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Histopathological Effects of Melatonin in Hyperammonemia Induced Wistar Rats

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Abstract

Exposure to ammonium acetate induces acute and chronic renal injuries as well as oxidative stress in rats. The aim of this study was to evaluate the effect of exogenous melatonin (MLT) treatment on ammonium acetate induced oxidative stress and hepatotoxicity in Wistarrats using biochemical parameters and histopathology. For histopathological evaluation, liver of all rats were excised and processed for light microscopy. Liver sections of ammonium acetate treated group showed changes in microanatomy. In contrast, these deleterious histopathological alterations resulting from ammonium acetate induced hepatotoxin were absent after MLT treatment in ammonium acetate treatment+MLT treated group of rats. In conclusion, our results demonstrated that MLT through its antioxidant activity effectively ameliorated ammonium acetate induced hepatotoxicity.

Keywords: Melatonin; Ammonium Acetate; Hepatotoxicity; Histopathology; Antioxidants.

Introduction

Ammonia is a catabolic product of protein and nitrogenous compounds that is formed in mammal and humans. At high levels, ammonia is neurotoxic, it affects the functions of the central nervous system, and leads to coma and death [Plum et al 1976]. Hyperammonemia caused by insufficient removal of ammonia in the liver [Meijer et al 1990] or portacaval shunting[Butterworth RF 1987], leads to an increase in ammonia level in the brain [Butterworth RF 1987], which is responsible for development of hepatic encephalopathy [Adams et al 1953, Butterworth 1995]. Ammonia intoxication impairs mitochondrial function [Kosenko et al 1997], which could lead to decreased ATP synthesis and also to increased formation of free radicals [Kosenko et al 1999]. The major toxic effects of ammonia likely involve changes in cellular pH and the depletion of certain citric acid cycle intermediates, in particular á -ketoglutarate. It has been reported that sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress condition. Melatonin (N-acetyl-5-methoxy-tryptamine) is the chief secretory product of the pineal gland. It is present in virtually all organisms ranging from bacteria [Manchester et al 1995] to mammals [Poeggeler et al 1991]. Recently, it has been reported that a variety of other tissues including retina [Pang et al 1986], Harderian gland [Menendez et al 1987], ovary [Itoh et al 1997], testes [Tijimes et al 1996] and bone marrow [Conti et al 2000, Tan et al 1999] may also synthesize melatonin. Melatonin is and endogenous free radical scavenger [Tan et al1993] and a broad spectrum antioxidant [Reiter et al 1998]. It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxy nitrite anion, singlet oxygen and nitric oxide [Reiter et al 1999]. Melatonin, which

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shows extreme diffusibility through membranes, is important for its scavenging action, since it could enter all cells and every subcellular compartment.

Melatonin (MLT) N- acetyl – 5- methoxytryptamine is a hormone found in all living creatures from algae (Caniato et al 2003) to humans, at levels that vary in a diurnal cycle.

MLT is produced by pinealocytes in the pineal gland. Melatonin participates in many important physiological functions, including anti-inflammatory (Cuzzocrea and Reiter 2002), also it is a powerful antioxidant that can easily cross cell membranes and the blood brain barrier (Hardeland 2005). MLT as an antioxidant is effective in protecting membranes lipids, nuclear DNA and protein from oxidative damage both invivo and invitro (Reiter et al, 1998a, Lussardi et al 2000, Altkinson et al 2003).

Melatonin may exert certain biologic effect such as the inhibition of stress – induced immuno-depression by augmenting the immune response (Maestroni ,1993).

Materials and Methods

Adult male Wistar rats (weighing 180–220 g), obtained from National Center for Laboratory Animal Sciences, Hyderabad, were kept at room temperature (32 ± 2°C) at L:D (12:12) cycles. All studies were conducted in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" [18]. Animals were randomized and separated into four groups (Group I – control, Group II – ammonium acetate-treated, Group III – ammonium acetate- and melatonin-treated, Group IV – melatonin-treated; n = 6 in each group). Food pellets (Kamadhenu Agencies, Bangalore, India) and water were available *ad libitum* to animals. Melatonin (salt form) was purchased from Sisco Research Laboratories Private Limited, Mumbai, India. Ammonium acetate and all other chemicals used in this study were of analytical grade. Group I animals served as controls. Group II animals were administered with ammonium acetate intraperitoneally (*ip*) (100 mg/kg) every day for 45 days [Hilgier et al 1990]. Group III animals were treated with ammonium acetate as Group II animals along with melatonin (5 mg/kg) (*ip*) [Liu 2000]. Group IV animals received melatonin (5 mg/kg) (*ip*) for 45 days. Biochemical determinations were performed after 45 days of ammonium acetate and/or melatonin administration. At the end of experimental period (45 days) animals from all the groups were sacrificed by cervical dislocation.

Preparation of Tissue Homogenate

The tissue was rinsed in cold physiological saline, cleaned of gross adventitial tissue, blotted dry and stored in a Biofreezer at -80^{0}C until analysed. Liver tissue was homogenized with Potter Elvejham homogenizer, 10% homogenates, were prepared in 6.7mμ phosphate buffer, pH7.4 and centrifuged at 10,000rpm for 10 min at 4^{0}C and the resultant supernatant was used for measurement of antioxidant enzymes.

Assay of Antioxidant Enzymes

Superoxide Dismutase Activity (SOD)

Liver SOD activity was assayed by the method of Kakkar *et al.,* (1984). Reaction mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 mM, pH 7.0), 0.1 ml of phenazine methosulphate (PMS) (186 μM), 0.3 ml of nitro blue tetrazolium (NBT) (300 μM). 0.2 ml of the supernatant obtained after centrifugation (1500 x g, 10 min followed by 10,000 x g, 15 min) of 10% liver homogenate was added to reaction mixture. Enzyme reaction was initiated by adding 0.2 ml of NADH (780 μM) and stopped precisely after 1 min by adding 1 ml of glacial acetic acid. Amount of chromogen formed was measured by recording color intensity at 560 nm. Results are expressed as units/mg protein.

Glutathione Peroxidase Activity (GSH-Px)

Glutathione peroxidase (GSH-Px) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide (Tappel, 1978). 100 μL of enzyme sample was incubated for five minutes with 1.55 ml stock solution (prepared in 50 mM Tris buffer, pH 7.6 with 0.1 mM EDTA) containing 0.25 mM GSH, 0.12 mM NADPH and 1 unit glutathione reductase. The reaction was initiated by adding 50 μL of cumene hydroperoxide (1 mg/ml), and the rate of disappearance of NADPH with time was determined by monitoring absorbance at 340 nm. One unit of enzyme activity is defined as the amount of enzyme that transforms 1 μmol of NADPH to NADP per minute. Results are expressed as units/mg protein.

Catalase Activity (CAT)

The activity of CAT was measured in the liver

using its perioxidatic function according

to the method of Johansson and Borg, (1988). 50 ìL potassium phosphate buffer (250 mM, pH 7.0) was incubated with 50 µl methanol and 10 µL hydrogen peroxide (0.27%). The reaction was initiated by addition of 100 ìL of enzyme sample with continuous shaking at room temperature (20°C). After 20 minutes, reaction was terminated by addition of 50 µL of 7.8 M potassium hydroxide. 100 µL of purpald (4-Amino-3-hydrazino-5- mercapto-1,2,4-triazole, 34.2 mM in 480 mM HCl) was immediately added, and the mixture was again incubated for 10 minutes at 20°C with continuous shaking. Potassium peroxidate (50 µL 65.2 mM) was added to obtain a colored compound. The absorbance was read at 550 nm in a spectrophotometer. Results are expressed as micromoles of formaldehyde produced/mg protein.

Reduced Glutathione (GSH)

Reduced glutathione (GSH) level in the liver was assayed following the method of Ellman (1959). The homogenate (720 µl) was double diluted and 5% TCA was added to it to precipitate the protein content of the homogenate. After centrifugation (10, 000 x g for 5 minutes) at 4°C the supernatant was taken, 5,5´-dithiolbis-2-nitrobenzoic acid (DTNB) solution (Ellman's reagent) was added to it and the absorbance was measured at 412nm on a spectrophotometer. A standard graph was drawn using different concentrations of standard GSH solution (1 mg/ml). With the help of the standard graph, GSH contents in the homogenates of the experimental animals were calculated.

Thiobarbituric Acid Reactive Substances

(TBARS)

The product of the reaction between malondialdehyde (MDA) and thiobarbituric acid

reactive substances (TBARS) were measured by the method of Ohkawa *et al*., (1997). The reaction mixture consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10% (w/v) of homogenate. The mixture was brought to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1 v/v) was added and centrifuged at 3000 rpm for 10 minutes. The organic layer was taken out and absorbance of the clear upper (n-butanol) layer was measured using Shimadzu UV-1601 (Japan) spectrophotometer at 532 nm. TBARS were quantified using an extinction coefficient of 1.56 x 105 cm-1 M-1 and expressed as nmol of TBARS/ mg tissue protein. Tissue protein was estimated using Biuret method of protein assay and the liver MDA content expressed as nmol of MDA per mg protein.

Procedure for Histopathology

Liver tissues were excised from sacrificed animals, individually weighed, and thin liver slices were cut, fixed in 4% paraformaldehyde and were sequentially embedded in paraffin wax blocks. Tissue sections of 5 ìm thick were cut, and stained with hematoxylin eosin (H-E) and masson's trichrome (Suzuki and Suzuki, 1998) for conventional morphological evaluation, then examined under light microscope (BX50; Olympus, Tokyo). The images were obtained by a digital camera system (Pixcera Co., Osaka, Japan) attached to the microscope. The scoring was done as none (-), mild (+), moderate (++) and severe (+++).

Results

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group II is compared with Group I (^{a} $p < 0.001$), Group III is compared with Group II ($p < 0.001$), Group IV is compared with Group I; ns not significant. The units for TBARS is n moles/10g tissue, GSH is mg/g tissue, SOD is 50% inhibition of NBT redn /min/mg/protein and CAT is µmoles of H_2O_2 consumed/min/mg/protein. GPx µg of GPx consumed/ min /mg/protein.

Table 1: Changes in the levels of TBARS and antioxidants in liver

	TBARS	GSH	SOD	CAT	GPx
Group I	1.77 ± 0.08	23.62 ± 1.97	4.94 ± 0.49	77.13 ± 7.43	12.03 ± 1.24
Group II	3.73 ± 0.32^{xxx}	11.30 ± 0.91^{xxx}	2.22 ± 0.09^{xxx}	34.28 ± 2.35^{xxx}	4.71 ± 0.25^{xxx}
Group III	2.42 ± 0.16^{xxx,a}	18.23 ± 0.79^{xxx,a}	4.40 ± 0.49^{xxx,a}	68.62 ± 3.32^{xxx,a}	8.78 ± 0.64^{xxx,a}
Group IV	1.73 ± 0.10^{ns}	23.14 ± 1.61^{ns}	5.03 ±0.51^{ns}	74.80 ± 6.06^{ns}	12.00 ± 1.16^{ns}

Table 2: Histopathological changes in liver

Microscopic Observation	Group I (Control)	Group II (Ammonium acetate treated)	Group III (Ammonium acetate& melatonin treated)	Group IV (Melatonin treated)
Hepatic damage	A	+++	A	A
Inflammatory collections	A	+++	A	A
Sinusoidal dilatation	A	+++	++	+

A - Absent
\+ - Present
++ - Moderately present
+++ - Highly present

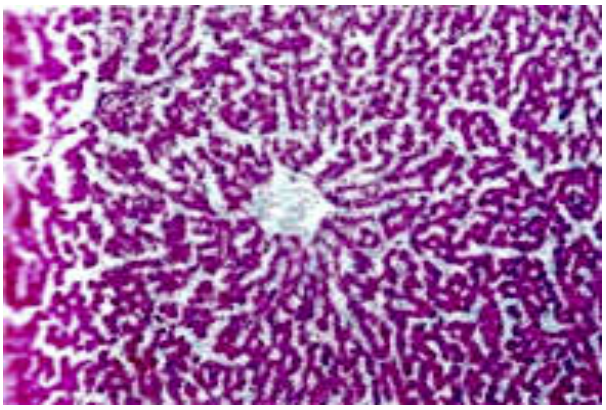

Fig. 1: Normal architecture of liver (control) H&EX 20

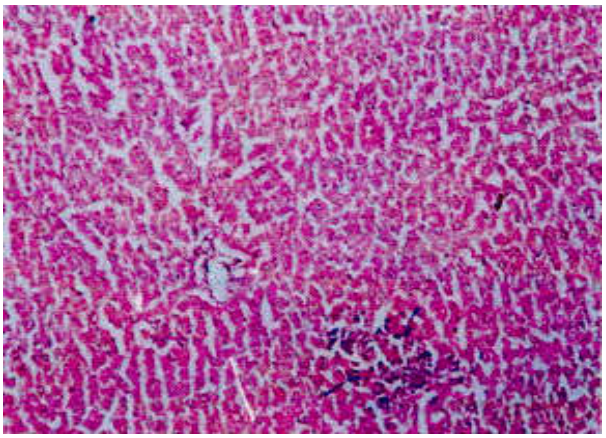

Fig. 2: Liver section showing focal hepatic damage with inflammatory collections (ammonium acetate treated) H&EX20

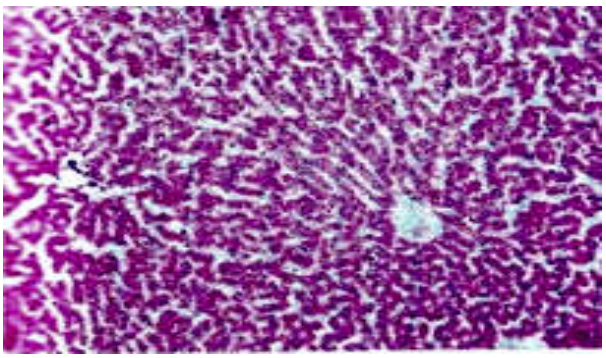

Fig. 3: Liver section showing marked reduction in inflammatory collection (ammonium acetate & melatonin treated) H&EX20

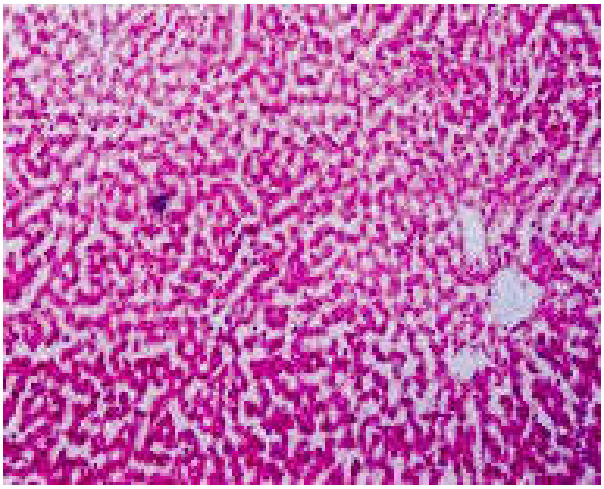

Fig. 4: Liver section of melatonin treated rat showing normal architecture

Discussion

Reports have shown that excess ammonia induces nitric oxide synthase which leads to enhanced production of nitric oxide, leading to oxidative stress and liver damage [Kosenko et al 1999, Schliess et al 2002]. The decrease in urea and ammonia in melatonin-treated rats maybe due to the antioxidant potential of melatonin. Melatonin has been proved to be an effective free radical scavenger [Reiter et al 1998, Tan et al 1993], by inhibiting the pro-oxidant enzyme nitric oxide synthase [Pozo et al1994].

Ammonia intoxication enhances lipid peroxidation and leads to the formation of free radicals [Kosenko et al 1997, Vidhya et al 2003]. This might account for the increased levels of TBARS (which is a measure of lipid peroxidation and an index of membrane oxidative damage) and decreased vitamin C and E levels in ammonium acetate-treated rats. Ammonia intoxication depletes the level of glutathione (GSH) [Kosenko et al 2000]. The protective capability of antioxidants against free radical-induced damage is increased if the scavenging molecule can be recycled. Mahal et al. (1999) reported regeneration of melatonin from the one-electron oxidized melatonyl radical by both ascorbate and urate. Melatonin directly scavenges hydrogen peroxide to form N1-acetyl-N2-formyl-

5methoxykynuramine, which, by the action of CAT forms N1-acetyl-5-methoxykynuramine [Tan et al 2000]. These biogenic amines could also scavenge hydroxyl radical and reduce lipid peroxidation.

In our study, the decreased activities of antioxidant enzymes (SOD and CAT, GPx and GSH) in ammonium acetate treated group may be due to the inhibition of these enzymes by nitric oxide. It is known that ammonia-induced inhibition of antioxidant enzymes is mediated by the activation of NMDA receptors of nitric oxide synthase and formation of nitric oxide which inhibits the activities of antioxidant enzymes [Kosenko et al 1999]. The indole moiety of the melatonin molecule is the reactive center of interaction with oxidants due to its high resonance stability and low activation energy barrier towards the free radical reactions. The methoxy and amide side chains also contribute significantly to melatonin's antioxidant capacity. The methoxy group in C5 appears to keep melatonin from exhibiting pro-oxidative capacity [Tan et al 2002].

Control Rats

The liver of control rats of all experimental groups examined by the light microscope showed that same normal hepatic structure. The structural unit of liver is the hepatic lobule, which is made up of cords or strands of cells forming a network around a central vein. The liver strands are alternating with narrow sinusoids. These sinusoids have irregular boundaries composed of a single layer of fenestrated endothelial cells and large irregularly phagocytic cells called as Kupffer cells. The hepatocytes are polyhedral in shape with relatively large size and granular cytoplasm. Each cell has a centrally located nucleus and binucleate hepatocytes were observed. Outside the hepatic lobules, collagen fibres are distributed and branches of portal vein, hepatic artery and bile duct are present.

Ammonium Acetate Treated Rats

Ammonium acetate treatment in rats results in loss of normal architecture of the liver with marked cytoplasmic vacuolation and sinusoidal dilatation was also observed. Microscopic foci of hepatic injury were observed with small hepatocytes admixed with macrophages, Cirrhosis and necrosis was also observed. Acute inflammation in blood vessels was detected.

Ammonium Acetate and Melatonin Treated Rats

The histological examination of liver of ammonium acetate and melatonin treated group showed less necrosis and less inflammation. It exhibited some histological regeneration and the reversed cirrhosis condition.

Melatonin Treated Rats

These rats showed no inflammation but a mild sinusoidal dilatation was observed in them. These rats showed no changes and were similar to that of control rats.

Conclusion

Melatonin is a very potent endogenous free radical scavenger. Receptor- dependent actions of melatonin, e.g. an antioxidative enzyme induction, oxide synthase which generates nitric oxide (ii) directly scavenging a variety of radicals and reactive oxygen species, (iii) inducing antioxidative enzymes which reduce steady state levels of reactive oxygen species. It reacts with highly toxic radicals and provides protection against oxidative damage. Melatonin acts as a primary non enzymatic antioxidative defense against the devastating action of free radicals (Abdel Wahab 1997). Melatonin has been proved to be an effective free radical scavenger [Reiter et al 1998, Tan et al 1993], by inhibiting the pro-oxidant enzyme nitric oxide synthase [Pozo et al 2000].

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