Research Article

HEPATOPROTECTIVE INFLUENCES OF MELATONIN ON THE LEVELS OF ANTIOXIDANTS AND LIPID PEROXIDATION IN HYPERAMMONEMIC WISTAR RATS

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ABSTRACT

The antioxidant potential of melatonin (MLT) on hyperammonemia induced by ammonium acetate treatment was studied in rats. In liver tissue, the levels of thiobarbituric acid reactive substances and lipid profile variables was observed to be increased significantly in ammonium acetate treated rats and decreased significantly in rats treated with melatonin and ammonium acetate. Further, enzymatic, superoxide dismutase, catalase and glutathione peroxidase and non-enzymatic, reduced glutathione antioxidants in liver tissues decreased significantly in ammonium acetate treated rats and increased significantly in rats treated with melatonin and ammonium acetate. These biochemical alterations could be due to the ability of melatonin to (i) scavenge a variety of radicals and reactive oxygen species (ii) induce antioxidative enzymes which reduce steady state levels of reactive oxygen species and (iii) stabilize cell membranes which assist them in reducing oxidative damage and thus could prevent oxidative stress in rats.

INTRODUCTION

Ammonia is a catabolic product of protein and nitrogenous compounds that is formed in mammals and humans. At high levels, ammonia is neurotoxic; it affects the functions of the central nervous system, and leads to coma and death (1). Hyperammonemia, caused by insufficient removal of ammonia in the liver (2) or portacaval shunting (3), which is responsible for the development of hepatic encephalopathy (4). Ammonia intoxication impairs mitochondrial function (5), which could lead to decreased ATP synthesis and increased formation of free radicals (6). The major toxic

effects of ammonia likely involve changes in cellular pH and the depletion of certain citric acid cycle intermediates, in particular α-keto-glutarate. Sustained Hyperammonemia in mice leads to increased lipid peroxidation in liver, reflecting an oxidative stress condition (7, 8). Melatonin (*N*-acetyl-5-methoxy-tryptamine) is the main secretory product of the pineal gland. It is present in virtually all organisms ranging from bacteria (9) to mammals (10). Further, Melatonin is an endogenous free radical scavenger (11) and a broad spectrum antioxidant (12). It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide (13). Melatonin, which shows extreme diffusibilty through membranes, is important for its scavenging action, since it could enter all cells and every subcellular compartment.

Systematic investigations of the levels of lipid peroxidation products and the levels of enzymic and non-enzymic antioxidants under the conditions of hyperammonemia are lacking. The present study deals with the levels of thiobarbituric acid reactive substances (TBARS-the products of lipid peroxidation) and the levels of catalase, superoxide dismutase and glutathione peroxidase (enzymatic antioxidants) and reduced glutathione (non-enzymatic antioxidant) in the tissue under the condition levels of hyperammonemia and during melatonin treatment in rats. Furthermore, the levels of lipid profile variables (free fatty acids, triglycerides, phospholipids and cholesterol) in all the groups were investigated.

MATERIALS AND METHODS

Adult male Wistar rats (180-220g), obtained from National Centre for Laboratory Animal Sciences, Hyderabad, were maintained in polypropylene cages in a controlled environment (22-24^{0}C) under 12:12h light dark cycles. Standard pellet diet purchased from (Kamadhenu Agencies, Bangalore, India) and water were provided *ad libitum*. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (14). Melatonin was purchased from Sisco Research Laboratories Private Limted, Mumbai, India. Ammonium acetate and all other chemicals used in this study were of analytical grade.

The animals were divided into four groups of six rats each and all were fed with the standard pellet diet. Group I animals served as controls. Group II animals were administered with ammonium acetate intraperitonealy (100 mg/kg) every day for 45days (15) Group III animals were treated with ammonium acetate as group II animals along with melatonin (5mg/kg) intraperitonealy (16). Group IV animals received melatonin (5 mg/kg) intraperitonealy throughout the experiment.

The experiment was terminated after 45 days and all animals were killed by cervical decapitation. Blood samples were collected from each group of rats. Rats were sacrificed by cervical dislocation and liver samples were collected and stored at -80^0 C. Liver samples from each rat were homogenized in freshly prepared phosphate buffer saline. Tissue homogenate was used for the estimation of TBARS (17). Remaining volume of homogenate was centrifuged at 5000g for 15 min at 4^{0}C. The supernatant was collected and used for the estimation of antioxidants such as catalase, superoxide dismutase, glutathione peroxidase and reduced glutathione. The extent of lipid peroxidation (TBARS) in liver was determined by measuring malondialdehyde content based on the reaction with thiobarbituric acid (TBA) (17). Data were expressed nmoles per 100 gm tissue. Catalase activity was determined by measuring the decomposition of hydrogen peroxide at 240nm (18). Data was expressed as units per mg protein. Tissue glutathione content in liver homogenate was measured by biochemical assay using dithionitrobenzoic acid (DTNB) (19). Superoxide Dismutase has been assayed by a spectrophotometric method based on the inhibition of a superoxide – driven NADH oxidation (20). Data was expressed as 50% inhibition of NBT reduction /min/mg protein. Glutathione Peroxidase activity was assayed by following the oxidation of NADH at 340nm in the presence of glutathione reductase which catalyzed the reduction of GSSG formed by the peroxidase (21). Data was expressed as µg of GPx consumed min/mg protein. Protein concentration was determined by the method of Bradford (22).

Lipids from the tissues were extracted by the method of Folch (23). Total cholesterol was determined by the method of Zlatkis (24). Lipid extract was treated with ferric chloride acetic acid reagent to precipitate the proteins. The protein free supernatant was treated with concentrated sulphuric acid a reddish purple color formed was read in a Spectronic20 colorimeter at 560nm.Values are expressed as mg/100g tissue or mg/dl. Phospholipids were determined by the method of Zilversmit (25). The formation of stable blue color, which was read in a colorimeter at 680nm.The amounts of phospholipids are expressed as mg/100g tissue or mg/dl. Triglycerides were determined by the method of Foster (26).The absorbance of yellow colored compound was read in a Spectronic20 colorimeter at 405nm. The triglyceride content is expressed as mg/100g tissue or mg/dl. Free fatty acids were determined by the method of Falholt (27). Non esterified free fatty acids were estimated by the method of copper soap formation Falholt (27). The amounts of free fatty acids are expressed as mg/100g tissue or mg/dl.

The data were analyzed using an analysis of variance (ANOVA) and the group means were compared by Least Significant Difference (LSD) test. The results were considered statistically significant if the p-value was 0.05 or less.

RESULTS AND DISCUSSION

Ammonia is removed either in the form of urea in periportal hepataocytes and/or as glutamine in perivenous hepatocytes (28). Elevated levels of ammonia, in ammonium acetate treated rats may be due to the tissue damage caused by ammonia induced free radical generation, leading to oxidative stress and tissue damage. (6,8,30). Melatonin is an effective free radical scavenger (13) which by its antioxidant potential decreases the ammonia levels of, Under hyperammonemia conditions, elevated levels of ammonia result in the production of free radicals such as hydroxyl radicals, superoxide radicals, peroxyl radicals, alkoxyl radicals and reactive nitrogen species.

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^{xxxa}	18.23 ± 0.79^{xxx,a}	4.40 ± 0.49^{xxx,a}	68.62 ± 3.32^{xxxa}	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}

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group II is compared with Group I (^{a} p <o.oo1), Group III is compared with Group II (p < 0.001), Group IV is compared with Group I; ns not significant. The unites for TBARS is nmoles/10g tissue, GSH is mg/g tissue, SOD is 50% inhibition of NBT redn /min/mg/protein and CAT is µ moles of H_2O_2 Consumed/min/mg/protein. GPx µg of GPx consumed/ min/mg/protein.

Elevated levels of TBARS have been observed in the liver tissue of ammonium acetate treated rats indicating the increased levels of lipid peroxidation (Table 1). It is a well-established fact that ammonia intoxication enhances lipid peroxidation and generates free radicals .The levels of TBARS in ammonium acetate and melatonin treated rats were significantly decreased when compared to group 2 rats. This suggests that melatonin could offer protection against lipid peroxidation (31).

Table 2: **Changes of lipid profiles in liver tissue**

	Free fatty acid	Phospholipids	Triglycerides	Cholesterol
Group I	596.76 ± 57.53	950.18 ± 102.82	342.84 ± 29.04	348.62 ± 32.67
Group II	910.75 ± 73.13^{xxx}	1618.84 ± 57.37^{xxx}	653.06 ± 32.84^{xxx}	565.86 ± 55.47^{xxx}
Group III	748.06 ± 54.69^{xxx,a}	1299.87 ±98.98^{xxx,a}	422.78 ± 30.14^{xxx,a}	458.83 ± 33.75^{xxx,a}
Group IV	573.30 ± 61.07^{ns}	941.01 ± 100.33^{ns}	458.83 ± 33.75^{xxx,a}	340.62 ± 30.41^{ns}

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group II is compared with Group I (^{a} p <o.oo1). Group III is compared with Group II (p < 0.001). Group IV is compared with Group I; ns not significant. Free fatty acid, Phospholipids, Triglycerides, Cholesterol are expressed as mg/100g of tissue.

The non-enzymatic antioxidant glutathione is a scavenger of hydroxyl radicals and singlet oxygen (32). It has been reported that ammonia intoxication induces depletion of glutathione and an increase in lipid peroxidation (5).Reports have also shown that ammonia intoxication leads to the increased formation of nitric oxide which results in the oxidation of glutathione (GSH) to glutathione disulphide (GSSG) and to mixed glutathione disulphides (GSSR) resulting in depletion of GSH and increased free radical formation (33). Group III rats compared to Group II rats showed elevated levels of glutathione. This is because, under hyperammonemic conditions, melatonin increases the levels of glutathione, an important intracellular antioxidant, by stimulating its rate-limiting enzyme, Ɣ-glutamylycysteine synthase (34).

In our study, the decreased activities of antioxidant enzymes (SOD, CAT, GPx) in the 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 that leads to increased intracellular calcium levels, which in turn activate neuronal nitric oxide synthase, leading to the formation of nitric oxide which inhibits the activities of antioxidant enzymes (6). Under hyperammonemic conditions, melatonin causes an increase in the gene expression and activities of the antioxidant enzymes such as glutathione peroxidase, glutathione reductase and superoxide dismutase (35,36) which results in the elevated levels of these enzymes in Group II rats. The elevated levels of these enzymes might protect against oxidative damage caused by the free radical formation (37). Melatonin directly

scavenges hydrogen peroxide to form N^1 –acetyl-N^2 –forms N^1 –acetyl-5-methoxy Kynuramine (38). These biogenic amines could also scavenge hydroxyl radicals and reduce lipid peroxidation. Ammonium acetate may deplete the levels of α-KG and other Krebs cycle intermediates (39) and thus elevate the levels of acetyl coenzyme A. The elevated levels of acetyl coenzyme A may increase the levels of lipid profile variables (free fatty acids, triglycerides, phospholipids and cholesterol) as observed in our study (Table 2). The decreased α-KG levels in rats treated with ammonium acetate might be reversed during treatment with melatonin, since melatonin was found to reduce these levels (40).

CONCLUSION

Our results suggest that melatonin could control the oxidative stress caused by hyperammonemia by (i) directly scavenging a variety of radicals and reactive oxygen species (ii) inducing antioxidative enzymes which reduce steady state levels of reactive oxygen species and by (iii) stabilizing cell membranes which assist them in reducing oxidative damage.

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