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

Peter Jasmin Lena, Perumal Subramanian

Perumal Subramanian, Department of Biochemistry, Faculty of Science, Annamalai University,

Annamalainagar – 608002, TamilNadu, India

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.

1. 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 (Plum et al. 1976). Hyperammonemia, caused by insufficient removal of ammonia in the liver (Meijer et al.1990) or portacaval shunting (Butterworth et el. 1987), which is responsible for the development of hepatic encephalopathy (Butterworth et al. 1995). Ammonia intoxication impairs mitochondrial function (Kosenko et el. 1997) which could lead to decreased ATP synthesis and increased formation of free radicals (Kosenko et al. 2000). 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 and brain, reflecting an oxidative stress condition (Connor and Costell 1990; Dakshayani et al. 2002). 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 (Manchester et al. 1995) to mammals (Poeggeler et al. 1991). Further, Melatonin is an endogenous free radical scavenger (Tan et al. 1993) and a broad spectrum antioxidant (Reiter et al.1993). It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide (Reiter et al.1999). 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-enzymatic 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 conditions 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 (180-220 g), obtained from National Centre for Laboratory Animal Sciences, Hyderabad, were maintained in polypropylene cages in a controlled environment (22-240 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 (National Institute Guide, 1985). 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 (Hilgier et al. 1990). Group III animals were treated with ammonium acetate as group II animals along with melatonin (5mg/kg) intraperitoneally (Liu and Ng 2000). 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. Biochemical determinations were done by the methods mentioned in Table 1

The data were analysed 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.

Table1**: Biochemical determinations and methods**

|  |  |  |
| --- | --- | --- |
| Parameter | Studies carried out in Tissue | Method |
| Thiobarbituric acid reactive substances | liver | Nichans and Samuelson (1951) |
| Reduced glutathione | liver | Ellman (1959) |
| Superoxide dismutase | liver | Kakkar et al. (1984) |
| Catalase | liver | Sinha (1972) |
| Glutathione peroxidase | liver | Rotruck et al. (1973) |
| Free fatty acids | liver | Falholt et al (1973) |
| Phospholipids | liver | Zilversmit et al. (1950) |
| Triglicerides | liver | Foster and Dunn (1973) |
| Cholestrol | liver | Zlatkis et al. (1953) |

Results and Discussion

Ammonia is removed either in the form of urea in periportal hepataocytes and/or as glutamine in perivenous hepatocytes (Nelson and Cox 2000) 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 (Kosenko et al. 2000; Dakshayani et al. 2002; Vidya et al.2003). Melatonin is an effective free radical scavenger (Reiter et al. 1999), which by its antioxidant potential decreases the ammonia levels of, Under hyperammonemic 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: 2

Changes in the levels of TBARS and antioxidants in liver

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameter | Group I | Group II | Group III | GroupIV |
| TBARS  (nmoles /100g tissue) | 1.77 ± 0.08 | 3.73 ± 0.32xxx | 2.42 ± 0.16xxxa | 1.73 ± 0.10ns |
| GSH  (mg/g tissue) | 23.62 ± 1.97 | 11.30 ± 0.91xxx | 18.23 ± 0.79xxx,a | 23.14 ± 1.61ns |
| SOD  (50% inhibition of NBT redn /min/mg/protein) | 4.94 ± 0.49 | 2.22 ± 0.09xxx | 4.40 ± 0.49xxx,a | 5.03 ±0.51ns |
| CAT μ moles of H 2 o 2  Consumed/min/mg/protein) | 77.13 ± 7.43 | 34.28 ± 2.35xxx | 68.62 ± 3.32xxxa | 74.80 ± 6.06ns |
| GPx μg of GPx consumed/ min/mg/protein | 12.03 ± 1.24 | 4.71 ± 0.25xxx | 8.78 ± 0.64xxx,a | 12.00 ± 1.16ns |

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

Elevated levels of TBARS have been observed in the liver tissue of ammonium acetate treated rats indicating the increased levels of lipid peroxidation. It is a well-established fact that ammonia intoxication enhances lipid peroxidation and generates free radicals (Kosenko et al. 2000; Dakshayani et al.2002; Vidya et al.2003). 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 peroxidatio (Lastra et al.1997).

The non-enzymatic antioxidant glutathione is a scavenger of hydroxyl radicals and singlet oxygen (Halliwell and Gutteridge 1999). It has been reported that ammonia intoxication induces depletion of glutathione and an increase in lipid peroxidation (Kosenko et al. 1999). Reports have also shown that ammonia intoxication leads to the increased formation of nitric oxide which results in the oxidation of glutathione (GSE) to glutathione disulphide (GSSG) and to mixed glutathione disulphides (GSSR) resulting in depletion of GSH and increased free radical formation (Luperchio et al.1996). Group 3 rats compared to group 2 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, ϒ-glutamylycysteine synthase (Urata et al. 1999).

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 (Kosenko et al.2000) 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 (Barlow et al. 1995; Antolin et al. 1996) which results in the elevated levels of these enzymes in group 3 rats. The elevated levels of these enzymes might protect against oxidative damage caused by the free radical formation (Reiter et al. 2003).

Melatonin directly scavenges hydrogen peroxide to form *N*1 –acetyl-*N*2 –forms *N*1 –acetyl-5-methoxy Kynuramine (Tan et al. 2000). 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 (Yamamoto 1989) 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. 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 (Baydas et al. 2002).

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.

Table 3: Changes of lipid profiles in liver tissue

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Parameter | GroupI | GroupII | GroupIII | GroupIV |
| Free fatty acid  (mg/100g tissue) | 596.76 ± 57.53 | 910.75 ± 73.13xxx | 748.06 ± 54.69xxx,a | 573.30 ± 61.07ns |
| Phospholipids  (mg/100g tissue) | 950.18 ± 102.82 | 1618.84 ± 57.37xxx | 1299.87 ±98.98xxx,a | 941.01 ± 100.33ns |
| Triglycerides  (mg/100g tissue) | 342.84 ± 29.04 | 653.06 ± 32.84xxx | 422.78 ± 30.14xxx,a | 458.83 ± 33.75xxx,a |
| Cholesterol  (mg/100g tissue) | 348.62 ± 32.67 | 565.86 ± 55.47xxx | 458.83 ± 33.75xxx,a | 340.62 ± 30.41ns |

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.

3. References:

PlumF, Hindfelt B, In : Vinken PJ., Bruyn GW., Klawans HI : Eds. Metabolic and deficiency disease of nervous system : the neurological complications of liver disease. New York, Elsevier 1976,349

Meijer AJ, Lamers WH, Chamuleau RAFM (1990) Nitrogen metabolism and ornithine cycle function Physiol Rev 70 :701 – 748.

Butterworth RF, Giguere JF, Michaud J, Lavoie J, Pomier-Layrargues G (1987) Ammonia: key factor in thr pathohenesis of hepatic encephalopathy. Neurochem Pathol 6: 1-12

Butterworth RF (1995) Hepatic encephalopathy. Neurologist 1: 95-104.

Kosenko E., KaminskyY, Kaminsky AValencia M., Lee L. Hermenegildo C., Felipo V Superoxide production and antioxidant enzymes in ammonia intoxication in rats. Free Radical Res 1997 27, 637-644.

Kosenko E., Felipo V., Montiliu C, Grisolia S., Kaminsky Y: Effects of acute hyperammonemia in vivo on oxidative metabolism in non synaptic rat brain mitochondria. Metab. Brain Dis., 1997, 12, 69-82.

O ‘Connor JE, Costell M (1990) New roles of carnitine metabolism in ammonia cytotoxicity In : Grisolia S, Felipo V, Minana MD (eds) : Cirrhosis, hepatic encephalopathy and ammonia toxicity. Plenum press, New York pp 183 – 195

Dakshayani KB, Velvizhi S, Subramanian P (2002) Effects of ornithine α-ketoglutarate on circulatory antioxidants and lipid peroxidation products in ammonium acetate treated rats. Ann Nutr Metab 46: 93-96.

Manchester LC, Poeggeler B, Alvares FL, Ogden GB, Reiter RJ (1995) Melatonin immunoreactivity in the photosynthetic prokaryote, Rhodospirillum rubrum : implications for an ancient antioxidant system. Cell Mol Biol Res 41 :391 – 395.

Tan DX, Chen LD, Poeggler B, Manchester LC, Reiter RJ (1993) Melatonin : a potent endogenous hydroxyl radical scavenger Endocr J. 1:57-60.

Reiter RJ, Tan DX, Cabrera J,D’ Arpa D, Sainz Rm, Mayo JC,Ramos S (1999) The oxidant/antioxidant network role of melationin. Biol Signals recept 8:56-63.

National Institute of Health Guide for the Care and Use of Laboratory Animals DHEW publication (NIH), revised (1985) Office of Science and Health Reports, DRR/NIH Bethesda, USA.

Hilgier W., Albrecht J., Lisy V., Stastny F (1990) The effect of acute and repeated hyperammonemia on gamma – glutamyl transpeptidase in homogenates and capillaries of various rat brain regions. Mol Chem Neuropathol 13: 47 – 56.

Liu F, Ng TB (2000) Effect of pineal indoles on the activities of the antioxidant defence enzymes superoxide dismutase, catalase and glutathione reductase and levels of reduced and oxidized glutathione in rat tissues. Biochem Cell Biol 78 : 447 – 453.

Nichans WG, Samuelson B (1968) Formation of malondialdehyde from phospholipid arachidonate during microsomal lipid peroxidation Eur J Biochem 6 :126 -130.

Ellman GL. (1959) Tissue sulphydryl groups. Arch Biochem Biophys 82: 70 – 79

Kakkar P., Das B., Viswanathan DN : A modified spectrophotometric assay of superoxide dismutase . Indian J Biochem. Biophys., 1984, 21 : 130 – 132.

Sinha ,Ka,: Colorimetric assay of catalase. Anal.Biol.Chem., 1972 47, 389 – 394.

Rotruck JT, Pope AL, Gauther HE, Swanson AB, Hafeman DG and Hoekstna WG, (1973). Selenium : Biochemical roles as component of glutathione peroxidase. Science 179 :588 – 590.

Falholt K, Falholt W, Lund B (1973) An easy colorimetric method for routine determination of free fatty acids in plasma. Clin Chim Acta 19:338.

Zilversmit DB, Mc Chandless EL,Davis AK (1950). Microdetermination of plasma phospholipids by trichloroacetic acid precipitation. J.Lab Clin Med 35:155

Foster CS, Dunn O (1973) Stable reagents for determination of serum triglycerides by a colorimetric Hantsch condensation method. Clin Chim Acta 19: 338 – 340.

Zlatkis A, Zak B,Boyle AJ (1953) A new method for the direct determination of serum cholesterol. J Lab Clin Med 45:486.

Nelson NL, Cox MM (2000) Lehninghr Principles of Biochemistry Macmillan Worth Publishers, Hampshi re,UK

Vidya M, Subramanian P (2003) Enhancement of circulatory antioxidants by α- ketoglutarate during sodium valproate treatment in Wistar rats. Pol J Pharmacol 55 : 31 – 36

Lastra Ld, Cazeba J, Motiva V, Martin Mj (1997) Melatonin protects against gastric ischemia-reperfusion injury in rats. J Pineal Res 23:47-52.

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine 3rd een.Oxford Science Publications, UK

Luperchio S, Tamir S,Tannenbaum SR (1996) NO-induced oxidative stress and glutathione metabolism in rodent and human cells. Free Radical Biol Med 21:513-519

Urata Y, Honma S,Goto s, Todoroki S, Ueda T, Cho S, Honma K., Kondo T, (1999) Melatonin induces ϒ-glutamycystenine synthase mediated by activator protein-1 in numan vascular endothelial cells. Free Radical Biol Med 27: 838-847

Barlow-Walden LR,Reiter RJ, Abe M, Pablos M, Pelaez Ma, Chen LD (1995) Melatonin stimulates brain glutathione peroxidase activity. Neurochem Int 26: 497 -502

Antolin I, Rodriguez C, Sainz RM (1996) Neurohormone melatonin prevents cell damage effect on gene expression for antioxidant enzymes. FASEB J 10: 882 -890.

Reiter RJ and Tan DX (2003). Melatonin: a novel protection agent against oxidative injury of the ischemic reperfused heart. Cardiovasc Res 58:10 – 19.

Yamamoto H (1989) Hyperammonemia: increased brain neutral and aromatic amino acid levels and encephalopathy induced by cyanide in mice. J Toxicol Appl Pharmacol 99: 415-420.

Tan DX, Manchester LC , Reiter RJ, Plummer BF, Limson J, Weintraub ST,Qi W (2000) Melatonin directly scavenges H2O2: a potentially new metabolic pathway of melatonin biotransformation. Free Radical Biol Med 29:1177-1185

Baydas G, Yilmaz O, Celik S, Yasar A, Felit Gursu M (2002) Effects of certain micronutrients and melatonin on plasma lipid peroxidation and homocysteine levels in rats. Arch Med Res 33: 515 – 519.