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Nephroprotective effects of melatonin in hyperammonemia induced oxidative stress in Wistar rats

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Abstract: The antioxidative action of melatonin on ammonium acetate induced hyperammonemia was studied in rats. In kidney 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. Enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase and non -enzymatic antioxidants such as reduced glutathione in kidney tissues decreased significantly in ammonium acetate treated rats and increased significantly in rats treated with melatonin and ammonium acetate. These biochemical alterations during melatonin treatment could be due to i) scavenging action of free radicals ii) induction of antioxidative enzymes that reduces the concentration of reactive oxygen species iii) inhibition of nitric oxide synthase Iv) cell membrane stabilization which assists them in reducing oxidative damage and thus prevents oxidative stress in rats.
Keywords: melatonin, antioxidants, thiobarbituric acid reactive substances, lipid peroxidation, kidney.

Introduction:

In mammals and humans, ammonia is the end product of protein metabolism. Ammonia is neurotoxic at high levels, it affects the functions of the nervous system and leads to coma and death[1]. Insufficient removal of ammonia causes hyperammonemia[2] or portacaval shunting[3] leads to increased ammonia levels in the brain, 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]. Changes in cellular pH and the depletion of certain citric acid cycle intermediates in particular alpha ketoglutarate , are the major toxic effects of ammonia. Prolonged htperammonemia condition in mice, results in increased lipid peroxidation in kidney[7,8]. Melatonin (N- acetyl – 5- methoxy tryptamine) is the main secretory product of the pineal gland. It is present virtually in all organisms ranging from bacteria [9] to mammals[10]. Melatonin is a broad spectrum antioxidant [11] and an endogenous free radical scavenger [12]. It detoxifies a variety of free radicals and reactive oxygen species including the hydroxyl radicals, peroxy nitrite anion and nitric oxide[13]. Melatonin is permeable to membranes by which it could enter the cells and subcellular compartment, to impart its scavenging action. However, the antioxidant potential of melatonin during hyperammonemia has not been investigated so far. 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 kidney under induced hyperammonemic condition 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.

Experimental:

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 (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 intraperitoneally (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) intraperitoneally[16]. Group IV animals received melatonin (5 mg/kg) intraperitoneally 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. Rat were sacrificed by cervical dislocation and kidney samples were collected and stored at -80^0 C. Kidney 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 wasread 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:

In the form of urea, ammonia is removed in the periportal hepatocytes and or as glutamine in perivenous hepatocytes[28]. In the ammonium acetate treated rats, increased levels of ammonia shows that the tissue damage caused by hyperammonemia induced free radical formation, leading to oxidative stress and tissue damage [6,8,29,30]. Melatonin is an effective free radical scavenger [13], which by its antioxidant potency decreases the ammonia levels. As a consequence of hyperammonemia, free radicals such as hydroxyl radicals, superoxide radicals, peroxyl radicals, alkoxyl radicals and reactive nitrogen species levels are elevated.

Table1: Changes in the levels of TBARS and antioxidants in kidney

	TBARS	GSH	SOD	CAT	GPx
Group I	1.77 ± 0.12	21.94 ± 2.00	4.82 ± 0.43	57.56 ± 5.71	8.64 ± 0.66
Group II	3.13 ± 0.19^{xxx}	9.16 ± 0.58^{xxx}	2.81 ± 0.17^{xxx}	26.25 ± 2.29^{xxx}	4.21 ± 0.16^{xxx}
Group III	2.21 ± 0.16^{xxxa}	13.64± $0.69^{xxx,a}$	4.15 ± $0.24^{xxx,a}$	62.4 ± 4.22^{xxxa}	7.31 ± $0.39^{xxx,a}$
Group IV	1.67 ± 0.14^{ns}	21.58± 1.27^{ns}	4.87 ±0.43^{ns}	55.18 ± 5.48^{ns}	8.59 ± 0.77^{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 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 μ moles of H_{2O_2} consumed/min/mg/protein. GPx μg of GPx consumed/ min /mg/protein.

The levels of TBARS in ammonium acetate and melatonin treated rats were significantly decreased when compared to group II, which suggests that melatonin rendered protection against lipid peroxidation[30]. Glutathione is a non enzymatic antioxidant and scavenges hydroxyl radicals and singlet oxygen[31].

Reports have shown that the ammonia intoxication induces depletion of glutathione and an increase in lipid peroxidation[5]. It has been reported that ammonia intoxication leads to the increased formation of nitric oxide, which results in the oxidation of glutathione (GSH) and to glutathione disulphides resulting in depletion of GSH and increases free radical formation[32]. Group III rats when Compared to Group II rats showed elevated levels of glutathione,which is because of melatonin's ability to increase the levels of glutathione by stimulating its rate limiting enzyme γ – glutamyl cysteine synthase[33].

In the present study, in ammonium acetate treated rats, the decreased activities of antioxidant enzymes (SOD, CAT &GPX) may be due to inhibition by nitric oxide. It is known that ammonia 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 activates neuronal nitric oxide synthase, leading to the formation of nitric oxide which inhibits the activities of antioxidant enzymes[6].

Under hyperammonemic conditions, melatonin is found to increase the gene expression and activities of antioxidant enzymes such as glutathione peroxidase, glutathione reductase and superoxide dismutase[34,35] that results in Group II rats. The elevated levels of these enzymes might protect against oxidative damage caused by free radical formation[36].

The indole moiety present in the melatonin molecule is the reactive centre of interaction with oxidants, because of its high resonance stability and low activation energy barrier towards free radical reactions. The methoxy and amide side chains of melatonin contributes to its antioxidant capacity. The methoxy group in C_5 melatonin prevents from exhibiting pro-oxidative capacity [36]. Melatonin scavenges hydrogen peroxide and forms N^1 – acetyl – N^2 – forms N^1 – acetyl 5 -methoxy kynuramine[37]. 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[38] 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[39].

Table 2: Changes of lipid profiles in kidney tissue

	Free fatty acids	Phospholipids	Triglycerides	Cholesterol
Group I	433.18 ± 39.97	884.53 ± 99.15	476.96 ±32.12	639.91 ± 43.96
Group II	771.73 ± 49.38^{xxx}	1404.12± 109.58^{xxx}	704.81 ± 37.35^{xxx}	972.25 ± 71.42^{xxx}
Group III	535.58± $38.59^{xxx,a}$	1095.05±$105.10^{xxx,a}$	491.50 ± $46.56^{xxx,a}$	829.02 ± $18.30^{xxx,a}$
Group IV	422.09 ± 39.81^{ns}	878.67 ± 85.68^{ns}	470.20 ± 32.55^{ns}	636.26 ± 41.96^{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 acids, Phospholipids, Triglycerides, Cholesterol are expressed as mg/100g of tissue.

Conclusion:

Receptor – dependent actions of melatonin such as an antioxidative enzyme induction, seem to contribute to the antioxidant potential of melatonin. Our results suggests that melatonin could control the oxidative abuse by i) the direct scavenging action of radicals and reactive oxygen species ii) inhibition of nitric oxide synthase which generates nitric oxide iii)induction of antioxidative enzymes which reduces steady state levels of reactive oxygen species, iv)stabilization of cell membranes which assists in reduction of oxidative damage.

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