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Silymarin, a flavonoid antioxidant, protects streptozotocin-induced lipid peroxidation and β-Cell damage in rat pancreas

Manju Sharma[1,*], Tarique Anwer[1], K K Pillai[1], Syed Ehtaishamul Haque[1], A K Najmi[1] and Yasmin Sultana[2]

[1]Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), New Delhi-110 062, India; [2]Department of Pharmaceutics, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), New Delhi-110 062, India

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SUMMARY

The present study is aimed at finding the influence of silymarin (a flavonoid) (25 mg/kg & 50 mg/kg) in streptozotocin (STZ)-induced diabetic rats. Type 2 diabetes was induced by single intraperitoneal injection of STZ (100 mg/kg) to 3 days old rat pups. Silymarin was administered for 15 days after the animals were confirmed diabetic (75 days after STZ injection). Blood glucose, glycosylated hemoglobin (HbA_{1c}), lipid peroxides (LPO) levels and reduced glutathione (GSH) contents in pancreas and liver were estimated following the established procedures. Biochemical observations were further substantiated with histological examination of pancreas. Blood glucose and HbA_{1c} levels, which were elevated by STZ, were lowered to physiological levels by the administration of silymarin. The levels of LPO were significantly increased in STZ-induced diabetic rats. Silymarin reduced the LPO levels in both pancreas and liver. GSH contents which were reduced significantly in pancreas and liver of STZ-induced diabetic rats were brought back to near normal levels by silymarin treatment. Multifocal necrotic and degenerative changes of pancreas in STZ-diabetic rats were minimized to near normal morphology by administration of silymarin as evident by histopathological examination. Silymarin showed a dose dependent protective effect on STZ-induced β-cell damage. It could be attributed to the antioxidative and free radicals scavenging properties of the flavonoid. Thus, it may be considered as a natural antioxidant with potential therapeutic application in the treatment of type 2 diabetes.

Key words: Silymarin; Flavonoid; Streptozotocin; Lipid peroxidation

INTRODUCTION

Type 2 diabetes mellitus is among the most common disorders in developed and developing countries. The existence of an oxidative stress in diabetes is still debated (Baynes, 1991; Oberley, 1998). Lipid peroxidation has been implicated in the pathogenesis of naturally occurring and chemically induced diabetes (Wolff *et al.,* 1990). Generation of free radicals has been proposed to be a major mechanism involved in the streptozotocin (STZ) cytotoxicity. STZ is frequently used to induce diabetes mellitus in experimental animals

*Correspondence: Manju Sharma, Department of Pharmacology, Faculty of Pharmacy, Jamia Hamdard (Hamdard University), New Delhi - 110 062, India. Tel: +01126058682, Extn-5648; E-mail: manju_sharma72 @yahoo.com

through its toxic effects on pancreatic β-cells (Yamagishi *et al.,* 2001; Stefek *et al.,* 2002; Kalendar *et al.,* 2002; Kim *et al.,* 2003). The cytotoxic action of STZ is associated with the generation of reactive oxygen species causing oxidative damage (Szkudelski, 2001). Diabetes manifested by experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of antioxidative defense system (Baynes and Thorpe, 1997). Increased oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the etiology of diabetic complications (Baynes, 1991). Oxidant stress may be increased in diabetes owing to a hyper production of reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) and/or a deficiency in the anti-oxidant defense system. An impaired radical scavenger function has been linked to the decreased activity of enzymatic and non-enzymatic scavengers (Loven *et al., 1990*). Some authors have demonstrated the therapeutic potential of antioxidant treatment in type 2 diabetes mellitus. Chemicals with antioxidant properties and free radical scavengers may help in the regeneration of β-cells and protect pancreatic islets against cytotoxic effects of STZ (Alvarez *et al.,* 2004).

Silymarin is a flavonoid extracted from the milk thistle of *Silybum marianum*. It is a free radical scavenger and a membrane stabilizer that prevents lipid peroxidation (LPO) and its associated cell damage in some experimental models. This compound has shown protective effects against the oxidative peroxidation of cells in several models such as hepatotoxicity produced by CCl_4 (Muriel and Mourelle, 1990), paracetamol (Muriel *et al.,* 1992) and bromobenzene (Paya *et al.,* 1993). In these models, silymarin functioned as a free radical scavenger, increasing available GSH (Reduced glutathione (as a detoxificant of intermediary oxygen reactive products of lipid peroxidation)) and preventing membrane alterations. Silymarin has also showed its protective effect in cirrhotic diabetic patients (Velussi *et al.,* 1997) and in exocrine pancreas (Schonfeld *et al.,* 1997). Flavonoids are known for their strong scavenging effect on free radicals and may also be able to suppress the formation of free radicals by binding of heavy metal ions, which are known to catalyze many processes leading to the appearance of free radicals (Bosisio *et al.,* 1992). Furthermore, cytoprotective effect of silymarin in pancreatic β-cell against cytokine-mediated toxicity is recently been studied (Matsuda *et al.,* 2005). Since the free radicals mediated LPO is caused by STZ, therefore the protective effect of silymarin was studied in STZ-induced lipid peroxidation and β-cell damage in rat pancreas.

MATERIALS AND METHODS

Animals

Healthy albino Wistar rats (150–180 g) were kept for breeding. The animals were maintained under controlled condition of illumination (12/12 h light/ darkness) and temperature 20-25°C. They were housed under ideal laboratory conditions, maintained on standard pellet diet (Lipton rat feed, Ltd; Pune) and water *ad libitum* throughout the experimental period. The experimental study was approved by the Institutional Animal Ethics Committee of Jamia Hamdard, New Delhi, India.

Drugs and chemicals

Silymarin (Silybon) was purchased from Micro-Labs Ltd., India. STZ was purchased from Sigma Chemicals (USA). Glucose kit was procured from Span diagnostics, Surat, India. All the other chemicals used were of analytical grade.

Induction of experimental diabetes

To induce type 2 diabetes mellitus, STZ (100 mg/kg) in citrate buffer (pH-4.5) was administered intraperitoneally to 3 days old rat pups. Another group of pups received only citrate buffer. 75 days

after STZ treatment, development of diabetes was confirmed by measuring blood glucose level. Rats with fasting-glucose levels of 150 mg/dl or higher were considered to be diabetic.

Experimental design

The rats were divided into four groups comprising of six animals in each group as follows: Group I: Normal control; Group II: Diabetic control; Group III: Diabetic treated, received silymarin (25 mg/kg, p.o) for 15 days; Group IV: Diabetic treated, received silymarin (50 mg/kg, p.o) for 15 days. On the last day of experiment, blood samples were collected for biochemical estimations. Later the animals were sacrificed and liver and pancreas were removed, cleaned and washed in ice-cold normal saline for biochemical study.

Determination of blood glucose

Blood glucose level was estimated by glucose oxidase (Braham and Trinder, 1972) method using a commercial diagnostic kit from Span diagnostic Ltd, Surat, India.

Determination of glycosylated hemoglobin (HbA_{1c})

HbA_{1c} was estimated by a colorimetric assay method as described by Pecoraro *et al.* (1979).

Determination of LPO

LPO was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids (Ohkawa *et al*., 1979). Tissues were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) that contained KCl (1.17% w/v), using motor driven Teflon pestle. Aliquot of 1 ml of the suspension medium was taken from the supernatant obtained after the centrifugation of tissue homogenate (10% w/v) at 10,500 × g. 0.5 ml of 30% TCA followed by 0.5 ml of 0.8% TBA was then added to it. The tubes were kept in shaking water bath for 30 min at 80°C. After 30 min of incubation tubes were taken out and kept in ice cold water for 10 min. These were then centrifuged at 800 × g for 15 min. The absorbance of supernatant was read at 540 nm at room temperature against appropriate blank. The concentration of MDA was measured from the standard calibration curve prepared by using tetraethoxypropane. Protein was estimated by the method of Lowry *et al.* (1951). Lipid peroxidation was expressed as nmoles of MDA per milligram of protein.

Determination of GSH

GSH content was estimated by method of Sedlak & Lindsay (1968). The tissues were homogenized in 0.02 M EDTA. Aliquots of 5 ml of the homogenates were mixed in test tube with 4 ml of cold distilled water and 1 ml of 50% TCA. The tubes were shaken for 10 min using vortex mixer and the centrifuged at 1,200 × g for 15 min. Following centrifugation 2 ml of supernatant was mixed with 4 ml of 0.4 M tris buffer (pH 8.9). The whole solution was mixed and 0.1 ml of 0.01 M DTNB {5, 5'-Dithiobis (2-nitrobenzoic acid)} was added to it. The absorbance was read within 5 min of addition of DTNB at 412 nm using UV-spectrophotometer (Shimadzu, UV-1601, Japan) against a reagent blank with no homogenate.

Histopathological examination

The pancreas was isolated immediately after sacrificing the animal and washed with ice-cold saline. It was then fixed in 10% neutral buffered formalin solution. Sections of 3-5 μm thickness were stained with hematoxylin and eosin (H.E.) for histopathological examination.

Statistical analysis

Data were expressed as the mean ± standard error (S.E.) of the means. For a statistical analysis of the data, group means were compared by one-way analysis of variance (ANOVA) with post hoc analysis. The Tukey-Karmer test post hoc was applied to identify significance among groups. $P < 0.05$ was considered to be statistically significant. GraphPad software, Inc. (version 3.06) was used for statistical analysis.

RESULTS

Table 1 shows the effect of silymarin on blood glucose level. Significant increase in blood glucose level was observed in animals treated with STZ. Administration of two different doses (25 mg/kg and 50 mg/kg) of silymarin reduced the blood glucose level significantly ($P < 0.001$) when compared with diabetic control rats. Silymarin showed antihyperglycemic effect in a dose dependent manner.

Table 1 shows the effect of silymarin on HbA_{1c} level. Significant ($P < 0.001$) increase in HbA_{1c} level was observed in diabetic control rats when compared with normal control rats. Oral administration of silymarin at two different doses (25 mg/kg and 50 mg/kg) reduced the HbA_{1c} level significantly ($P < 0.05$ - $P < 0.01$) in a dose-dependent manner.

As shown in the Table 2, the level of MDA, a secondary product of LPO was found to be significantly ($P < 0.001$) higher in both pancreas and in liver of diabetic control rats when compared with normal control rats. Silymarin (25 mg/kg and 50 mg/kg) treatment produced a significant ($P < 0.05$ - $P < 0.001$) reduction in LPO levels in both pancreas and liver of diabetic treated rats when compared with diabetic control rats. Silymarin (50 mg/kg) showed better protective effect in terms of lowering LPO levels.

As shown in the Table 3, diabetic control rats showed significant decrease in pancreatic and hepatic GSH content when compared with normal control rats. Administration of silymarin in two doses (25 mg/kg and 50 mg/kg) increased the GSH contents significantly ($P < 0.05$ - $P < 0.001$) in diabetic treated rats when compared with diabetic control rats. Silymarin (50 mg/kg) showed better protective effect in terms of increasing GSH contents.

Section of rat pancreas from normal control group showed, normal pancreatic acini and β-cells sof islets of Langerhans (Fig. 1A). STZ diabetes resulted in degenerative and lytic changes in the islets of Langerhans of the pancreas (Fig. 1B) and

Table 1. Effect of silymarin on blood glucose and glycosylated hemoglobin levels in STZ-induced diabetic rats

Group	Treatment	Blood glucose (mg/dl)	Glycosylated hemoglobin HbA_{1c} (%)
I	Normal Control	82.42 ± 5.90	5.03 ± 0.67
II	STZ (100 mg/kg, i.p)	259.99 ± 23.64^{x}	10.73 ± 0.49^{x}
III	STZ+Silymarin (25 mg/kg/day, p.o)	99.90 ± 2.62***	7.77 ± 0.68*
IV	STZ+Silymarin (50 mg/kg/day, p.o)	89.17 ± 3.32***	7.53 ± 0.69**

The data are expressed in mean ± S.E.M. (n = 6) in each group. $^{x}P < 0.001$ compared with the corresponding value for normal control group (group I). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding value for silymarin treated groups (group III & IV).

Table 2. Effect of silymarin on lipid peroxidation (MDA) levels in pancreas and liver of STZ-induced diabetic rats

Group	Treatment	Pancreatic tissue MDA (nmoles/mg protein)	Hepatic MDA (nmoles/mg protein)
I	Normal Control	0.158 ± 0.012	4.14 ± 0.29
II	STZ (100 mg/kg, i.p)	0.95 ± 0.047^{x}	8.24 ± 0.68^{x}
III	STZ+Silymarin (25 mg/kg/day, p.o)	0.715 ± 0.071**	6.05 ± 0.35*
IV	STZ+Silymarin (50 mg/kg/day, p.o)	0.558 ± 0.052***	5.45 ± 0.40**

The data are expressed in mean ± S.E.M. (n = 6) in each group. $^{x}P < 0.001$ compared with the corresponding value for normal control group (group I). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the corresponding value for silymarin treated groups (group III & IV).

Table 3. Effect of silymarin on GSH levels in pancreas and liver of STZ-induced diabetic rats

Group	Treatment	Pancreatic GSH (nmoles/mg protein)	Hepatic GSH (nmoles/mg protein)
I	Normal Control	0.98 ± 0.29	2.52 ± 0.22
II	STZ (100 mg/kg, i.p)	0.628 ± 0.47^{x}	1.698 ± 0.49^{x}
III	STZ+Silymarin (25 mg/kg/day, p.o)	0.728 ± 0.65**	1.82 ± 0.31*
IV	STZ+Silymarin (50 mg/kg/day, p.o)	0.86 ± 0. 33***	2.195 ± 0.26**

The data are expressed in mean ± S.E.M. (n = 6) in each group. $^{x}P < 0.001$ compared with the corresponding value for normal control group (group I). $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.001$ compared with the corresponding value for silymarin treated groups (group III and IV).

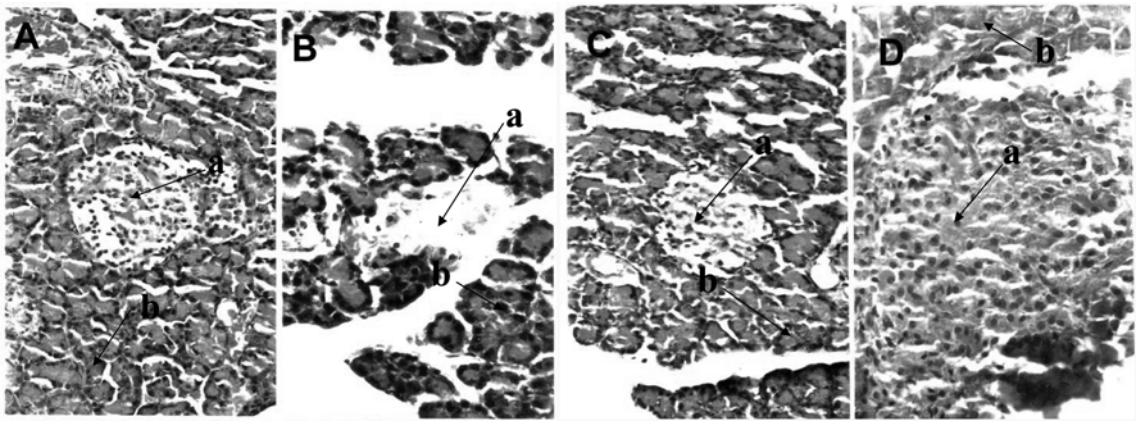

Fig. 1. Hematoxylin and eosin-stained sections of rat pancreas. **(A)** Section of rat pancreas from normal control rats showing (a) normal islets of Langerhans comprising of b-cells and (b) lobules of pancreatic acini and blood vessels **(B)** Section of pancreas from STZ treated diabetic rats showing (a) pancreatic islets with depletion of b cells and (b) lobules of pancreatic acini with areas of fibrosis **(C)** Section of pancreas from STZ + Silymarin (25 mg/kg) treated rats showing (a) regeneration of β-cells with (b) mild fibrosis of pancreatic acini **(D)** Section of pancreas from STZ + Silymarin (50 mg/kg) treated rats showing (a) normal b cells with marked hyperplasia and enlargement of pancreatic islets and (b) well brought out pancreatic acini. (A&C - H.E × 200; B&D - H.E × 400).

there were depletion of β-cells. Administration of silymarin (25 and 50 mg/kg) improved the histological picture of pancreas as evidenced by an increase in the size of the islets. These changes were consistently observed in all animals in these groups (Fig. 1C and 1D). However, silymarin (50 mg/kg) treated animals had better protective effect as evidenced by minimal necrotic changes (Fig. 1D) and evidence of hyperplasia marked by increase in cellular contents.

DISCUSSION

It has been suggested that oxidative stress plays an important role in tissue damage associated with diabetes (Giugliano *et al*., 1996). The sources of oxygen derived free radicals in diabetes are not known, but it is possible that the source may be from autoxidation of glucose (Wolff and Dean, 1987) and non-enzymatic glycation (Ceriello *et al*., 1992). STZ a naturally occurring nitrosamide has relative pancreatic islets β-cell cytotoxicity in animals. The precise mechanism of action of STZ is unknown, but there is evidence that it interferes with cellular metabolite oxidative mechanisms (Okamoto, 1985). Generation of oxygen free radicals has been proposed to be a major mechanism involved in the cytotoxicity (Takasu *et al*., 1991). Pancreatic islets possess very low free radical scavenging enzyme and hence are vulnerable to

free radical toxicity (Wohaieb and Godin, 1987). The extent of tissue damage induced by O_2 radical depends on the balance of O_2 radical formation and the endogenous antioxidant defense mechanism (Machlin and Bendich, 1987; Barnes, 1990). Pascual *et al*. (1993) in chemiluminescence studies showed the protective activity of silymarin on free radicals. Muriel *et al*. (1992) showed that the increase of MDA products induced by paracetamol was diminished by silymarin administration. It is also reported that silymarin prevents the damage induced by oxidative agents in hepatic membranes (Greimel and Koch, 1977), mitochondria and microsomes (Bindoli *et al*., 1977). These effects of silymarin in the area of hepatocyte protection may contribute to explaining why this compound has a protective effect on pancreatic LPO level, with the recovery of the β-cell function. This may contribute to the regulation of plasma glucose.

Diabetic control rats exhibited persistent hyperglycemia. Silymarin (25 mg/kg and 50 mg/kg) treatment to diabetic rats produced a significant decrease in blood glucose level. In uncontrolled and poorly controlled diabetes, there is an increased glycosylation of a number of proteins including hemoglobin and β-crystalline of lens (Alberti and Press, 1982). Measurement of HbA_{1c} has proven to be particularly useful in monitoring the effectiveness of therapy in diabetes (Goldstein, 1995). Agents with antioxidant or free radical scavenging property may inhibit oxidative reactions associated with glycation (Elgawish, 1996). Administration of silymarin (25 mg/kg and 50 mg/kg) to diabetic rats significantly reduced the glycosylation of hemoglobin by virtue of its free radical scavenging property and thus may decreases the level of HbA_{1c}.

MDA is the most commonly used marker of LPO in diabetes (Ikuo *et al*., 1991; Gallou *et al*., 1993). Several studies have shown elevated levels of LPO in both type 1 and type 2 diabetics (Sato *et al*., 1979; Griesmacher *et al*., 1993). Administration of silymarin resulted in lowering of LPO levels both in pancreas and liver. These observations demonstrate the scavenging effect of silymarin on free radicals produced by pancreas in response to STZ toxicity.

GSH, the cellular antioxidant was found to be depleted in animals treated with STZ, in agreement with reports of earlier studies (Murakami *et al*., 1989; Mukherjee *et al*., 1994). However, in animals treated with silymarin, GSH contents were increased suggesting its protective effect. Cellular defenses against free radicals are many and varied. The findings of elevation of GSH contents by silymarin suggest GSH-dependent detoxification of free radicals. The glutathione redox cycle is regulated by the intracellular content of GSH, oxidized glutathione (GSSG) and by, glutathione peroxidase (GPX) and glutathione reductase. A decrease in the activity of glutathione reductase leading to the decrease in the regenerating activity of GSSG to GSH was reported in STZ induced diabetic rat (Pescarmona, 1982). Moreover, in diabetics the increased sorbitol synthesis causes NADPH depletion, which when deficient also limits the reduction of GSSG to GSH (Reddi, 1978). Therefore, the great decrease in GSH may profoundly impair free radical scavenging activity, resulted in exacerbated cell damage after exposure to free radicals generated by glucose autoxidation. The role of silymarin in this context requires further study.

Biochemical observations were in keeping with the morphological changes in β-cells of pancreas. The necrotic and infiltrative changes of pancreas, which were consistently observed in animals treated with STZ, were reduced to a minimal with silymarin treatment, further substantiating the protective effect of silymarin in restoring the activity of islets of Langerhans.

In summary, the present study demonstrates the protective effect of silymarin against STZ induced LPO and β-cell damage in rat pancreas. The protective action could be attributed to the scavenging action of silymarin on free radicals

induced by STZ toxicity. However, detailed mechanistic action on different free radicals by silymarin requires further study. Further investigations are warranted to explore the full potential of silymarin for the treatment of type 2 diabetes mellitus and its complications.

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