

The Role of Silymarin in Prevention of Alloxan-Induced Diabetes Mellitus in Balb/C Mice

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Abstract: The aim of this study was to investigate the role of silymarin in the protection of pancreatic cells from the effect of alloxan in Balb/c mice. It has been proposed that lipid peroxidation caused by free radicals may be involved in alloxan-induced diabetes mellitus. Alloxan elicited pancreatic lipid peroxidation which precedes the appearance of hyperglycemia in Balb/c mice. We have studied the effects of the free radical scavenger, silymarin on Balb/c mice pancreas, the effect of this flavonoid on pancreatic, hepatic and blood glutathione together with the pancreatic malondialdehyde (MDA) concentrations in response to alloxan. Our results showed that silymarin increases pancreatic and blood GSH without changes in either hepatic GSH or in blood glucose. Silymarin has prevented the increase in lipid peroxidation, lactate dehydrogenase (LDH) and gamma-glutamyl transferases (GGT) levels in the serum induced by alloxan. Also, silymarin has blunted the sustained increment in plasma glucose induced by alloxan.

Key words: Alloxan • Silymarin • Diabetic Mellitus • Antioxidant Enzymes

INTRODUCTION

Flavonoids are a group of natural products, phenolic compounds of plant origin [1]. Silymarin is a flavonoid complex consisting of silybin, which is the most active component, silydianin and silychristina [2]. Silymarin is derived from milk thistle (*Silybum marianum*) [3], which has anti-inflammatory, cytoprotective and anticarcinogenic effects that suppress the production of reactive oxygen species, ROS in tissues [4]. It was found by many investigators that silymarin is capable of protecting liver cells directly by stabilizing the membrane permeability through inhibiting lipid peroxidation [5], preventing liver glutathione depletion [6], activating antioxidant enzymes in different tissues and protecting DNA [7]. It was found by several studies [8-11] that the number of hydroxyl (-OH) substitutions are a critical factor in ROS scavenging activity of silymarin with more -OH groups exhibited more potent antioxidant activity [12,13]. The antioxidant nature of silymarin is defined mainly by the presence of a catechol group

(dihydroxylated \hat{a} -ring) capable of readily donating hydrogen electron) to stabilize a radical species [14]. 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 [15].

It has been reported that ROS and increased oxidative stress might play an important role in the development of diabetic complications [4,16,17]. Alloxan is a commonly used chemical to generate diabetic animals in the laboratory for its ability to destroy insulin-producing \hat{a} -cells [18]. It is generally accepted that free radicals, especially superoxide radicals, induced by alloxan cause cellular damage, that is key to its role as a diabetogen [19,20].

The aim of this study was to evaluate the effect of the antioxidant silymarin on the alloxan-induced diabetes mellitus, since its potential protective effects have been previously addressed in other models of cell damage induced by drug.

MATERIALS AND METHODS

Alloxan, silymarin and other chemicals were purchased from Sigma Chemical Co. (Malaysia). All other reagents were of analytical grade, obtained from local dealers.

Animal Treatment: 40 male Balb/c mice (28-34 g body weight) were obtained from our animal facility. They were fed standard chow and maintained at 22-24°C, 12-12h dark/light periods and water *ad libitum*. The animals were divided into four groups (10 mice each): (i) a control group (C) without alloxan or silymarin treatment. (ii) a silymarin group (S), which received oral dose (200 mg/kg b.wt. daily for 10 days), the vehicle used for silymarin was carbopol, 0.5% orally as recommended by Soto *et al.* [21]. (iii) an alloxan group (A), which received a single i.v. dose of alloxan (100 mg/kg b.wt. in isotonic saline daily for 10 days). (iv) a silymarin plus alloxan group (AS) which received silymarin at the same doses and schedule as group 2, together with alloxan (100 mg/kg b.wt.i.v.) one single dose, given 60 min after silymarin dose as recommended in literature [22]. All animals were sacrificed after 10 days by anaesthetizing them with sodium pentobarbital (50 mg/kg i.p.). Blood was collected immediately by cardiac puncture, plasma isolated and kept at -80°C for further investigation.

Biochemical Studies: The degree of lipid peroxidation (LPO) was estimated in pancreas and liver homogenates (1:9 w/v in distilled water) by measuring malondialdehyde (MDA) formation using the thiobarbituric acid method described previously [23]. To avoid spontaneous peroxidation, all manipulations were carefully performed to maintain the samples below 0°C. Aliquots of each homogenate (5 mg of protein) in 1 ml of 0.15 M tris pH 7.4 were incubated for 30 min at 37°C, then 2 ml of 0.375% W/V thiobarbituric acid in 15% W/V trichloroacetic acid were added. The samples were kept for 45 min in a bath of boiling water. The colored complex formed was extracted with pure butanol-pyridine (15: 1 V/V) and absorbance measured at 532 nm. The extinction coefficient of the malondialdehyde-color complex was $1.5 \times 10^{-5} \text{ cm}^{-1} \text{M}^{-1}$.

Lactate dehydrogenase (LDH) and creatine phosphate kinase (CPK) activities were determined using the method recommended before [24]. Total LDH activity was assessed according to the method designed by Henry [25]. The method depends on the reaction of lactate with NAD and NADH formed was measured spectrophotometrically at 340 nm. The increase in

absorbance is measured at 1-min intervals for 3 min. Plasma total LDH activity was calculated as units per liter (U/L). Total CPK activity was determined according to the method reported in literature [26]. The method is based on the transphosphorylation of ADP to ATP through a series of coupled enzymatic reactions. plasma total CPK activity was calculated as units per liter (U/L). Serum glucose was measured in 50 µl of the serum using the orto-toluidine method [27]. The serum insulin concentrations were measured according to Soto *et al.* [28] method.

Amounts of ROS in plasma, liver and pancreas homogenates were measured using 2,7 dichlorofluorescein diacetate (DCFDA) that gets converted into highly fluorescent DFC by cellular peroxides (including hydrogen peroxide). The assay was performed as described in literature [29].

Superoxide dismutase (SOD) activity was measured in a 10-500 µl of sample (approx. 10-250 µg protein) by the method described before [30]. Catalase (CAT) activity was measured in 0.1 ml of supernatant containing 200-500 µg of protein [31]. GSHpx activity was measured in an aliquot of supernatant containing 200-500 µg of protein [32].

Statistical Analysis: Results are expressed as mean± standard deviation. For comparison between groups, data were analyzed by one-way ANOVA; $P= 0.05$ was considered statistically significant.

RESULTS

The present results revealed non significant alterations in the body weights of mice of the various treated groups. Alloxan treated mice liver showed a little increase in the liver body mass index ratio due to massive intra-hepatic hemorrhage and pooling of blood in the liver. There was no any significant alteration of pancreas weight or color in alloxan group.

Levels of ROS in the blood, liver and pancreas were increased on alloxan exposure. Administration of silymarin post alloxan exposure was beneficial in significantly reducing ROS levels in these tissues towards normal (Table 1).

Table 1: Results of the effect of alloxan and silymarin on the ROS level in the Serum, liver and pancreas of Balb/c mice

	C group	S group	A group	AS group
ROS in serum µM/ml blood	6.7	6.1	22.8	7.1
ROS in liver (FIU)	480	466	662	496
ROS in pancreas (FIU)	461	456	601	488

Table 2: Results of the effect of alloxan and silymarin on Balb/c mice serum, liver and pancreas

	C group	S group	A group	AS group
LPO in the liver (μM)	0.071 \pm 0.01	0.066 \pm 0.01	2.12 \pm 0.22	0.74 \pm 0.17
LPO in the pancreas (μM)	0.044 \pm 0.01	0.045 \pm 0.01	1.68 \pm 0.16	0.21 \pm 0.09
LDH in the serum (U/L)	316.23 \pm 4.021	318.11 \pm 4.112	986.61 \pm 7.556	522.67 \pm 4.466
CPK in the serum (U/L)	91.08 \pm 1.68	90.88 \pm 1.74	1124.28 \pm 7.87	177.22 \pm 2.69
Serum glucose (mmol/L)	6.8 \pm 0.88	7.16 \pm 0.66	42.41 \pm 1.46	11.16 \pm 1.02
Serum insulin (ng/ml)	0.92 \pm 0.02	1.0 \pm 0.05	0.143 \pm 0.07	0.99 \pm 0.03
SOD activity (Umg/protein)	137 \pm 1.667	141 \pm 1.821	28 \pm 0.870	128 \pm 1.51
CAT activity (k seg ⁻¹ mg/protein)	0.048 \pm 0.007	0.052 \pm 0.007	0.011 \pm 0.002	0.039 \pm 0.024
GSHpx activity ($\mu\text{mol NADPH min}^{-1}\text{mg/protein}$)	0.17 \pm 0.015	0.19 \pm 0.021	0.08 \pm 0.006	0.14 \pm 0.22

Serum glucose in normal mice was 4.28 \pm 0.212 mmol/l. At the 3rd day after alloxan administration this value was increased to 42.2 \pm 2.88 mmol/l. Our experiments showed that this value was maintained at similar level. Silymarin treatment decreased serum glucose to near normal level (6.70 \pm 1.581 mmol/l) (Table 2). Silymarin alone or vehicle treatment did not change serum glucose levels.

Serum insulin value of control Balb/c mice was 1.0 \pm 0.05 ng/ml. In contrast, in alloxan treated mice, serum insulin decreased significantly at the third day of alloxan administration (0.09 \pm 0.006 ng/ml). The value was almost constant at the rest days of experiment (Table 2). The serum insulin values found in Balb/c mice treated simultaneously with alloxan and silymarin were similar to those found in the control group. Treatment only with silymarin or vehicle did not change insulin serum levels.

Antioxidant enzymes, SOD, CAT and GSHpx were significantly decreased in diabetic Balb/c mice group throughout the course of the experiments. Silymarin treatment blocked changes in enzyme activities (Table 2). LDH and CPK activities were significantly increased in the serum of alloxan treated Balb/c mice while in silymarin treatment the activities of these enzymes were decreased to near normal levels.

DISCUSSION

The main finding of this study was that silymarin prevented a rise in LDH, CPK, plasma glucose and pancreatic lipid peroxidation induced by alloxan in Balb/c mice. This result has suggested a protective effect of silymarin against alloxan action.

Scientists found that silymarin increases the pancreatic activities of SOD, CAT and GSHpx [33] and this will confirm our findings. Our results showed that silymarin blocked alloxan-induced decreases in the activities and changes in expression levels of these antioxidant enzymes.

Alloxan directly generates ROS [34] and the hyperglycemia induced by this compound also produces ROS from the electron transport chain and glucose auto-oxidation [35]. Furthermore, PKC activated by superoxide anion induces cellular ROS [36] which can damage liver, pancreas and kidney [37] and activate signalling pathways (PKC, mitogen – activated protein kinase), transcription factors (nuclear factor-kappa B, activated protein-1) and regulate transforming growth factor B-1, angiotensin II, monocyte chemoattractant protein-1 and plasminogen activator inhibitor-1 [38]. ROS also promote the formation of advanced glycation end-product.

Several researchers have proposed that free radicals produced by the reduction of alloxan to dialuric acid [39]. An oxygen reduction cycle would then take place in which anionic superoxide radicals would be produced during the oxidation of the dialuric acid [40].

It has been shown that silymarin prevents the damage induced by oxidative agents in hepatic membranes [41], microsomes and mitochondria [42]. These observations of the effect of silymarin in the area of hepatocyte protection may contribute to explaining why this compound has a protective effect on pancreatic lipid peroxidation with the recovery of the β -cells function. This, in turn, may contribute to the regulation of plasma glucose.

The glutathione reacts with free radicals and in crucial substrate for glutathione peroxidase and glutathione-S-transferase which take part in the cellular defence mechanisms against intermediate oxygenated products of the metabolism. The effects of silymarin on plasma glucose and pancreatic lipid peroxidation produced by alloxan may be related to the significant rise in pancreatic and plasma glutathione induced by this drug. In addition, Paolisso et al. [43] have proposed that the ratio of GSH/GSSG plays a critical role in the glucose homeostasis of diabetes. It has been suggested that thiol groups are important in the intracellular and membranous redox state of the secondary function of

β -pancreatic cells. Table 1 shows that silymarin induced an increase in pancreatic glutathione content which may induce the GSH/GSSG ratio and therefore improve plasma glucose regulation.

In summary, this study suggests that the induction of diabetes mellitus by alloxan in Balb/c mice may be prevented by silymarin administration. This flavonoid had a favourable effect on the pancreatic damage produced by the production of free radicals. This is the case in the experimental model of diabetes mellitus by alloxan and is probably the case in human diabetes mellitus type I.

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