

RESEARCH ARTICLE

Effect of morin on glycoproteins and membrane bound enzymes in streptozotocin-induced diabetic rats

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Abstract

This study investigated the effect of morin on membrane bound enzymes and glycoproteins in normal and streptozotocin (STZ)-induced diabetic Wistar rats. Diabetes was induced in rats by an intraperitoneal injection of STZ, dissolved in citrate buffer (0.01 M, pH 4.5) and injected to overnight fasted rats with a dose of STZ 50 mg/kg. Morin was dissolved in carboxy methyl cellulose (CMC) at a dose of 100 mg/kg and given to rats orally for a period of 45 d. In diabetic rats, the levels of glycoproteins were significantly increased in plasma and tissues (liver and kidney) and activities of membrane bound enzymes significantly decreased in liver and kidney. In addition, morin treatment significantly prevents the histopathological alterations in STZ-induced rats. Thus, morin exhibits beneficial role on membrane bound enzymes and glycoproteins in STZ-induced diabetic rats.

Keywords: Morin, streptozotocin, glycoproteins, antioxidants, diabetes, free radicals.

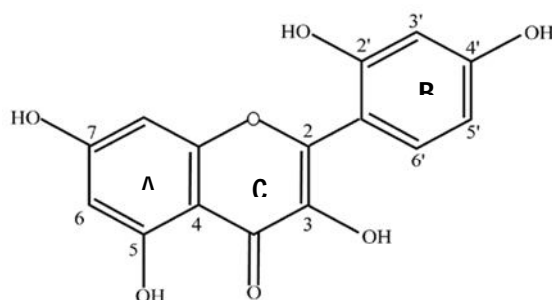
Introduction

Diabetes mellitus is a serious metabolic disorder with micro and macrovascular complications that results in significant morbidity and mortality. The prevalence of diabetes is strongly associated with a sedentary lifestyle, high calorie nutrition and obesity. It has been estimated that the global burden of type 2 diabetes mellitus in 2010 were 285 million people, which is projected to increase to 438 million in 2030; a 65% increase (Snehalatha and Ramachandaran, 2009). The effects of streptozotocin (STZ) on glucose as well as insulin homeostasis reflect the toxin-induced abnormalities in beta cell function.

Streptozotocin administration leads to impairment of functional beta cells, deficiencies in terms of gene expression and protein production lead to the deterioration of both glucose transport and metabolism (Coskun *et al.*, 2005). The toxic effect of STZ and chemically related alkylating compounds requires their uptake into the cells. Streptozotocin is selectively accumulated in pancreatic beta cells via the low-affinity GLUT2 glucose transporter in the plasma membrane (ADA, 2007). Flavonoids are widely distributed group of plant phenolics, which are abundant in foods. Phytochemicals, due to their phenolic ring and hydroxyl substituents can function as effective antioxidants due to their ability to quench free radicals. Morin (2',3,4',5,7-pentahydroxyflavone), (Fig. 1) is a pigment found in yellow Brazil wood (Liu and Sheu, 1989), which is predominantly present as glycosides and distributed in mulberry fruit and herbs (Elangovan *et al.*, 1994).

Morin has been reported to possess various pharmacological properties including prevention of coronary artery diseases (Tanaka *et al.*, 1990), inhibit tumor proliferation (Wu *et al.*, 1994) and protect human erythrocytes, ventricular myocytes, and saphenous vein endothelial cells (Kok *et al.*, 2000), prevent LDL-oxidation (Liu and Sheu, 1989), as well as scavenge free radicals (Zeng *et al.*, 1994; Vishnukumar *et al.*, 2012). As there are no available reports detailing the role of morin on membrane bound enzymes and glycoproteins in STZ-induced diabetes, this study examined the role of morin on membrane bound enzymes (total ATPase, sodium/potassium ATPase, calcium ATPase and magnesium ATPase) in liver and kidney and glycoproteins (hexose, hexosamine, fucose and sialic acid) in plasma and tissues (liver and kidney). In addition, the effect of morin on histopathology of liver and kidney were also studied.

Fig. 1. Structure of morin.



Materials and methods

Drugs and chemicals: Streptozotocin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Carboxy methyl cellulose (CMC) sodium salt was obtained from S.D. Fine Chemicals, Mumbai, India. Morin was purchased from Hi-media Laboratories Pvt., Ltd, Mumbai. All other chemicals used in the study were of analytical grade.

Experimental animals: Male albino Wistar rats (150-200 g) obtained from Venkateswara Enterprises, Bangalore were used in this study. The animals were housed in polypropylene cages (47 x 34 x 18 cm) lined with husk. It was renewed every 24 h. The animals were fed on a standard pellet diet (Sri Durga Seeds and Foods, Bangalore, India) and water *ad libitum*. The standard pellet diet comprised 21% protein, 5% lipids, 4% crude fibre, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamin and 55% nitrogen free extract (carbohydrates). It provides metabolisable energy of 3,600 kcal. The experimental animals were maintained in a controlled environment (12:12 h light/dark cycle) and temperature ($30 \pm 2^\circ\text{C}$). All the experiments were carried out according to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), New Delhi, India and approved by the institutional animal ethical committee of Vinayaka Missions University (Approval No. Biotech/03/2008).

Induction of experimental diabetes: Streptozotocin was freshly dissolved in citrate buffer (0.01 M, pH 4.5) and overnight fasted rats were intraperitoneally injected with STZ (50 mg/kg).

Experimental groups: In this experiment, a total of 24 rats divided in to 4 groups of 6 rats each. Morin was dissolved in carboxy methyl cellulose (CMC) at a dose of 100 mg/kg given to rats orally for a period of 45 d (Vishnukumar *et al.*, 2012).

Group 1: Normal control rats

Group 2: Normal rats + Morin (100 mg/kg)

Group 3: STZ induced diabetic rats (50 mg/kg)

Group 4: Diabetic rats + Morin (100 mg/kg)

After 45 d of treatment period, the fasted rats were sacrificed by cervical decapitation and the blood was collected and serum separated. The liver and kidney were weighted and 10% tissue homogenate was prepared with 0.025 M, Tris-HCl buffer pH 7.5. After centrifugation at 2,000 rpm for 10 min, the clear supernatant was used for the analysis of various biochemical parameters.

Biochemical analysis: The tissue samples were defatted prior to estimation. A weighed amount of defatted tissue was suspended in 3.0 mL of 2 N HCl and heated at 90°C for 4 h. The sample was cooled and neutralized with 3 mL of 2 N NaOH.

Aliquots from this were used for the estimation of hexose, hexosamine, fucose and sialic acid. Protein bound hexose was estimated by the method of Dubois and Gilles (1956). To 0.1 mL of sample, 5.0 mL of 95% ethanol was added, mixed and then centrifuged. The precipitate was dissolved in 1.0 mL of 0.1 N NaOH. To all the tubes, 8.5 mL of orcinol-sulfuric acid reagent was added and kept in a water bath at 90°C for 15 min. The tubes were cooled in tap water and the colour developed was read at 540 nm. Protein bound hexosamine was estimated according to the method of Wagner (1979). To 1.0 mL of sample, added 2.5 mL of 3 N HCl and kept for 6 h in a boiling water bath and then neutralized with 6 N NaOH. To 0.8 mL of the neutralized sample, added 0.6 mL of acetyl acetone reagent. Then, the tubes were heated in a boiling water bath for 30 min. After cooling, 2.0 mL of Ehrlich's reagent was added and mixed well. The intensity of the colour developed was read at 540 nm.

Fucose was estimated by the method of Dische and Shettles (1948). To 0.5 mL of sample, 4.5 mL of H_2SO_4 was added and heated in a boiling water bath for 3 min, cooled and 0.1 mL of cysteine reagent was added. After 75 min in dark, the absorbance was read at 393 and 430 nm. Sialic acid was estimated by the method of Warren (1959). To 0.5 mL of sample, 0.2 mL of distilled water and 0.25 mL of periodic acid were added and incubated at 37°C for 30 min. To this, 0.2 mL of sodium m-arsenate and 2.0 mL of TBA were added and heated in a boiling water bath for 6 min, cooled and 5.0 mL of acidified butanol was added. The absorbance was read at 540 nm.

The activity of Na^+/K^+ -ATPase was assayed according to the procedure of Bonting (1970). The incubation mixture contained 1.0 mL of buffer, 0.2 mL of magnesium sulphate, 0.2 mL of potassium chloride, 0.2 mL of sodium chloride, 0.2 mL of EDTA, 0.2 mL of ATP and 0.2 mL of tissue homogenate. The contents were incubated at 37°C for 15 min. One mL of ice-cold 10% TCA was added at the end of 15 min to arrest the reaction. The amount of phosphorus liberated was estimated as described by Fiske and Subbarow (1925). Supernatant (1.0 mL) was made up to 4.0 mL with distilled water and 1.0 mL of 2.5% ammonium molybdate was added. This was incubated at room temperature for 10 min and 0.4 mL of aminonaphthol sulfonic acid was added. The colour developed was read spectrophotometrically at 640 nm after 20 min. The activity of Ca^{2+} -ATPase was assayed according to the method of Hjerten and Pan (1983). The incubation mixture contained 0.1 mL of buffer, 0.1 mL of calcium chloride, 0.1 mL of ATP, 0.1 mL of distilled water and 0.1 mL of tissue homogenate. The contents were incubated at 37°C for 15 min. The reaction was then arrested by the addition of 0.5 mL of ice-cold 10% TCA. The amount of phosphorus liberated was estimated according to the method of Fiske and Subbarow (1925).



Table 1. Effect of morin on the levels of serum glycoproteins (hexose, hexosamine, fucose and sialic acid) in normal and streptozotocin-induced diabetic rats.

Groups	Hexose (mg/dL)	Hexosamine (mg/dL)	Fucose (mg/dL)	Sialic acid (mg/dL)
Normal control rats	78.3±5.8 ^a	63.8±4.1 ^a	33.4±2.5 ^a	55.2±4.6 ^a
Normal + Morin (100 mg/kg)	79.2±6.7 ^a	62.5±3.9 ^a	32.7±2.0 ^a	54.7±4.1 ^a
Diabetic control rats (50 mg/kg)	135.2±8.2 ^b	102.6±7.1 ^b	55.6±3.7 ^b	88.4±7.5 ^b
Diabetic + Morin (100 mg/kg)	92.6±8.1 ^c	77.3±5.7 ^c	41.2±3.3 ^c	63.3±5.0 ^c

Each value is mean ± S.D. for six rats in each group;
 Values not sharing a common superscript (a-c) differ significantly with each other (P<0.05, DMRT).

Table 2. Effect of morin on the levels of liver glycoproteins (hexose, hexosamine, fucose and sialic acid) in normal and streptozotocin-induced diabetic rats.

Groups	Hexose (mg/g defatted tissue)	Hexosamine (mg/g defatted tissue)	Fucose (mg/g defatted tissue)	Sialic acid (mg/g defatted tissue)
Normal control rats	41.2±3.1 ^a	22.5±1.4 ^a	16.8±0.91 ^a	12.4±0.73 ^a
Normal + Morin (100 mg/kg)	40.9±3.5 ^a	21.7±1.5 ^a	16.2±0.64 ^a	11.7±0.52 ^a
Diabetic control rats (50 mg/kg)	65.4±4.1 ^b	38.2±2.2 ^b	28.3±1.9 ^b	19.0±1.3 ^b
Diabetic + Morin (100 mg/kg)	48.3±3.2 ^c	26.6±2.0 ^c	20.1±1.6 ^c	16.2±1.1 ^c

Table 3. Effect of morin on the levels of kidney glycoproteins (hexose, hexosamine, fucose and sialic acid) in normal and streptozotocin-induced diabetic rats.

Groups	Hexose (mg/g defatted tissue)	Hexosamine (mg/g defatted tissue)	Fucose (mg/g defatted tissue)	Sialic acid (mg/g defatted tissue)
Normal control rats	32.5±2.7 ^a	24.3±1.8 ^a	14.5±0.9 ^a	9.5±0.5 ^a
Normal + Morin (100 mg/kg)	31.4±2.2 ^a	23.8±1.5 ^a	14.2±1.0 ^a	9.1±0.4 ^a
Diabetic control rats (50 mg/kg)	56.8±3.9 ^b	40.4±2.1 ^b	26.0±2.2 ^b	16.5±1.3 ^b
Diabetic + Morin (100 mg/kg)	38.6±2.9 ^c	29.5±1.7 ^c	19.2±1.3 ^c	12.5±0.6 ^c

Table 4. Effect of morin on membrane bound enzymes in liver of normal and streptozotocin-induced diabetic rats.

Groups	Total ATPase*	Na ⁺ /K ⁺ -ATPase*	Ca ²⁺ -ATPase*	Mg ²⁺ -ATPase*
Normal control rats	5.23±0.31 ^a	1.57±0.10 ^a	3.13±0.17 ^a	0.77±0.003 ^a
Normal + Morin (100 mg/kg)	5.30±0.27 ^a	1.54±0.10 ^a	3.05±0.13 ^a	0.80±0.005 ^a
Diabetic control rats (50 mg/kg)	2.55±0.19 ^b	0.86±0.05 ^b	1.16±0.09 ^b	0.37±0.002 ^b
Diabetic + Morin (100 mg/kg)	4.56±0.29 ^c	1.40±0.12 ^c	2.78±0.15 ^c	0.66±0.0043 ^c

*Units: μmoles of phosphorus liberated/min/mg protein.

Table 5. Effect of morin on membrane bound enzymes in kidney of normal and streptozotocin-induced diabetic rats.

Groups	Total ATPase*	Na ⁺ /K ⁺ -ATPase*	Ca ²⁺ -ATPase*	Mg ²⁺ -ATPase*
Normal control rats	7.13±0.42 ^a	3.13±0.19 ^a	1.83±0.12 ^a	1.94±0.14 ^a
Normal + Morin 100 mg/kg	7.23±0.63 ^a	3.09±0.25 ^a	1.84±0.09 ^a	1.89±0.15 ^a
Diabetic control rats (50 mg/kg)	3.55±0.25 ^b	1.25±0.10 ^b	0.76±0.05 ^b	0.97±0.16 ^b
Diabetic + Morin 100 mg/kg	6.55±0.50 ^c	2.57±0.19 ^c	1.60±0.13 ^c	1.77±0.14 ^c

*Units: μmoles of phosphorus liberated/min/mg protein.

The activity of Mg²⁺-ATPase was assayed according to the method of Ohnishi *et al.* (1982). The incubation mixture contained 0.1 mL of buffer, 0.1 mL of magnesium chloride, 0.1 mL of ATP, 0.1 mL of distilled water and 0.1 mL of tissue homogenate. The reaction mixture was incubated at 37°C for 15 min. The reaction was arrested by the addition of 0.5 mL ice cold 10% TCA. The amount of phosphorous liberated was estimated by the method of Fiske and Subbarow (1925). Protein in the enzyme extract was determined by the method of Lowry *et al.* (1951).

Histopathological studies: For histopathological studies, tissues were fixed with 10% buffered formalin for 2 h and processed. Liver and kidney were sectioned at 5μm thickness using a microtome. The paraffin sections were stained with haemotoxylin and eosin (H&E). The photographs were taken in a light microscope.

Statistical analysis: Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Using SPSS software package, version 9.05 p values <0.05 were considered as significant.

Results

The levels of glycoproteins (hexose, hexosamine, fucose and sialic acid) in serum, liver and kidney of normal and STZ-induced rats are presented in Tables 1, 2 and 3. Significantly higher levels of these glycoproteins were observed in the serum and tissues of diabetic control rats. Treatment with morin (100 mg/kg) to diabetic rats resulted in a significant reduction of hexose, hexosamine, fucose and sialic acid in serum and tissues, when compared with diabetic control rats. The activities of membrane bound ATPases in the liver and kidney of normal control and experimental group of rats are summarized in Tables 4 and 5 respectively. There were a marked decrease in the activities of membrane bound phosphatases such as total ATPases, Na^+/K^+ -ATPases, Mg^{2+} -ATPases and Ca^{2+} -ATPases in the liver and kidney of STZ-induced diabetic rats. Oral administration of morin to diabetic rats for a period of 45 d significantly increased the activities of these enzymes towards near normalcy. Histopathological changes were observed in the tissues of STZ-induced rats as compared to those of untreated control rats. The liver samples of STZ-induced rats (Fig. 2c) showed severe fatty changes and characteristic displacement of nucleus in hepatocytes. Diabetic rats treated with morin (100 mg/kg) showing mild vacuolar changes and mild congestion (Fig. 2d). Diabetic rat kidney showing degenerating tubules, with disturbed lining epithelium with inflammatory cells (Fig. 3c). Diabetic rat treated with morin kidney showing apparently normal tubular structures with intact lining epithelium (Fig. 3d). Normal rats treated with morin (100 mg/kg) didn't show any pathological alteration in liver and renal tissues (Figs. 2b and 3b), administration of morin didn't have any pathological effect under normal conditions. In all the parameters studied, oral administration of morin (100 mg/kg) to normal rats for a period of 45 d showed minor effects, but none was statistically significant. Morin at a dose of 100 mg/kg showed a significant effect in STZ-induced diabetic rats.

Discussion

Increased glycosylation of various proteins in diabetic patients had been reported earlier (Rahman *et al.*, 1990). In this study, we have observed increased levels of hexose, hexosamine, fucose and sialic acid in serum and tissues of STZ-treated diabetic rats. Increased serum glycoprotein components have been associated with the severity and duration of diabetes. In hyperglycemia, free amino groups of proteins react slowly with the carbonyl groups of reducing sugars such as glucose, to yield a schiff-base intermediate and these schiff-base intermediates undergo Amadori rearrangement to stable ketoamine derivative (fucosamine) (Bucala, 1999). Fucose is a member of the group of 8 essential sugars the body requires for optimal function of cell-to-cell communication and its metabolism appears to be altered in various diseases such as diabetes mellitus (Mondoa *et al.*, 2001). Elevated levels of fucose in experimental diabetes were reported by researchers (Prakasam *et al.*, 2005; Latha and Pari, 2005).

A raise in fucose levels could be due to increased glycosylation in the diabetic state (Hunt *et al.*, 1991).

Fig. 2a. Control rat liver showing normal architecture (400x).

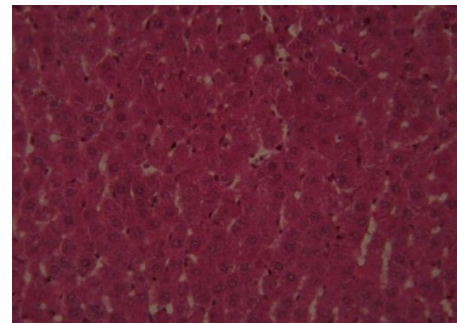


Fig. 2b. Normal rat treated with morin (100 mg) liver showing normal appearance of the hepatocytes (400x).

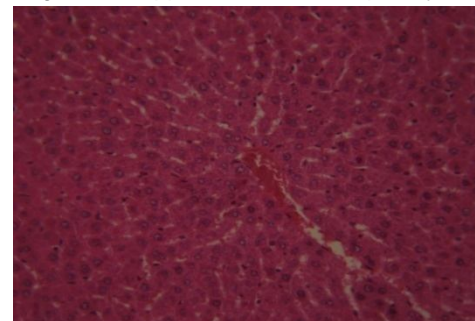


Fig. 2c. Diabetic control rat liver showing severe fatty changes and characteristic displacement of nucleus in hepatocytes.

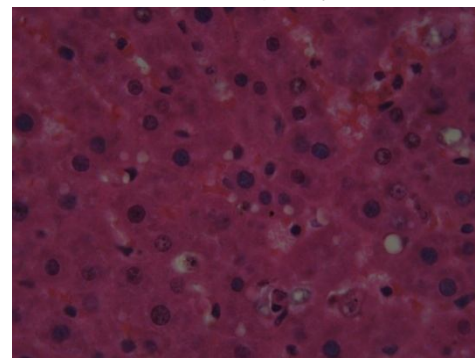
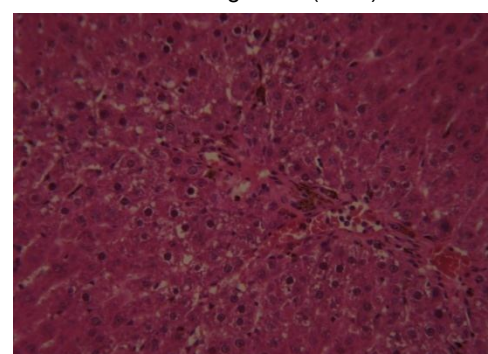


Fig. 2d. Diabetic rat liver treated with morin (100 mg/kg) showing mild vacuolar changes and mild congestion (400x).



Sialic acid is found in a wide variety of substances and tissues in animals and humans, occurring most abundantly in glycoproteins and glycolipids. Sialic acid bound to membrane glycoproteins and glycolipids apparently enters the circulation by either shedding or cell lysis (Sheshadri, 1994). Increased levels of sialic acid were reported in STZ-diabetic rats and in diabetic patients (Ekin *et al.*, 2003). In the diabetic state, deficiency of insulin secretion causes derangement of glycoprotein metabolism that result in the basal membrane thickening. Excess availability of glucose in the hyperglycemic state accelerates the synthesis of basement membrane components, i.e., glycoproteins (Kamalakannan and Prince, 2006).

Administration of morin (100 mg/kg) to diabetic rats significantly decreased the levels of glycoproteins in serum, liver and kidney. Blood glucose homeostasis is due to increased secretion of insulin observed in morin-treated diabetic rats could be responsible for the decreased glycoproteins in plasma, liver and kidney. In this context, other researchers also reported that decrease in hyperglycemia could lead to a decrease in glycoprotein levels (Prakasam *et al.*, 2005; Latha and Pari, 2005). Agents with antioxidant or free radical scavenging property may inhibit oxidative reactions associated with glycation. Morin was shown to inhibit glycosylation reactions by reducing the generation of reactive oxygen species. Morin decreased glycosylated haemoglobin levels, decreased lipid peroxidation and improved antioxidant status in plasma of STZ-diabetic rats. The results of the present study indicate that morin has a beneficial effect on the levels of glycoproteins in the diabetic state in addition to its antihyperglycemic and antioxidant properties. Assay of membrane associated enzyme activities like ATPases indicate the alterations of the membrane under diseased status. ATPases are intimately associated with the plasma membrane and participates in the energy requiring translocation of sodium, potassium, calcium and magnesium (Mourelle and Franco, 1991). Higher level of lipid peroxidation process ultimately impairs the membrane function by decreasing its fluidity and altering the activities of membrane-bound enzymes and receptors.

In this study, we have observed decreased activities of membrane bound enzymes. Alterations in Na^+/K^+ -ATPase alters the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism which may play a vital role in regulating the cellular calcium levels (Trump *et al.*, 1984). Calcium dependent adenosine triphosphatase (Ca^{2+} -ATPase) is the major active calcium transport protein responsible for the maintenance of intracellular calcium levels in various tissues. Since Na^+/K^+ -ATPase is a 'SH' group containing enzyme and is lipid dependent, the inactivation of Na^+/K^+ -ATPase could be due to enhanced lipid peroxidation by free radicals (Paritha and Devi, 1997).

Fig. 3a. Normal control rat kidney showing intact tubular structure (400x).

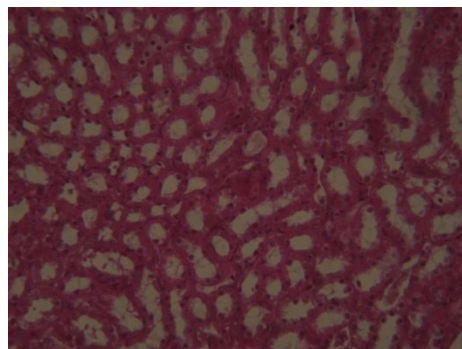


Fig. 3b. Normal rat kidney treated with morin (100 mg/kg) showing normal architecture of the renal cells (400x).

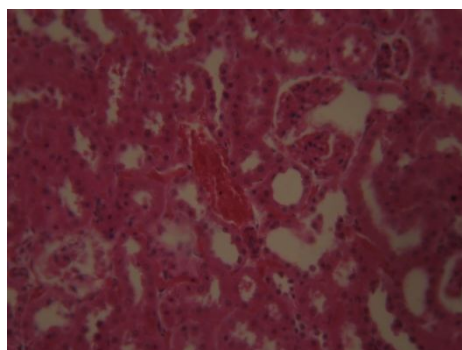


Fig. 3c. Diabetic rat Kidney showing degenerating tubules with disturbed lining epithelium with inflammatory cells (400x).

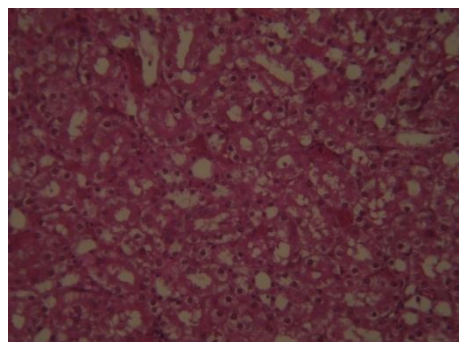
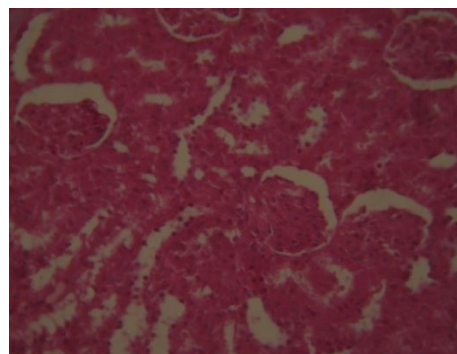


Fig. 3d. Diabetic rat kidney treated with morin (100 mg/kg) showing apparently normal tubular structures with intact lining epithelium (400x).



Pretreatment with morin increased the activities of membrane bound enzymes in STZ-administered rats. This could be due to the ability of morin to protect the 'SH' groups from the oxidative damage through the inhibition of peroxidation of membrane lipids. Minimizing the alterations in the activities of these enzymes indirectly indicates the membrane stabilizing action of morin in diabetic rats. Histopathological changes were observed in the tissues of STZ-induced rats as compared to those of untreated control rats. The liver samples of STZ-induced rats showed severe fatty changes and characteristic displacement of nucleus in hepatocytes. It is well known that during diabetic conditions the hepatocytes also undergo pathological alterations. Diabetic rats treated with morin showed mild vacuolar changes and congestion. Diabetic rat kidney showed degenerating tubules, with disturbed lining epithelium with inflammatory cells. Diabetic rats treated with morin showed apparently normal tubular structures with intact lining epithelium. Normal rats treated with morin didn't show any pathological alterations in both liver and kidney, which indicates that administration of morin didn't have any pathological effect under normal conditions. Administration of flavonoids was reported to possess reducing the pathological alterations in diabetic rats in experimental studies.

Morin, a flavonoid significantly reduces the pathological alterations in STZ-induced rats. The presence of the hydroxyl group at C-3 position of the skeletons of morin has been shown to be responsible for the potent inhibitory action on lipid peroxidation (Affana's *et al.*, 1989; Mora *et al.*, 1990; Ratty and Das, 1998; Morel *et al.*, 1993). When the double bond between the carbons 2 and 3 of the C ring is hydrogenated, antiperoxidative potential decreases (De Whalley *et al.*, 1990; Cholbi *et al.*, 1991). The carbonyl group is essential for the antiperoxidant activity (Cholbi *et al.*, 1991; Ratty and Das, 1998). The hydroxyl groups at the C-5 and C-7 positions of the A-ring and C-4' of the B-ring and C-3 of the C-ring seem to contribute to the antioxidant action. The flavonoids that have hydroxyl groups at C-2' position, such as morin, have shown antiperoxidative properties (Cholbi *et al.*, 1991). The hydroxyl groups at the C-3 and C-5 of the flavonoid skeleton, like in quercetin and morin, can form chelators from iron ions.

Conclusion

This study investigated the effect of morin on membrane bound enzymes and glycoproteins in normal and streptozotocin-induced diabetic Wistar rats. The hydroxyl and carbonyl group of morin could be responsible for its antilipoperoxidative, antioxidative and metal chelating properties and these properties indirectly helps to maintain the levels of membrane bound enzymes and glycoproteins in STZ-induced diabetic rats showing the membrane stabilizing property of morin.

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