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RESEARCH ARTICLE

PROTECTIVE EFFECT OF MORIN AGAINST ETHANOL INDUCED RENAL DAMAGE IN MALE ALBINO RATS

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ABSTRACT

Morin, a bioflavonoid with antioxidant property, is a constituent of many herbs and fruits that are used as herbal medicines. It exhibits many biological activities and possibly even has protective effects against chronic renal diseases. The present study aims to study the protective effect of morin against ethanol-induced biochemical changes in kidney. Male albino rats were divided into four groups as follow; (1): control group, (2): control rats treated with morin (3) ethanol fed rats (4) ethanol fed rats treated with morin. Administration of ethanol (6g/kg BW) daily for a period of 60 days causes significant elevation in the levels of nephrotic markers such as urea, uric acid, creatinine and also significant elevation of kidney lipids such as total cholesterol, triglyceride, free fatty acid, and phospholipids. Ethanol also causes the significant decreased in the activities of membrane bound ATPases. However, ethanol fed rats treated with morin (60 mg/ kg BW, post orally once daily for a period of 30 days) significantly reduced the level of nephrotic markers, the lipids levels and increased the activities of membrane bound enzyme. The results of the present study strongly indicate that morin has potent reno-protective effect against ethanol induced oxidative tissues damage in experimental animals.

INTRODUCTION

Ethanol, most frequently abused drug, is a small molecule and soluble in both water and lipids. Ethanol permeates in all the tissues, thereby giving it the ability to influence a wide range of cellular targets and virtually all organs, including kidney (Lieber, 1995). A number of reports support the hypothesis that habitual consumption of large amounts of alcohol has a variety of deleterious effects on the kidney (Cecchin and De Marchi, 1996). Ethanol may cause functional abnormalities in the kidney depending on the quantity ingested. Thus, consumption of more than two standard drinks per day (24 g ethanol/day) is associated with an increased risk of kidney failure in the general population (Perneger *et al*., 1999). Ethanol oxidation causes increased production of reactive oxygen species (ROS), which are mediators of the tissue damage following ethanol intoxication. Oxidative injury may alter the structure and function of the glomerulus mainly due to the effect of ROS on mesangial and endothelial cells (Martin-Mateo, 1999). It has been shown that increased activities of alcohol dehydrogenase in the kidney and might responsible for the toxic effect of alcohol on the kidney. Flavonoids comprise a class of natural products which are found in fruits, nuts, seeds, herbs, and spices. They are consumed as a part of the human diet, and have considerable interest due to their broad pharmacological activity (Galvez *et al*., 2001). Plant flavonoids are emerging as potent therapeutic drugs effective against wide range of free radical mediated diseases. Morin (2',3,4',5,7-pentahydroxyflavone), a kind of bioflavonoid, occurs in fig, guava leaves, onion, apple, and other members of the Moraceae family, which are used as dietary agents and herbal medicine. It is also widely distributed in tea, cereal grains and a variety of fruits and vegetables. (Lotito and Frei, 2006 and Xie *et al*., 2006). Morin, with potent antioxidant and metal ion chelating capacities, possesses various biological effects including anti-inflammatory, anti-neoplastic, and cardio-protective activities (Middleton *et al*., 2000). Morin has renoprotective effect against imipenem (Lim *et al*., 2008), DOCA salt (Prahalathan *et al*., 2012), gentamycin (Khattab, 2012) and improves the kidney function through the regulation of organic ion transporters (Wang *et al*., 2010). The effect of morin on an experimental model of nephrotoxicity induced by chronic ethanol treatment is not known. Hence, the present study was undertaken to establish the protective effect of exogenous administration of morin in an animal model of ethanol-induced nephrotoxicity by assessing serum urea, creatinine, uric acid, lipids levels and membrane bound ATPase.

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MATERIALS AND METHODS

Chemicals

Morin hydrate was purchased from Sigma Chemicals Co., St. Louis, Mo, USA. Absolute ethanol was obtained from Himedia, Pvt. Lt., Mumbai, India and all the other chemicals and solvents used were of analytical grade.

Experimental Animals and diet

Male albino Wistar rats (150-180) g were obtained from the Central Animal House, Rajah Muthiah Medical College and Hospital, Chidambaram, The animals were housed in polypropylene cages in a well-ventilated room and maintained in a 12-h light/12-h dark cycle, 50% humidity and 30° C. The animals had free access to standard pellet diet (Pranav Agro Industries Ltd, Bangalore, India) and water *ad libitum*. This study was approved by Institutional Animal Ethics Committee, Rajah Muthiah Medical College (Registration Number: 166/1999/CPCSEA,) and the study was conducted in accordance with the Guidelines of "Committee for the purpose of control and supervision on experimental animals" (CPCSEA, 2004).

Oral administration of Morin

Morin was freshly suspended in water (Prahalathan *et al*., 2012) and administered to rats orally using an intragastric tube.

Experimental Design

The animals were divided into four groups of six rats each and were maintained as follows

Group I: Normal control rats, received glucose from a 40% stock solution twice in a day, which was isocaloric to ethanol.

Group II: Rats received isocaloric glucose from a 40% stock solution every day and aqueous morin (60mg/ kg BW) via intubation from the 30th day.

Group III: Rats received ethanol (6g/kg BW) from a 30% stock solution twice in a day for a period of 60 days.

Group IV: Rats received ethanol (6g/kg BW) and co-treated with morin (60 mg/kg BW) from the 30th day along with ethanol.

The overall duration of the experiment was 60 days. At the end of the experimental period the animals were fasted over night, anesthetized with ketamine hydrochloride (30 mg/kg, i.p) and then sacrificed by cervical decapitation. Blood was collected in dry test tubes containing heparin as the anticoagulant. Plasma was separated by centrifugation at 1000xg for 10 minutes and used for various biochemical estimations. The kidney of animals were dissected carefully, cleared off blood and immediately transferred to ice-cold containers containing physiological saline and homogenized in 0.1M Tris-HCl buffer, pH 7.4.

Biochemical estimations

Serum urea was estimated by using the diagnostic kit based on the method of Fawcett and Scott (1960). Serum creatinine was estimated using the diagnostic kit based on the method of Tietz (1987) using Jaffe's (1886) colour reaction. Serum uric acid was estimated by using the diagnostic kit based on the enzymic method described by Caraway (1995). Lipids were extracted from the kidney by the method of Folch *et al* (1957). Total cholesterol (TC) was estimated by the enzymic method described by Zlatkis *et al*., (1953) TG and free fatty acids (FFA) in the kidney were estimated according to the method of Foster & Dunn, 1973 and Falholt *et al*., (1973) respectively. Phospholipids (PL) were estimated by the method of Zilversmit & Davis, (1950). Total ATPases activity was determined by the method of Evans (1969), while Ca^{2+} ATPase activity was evaluated by the method of Hjertan and Pan *et al*., (1983). Mg^{2+}ATPase was assessed according to Ohnishi *et al*. (1982) and Na^{+}/K^{+} ATPase was measured by the method of Bonting, (1970). Protein content in the tissues was determined by the method of Lowry *et al.* (1951).

Histopathological investigation

Kidney were excised, washed and placed in 10% formalin. They were later sectioned with a microtome, dehydrated in ethanol, embedded in paraffin wax. Five micrometer thick sections were stained with hematoxylin and eosin (H&E) and studied by a routine light microscope.

Statistical analysis

All the grouped data were evaluated statistically and the significant changes caused by the treatment was determined using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) by using SPSS 17 for Windows. The results are expressed as means ± SD of six rats from each group. The level of statistical significance was set at $p < 0.05$.

RESULTS

Effect of morin on renal function marker enzymes

Effect of morin on the levels of serum nephrotic marker such as urea, uric acid and creatinine in the control and experimental rats were given in Table 1. The nephrotic markers were significantly ($p < 0.05$) elevated in ethanol-fed rats when compared to control rats. Oral administration of morin at a dose of 60 mg/kg BW to ethanol-fed rats significantly ($p < 0.05$) decreased the levels of these renal function markers. Control rats treated with morin did not showed any significant changes when compared to the normal control rats.

Effect of morin on Lipid profile levels

Figure 1 shows the levels of lipid in the kidney of control and experimental animals. Ethanol-fed rats showed elevated levels of TC, TG, PL and FFA when compared to control rats. Ethanol-fed rats when treated with morin showed significantly decreased levels of TC, TG, PL and FFA when compared to untreated ethanol-fed rats (Group 3). Control rats treated with morin alone did not showed any significant changes when compared to normal control rats.

Table 1. Effect of Morin on Kidney Markers in control and experimental animals

Groups	Kidney Markers (mg/dL)		
	Urea	Uric acid	Creatinine
Control	29.08 ± 2.15^{a}	1.84 ± 0.67^{a}	0.43 ± 0.03^{a}
Control + Morin (60mg/kg BW)	$31.20 \pm 2.41^{a,c}$	2.06 ± 0.22^{a}	0.51 ± 0.037^{a}
Ethanol (6g/kg BW)	65.40 ± 4.44^{b}	5.02 ± 0.49^{b}	1.35 ± 1.24^{b}
Ethanol + Morin (60mg/kg BW)	35.68 ± 2.91^{c}	2.89 ± 0.19^{c}	0.75 ± 0.06^{c}

Values are means ± SD for six rats:
Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT).

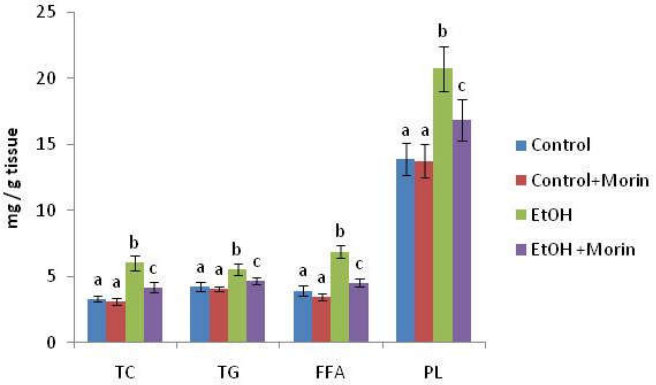

Fig.1. Values are means ± SD for six rats: Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT); EtOH: Ethanol. TC-Total cholesterol, TG - Triglyceride, FFA-Free Fatty acids, PL- Phospholipids.

Effect of morin on the activities of membrane bound ATPases in kidney

Tables 2 shows the activities of membrane bound ATPases in kidney of control and experimental animals. Significant reductions in the activities of total ATPases, Ca^{2+}ATPase, Na^{+}/K^{+}ATPase and Mg^{2+}ATPase were observed in the kidney of ethanol fed rats. Morin

Table 2. Activities of membrane bound ATPase in kidney of control and experimental animals

Groups	Control	Control + Morin	Ethanol	Ethanol + Morin
Total ATPase ($U^{\clubsuit}$)	1.42 ± 0.10^{a}	1.45 ± 0.09^{a}	0.90 ± 0.12^{b}	1.24 ± 0.10^{c}
Ca^{2+}ATPase ($U^{\clubsuit}$)	0.38 ± 0.04^{a}	0.40 ± 0.02^{a}	0.19 ± 0.09^{b}	0.29 ± 0.03^{c}
Na^{+}/K^{+} ATPase ($U^{\clubsuit}$)	0.51 ± 0.04^{a}	0.52 ± 0.04^{a}	0.35 ± 0.05^{b}	0.41 ± 0.03^{c}
Mg^{2+}ATPase ($U^{\clubsuit}$)	0.65 ± 0.05^{a}	0.69 ± 0.04^{c}	0.38 ± 0.04^{b}	0.52 ± 0.04^{c}

$U^{\clubsuit}$: µmoles of phosphorous liberated/ mg protein. Values are means ± SD for six rats
Values not sharing a common superscript differ significantly at $p < 0.05$. Duncan's Multiple Range Test (DMRT).

supplementation to ethanol-treated rats caused significant elevations in these enzyme activities and the values were significantly brought to normal levels, when compared to the untreated ethanol fed rats.

Effect of morin on histological changes in kidney of control and experimental animals

Microscopically, kidney sections from control rats revealed the normal structure of renal parenchyma (Fig 2a). Kidney of ethanol fed rats showed severe changes as evidenced by glomerulnephritis and haemorrhage area in parenchyma, scattered inflammatory cell infiltrate in parenchyma and focal interstitial nephritis associated with mononuclear cells infiltration (Fig 2c). Kidney of rats received morin alone showed no histopathological alterations (Fig 2b). Concerning kidney sections of ethanol fed rats treated with morin showed slight congestion of peritubular capillaries (Fig 2d) and appear to be near normal.

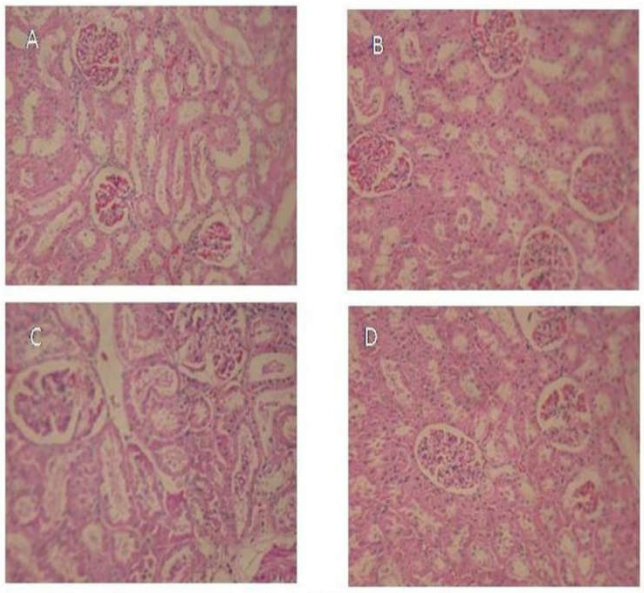

Fig.2. Histopathological changes in the kidney (A-D; H&E 20$^{\times}$). A, control rat showing normal glomeruli and tubules. B, control + morin (60mg/kg) treated rat showing normal kidney architecture. C, represent kidney sections of ethanol-fed rats shows segmented glomerulonephritis and hemorrhage areas in parenchyma and scattered inflammatory cell infiltrate in parenchyma. D, ethanol + morin (60 mg/kg) treated rat showing mild swelling and near normal architecture

DISCUSSION

In our laboratory, a preliminary study was conducted with different doses of morin (15, 30, 60 and 120 mg/kg BW) to determine the dose-dependent effect of morin against ethanol induced toxicity rats. Among the doses the 60 mg/kg dose was found to the most effective. We believe that the concentration of low dose of morin (15 mg/kg BW) might not be sufficient to scavenge the radicals, yet the higher concentration of morin (120 mg/kg BW) might have resulted in the production of more by-products that would have interfered with the activity. In continuation of our research work, in this study we have chosen only 60 mg/kg BW. Ethanol and its metabolites are excreted into urine, and its content in the urine is higher than that of the blood and the liver. Chronic ethanol administration decreases the renal tubular reabsorption and reduces renal function. Functional abnormalities of renal tubules may be associated with ethanol-induced changes in membrane composition and lipid peroxidation. Because of high content of long-chain polyunsaturated fatty acids, kidney is highly sensitive to oxidative damage (Rodrigo and Rivera, 2002). The kidney seems to be the only vital organ generally spared in chronic alcoholics without advanced alcoholic liver disease or hepato-renal syndrome. But, regular alcohol consumption raises the blood pressure, which per se is a risk factor for renal damage (Heidland *et al*., 1985). Large amounts of ethanol have deleterious effects on the kidney.

Kidney function markers such as urea, creatinine and uric acid were used to determine the effect of morin on the functional status of kidney in ethanol-fed rats. The results from the present study showed that ethanol administration significantly increased the levels of urea, creatinine and uric acid in serum, which are considered as significant markers of renal dysfunction. The levels of these renal function markers were significantly lower in the case of ethanol-administered rats treated with morin than those given ethanol alone indicating that the degree of ethanol induced renal toxicity was of lesser magnitude in the morin treated group. It shows that morin, to an extent, preserves the functional capacity of the kidney from the adverse effects of ethanol. Long-term ethanol consumption is associated with modifications of fatty acid metabolism. One and two month ethanol treatment led to a 3 to 4 fold rise of the cytochrome P450 (CYP) 2E1 protein in kidney microsomes. Ethanol intake does not act on the kidney microsome capability to hydroxylate unsaturated fatty acids. CYP2E1 is strongly inducible by ethanol and therefore accounts for the tolerance of this hepatotoxicant (Amet *et al*., 2000). While in another study ethanol appeared to induce CYP2E1 in the kidney (Ronis *et al*., 1998).

Ethanol gets oxidized to acetaldehyde through alcohol dehydrogenase, which is associated with the reduction of nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH) and produces a striking redox change associated with metabolic disorders. Fatty acid (FA) accumulation in the tissues of ethanol fed rats may be directly because of lipid breakdown and indirectly owing to the oxidation of ethanol by the liver to acetate and its conversion to FA, which is a means to remove excess hydrogen generated by ethanol. Administration of morin showed a protection against lipid alterations in kidney, which denotes a broad spectrum of protective activity. Morin diminishes the hypercholesterolemia, which is mainly because of their hypolipidimic properties, which in turn, suppress their influence on lipid metabolism. Morin, a major phenolic compound of the diet, has the capability to modulate the cholesterol and triglyceride (Ricardo *et al*., 2001) level and the previous report has been shown that morin preserve the hypertensive rats kidney by decreasing the lipid levels (Prahalathan *et al*., 2102) and thus decrease the oxidative stress. Multiple functional abnormalities of renal tubules may be associated with ethanol-induced changes in membrane composition and lipid peroxidation of epithelial cells. Ethanol interferes with the carrier function of cell membrane against the concentration (Bonilla *et al*., 1991). In a study, renal Ca^{2+} ATPase, Na^{+} K^{+}ATPase and Mg^{2+}ATPase activies were decreased in ethanol fed rats (Pushpakiran *et al*., 2005). Ethanol affects the selectivity of the Na^{+} K^{+}ATPase for Na^{+} and/or for K^{+}, enhancing the Na^{+} affinity for the K^{+} sites and/ or reducing the K^{+} affinity for its own sites (Rothman *et al*., 1994). The

Na^+ and the Na^+ K^+ATPase activities of basolateral plasma membrane from rat kidney proximal tubular cells are affected differentially by ethanol (Rothman *et al*., 1992). The Ca^{2+}ATPase are very sensitive to the oxidative damage (Moore *et al*., 1989). Kidney oxidative stress was effectively modulated by morin administration, morin significantly improved the status of kidney antioxidants and decreased the level of lipid peroxidation, thus may be attributed to antioxidant effects of morin against oxidative stress in the kidney (Subash and Subramanian, 2011). Intake of antioxidants avoided or minimized kidney injury by reducing oxidative stress (Prahalathan *et al*., 2012). It is well documented that localization of flavonoids within the membranes may modify membrane fluidity and lipid peroxidation of the membrane. Previous studies shown that morin decrease the lipid peroxidation and preserve the structural integrity of the membrane (Subash and Subramanian, 2008) which might be responsible for the observed increased activities of these enzymes on morin administration. Histopathological analysis of kidney of control rats tissue revealed normal section and healthy cells. However, histology of ethanol-fed rats kidney showed alteration in the glomeruli and tubules. Renal cells and renal parenchyma cells are also damaged. Ethanol induces kidney CYP450, enhances lipid peroxidation and which might be responsible for the tissue damage in ethanol fed rats. Co-administration of morin to ethanol fed rats showed less damage in the kidney. The administration of morin may mope up free radicals generation by ethanol and its metabolism and it may be responsible for the healthy state of renal cells. It shows that morin has protective effect on kidney against ethanol toxicity. There was no evidence of pathological changes in the morin alone-treated control group. Major findings from the present study demonstrated that morin possesses nephroprotective, antihyperlidemic and membrane stabilizing properties against ethanol induced nephrotoxicity and hyperlipidemia as evidenced by a considerable decrease in renal functions markers, decreased lipid levels and increased levels of membrane bound ATPases. Further studies are under way in our laboratory to elucidate the exact mechanism of action of morin.

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