



RESEARCH ARTICLE

Silymarin Improves Pancreatic Endocrine Function in Rats

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ABSTRACT

The protective effects of silymarin, a free radical scavenger on normal and alloxan-induced diabetic rats were studied for four weeks. Some endocrine pancreatic function and hematological parameters were measured. Histological structure of pancreas, Pancreatic activities of superoxide dismutase (SOD) and catalase, levels of lipid peroxidation product malondialdehyde (MDA) and reduced glutathione (GSH) were also estimated. A single s/c injection of alloxan (150mg/kg BW) elicited a significant decrease in serum insulin, activities of SOD and catalase, content of GSH and a significant increase in pancreatic MDA, blood glucose level, and glycated hemoglobin%. In addition to adverse effects on beta cell function indices, hematological parameters and marked necrotic changes in the pancreatic ultrastructure compared to control rats. The repeated oral intake of silymarin (100 mg/kg BW) for 4 weeks interrupted by a single s.c. injection of alloxan improved significantly the previously mentioned parameters compared to their values in alloxan treated group. This improvement indicates a partial restoration of beta cell function relieving the alloxan induced damage. In conclusion, silymarin administration caused an improvement in normal rats pancreatic function and was able to reduce the pancreatic damage induced by alloxan which might be contributed to its direct cytoprotective effect on beta cells or simply related to restoration of some of the antioxidant capacity in pancreatic tissue.

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INTRODUCTION

Silymarin a natural hepatoprotector is a standardized extract from the ripe seeds of *Silybum marianum* (Milk Thistle). Silymarin is a mixture of flavonolignanes where, silibinin is the main component. Although, silymarin performs its actions in different ways, several numbers of these actions are bounded to its antioxidant activities (Saller *et al.*, 2007; Muhammad *et al.*, 2012; Ahmad *et al.*, 2012). Silybin have important and protective activities against oxidative stress on various organs, such as liver, pancreas and gastrointestinal tract and seems to be beneficial also in treatment of pancreatic problems and balancing blood glucose level (Kren and Walterova, 2005). Silymarin has anti-inflammatory, cytoprotective, and anticarcinogenic effects that inhibit the production of reactive oxygen species (ROS) in tissues. It has been used for long time in the treatment of liver diseases because of their ability to scavenge free radicals and to chelate metal ions. The number of hydroxyle (-OH) substitution was found to be a critical factor in ROS scavenging activity of

silymarin with more -OH groups exhibited more potent antioxidant activity (Shaker *et al.*, 2010). The antioxidant effect of silymarin is due to the presence of a -ring catechol group (dihydroxylated-ring) which has the ability to donate hydrogen electron to stabilize a radical species (Kiruthiga *et al.*, 2010). The presence of 2,3 unsaturation in conjugation with a 4-oxo-function in the C ring and the presence of functional groups capable of binding transition metal ions, such as iron also responsible for the antioxidant nature of silymarin (Abdel-Zaher *et al.*, 2008).

Islets of Langerhans are organelles present within the pancreas responsible for the production of insulin, glucagon, somatostatin and pancreatic polypeptide upon stimulation. The major interest of islet research is, to reach to a cure and/or better management of diabetes mellitus. It is clear that the destruction of islets is caused by several mechanism of cell death (Bhonde *et al.*, 2007). Beta cell death appears to be ultimately caused by receptor mediated mechanisms and/or by secretion of cytotoxic molecules like granzymes and perforin. Moreover, toxic molecules such as ROS: superoxide

radicals, hydroxyl radicals, and nitric oxide play a role in islets cell death by inducing deoxyribonucleic acid (DNA) damage, which leads to poly adenosine diphosphate ribose polymerase (PARP) activation causing an increase in nicotinamide adenine dinucleotide (NAD) consumption, depletion of which compromises adenosine triphosphate (ATP) production in cells (Burkart *et al.*, 1999). Alloxan is commonly used to generate diabetic animal in laboratory for its ability to destroy pancreatic beta cells through generation of free radicals especially superoxide radicals (Ramasamy and Agarwal, 2008). The objective of the present work was to study the possible protective role of silymarin on endocrine pancreatic function in normal and alloxan induced diabetic rats.

MATERIALS AND METHODS

Silymarin was friendly provided by Medizen Pharmaceutical plant Co. (Borg El-Arab, Egypt). The diagnostic kits assaying the levels of the antioxidants enzymes, GSH and MDA were obtained from Bio-diagnostic Co, Egypt. Alloxan and other chemicals and kits were purchased from Sigma Chemical Co. St. Louis, USA.

Animals and experimental design: Thirty two 5 months old Albino male rats weighing 180-200 g were obtained from the Medical Research Institute, Alexandria University, Egypt. Rats received humane care in compliance with the guidelines of animal care of the National Institutes of Health (NIH) and the local committee of the Faculty of Veterinary Medicine, Alexandria University approved this study. Rats were acclimatized two weeks prior to the experiments and were housed in plastic cages with free access to water and standard laboratory diet containing 0.5% NaCl 22% protein and 4-6% dietary fat purchased from (Damanhour Feed Co, Behera, Egypt). Rats were kept at a natural humidity, light cycle and room temperature 22-25°C.

Rats were divided into four equal groups. Group-I (control group) received saline by gavage (2 ml/kg BW) for 4 weeks interrupted by a single s/c injection of saline (2 ml/kg BW) after two weeks. Group-II received 100 mg/kg BW silymarin (EL-Maddawy and Gad, 2012) in 2 ml/kg BW saline by gavage for 4 weeks interrupted by a single s/c injection of saline (2 ml/kg BW) after two weeks. Group-III received saline (2 ml/kg BW) orally for 2 weeks. The animals were fasted overnight and diabetes was induced by a single s.c. injection of alloxan at a dose of 150 mg/kg BW (Al-Jassabi *et al.*, 2011) in 2 ml/kg BW saline. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced transient hypoglycemia followed by another 2 weeks of oral administration of saline. Group-IV received silymarin (100 mg/kg BW in 2ml/kg saline) by gavage for 4 weeks interrupted by a single s.c. injection of alloxan (150 mg/kg BW in 2 ml/kg BW saline) after two weeks in the same treatment regimen as in group-III.

Blood/serum collection and analysis: Individual 12 hours fasting blood samples were collected via retro-orbital bleeding after 24h from the end of the experiment. Two blood samples from each rat were collected one

sample was collected on EDTA for hematological studies and the other was left to clot and centrifuged at 3000 rpm for 15 min to obtain serum which was stored at -20°C for biochemical parameters. Hemoglobin concentration, PCV percent, RBCs and WBCs counts were estimated automatically using a 17 parameter, 3 part leukocyte differential veterinary hematology analyzer (Boule medical for multispecies veterinary applications, Stockholm, Sweden).

Serum glucose level and glycated hemoglobin were determined using kits (Cat #; 441Sigma Chemical Co. St. Louis, USA). Quantitative determination of insulin in rat serum was performed using a High Range rat's Insulin ELISA kits (Cat #; 80-INSRTH-E01, E10 American Laboratory Products Company, USA).

Pancreatic parameters: All rats were euthanized at the end of the experiment. After animal dissection, pancreas was rapidly removed, grossly examined and weighed. The index weight (I.W.) of each pancreas was calculated according to (Matousek, 1969) where, I.W. = organ weight (g)/body weight (g) x 100.

Parts of pancreatic tissues were kept frozen at -70°C for estimation of the levels of MDA, GSH and activities of SOD and catalase. SOD and catalase activity was estimated following the method of Nishikimi *et al.* (1972) and (Aebi, 1974), respectively. Level of MDA and GSH was measured according to the method of (Ohkawa *et al.*, 1979) and (Glatzle *et al.*, 1974), respectively.

The other parts of pancreatic specimens were collected and fixed in 10% neutral buffered formalin solution. Five micron thick paraffin sections were prepared and stained with hematoxylin and eosin (HE) and then examined histopathologically (Bancroft *et al.*, 1996).

Homeostasis model assessment of bet-cell function index (HOMA-Index): The HOMA-bet-cell index was calculated from plasma fasting insulin and glucose concentration according to the Matthews's formula (Matthews *et al.*, 1985):

$$\text{HOMA-Beta cell Index} = 20 \times \text{fasting plasma insulin} (\mu\text{U/ml}) / \text{fasting plasma glucose} (\text{mmol/L}) - 3.5.$$

The Insulin sensitivity-Indices: (glucose/insulin ratio) and fasting insulin⁻¹ was calculated from plasma fasting insulin and glucose concentration (Legro *et al.*, 1998).

Fasting glucose to insulin = fasting plasma glucose (mmol/L)/fasting plasma insulin (μU/ml)
Fasting insulin⁻¹ = 1 /fasting plasma insulin (μU/ml).

Statistical Analysis: Results were statistically analyzed by ANOVA followed by Duncan's multiple range test. Data are presented as means plus or minus the standard error. The minimum level of significance was set at P<0.05.

RESULTS

Pancreatic parameters: Alloxan and alloxan +silymarin caused a significant (P≤0.05) increase in LPO represented by MDA level and a decrease in GSH content, SOD and

catalase activities compared to other groups. However, administration of silymarin+alloxan reduced the intensity of increased LPO caused by alloxan and partially restored antioxidant status (Table 1).

Regarding to pancreas index weight there was a significant reduction ($P \leq 0.05$) in its value in alloxan and alloxan+silymarin treated groups compared to other groups. The reduction was less evident in alloxan+silymarin treated group (Table 1).

Hemato-Biochemical parameters and hormonal assay:

The obtained results showed that, there was a significant reduction in Hb g%, PCV%, RBCs and WBCs counts in alloxan and alloxan+silymarin treated groups compared to other groups. The reduction was less evident in alloxan+silymarin treated group (Table 2). Serum biochemical results showed that there was a significant increase ($P \leq 0.05$) in values of fasting blood glucose, fasting glucose to insulin ratio, Fasting insulin⁻¹ and glycated Hb in alloxan and alloxan+silymarin treated groups compared to other groups. The elevation was more pronounced in alloxan group (Table 3). Also, the obtained data revealed that there was a significant reduction in fasting insulin level in alloxan and alloxan+silymarin treated groups compared to other groups. The reduction was less evident in alloxan+silymarin treated group (Table 3). Moreover, Table 3 shows a significant increase ($P \leq 0.05$) in values of HOMA- beta- cell Index in silymarin treated group compared to the control, while there was a significant reduction in its values in alloxan and alloxan + silymarin treated rats as compared to control (Table 3).

Histopathological findings: The light microscopical examination of pancreas of the control rats (Group I) and silymarin treated rats (Group II) showed normal acini and

normal cellular population of the islets of Langerhans (Fig. 1A&B). However, in the alloxan treated rats (Group III), there was extensive damage and loss of architecture of the islets of Langerhans especially those around the central vessel (Fig. 1C). Marked reduction in the size and number of the islets together with severe reduction in the β -cells was demonstrated in these animals. Severe necrotic changes of the pancreatic islets, particularly the cells in the center of the islets, characterized by homogenous eosinophilic material with pyknosis or disappearance of nuclei were visible (Fig. 1D). Occasionally, pancreatic sections showed also congested blood vessels and leukocytic cellular infiltration in interlobular septa (Fig. 1E). Diabetic rats treated with silymarin (Group IV) showed partial restoration of normal cellular population and size of islets of Langerhans. Extensive damage and loss of architecture of the islets of Langerhans was occasionally seen. Moreover, degenerative and necrotic changes of islet cells characterized by cytoplasmic vacuolation with pyknosis or absence of nuclei were also detected (Fig. 1F).

DISCUSSION

In the present study alloxan induced severe damage of pancreatic β -cells, with the consequent lack of insulin secretion and hyperglycemia so it has been widely used to induce experimental diabetes mellitus, (Soto *et al.*, 2010). Alloxan directly generates ROS and the hyperglycemia induced by alloxan also generates ROS from electron transport chain and glucose auto-oxidation (Florence, 1995). ROS also induce the formation of advanced glycation end – products. Many studies have proposed that free radicals generation is caused by the reduction of alloxan to dialuric acid leading to formation of oxygen reduction cycle in which anionic super radicals would be

Table 1: Effect of silymarin alone or in combination with alloxan on index weight of pancreas, pancreatic lipid peroxidation (MDA) level, reduced glutathione (GSH) content, and catalase and superoxide dismutase (SOD) activities of rats

Parameter Group	Index weight of pancreas (g/100g.b.wt.)	Pancreatic LPO (nmolMDA/g wet tissue)	Pancreatic GSH (μ mol/g wet tissue)	Pancreatic catalase (unit/g wet tissue)	Pancreatic Superoxide dismutase (unit/g wet tissue)
Control	0.40 \pm 0.00a	3.23 \pm 0.19c	10.03 \pm 0.18a	7.23 \pm 0.12a	262.43 \pm 8.01a
silymarin	0.40 \pm 0.01a	3.47 \pm 0.16c	10.07 \pm 0.12a	7.38 \pm 0.14a	263.50 \pm 5.77a
Alloxan	0.24 \pm 0.01c	10.58 \pm 0.31a	5.67 \pm 0.15c	1.90 \pm 0.23c	166.43 \pm 3.87c
Alloxan + silymarin	0.32 \pm 0.01b	5.86 \pm 0.32b	8.04 \pm 0.06b	4.66 \pm 0.16b	206.95 \pm 1.84b

Values (mean \pm SE) with different letters in a column differ significantly ($P \leq 0.05$).

Table 2: Effect of silymarin alone or in combination with alloxan on hemoglobin (Hb g%), packed cell volume (PCV %), red blood cells (RBCs) and white blood cells (WBCs) counts of rats.

Parameter Group	Hbg(%)	PCV%	RBCs ($\times 10^6$ cmm)	WBCs ($\times 10^3$ cmm)
Control	12.50 \pm 0.11a	43.75 \pm 0.80a	6.34 \pm 0.04a	11.73 \pm 0.07a
silymarin	12.43 \pm 0.13a	43.75 \pm 0.80a	6.40 \pm 0.02a	11.67 \pm 0.08a
Alloxan	10.30 \pm 0.08c	32.50 \pm 0.73c	4.71 \pm 0.12c	9.86 \pm 0.10c
Alloxan + silymarin	11.93 \pm 0.07b	38.75 \pm 0.75b	5.91 \pm 0.10b	11.15 \pm 0.11b

Values (mean \pm SE) with different letters in a column differ significantly ($P \leq 0.05$).

Table 3: Effect of silymarin alone or in combination with alloxan on fasting glucose level, fasting insulin level, fasting glucose to insulin ratio, Fasting insulin⁻¹, HOMA-beta-cell Index and glycated Hb% of rats.

Group	Fasting glucose level (mmol/L)	Fasting insulin level(μ U/ml)	Fasting glucose to insulin ratio	Fasting insulin ⁻¹	HOMA- beta-cell Index	Glycated Hb (%)
Control	4.35 \pm 0.11c	9.07 \pm 0.09a	0.49 \pm 0.01c	0.11 \pm 0.00c	206.00 \pm 18.6 b	4.74 \pm 0.12c
silymarin	3.88 \pm 0.02c	9.80 \pm 0.10a	0.40 \pm 0.01c	0.10 \pm 0.00c	526.00 \pm 22.70 a	4.98 \pm 0.07c
Alloxan	21.10 \pm 0.48a	2.43 \pm 0.06c	8.51 \pm 0.11a	0.41 \pm 0.01a	2.82 \pm 0.04c	13.93 \pm 0.24a
Alloxan + silymarin	7.17 \pm 0.20b	5.27 \pm 0.16b	1.32 \pm 0.07b	0.19 \pm 0.01b	32.40 \pm 2.70c	8.75 \pm 0.18b

Values (mean \pm SE) with different letters in a column differ significantly ($P \leq 0.05$).

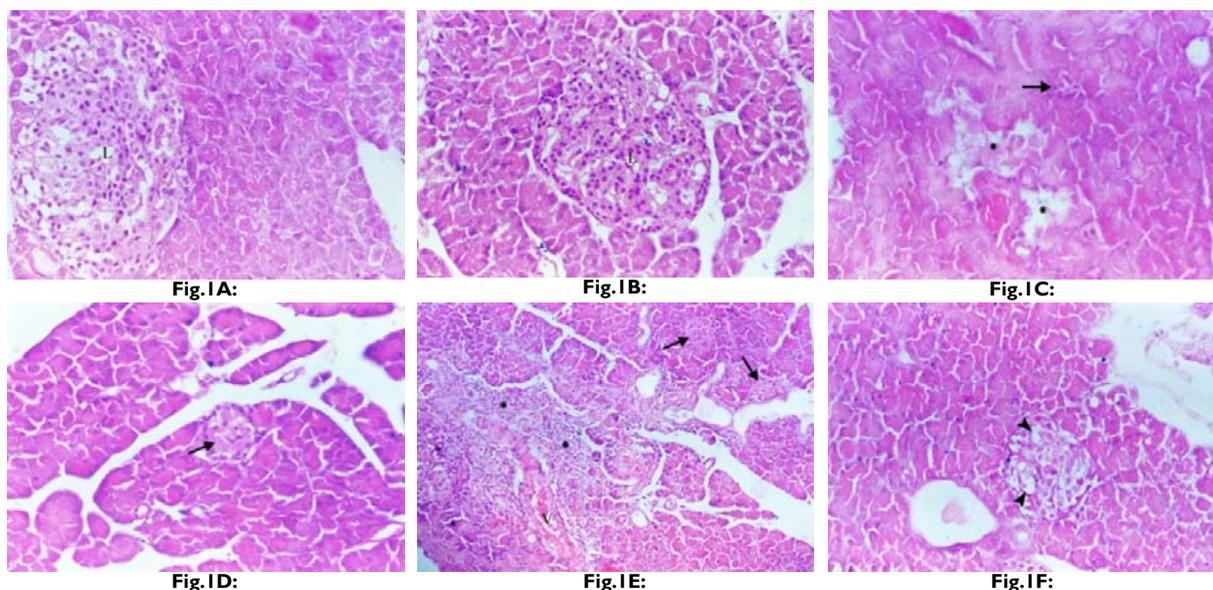


Fig. 1: Pancreas of rat. A) Pancreas of control rat showing normal cellular population of the islet of Langerhans (L). B) Pancreas of silymarin treated rat showing normal histological appearance of the islet of Langerhans (L). C) Pancreas of alloxan treated rat showing extensive damage and loss of architecture (asterisk) of the islets of Langerhans. Note also marked atrophy of the islet (arrow) with severe reduction in the β -cells. D) Pancreas of alloxan treated rat showing necrosis of the islet characterized by homogenous eosinophilic material (arrow) with pyknosis or disappearance of nuclei. E) Pancreas of alloxan treated rat showing congested blood vessels (V) and leukocytic cellular infiltration (asterisk) in interlobular septa accompanied by reduction in the size of the islets (arrows). F) Pancreas of silymarin treated diabetic rat showing cytoplasmic vacuolation (arrow heads) of islet cells with pyknosis or absence of nuclei. H&E stain. A-D and F= $\times 400$; E= $\times 200$.

produced during the oxidation of the dialuric acid (Das *et al.*, 2012). ROS play a relevant role in the etiology and pathogenesis of diabetes and its long-term effects. (Soto *et al.*, 2010). This is a strong support for the findings in our present investigation where, there was a significant increase in blood glucose level and pancreatic MDA and glycated hemoglobin% in alloxan treated rats compared to their levels in control and silymarin treated groups.

Silymarin+alloxan treated group (g IV) was significantly able to reduce the pancreatic β -cells damage maintaining the pancreatic activities of antioxidant enzymes significantly higher than their activities in alloxan induced diabetic rats (g III) and that agrees with the previous findings of (Soto *et al.*, 2010). The increase in the activities of antioxidant enzymes has been reported in some organs of diabetic rats, probably as defense mechanisms against increased free radicals caused by the induced hyperglycemic state (Soto *et al.*, 2010). It has been reported that silymarin protects against oxidative stress induced renal and hepatic damage (EL-Maddawy and Gad, 2012), these protective effects suggest a cytoprotective effect of silymarin on other organs including pancreas (Al-Jassabi *et al.*, 2011). Silymarin can increase serum insulin, reduces serum glucose and causes rise of antioxidant enzymes and glutathione, as well as recovery of endocrine function and pancreatic morphology in diabetic models (Soto *et al.*, 2010). Also, Jose *et al.* (2011) recorded that silymarin has established efficacy in controlling blood glucose level in diabetes patients with liver diseases. Moreover, Fallahzadeh *et al.* (2012) concluded that silymarin decreases urinary excretion of TNF- α , albumin and MDA in people suffering from diabetes.

The improvement of hematological parameters in silymarin +alloxan treated group compared to alloxan

treated group could be related to the powerful antioxidant activity of silymarin (Saller *et al.*, 2007). ROS, have been involved in the mechanism of RBCs damage in diabetic patients as a result, hematological complications develop in the function, morphology and metabolism of erythrocytes, leukocytes and platelets (Dallak and Bin-Jaliah, 2010). Oyedemi *et al.* (2011) reported the occurrence of anemia in diabetes mellitus which might be contributed to the increased non-enzymatic glycosylation of RBCs membrane proteins. Oxidation of these proteins and hyperglycaemia in diabetes mellitus causes an increase in the production of lipid peroxides that leads to decrease in Hb and PCV values in this study.

Beta cell indices can be a good indicator for beta cell function and insulin sensitivity from a single fasting blood sample. The present investigation revealed a significant increase in beta cell function index in silymarin administered intact rats (g II) compared to control group which might be attributed to the numerical decrease in blood glucose level and the increase in serum insulin. There was a dramatic decrease in beta cell function index and increase in insulin sensitivity (I/S) indices in alloxan induced diabetic rats (g III) compared to control which might be due to loss of beta cell function and severe decrease in insulin secretion and hyperglycemic state. These indices were improved significantly when silymarin was administered before and after alloxan induction of diabetes revealing a protective effect of silymarin on pancreatic beta cell function where there was a significant decrease in fasting glycated Hb%, blood glucose level and a significant increase in serum fasting insulin level compared to alloxan-diabetic rats. There is a close inverse relationship between insulin secretion and insulin sensitivity.

The reduction in index weight of pancreas in alloxan-induced diabetes might be related to the phosphorus depletion of soft tissues induced by alloxan and negative phosphate balance and nitrogen balance (Kaleem *et al.*, 2008). The improvement in index weight of pancreas in silymarin+alloxan treated group may be due to its direct effects on protein metabolism and improved lipid metabolism.

Conclusion: This study reported silymarin enhancement of pancreatic antioxidant system, which plays a key role in the defense mechanism against pancreatic damage caused by free radicals, in addition to its direct cytoprotective effects electing a degree of protection and improving the pancreatic beta-cell function in intact and diabetic rats.

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