and Aruoma 1991). Cells exposed to ionizing radiation can develop prolonged genetic instability that is manifested in multiple ways, including delayed reproductive death, an increased rate of point mutations, and chromosome rearrangement (Murnane 1996; Morgan et al. 1996). The injury of ionizing radiation to living cells is, to a large extent, due to oxidative stress. Interactions of ionizing radiation with DNA consist of the ionization of DNA and its reaction with surrounding water molecules, followed by decomposition of molecular structure of DNA by the induced radicals (Halliwell and Aruoma 1991; Wallace 1998; Ward 1998). In addition to DNA, lipids and proteins are also attacked by reactive oxygen species (ROS) specially hydroxyl radicals ($^{-}$OH) and the initiated chain reaction caused by ionizing radiation leads to the formation of a variety of degradation products in biological membranes including products of lipid breakdown which cause changes in membrane structure and function (Karbownik and Reiter 2000).

Since radiation-induced cellular DNA and membrane damage is primarily attributed to the damaging effects of ROS, molecules with free radical scavenging properties are particularly promising as radioprotectors. Dale and

colleagues (DALE et al. 1949) were first noted the ability of certain substances to provide protection against radiation-induced damage. Melatonin (N-acetyl-5-methoxytrptamine) is distributed ubiquitously in organisms and in all cellular compartments (EBADI 1984; REITER 1991). In human blood the physiological concentrations of radioimmunoassayable melatonin are approximately 0–20 pg/ml during the day and 40–200 pg/ml during the night (WALDHAUSER and DIETZEL 1985). Melatonin participates in several important physiological processes. It has been found to have effects in immune reactions and cancer growth (MAESTRONI 1993; BLASK 1993). Its scavenging free radical properties and thus antioxidant ability have been documented in "in-vitro" studies using human tissues and also in "in-vivo" animal experiments and the protective effects of melatonin against radiation induced oxidative and DNA damage have been demonstrated as well (TAN et al. 1993; BLICKENSTAFF et al. 1994; VIJAYALAXMI et al. 1995a, 1996, 1999; AHLERS et al. 1997; KAYA et al. 1999; BADR et al. 1999; KIM 2001). It is suggested that melatonin may scavenge the free radicals produced by ionizing radiation before they induce DNA damage and/or it also may stimulate the antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and glutathione reductase (GR) (SINGH et al 1990; VIJAYALAXMI et al. 1995b, 1998). Melatonin has been found also to decrease the formation of nitric oxide (NO) by inhibiting the activity of nitric oxide synthase (NOS) (BETTAHI et al. 1996; GUERRERO et al. 1997; POZO et al. 1997). It has also the stabilization effect on membrane fluidity (GARCIA et al. 1997). Although it has not been cleared yet, melatonin has been suggested to influence DNA repair enzymes directly and stimulate intracellular signals indirectly for the activation of genes related to DNA repair (KARBOWNIK and REITER 2000).

This study has been undertaken to investigate the effects of ionizing radiation on the DNA damage and lipid peroxidation and the radiopreventive ability of melatonin in rat brain. To provide data on the possible protective effects of melatonin on the ionizing radiation-induced oxidative DNA damage we have evaluated the DNA strand breakage in control, melatonin-treated, irradiated and irradiated plus melatonin-treated rat brains by the alkaline single cell gel electrophoresis or 'comet' assay which has been considered as a sensitive method for the detection of DNA single strand breaks and alkali-labile damage in individual cells (FAIRBAIRN et al. 1995). For the determination of lipid peroxidation levels, measurement of thiobarbituric acid reactive substances (TBARS) concentrations, a frequently used biomarker of lipid peroxidation, was performed in the same groups.

Materials and methods

Animals and treatments

Female Wistar albino rats (200 ± 10 g) were obtained from the Animal Care Unit of Hacettepe University and were group- housed in plastic cages with stainless- steel grid tops. The cages were placed in a room with controlled temperature (23 ± 2 °C) and a 12h light- dark cycle. The animals received standard laboratory chow and water ad libitum.

The rats were divided into four groups consisting 6–8 rats in each group.

Group 1 received 0.9% saline solution containing ethanol less then 0.5% (v/v) as control. Thirty minutes later, the animals were anesthetized and decapitated.

Group 2 received a single dose of 100 mg/kg, intraperitoneally (i.p.), melatonin (Boehringer Mannheim) (Melatonin was dissolved in a minimum volume of absolute ethanol and then diluted with 0.9% saline solution). The final concentration of ethanol in the aqueous melatonin solution provided to animals was less than 0.5% (v/v) and the animals were anesthetized and decapitated thirty minutes later.

Group 3 was stabilized under general anesthesia and irradiated with 1000 cGy using a Co-60 therapy machine specifically directed to the head.

Group 4 was pretreated with a single dose of 100 mg/kg, i.p., melatonin. Thirty minutes later, this group was also stabilized under general anesthesia and irradiated with 1000 cGy specifically directed to the head. The animals in the group 3 and 4 were decapitated thirty minutes after their exposure to ionizing radiation.

Ketamin (ketamin hydrochloride, 50 mg/kg [Ketalar® 5%, Parke-Davis] and xylazine 8 mg/kg [Rompun® 2%, Bayer]) mixture was used intraperitoneally for the anesthesia of the animals. After decapitation, whole brain were rapidly removed, washed in saline and divided into two portions in order to measure DNA damage and lipid peroxidation levels.

Comet assay

The basic alkaline comet technique of SINGH et al. (SINGH et al. 1994), as further described by ANDERSON et al. (ANDERSON et al. 1994) and COLLINS et al. (COLLINS et al. 1997) was applied to brain tissues taken from right parietal lobe of each animal. Brain tissues were minced gently into fine pieces in 1 ml Hank's balanced solution with 20 mM EDTA (TICE 1995). A total of 5–10 μl of the cell (approximately 10 000 cells) suspension were dissolved in 75 μl of 0.5% LMA (Boehringer Mannheim) and spread on fully frosted microscopic slides precoated with 110 μl of 0.5% NMA (Boehringer Mannheim) and maintained on an ice-cold flat tray for 5 min to solidify. Then the third layer of 0.5% LMA (75 μl) was added and allowed to solidify on ice for 5 min. The cells were lysed in high salt and detergent (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10; with 1% Triton-X 100 and 10% DMSO added just before use) for a minimum of 1 h at 4 °C, and placed in a horizontal gel electrophoresis tank side by side. The tank was filled with fresh electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH 13) to a level approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage. Electrophoresis was conducted at a low temperature (4 °C) for 20 min using 24 V and adjusting the current to 300 mA by

raising or lowering the buffer level and using a compact power supply (Power Pack P 25 Biometra Analytic GmbH). All of these steps were conducted under dimmed light (tank was covered with a black cloth) to prevent the occurrence of additional DNA damage. After electrophoresis, tris buffer (0.4 M Tris, pH 7.5) was added dropwise and gently to neutralize the excess alkali and the slides were allowed to sit for 5 min. The neutralizing procedure was repeated 3 times. The slides were stained with 65 μl ethidium bromide (Sigma) (20 μg/ml). For visualization of DNA damage, slides were examined at an ×1000 magnification using a 100× objective (oil immersion) on a fluorescence microscope (Zeiss, Germany). Images of 100 randomly selected cells, i.e. 50 cells from each of two replicate slides, were analyzed from each sample and the DNA damage was scored visually as described by ANDERSON et al. (ANDERSON et al. 1994) and COLLINS et al. (COLLINS et al. 1997). In the experiments, the cells were graded by eye into four categories on the basis of damage extent, i.e.; no damage, low damage, damaged and highly damaged. One slide reader, who is not aware of the treatment groups performed analysis in order to minimize variability due to subjective scoring. A scheme for visual scoring of the comets, which has been developed, by Collins and colleagues (COLLINS et al. 1997) was performed. DNA damage was scored based on 4 recognizable classes of comet, from class 0 (undamaged, no discernible tail) to class 3 (almost all DNA in tail, insignificant head). Each comet is given a value according to the class it is put into, so that an overall score can be derived for each gel, ranging from 0 to 300 arbitrary units.

Measurement of lipid peroxidation levels

The homogenization of left parietal lobe of each brain tissue was carried out in a Teflon- glass homogenizer in a volume of ice- cold buffer containing 140 mM KCl, 10 mM $NaHCO_3$, 3 mM KH_2PO_3 and 2 mM K_2HPO_4 (adjusted to pH 7.2) to obtain a 10% (w/v) whole homogenate. The concentration of TBARS in whole homogenates of cerebral tissues was determined as a measure of lipid peroxidation according to the method of Ohkawa and colleagues (OHKAWA et al. 1979). The results were expressed as nmol equivalent malondialdehyde per mg of protein. The protein concentrations were determined by the standard method of Lowry and colleagues (LOWRY et al. 1951).

Statistical analysis

The SPSS for Windows 10.0 computer program was used for statistical analysis. Statistical comparison of the results from controls without any treatment, irradiated, melatonin treated and irradiated plus melatonin treated groups were carried out by one-way analysis of variance (ANOVA) test and post hoc analysis of group differences was performed by LSD test (LOVELL et al. 1999). Results are expressed as mean ± S.E.M.

Results

The results of the DNA strand breakage in the brain cells of control, melatonin treated, irradiated and irradiated plus melatonin treated groups are given in figure 1. A significant increase in the DNA strand breakage was observed in the irradiated brain cells ($p < 0.05$) compared to the controls. The number of brain cells without DNA damage was significantly higher in the controls compared to that irradiated brain cells. Also DNA strand breakage was found to be significantly increased in irradiated brain cells compared to melatonin treated brain cells ($p < 0.05$) but melatonin pre-treatment resulted in a significant decrease in DNA strand breakage in irradiated brain cells ($p < 0.05$).

On the other hand, single fraction of radiation treatment (1000 cGy) caused a significant increase in TBARS concentrations of rat brain tissues (~220%) compared to the control and melatonin treated groups ($p < 0.01$). Pretreatment of rats with melatonin (100 mg/kg, i.p.) provided also significant protection against the elevation of lipid peroxidation levels induced by ionizing radiation ($p < 0.01$) (fig. 2).

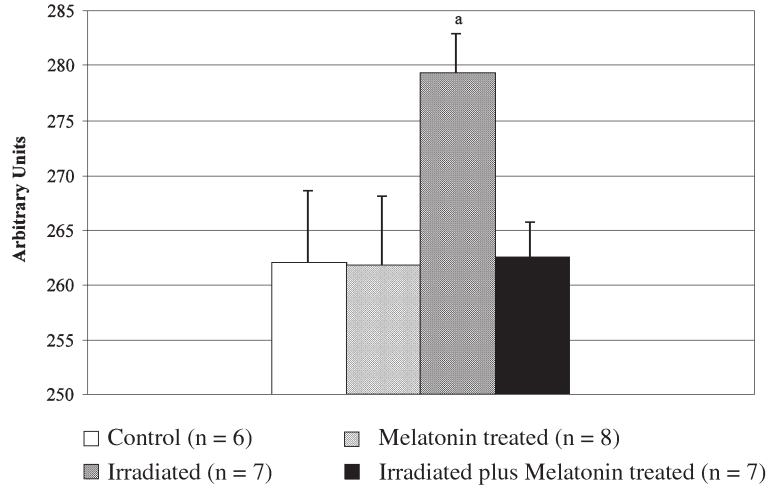

Fig. 1. DNA strand breakage in rat brain cells of the experimental groups. [a] $p < 0.05$ vs control, melatonin treated and irradiated plus melatonin treated groups. Values are given as mean ± S.E.M.

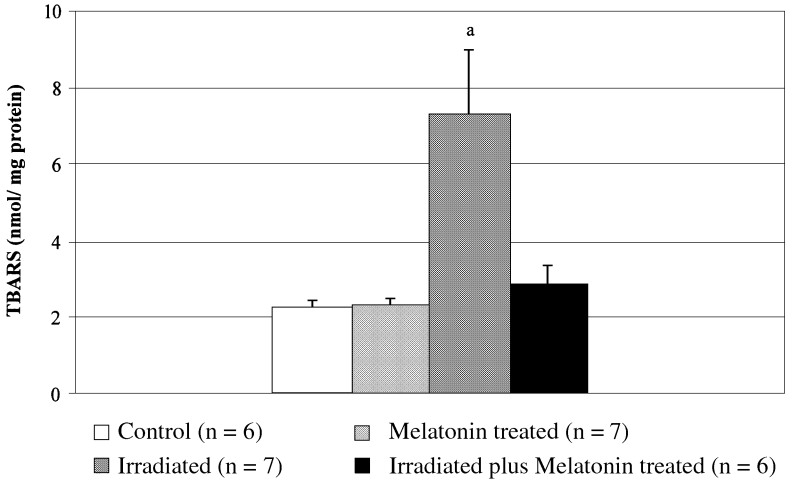

Fig. 2. TBARS concentrations in cerebral tissues of the experimental groups. [a] $p < 0.01$ vs control, melatonin treated and irradiated plus melatonin treated groups. Values are given as mean ± S.E.M.

Discussion

Several "in-vivo" and "in-vitro" studies have examined the radiation-induced free radical damage evidenced by the elevation of lipid peroxidation levels (Ronai and Benko 1984; Rejholcova and Wilhelm 1989; Zorlu et al. 1994; Ono et al. 1998; Kaya et al. 1999; Kilic et al. 2000; Karbownik et al. 2000; Przybyszewski et al. 2002). A time-dependent significant increase in lipid peroxidation levels was demonstrated in some studies (Ueda et al. 1996; Slyshenkow et al. 1998). However Karbownik and colleagues (Karbownik et al. 2000) did not found a significant increase in lipid peroxidation levels indicated as malondialdehyde (MDA) and 4-hydroxyalkenal concentrations in cellular membranes of liver, small intestine, lung and brain of male Sprague-Dawley rats measured 12h after exposure to whole body irradiation of 800 cGy. In their study a significant increase in DNA-adduct levels in liver tissue and a significant decrease in hepatic microsomal membrane fluidity was observed and they reported that the apparent lack of increased TBARS formation indicated the significance of the dose of ionizing radiation and the time of measurement. In our study, the observation of the elevated lipid peroxidation and oxidative DNA damage levels in a short time by single irradiation of 1000 cGy to whole brain has suggested that ionizing radiation may initiate an immediate free radical production effects. Brain tissue is reported to be highly vulnerable to free radical damage because of its high oxygen utilization, high concentrations of polyunsaturated fatty acids and transition metals such as iron, and low concentration of cytosolic antioxidants (Floyd and Carney 1993). Our study seems to be the first "in-vivo" study that has investigated the effects of brain irradiation on DNA damage and lipid peroxidation in rat cerebral tissue.

A number of different substances have been examined as to their protective effects against ionizing radiation induced oxidative damage, and it has been reported that molecules with direct free radical scavenging properties are particularly promising as radioprotectors possibly due to the observation that whole body irradiation decreases the total antioxidant capacity of the organism and depletes the levels of known antioxidants (Karbownik and Reiter 2000). Melatonin is known to be an effective antioxidant (Reiter 1995, 1996, 1998; Reiter et al. 1995) and to scavenge the highly toxic $^{-}$OH and other radicals produced by ionizing radiation (Tan et al. 1993, 1998; Ahlers et al. 1997; Stasika et al. 1998; Kaya et al. 1999). Because of its rather small size and high lipophilicity, melatonin crosses biological membranes easily, thus reaching all compartments in the cell (Reiter 1991). The findings of the present study which provide evidence of the preventive role of 100 mg/kg i.p. doses of melatonin against ionizing radiation induced DNA damage and lipid peroxidation are consisted with the results of the other "in-vitro" and "in- vivo" studies in other organs and animals (Blickenstaff et al. 1994; Ahlers et al. 1997; Vijayalaxmi et al. 1999; Kaya et al. 1999; Badr et al. 1999). Studies showed that melatonin at a high dose (up to 250 mg/kg body weight) was without observable toxicity and assisted animals in resisting the cytological damage normally inflicted by ionizing radiation. The radical scavenging abilities and the immunomodulatory properties of melatonin provided a reasonable explanation for the enhanced survival of whole body irradiated mice which were pretreated with melatonin (Blickenstaff et al. 1994; Vijayalaxmi et al. 1999). Vijayalaxmi and colleagues (Vijayalaxmi et al. 1999) carried out a study using CD2-F1 male mice that in the peripheral blood and bone marrow cells percentage of polychromatic erythrocytes reduced and the incidence of micronuclei increased after 24 h exposure of whole body to ionizing radiation at a dose of 150 cGy. Melatonin injection in doses of 5 mg/kg or 10 mg/kg 1 h prior to irradiation reversed the damaging effects of radiation in dose dependent manner.

In "in-vitro" investigations using human peripheral lymphocytes which were pre-treated with melatonin, it was found to exhibit significantly reduced radiation-

induced chromosomal damage as compared with those irradiated cells which were not treated with melatonin (VIJAYALAXMI et al. 1995a). VIJAYALAXMI and colleagues (VIJAYALAXMI et al. 1996) showed that irradiated lymphocytes from the volunteers which were collected after 300 mg of oral ingestion of melatonin exhibited a significantly reduced frequencies of chromosomal aberrations and micronuclei, as compared with similarly 100–150 cGy "in-vitro" irradiated cells before the oral intake of melatonin. The same authors examined the extent of primary DNA damage using alkaline comet assay under similar conditions on the same population of blood lymphocytes and found that 1 or 2 h after melatonin ingestion ionizing radiation-induced increases in DNA strand breakage decreased significantly. An inverse correlation between serum melatonin concentration and the number of damaged cells was reported (VIJAYALAXMI et al. 1998). It was also found that the survival curve and the percentage of survival at one year were significantly higher in patients with brain tumor glioblastoma treated with 60 Gy radiotherapy plus 20 mg/daily oral melatonin than those receiving radiotherapy alone. Moreover, radiotherapy related toxicity was lower in patients concomitantly treated with melatonin and it was suggested that melatonin not only prolongs the survival time but also improves the quality of life due to decreases of radiotherapy related toxic effects (LISSONI et al. 1996). The findings of the present study are consistent with the overall studies which indicates that melatonin can protect cells from radiation-induced oxidative damage.

Conclusion

Melatonin can be effective in reducing radiation-induced toxicity since 100 mg/kg, i.p., melatonin pre-treatment of rats were found to decrease the DNA damage and the lipid peroxidation in irradiated brain cells. Therefore, the radioprotective effects of melatonin against cellular damage caused by oxidative stress and its low toxicity make the substance a potential supplement in the treatment or co-treatment in medical such as radiotherapy of brain tumours or occupational settings where the effects of ionizing radiation are to be minimized.

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