Effect of morin on the levels of circulatory liver markers and redox status in experimental chronic hyperammonaemic rats

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ABSTRACT

Introduction: Hyperammonaemia is a major contributing factor to neurological abnormalities observed in hepatic encephalopathy and in congenital defects of ammonia detoxication. Ammonia toxicity results in free radical generation that leads to oxidative stress and tissue damage. Morin is a bioflavonoid, a constituent of many herbs and fruits that are used as herbal medicines and also several biological activities. Our aim was to investigate the effect of morin on circulatory liver markers, lipid peroxidation and antioxidant status in ammonium chloride (AC)-induced hyperammonaemic rats.

Methods: Male albino Wistar rats weighing 180–200 g were used for the study. The hyperammonaemia was induced by interaperitonial injection of AC (100 mg/kg body weight). Rats were treated with morin (30 mg/kg body weight) via oral administration. Administration of morin in hyperammonaemic rats reduced the levels of ammonia and urea. The antioxidant property of morin was studied by assessing the activities of thiobarbituric acid reactive substances (TBARS), hydroperoxides (HP) and liver markers (alanine transaminase, aspartate transaminase and alkaline phosphatase) and the levels of glutathione peroxidase, superoxide dismutase, catalase, reduced glutathione, vitamins A, C and E in AC-treated rats.

Results: Oxidative stress was effectively modulated by morin administration. Morin significantly improved the status of antioxidants and decreased the levels of ammonia, urea, TBARS, HP and liver markers enzymes, as compared to the AC-treated group.

Conclusion: The study offers evidence for the antihyperammonaemic, hepatoprotective and antioxidant effects of morin against oxidative stress induced by AC.

Keywords: antioxidants, hyperammonaemia, lipid peroxidation, liver markers, morin

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INTRODUCTION

Hyperammonaemia is defined as an elevated ammonia concentration in the blood, caused by an impairment of the liver function resulting in inadequate ammonia detoxification. In living organisms, ammonia is an important nitrogen substrate in several reactions, and plays an important role in nitrogen homeostasis of cells. Moreover, it is a product as well as precursor of various important nitrogen-containing metabolites, such as amino acids, which in turn are the smallest subunits of proteins.[1] Ammonia is neurotoxic when accumulated in excess, and hyperammonaemia is mainly responsible for the neurological alterations found in liver disease and hepatic encephalopathy, including impaired intellectual function.[2] Antiepileptic drugs, such as valproate and salicylate, cause hyperammonaemia and urea cycle disorders.[3,4] Ammonia toxicity results in free radical generation, that leads to oxidative stress-mediated tissue damage,[5-7] and elevated ammonia concentration in the brain exerts toxic effects on neural cells.[1]

The greatest disadvantage of the presently-available potent conventional or synthetic antihyperammonaemic agents/therapies lies in their toxicity and reappearance of symptoms after discontinuation. These drugs or therapies are sometimes inadequate and can have serious adverse effects.[8] Therefore, the screening and development of drugs for their antihyperammonaemic activity is still in progress, and there is thus a worldwide trend to go back to traditional medicinal plants and natural products. There is a need to search for appropriate protective agents against hyperammonaemia. This can be achieved by focusing on plants and plant products used in traditional medicine because of leads provided by natural products that may offer better treatment than currently-used drugs.

Flavonoids are ubiquitous and abundant in plants, and are considered very important for preventing a wide variety of diseases, including allergies, cardiovascular disease, certain forms of cancer, hepatic diseases and inflammation.[9] Morin (3, 5, 7, 2', 4' -pentahydroxyflavone; a yellowish pigment) is a bioflavonoid constituent of many herbs and fruits (Fig. 1). Bioflavonoids are used as herbal medicines, and exhibits various biological activities including antioxidation, cytoprotection, antimutagenesis

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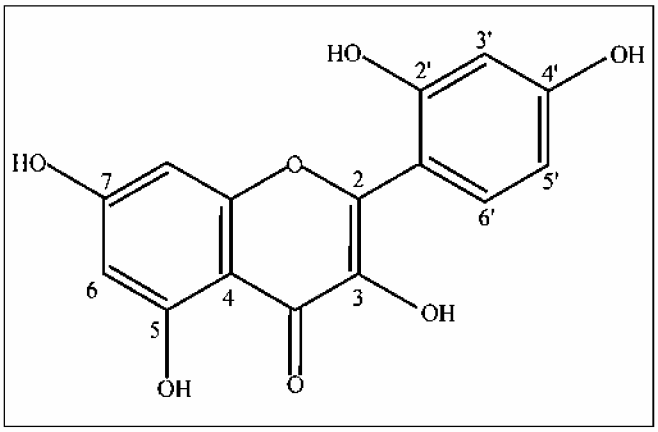

Fig. 1 Chemical structure of morin.

and anti-inflammation.[10,11] It was reported that morin could modulate the activities of the metabolic enzymes, including cytochrome P450,[12] and it is also an antioxidant that protects various human cells, like myocytes, endothelial cells, hepatocytes and erythrocytes, against oxidative damages.[13,14] Moreover, morin acts as a chemopreventive agent against oral carcinogenesis *in vitro* and *in vivo*.[15,16]

Although various traditional medicinal values have been attributed to morin, no biochemical studies have been carried out to shed light on the role of morin on circulatory liver markers and redox status in experimental hyperammonaemia. In the light of this, the present study was designed to analyse the effect of morin on circulatory (i) ammonia, urea, alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP); (ii) thiobarbituric acid reactive substances (TBARS) and hydroperoxides (HP); and (iii) enzymatic and nonenzymatic antioxidants, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), vitamins A, C and E during ammonium chloride (AC)-induced hyperammonaemic rats.

METHODS

Morin was purchased from the Sigma Chemical Co. (St Louis, MO, USA). AC was purchased from Sisco Research Laboratories (Mumbai, India). All other chemicals used in the study were of analytical grade. Adult male albino Wistar rats, weighing 180–200 g and bred in the Central Animal House, Rajah Muthiah Medical College, Annamalai University, India, were used. The animals were housed in polycarbonate cages in a room with a 12 h day-night cycle, temperature of 22°C ± 2°C and humidity of 45%–64%. Animals were fed with a standard pellet diet (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*. Studies were carried out in accordance with Indian National Law on Animal Care and Use, and ethical clearance was provided by Committee for the Purpose of Control and Supervision of Experiments on Animals of Rajah Muthiah Medical College and Hospital (Reg. no: 160/1999/CPCSEA), Annamalai University, Annamalainagar, India.

In the experiment, a total of 32 rats were used. The rats were divided into four groups of eight rats each. Group I rats received physiological saline and considered as controls, Group II normal rats were administered with morin (30 mg/kg body weight) using an intragastric tube,[17] Group III rats were treated with AC (100 mg/kg body weight; i.p.)[18] and Group IV rats were treated with AC (100 mg/kg) and morin (30 mg/kg) thrice in a week for eight weeks. At the end of eight weeks, the rats were fasted overnight and sacrificed by cervical dislocation after anaesthetising the animals with intramuscular injections of ketamine hydrochloride (30 mg/kg body weight). Morin was freshly dissolved in a small amount of ethanol and then diluted with physiological saline.[19] Hyperammonaemia was induced in Wistar rats by intraperitoneal injections of AC at a dose of 100 mg/kg body weight thrice a week for eight consecutive weeks.[18,20,21]

Activities of AST and ALT were assayed by the method of Reitman and Frankel.[22] 0.2 ml aliquot of serum with 1 ml of substrate (aspartate and α-ketoglutarate (KG) for AST: alanine and α-KG for ALT) in phosphate buffer (pH 7.4) was incubated for one hour for AST and 30 minutes for ALT. 1 ml aliquot of 2,4-dinitrophenylhydrazine (DNPH) solution was added to arrest the reaction and kept for 20 minutes at 25°C. After incubation, 1 ml of 0.4 N NaOH was added and the absorbance was read at 540 nm. Activities are expressed as IU/L. ALP was assayed by the method of King and Armstrong.[23] The ALP activity was assayed using disodium phenylphosphate as substrate. After preincubation of the buffer (0.1 M bicarbonate buffer, pH 10) with the substrate for 10 minutes, 0.2 ml of serum was added and incubated for 15 minutes at 25°C. The liberated phenols from the substrate reacted with Folin-Phenol reagent (1 ml). The suspension was centrifuged and collected as the supernatant. Aliquot of 10% sodium bicarbonate 2 ml was added to the supernatant and the colour that developed was read at 680 nm after 10 minutes. Activities of ALP are expressed as IU/L.

Blood ammonia levels were estimated by the method of Wolheim.[24] To 20 µL of the blood, 200 µL of triethanolamine buffered substrate and 150 µL of reduced nicotinamide adenine dinucleotide phosphate (NADPH)/glutamate dehydrogenase (GLDH)/buffered substrate were added, mixed well and the absorbance was read at 470 nm. Plasma urea levels were determined by the diacetyl monoxime method of Varley et al.[25] To 0.1 ml of plasma, 3.3 ml of water was added and mixed. Then 0.3 ml

Table I. Effect of morin on changes in the blood ammonia, plasma urea, TBARS, HP, serum AST, ALP and ALT of normal and experimental rats.

Group	Liver marker enzymes			Blood ammonia (μmol/L)	Plasma urea (mg/dL)	TBARS (nmol/ml)	HP ($\times 10^{-5}$ mmol/dL)
	AST (IU/L)	ALP (IU/L)	ALT (IU/L)				
Normal	72.46 ± 5.52[a]	75.26 ± 5.73[a]	24.75 ± 1.88[a]	84.80 ± 5.27[a]	9.95 ± 0.62[a]	2.78 ± 0.21[a]	8.10 ± 0.62[a]
Morin (30 mg/kg)	71.59 ± 5.45[a]	73.83 ± 4.78[a]	22.68 ± 1.73[a]	78.47 ± 4.95[a]	9.06 ± 0.57[a]	2.95 ± 0.22[a]	8.24 ± 0.54[a]
AC (100 mg/kg)	117.81 ± 9.02[b]	137.92 ± 10.56[b]	62.48 ± 4.78[b]	374.46 ± 22.10[b]	23.67 ± 1.40[b]	4.62 ± 0.35[b]	13.14 ± 1.01[b]
AC (100 mg/kg) + morin (30 mg/kg)	84.35 ± 6.44[c]	86.45 ± 6.60[c]	30.69 ± 2.34[c]	147.87 ± 10.80[c]	12.66 ± 0.92[c]	3.38 ± 0.26[c]	9.48 ± 0.73[c]

ANOVA followed by Duncan's multiple range test
Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$

of 10% sodium tungstate and 0.3 ml of 0.67 N sulphuric acid were added, mixed and centrifuged. To 2.0 ml of the supernatant, 2 ml of water, 0.4 ml diacetyl monoxime and 1.6 ml of sulphuric acid-phosphoric acid mixture were added and heated in a boiling water bath for 30 minutes and cooled, and the absorbance was read at 480 nm.

Thiobarbituric acid (TBA) was added to the plasma samples under acidic conditions and the absorbance of colour that developed after heating was estimated spectrophotometrically at 535 nm.[26] 1,1,3,3-tetramethoxypropane was used as an internal standard and the plasma concentration was expressed as nmol/dL of plasma. Estimation of plasma lipid HP was done by the method of Jiang et al.[27] In this method, oxidation of ferrous ion (Fe^{2+}) under acidic conditions in the presence of xylenol orange led to the formation of a chromophore, which absorbs at 560 nm.

SOD was assayed by the inhibition of formation of nicotinamide adenine dinucleotide (NADH)-phenazine methosulphate (PMS) nitroblue tetrazolium (NBT) formazan.[28] The reaction was initiated by the addition of NADH, and after incubation for 90 s, the reaction was stopped by the addition of glacial acetic acid. The colour formed at the end of the reaction was extracted into the butanol layer and measured at 520 nm. One unit of activity was taken as the enzyme concentration, which gave 50% inhibition of NBT reduction in one minute. CAT was assayed colorimetrically by the method of Sinha.[29] Dichromate in acetic acid was converted to perchromic acid and then to chromic acetate when heated in the presence of H_2O_2. The chromic acetate formed was measured at 620 nm. The catalase preparation was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of a dichromate-acetic acid mixture and the remaining H_2O_2 was determined colorimetrically as chromic acetate. One unit of activity was expressed as a mole of H_2O_2 consumed/min/mg protein.

GPx was estimated by the method of Rotruck et al.[30] A known amount of haemolysate was allowed to react with H_2O_2 in the presence of GSH for a specified time period, then the remaining GSH content was allowed to react with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and the yellow colour developed was measured at 412 nm. One unit of activity was expressed as μmol of glutathione consumed/min/mg Hb. Plasma vitamin A was estimated by the method of Bradely and Hornebeck.[31] Proteins were precipitated with ethanol and the carotenes were extracted into light petroleum. The intensity of the yellow colour due to carotene was read directly at 450 nm using a violet filter. Plasma α-tocopherol was estimated by the method of Baker et al.[32] This method involved the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red-coloured complex with 2,2'-dipyridyl. Absorbance of the chromophore was measured at 520 nm.

Plasma vitamin C was estimated by the method of Roe and Kuether.[33] The ascorbic acid was converted to dehydroascorbic acid by mixing with acid washed norit and was then coupled with DNPH in the presence of thiourea, a mild reducing agent. The coupled DNPH was converted into an orange red-coloured complex when treated with sulphuric acid, which was read colorimetrically at 520 nm. GSH in plasma was measured according to the method of Ellman.[34] This method was based on the development of a yellow colour when DTNB was added to compounds containing sulfhydryl groups. Values were given as mean ± standard deviation for eight rats in each group. Data was analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using the Statistical Package for Social Sciences

Table II. Effect of morin on changes in the enzymatic and non-enzymatic antioxidants of normal and experimental rats.

Group	Enzymatic antioxidants (in erythrocytes)			Vitamin A in plasma (mg/dL)	Vitamin E in plasma (mg/dL)	Vitamin C in plasma (mg/dL)	GSH in plasma (mg/dL)
	SOD[A]	CAT[B]	GPx[C]				
Normal	2.85 ± 0.22[a]	2.15 ± 0.16[a]	23.52 ± 1.79[a]	2.15 ± 0.16[a]	1.55 ± 0.12[a]	1.56 ± 0.12[a]	26.72 ± 2.03[a]
Morin (30 mg/kg)	2.92 ± 0.22[a]	2.10 ± 0.15[a]	24.32 ± 1.85[a]	2.09 ± 0.13[a]	1.53 ± 0.10[a]	1.62 ± 0.12[a]	25.15 ± 1.92[a]
AC (100 mg/kg)	1.76 ± 0.13[b]	1.45 ± 0.11[b]	12.66 ± 0.97[b]	1.49 ± 0.11[b]	0.96 ± 0.07[b]	0.97 ± 0.07[b]	15.53 ± 1.19[b]
AC (100 mg/kg) + morin (30 mg/kg)	2.45 ± 0.19[c]	1.82 ± 0.14[c]	20.06 ± 1.53[c]	1.92 ± 0.15[c]	1.28 ± 0.10[c]	1.37 ± 0.10[c]	22.15 ± 1.69[c]

ANOVA followed by Duncan's multiple range test. Values not sharing a common superscript (a, b, c) differ significantly at $p \leq 0.05$. [A]: amount of enzyme required to inhibit 50% of NBT reduction/mg Hb; [B]: micromoles of H_2O_2 consumed/min/mg Hb; [C]: micromoles of GSH utilised/gHb.

version 10.0 (SPSS Inc, Cary, NC, USA). The limit of statistical significance was set at $p \leq 0.05$.

RESULTS

The levels of blood ammonia, plasma urea, HP, TBARS and serum AST, ALT and ALP in control and experimental rats are shown in Table I. The levels of circulatory ammonia, urea, liver markers, HP and TBARS were significantly higher in AC-treated rats when compared with controls. Hyperammonaemic rats treated with morin significantly normalised the levels of ammonia, urea, liver markers and lipid peroxidation products, as compared with hyperammonaemic rats. The levels of circulatory antioxidants in control and experimental groups are given in Table II. The levels of vitamins A, C and E, GSH, GPx, SOD and CAT were significantly lower in AC-treated rats, and these levels were significantly normalised in hyperammonaemic rats treated with morin.

DISCUSSION

Ammonia is present in all living organisms as a product of degradation of proteins and other nitrogenous compounds. However, at higher levels, ammonia is toxic, leading to functional disturbances in the central nervous system that could lead to coma and death. To avoid the deleterious effects of ammonia, ureotelic animals detoxify ammonia by incorporating it into urea that is eliminated in urine. However, when the liver fails, or when blood is shunted past the liver, blood ammonia levels are elevated and brain function deteriorates.[35,36] In the liver, ammonia is removed either in the form of urea in periportal hepatocytes and/or as glutamine in perivenous hepatocytes.[36] Increased levels of circulatory ammonia and urea might indicate a hyperammonaemic condition in rats treated with AC,[4,7,37] which may be due to liver damage caused by ammonia intoxication. Administration of morin to AC-induced hyperammonaemic rats significantly decreased the levels of blood ammonia and urea.[17] The reduction in the levels of ammonia and urea during morin treatment shows the potent anti-hyperammonaemic effect of morin.[17]

In our study, the elevated levels of circulatory liver markers and lipid peroxidation products in AC-treated rats might be due to the liver damage caused by ammonia-induced free radical generation. Reports have shown that excess ammonia intoxication leads to excessive activation of N-methyl-D-aspartate (NMDA) receptors leading to neuronal degeneration and death.[38,39] The mechanisms by which excessive activation of NMDA receptors lead to neuronal degeneration and death are caused by increased Ca^{2+} concentration in the postsynaptic neuron.[40,41] Ca^{2+} binds to calmodulin and activates nitric oxide synthase, increasing the formation of nitric oxide (NO) that contributes to the neurotoxic process. Activation of NMDA receptors also leads to increased production of superoxide radical, which has been also proposed under *in vivo* conditions.[37,42,43] Superoxide and NO have the ability to generate hydroxyl radicals.[44] This leads to oxidative stress, which causes tissue damage.[5,7,45,46] Decreased levels of circulatory liver markers and lipid peroxidation products in morin-administered rats may be due to its free radical scavenging property[47] as it is an effective free radical scavenger.[48] Previous studies show that morin offers neuroprotection by inhibiting excess activation of NMDA receptors and NMDA receptor-mediated neurotoxicity.[49] The protective potent neuroprotective activity of morin could be of therapeutic value for the treatment of acute neuronal damage and disability.[49]

There is evidence to show that oxidative stress

and free radical production could be involved in the mechanism of ammonia intoxication,[5,7,20,21,50] which might have decreased the levels of enzymatic (GPx, SOD and CAT) and non-enzymatic (Vitamins A, C, E and GSH) antioxidants in AC-treated rats. Administration of morin significantly restored the levels of enzymatic and non-enzymatic antioxidants and it may be due to its potent antioxidant property, by offering a possible role in reducing the oxidative stress by inducing cellular antioxidant enzymes.[48] It was reported that flavanoids, such as morin, quercetin, kaemferol, are well-known potent free radical scavengers and antioxidants.[51] For instance, phenolic phytochemicals due to their phenolic ring and hydroxyl substituents can function as effective antioxidants due to their ability to quench free radicals. It is therefore believed that dietary phenolic antioxidants can scavenge harmful free radicals and thus inhibit their oxidative reactions with vital biological molecules[52] and prevent development of many pathophysiological conditions.

The possible mechanisms by which morin modulates liver marker levels and redox status during experimental hyperammonaemia might be due to the removal of excess ammonia, inhibition of NMDA receptor-mediated neurotoxicity and its antioxidant properties. In conclusion, our results indicate that morin could modulate the levels of liver markers and redox status and thus exerts potent hepatoprotective and antihyperammonaemic effects during AC-induced hyperammonaemia. However, the exact mechanism(s) is still unclear and further research is needed.

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