Sequential changes of serum and liver Subcellular oxidants and antoxidant concentrations in silymarin treated male rats

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Abstract

The present study aimed to investigate the role of silymarin as an antioxidant and/or it activity in induction of the endogenous antioxidants in intact adult male rats. Seventy males were randomly devided into control and silymarin treated groups (35 each), and were drenched with drinking water and silymarin suspension (200 mg/ kg b.w) daily for 40 days. Each group was allocated to 5 equal subgroups; sacrificed before treatment (0 day), and after 10, 20, 30, and 40 days of treatment. At the end of each period, males were anaesthesized, dissected and blood samples were obtained for assessment of MDA, SOD, CAT and GSH concentrations. liver samples (1 g) have been removed and homogenized for assessment of liver subcellular MDA, SOD, CAT and GSH concentrations. At the end of each periods, serum and liver subcellular MAD concentrations showed no significant changes between groups, whereas SOD, CAT, and GSH concentrations significantly increased at 10, 20, 30, and 40 day periods in silymarin treated males compared with control. It can be concluded that silymarin antioxidant activity is of pharmacological value not only as an antioxidant by itself but also as an inducer of endogenous enzymatic and non-enzymatic antioxidants even in normal intact male.

Keywords: Silymarin, Oxidants, Antioxidants
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التغيرات المتعاقبة لتراكيز المؤكسدات ومضاداتها في مصل وسوائل أكباد ذكور الجرذان المعاملة بالسليمارين

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الخلاصة

هدفت الدراسة الحالية الى التحري عن دور السليمارين بوصفه مضادا للأكسدة و/أو فعاليته في تحفيز انتاج مضادات الأكسدة الداخلية في ذكور الجرذان السليمة. تم توزيع ٧٠ جرذا ذكرا ناضجا على مجموعتي سيطرة ومعاملة بالسليمارين (٣٥ جرذا لكل منها) وجرعت ماء الشرب ومعلق السليمارين (٢٠٠ ملغم/كغم من وزن الجسم)، على التوالي، يوميا ولمدة ٤٠ يوما. قسمت كل مجموعة الى ٥ مجموعات ثانوية متساوية العدد، تمت التضحية بها قبل المعاملة (اليوم الصفر) وبعد ١٠ و ٢٠ و ٣٠ و ٤٠ يوما. في نهاية كل مدة، تم أخذ عينات دم لغرض قياس تركيز MDA و SOD و CAT و GSH في مصل الدم، وعينات من أكبادها لغرض قياس نفس المعايير في السوائل الخلوية للأكباد. في نهاية كل مدة، أظهرت تراكيز MDA في مصل الدم والسوائل الخلوية عدم وجود فروقات معنوية بين المجموعتين، بينما كانت تراكيز كل من SOD و CAT و GSH في مصل الدم والوائل الخلوية مرتفعة معنويا في مجموعة المعاملة بالمقارنة مع السيطرة ابتداءً من اليوم العاشر من المعاملة واستمرت في الارتفاع مع تقدم مدة المعاملة. يستنتج أن فعالية السليمارين المضادة للأكسدة ذات قيمة دوائية ليس بوصفها مضادة للأكسدة فحسب بل أنها تحفز انتاج مضادات الأكسدة الداخلية حتى في الحيوانات السليمة.

Introduction

Silymarin, as the polyphenolic fraction of *Silybum marianum* seeds as well as its main component silybinin, are used for hepatoprotection in animals and humans. Silymarin provides good anti-toxic protection in various experimental liver diorders in laboratory animals. Its effects comes from its antioxidative, antifibrotic, anti-inflammatory, membrane stabilizing, antilipid peroxidative, and liver regeneration. Preclinical studies recorded that silymarin offers multiple hepatoprotective actions (1). Silymarin is the main flavonoid (70%) found in the seeds, whereas the remaining 20-30 % are chemically undefined fraction, mostly composed of oxidized polyphenolic and polymeric compounds (2).

As stress is normally associated with a decrement of reduced glutathione (GSH) amount in the liver, any agent that can increase GSH concentration will provide an important protective activity against chemical stress (3,4). The antioxidant effect of silymarin was observed in ethanol intoxicated rats, by attenuation the depletion of GSH and the increment of plasma levels of ALT, AST and gamma-glutamyl transpeptidase (GGT) (5). It has been reported that silibinin and silymarin have important role as regulators of the amount of GSH in various organs. Valenzuela *et al.* (6) mentioned that intravenous injection of silibinin or intraperitoneal injection of silymarin causes significant elevation of GSH concentration in the liver, stomach, and intestine.

In veterinary medicine, silymarin preparations are used as feed supplementation for improvement of animal health and productivity, as well as its benifit as therapeutic agent (7,8). At calving, moderate to severe fatty liver dairy cows, in which impairement of liver functions that leads to ketosis, silymarin treatment compromised their health and milk production (8).

Rrecently, Alozy (9) concluded that silymarin has a positive beneficial effect, when given at the dose (200 mg/ kg, bw), as hepatoprotective, hypolipidemic, and antioxidant agent in streptozotocin-induced diabetic mature male rats. Therefore, further investigations are needed to be performed in order to determine whether silymarin act only as an exogenous antioxidant or it can induce the endogenous enzymatic and/or non-enzymatic antioxidants. To investigate this hypothesis, the present study has been conducted to evaluate the sequential antioxidant potency of silymarin as well as its activity in the induction of endogenous enzymatic and non-enzymatic antioxidants in intact adult male rats.

Materials and methods

Experimental animals

Mature male Wistar rats have been used in the experiment. Male rats were allowed one week to acclimate to the animal house environment before beginning of experiment. Animals were housed in polypropylene cages inside a well-ventilated room. Each cage consist of not more than five rats. Animals were fed on the standard chow and drinking water *ad libitum* throughout the experiment. Room temperature was maintained at 23±2 °C., the light-dark cycle was on a 12:12 hr with light on at 06:00 a.m. and off at 06:00 p.m. throughout the experimental period.

Preparations of Silymarin suspension

Milk thistle seeds (silymarin) were purchased from Natures manufacturers for herbal extract, USA. The seeds were grinded by electric coffee mill into powder. Forty gram of seeds were suspended in one litter of drinking water (20 mg/ 0.5 ml) in order to prepare the dose of 200 mg/ kg bw (10). Each 100 g of body weight need to be drenched 0.5 ml of silymarin suspension (20 mg/ 0.5 ml/ 100 g bw). According to the body weight, male rats were drenched the suitable dose of silymarin suspension.

Experimental design

70 mature male rats were randomly devided (35 each) to control (C) and treated (S) groups. Male rats were drenched with drinking water and silymarin suspension (200 mg/kg b.w) daily for 40 days. Each group has been allocated to five subgroups (periods), were sacrificed before treatment (0 day), after 10, 20, 30, and 40 days of treatment. At the end of each period, male were anaesthesized (by injection of 0.3ml ketamine + 0.1 ml of xylazine/ kg b.w. *ip*), dissected and blood samples were obtained from abdominal vein. Blood serum samples were separated (by centifugation at 3000 rpm for 5 minuts) and kept at –20 ºC until assessment of MDA, SOD, CAT and GSH concentrations. Liver samples (1 g) from each male have been obtained and kept at –20 ºC until the step of homogenization for assessment of liver subcellular MDA, SOD, CAT and GSH concentrations.

Preparation of subcellular fluid

Liver tissues were perfused with distilled water until a pink color was appeared. Tissues were homogenized by about 20 up and down strokes in a ground-glass tissue grinder. Sucrose (0.88 M) was used for homogenization, washing, and resuspension of the particulate fractions. Homogenates were fractionated by cooled ultracentrifuge for obtaining subcellular fluid (11).

Assessment of MDA concentration

By using the Thiobarbituric acid (TBA) method for determination of serum MDA, in which MDA reacts with TBA (thiobarbituric acid) to give a pink color that is read at 535 nm (12).

Assessment of total GSH

By using the 5'5-Dithiobis (2.nitrobenzoicacid) (DTNB) as a disulfide chromogen that is readily reduced by sulfihydryl group of GSH to an intensity yellow compound. The absorbance of the reduced chromagen is measured at 412 nm and is directly proportional to the GSH conc. (13).

Assessment of superoxide dismutase (SOD) activity in liver subcellular fluid

By using the modified photochemical Nitroblue tetrazolium (NBT) method in utilizing sodium cyanide as peroxidase inhibitor, SOD levels were assessed (14).

Determination of catalase (CAT) activity in liver subcellular fluid

According to Aebi (15) and Kakkar *et al*. (16), CAT activity was assessed by measuring the degradation rate of H_2O_2. The rate of disappearance of H_2O_2 was monitored spectrophotometrically at 230 nm.

Statistical Analysis

All the values are expressed as mean ± SE. Comparisons were performed using two way analysis of variance (ANOVA2) and newman- keuls to test all groups unpaired values. Differences were considered to be significant at the level of $P<0.05$. All statistical analysis were carried out using the GraphPad Prism (SAS Institute, Inc., USA).

Results

Serum oxidant-antioxidant concentrations

Serum GSH concentration

The results illustrated in table (1) showed no significant differences ($p>0.05$) in serum GSH concentration between the two experimental groups at pre-treatment and 10 day period of silymarin treatment. After 20, 30, and 40 days of treatment, the result of silymarin treated groups revealed significant increase ($p<0.05$) compared with control. In comparison between periods, control males showed no significant differences ($p>0.05$) between the experimental periods, whereas silymarin treated males recorded significant gradual increase ($p<0.05$) started at day 20, as days 20 and 30 day periods recorded no significant differences ($p>0.05$) between each other, but day 40 showed further significant increase ($p<0.05$) compared with other periods.

Serum MDA concentration

The results revealed no significant differences ($p>0.05$) between groups at all periods of treatment. Also the statistical analysis showed no significant differences ($p>0.05$) between experimental periods for each group (table-1).

Serum SOD concentration

The pre-treatment period registered no significant differences ($p>0.05$) in serum SOD concentration between the two experimental groups, whereas silymarin treatment showed significant increase ($p<0.05$) compared with control at all of the experimental periods. In comparison between periods, control males showed no significant differences ($p>0.05$), whereas silymarin treated males recorded significant gradual increase ($p<0.05$) started from day 10 and continued to day 40 of treatment (table-1).

Serum CAT concentration

The pre-treatment and 10 day periods registered no significant differences ($p>0.05$) in serum CAT concentration between the two experimental groups, whereas silymarin treatment showed significant increase ($p<0.05$) compared with control at 20, 30, and 40 day periods. In comparison between periods, control males showed no significant differences ($p>0.05$) between the experimental periods, whereas silymarin treated males recorded significant gradual increase ($p<0.05$) started from day 20 and continued to day 40 of treatment, as CAT serum concentration at the periods 20, 30, and 40 day recorded no significant differences between each other (table -1).

Liver subcellular oxidant-antioxidant concentrations

Liver subcellular MDA concentration

The results of liver subcellular MDA concentrations, illustrated in table (1), showed no significant differences ($p>0.05$) between experimental groups at all periods of treatment. Also the results recorded no significant differences ($p>0.05$) between experimental periods for each experimental group.

Liver subcellular GSH concentration

The pre-treatment period (0 day) registered no significant differences ($p>0.05$) in Liver subcellular GSH concentration between the two experimental groups, whereas silymarin treatment showed significant increase ($p<0.05$) compared with control at all of the remaining experimental periods (10, 20, 30, and 40 days of treatment). In comparison between periods, control males showed no significant differences ($p>0.05$) between the experimental periods, whereas silymarin treated males recorded significant gradual increase ($p<0.05$) started from day 10 period and continued in elevation at 20 day period. The statistical analysis showed no further significant increase

(p>0.05) at 30 and 40 day periods of treatment in comparison with 20 day period (table-1).

Liver subcellular SOD concentration

The pre-treatment period (0 day) registered no significant differences (p>0.05) of Liver subcellular SOD concentration between the two experimental groups, whereas silymarin treatment showed significant increase (p<0.05) compared with control at all of the remaining experimental periods (10, 20, 30, and 40 days of treatment). In comparison between periods, control males showed no significant differences (p>0.05) between the experimental periods, whereas silymarin treated males recorded significant gradual increase (p<0.05) started at day 10 period and continued in elevation at 20 day period. The statistical analysis showed no further significant increase (p>0.05) at 30 and 40 day periods of treatment in comparison with 20 day period (table-1).

Liver subcellular CAT concentration

The results illustrated in table (1) showed no significant differences (p>0.05) of liver subcellular CAT concentrations between the experimental groups at the pre-treatment period, whereas silymarin treatment group showed significant increase (p<0.05) started at 10 day of treatment in comparison with control and continued in its elevation until the end of experiment at 40 days of treatment. In comparison between periods, control males showed no significant differences (p>0.05) between the experimental periods, whereas silymarin treated males recorded significant gradual increase (p<0.05) started at day 10 and continued to day 40 of treatment. The concentrations of silymarin treated group at 10, 20, 30, and 40 periods recorded no significant differences (p>0.05) when compared with each other.

Table 1: effect of silymarin treatment on serum and liver subcellular MDA, GSH, SOD, and CAT concentrations in mature male rats

Parameters	Periods	Serum		Liver subcellular	
		Control	Silymarin Treated	Control	Silymarin Treated
MDA (µmole/ml) (µmole/g.)	0 d.	1.570 ± 0.015 Aa	1.560 ± 0.011 Aa	1.946 ± 0.125 Aa	1.970 ± 0.136 Aa
	10 d.	1.560 ± 0.016 Aa	1.640 ± 0.011 Aa	1.896 ± 0.121 Aa	1.960 ± 0.136 Aa
	20 d.	1.610 ± 0.089 Aa	1.650 ± 0.028 Aa	1.985 ± 0.086 Aa	2.018 ± 0.110 Aa
	30 d.	1.650 ± 0.085 Aa	1.600 ± 0.045 Aa	2.038 ± 0.090 Aa	2.004 ± 0.080 Aa
	40 d.	1.610 ± 0.036 Aa	1.660 ± 0.047 Aa	1.920 ± 0.110 Aa	2.014 ± 0.140 Aa
GSH (µmole/ml) (µmole/g.)	0 d.	2.220 ± 0.095 Aa	2.250 ± 0.070 Ca	3.146 ± 0.066 Aa	3.182 ± 0.080 Ca
	10 d.	2.204 ± 0.022 Aa	2.292 ± 0.071 Ca	3.096 ± 0.076 Ab	3.436 ± 0.096 Ba
	20 d.	2.436 ± 0.115 Ab	2.982 ± 0.143 Ba	3.164 ± 0.076 Ab	3.696 ± 0.094 Aa
	30 d.	2.620 ± 0.094 Ab	3.186 ± 0.127 Ba	3.160 ± 0.074 Ab	3.781 ± 0.079 Aa
	40 d.	2.550 ± 0.127 Ab	3.764 ± 0.202 Aa	3.314 ± 0.093 Ab	3.892 ± 0.081 Aa
SOD (U/ml) (U/g.)	0 d.	1.950 ± 0.011 Aa	1.940 ± 0.011 Ea	2.042 ± 0.075 Aa	2.102 ± 0.098 Ca
	10 d.	1.960 ± 0.007 Ab	2.180 ± 0.040 Da	2.098 ± 0.082 Ab	3.030 ± 0.160 Ba
	20 d.	1.980 ± 0.038 Ab	2.900 ± 0.198 Ca	2.040 ± 0.076 Ab	3.624 ± 0.085 Aa
	30 d.	1.910 ± 0.090 Ab	3.370 ± 0.135 Ba	2.086 ± 0.087 Ab	3.794 ± 0.096 Aa
	40 d.	2.080 ± 0.047 Ab	3.860 ± 0.047 Aa	2.040 ± 0.088 Ab	3.916 ± 0.082 Aa
CAT (U/ml) (U/g.)	0 d.	0.495 ± 0.029 Aa	0.504 ± 0.034 Ba	0.589 ± 0.015 Aa	0.606 ± 0.014 Ba
	10 d.	0.500 ± 0.032 Aa	0.515 ± 0.037 Ba	0.614 ± 0.075 Ab	0.767 ± 0.050 Aa
	20 d.	0.536 ± 0.027 Ab	0.674 ± 0.032 Aa	0.638 ± 0.013 Ab	0.790 ± 0.040 Aa
	30 d.	0.541 ± 0.017 Ab	0.680 ± 0.019 Aa	0.685 ± 0.017 Ab	0.846 ± 0.018 Aa
	40 d.	0.522 ± 0.024 Ab	0.690 ± 0.027 Aa	0.644 ± 0.050 Ab	0.890 ± 0.040 Aa

The results represented as mean ± SE, Different small letters denotes the abscence of significant differences (P>0.05) between groups, Different capital letters denotes the abscence of significant differences (P>0.05) between periods, C: male rats drenched with drinking water (0.5 ml), S: male rats drenched with Silymarin (200 mg/kg suspended in 0.5 ml of drinking water), 0 d, 10 d, 20 d, 30 d, and 40 d represent the period of treatment.

Discussion

In the present study, we aimed to find out whether silymarin can acts as an antioxidant in healthy adult male rats and/or by induction of endogenous antioxidants. The interesting present findings showed that silymarin administration had perfomed benift improvement of antioxidant activity even in the intact

animal model, not only in stressed animals, as mentioned by researchers (17-19).

In general, the modulatory effects of silymarin, that reveated in the present study, could attributed to its role that derives from its ability to counterarrest the action of free superoxide radicals, which are formed due to cell membrane lipid peroxidation (the damage the cell membranes), competitive inhibition through the hepatocytes external cell membrane modification; stimulation of hepatic cell metabolism, in addition to activating of RNA biosythesis of the ribosomes, and stimulating of protein biosynthesis. On the other hand, silymarin may diminishes the activity of Kupffer cells and increase the production of glutathione, and also inhibits its oxidation (20), where one of the most important potency of silymarin, as proved in the present study, was by increasing the serum and hepatocytes contents of GSH.

Changes in the enzymatic (SOD and CAT) and non- enzymatic (GSH) antioxidant biomarkers concentrations, found in the control male rats of the present study, were significantly improved by silymarin treatment, as the present results demonstred no significant changes of MDA concentration and significant increase of SOD, CAT, and GSH concentrations in sera and liver subcellular fluid of silymarin treated male rats compared with control, nearly at all periods of the study. It has been mentioned that antioxidant therapy may be of good value in case of oxidative stress such as diabetic animals. However, the classic antioxidants, such as vitamins C and E, do not seem to be helpful (21). Whereas using silymarin, as in the present study, may be considered as one of the new strategic protocol to counterarrest the generation of free superoxide radicals and other reactive oxygen and nitrogen species even in intact animals.

In normal metabolism, as healthy body's cells are usually in ordinary functions, there are considerable amounts of metabolites and free superoxide radicals production in mitochondria, as a result of energy production. The protection provided by silymarin appears by increasing the counteraction mechanism against free radicals by increasing the scavenging mechanism (22). Silymarin actions can be performed through its activity against lipid peroxidation. One of the most important potency of silymarin, as proved in the present study was by increasing the cellular content of GSH. Where GSH increased significantly in serum and subcellular fluid.

Our results evidenced a parallel increase in serum and liver subcellular concentrations of both SOD and CAT in silymarin treated male rats. SOD and CAT are two of the major scavenging enzymes that responsible for removing free radicals. Administration of silymarin, in the present study, increased the activity of these enzymes both in serum and liver subcellular fluid and may help to control free superoxide radical, as silymarin has been reported to be one of the potent known antioxidants (23). On the other hand, silymarin can act as antioxidant by itself, where silymarin administration may inhibits the microsomal peroxidation which is usually produced by NADPH-Fe2+-ADP and therefore will inhibits the formation of hydroxyl radicals. In one study, that performed on rat hepatic microsomes, it has been postulated that lipid peroxidation produced from Fe(III)/ascorbate can be inhibited by silymarin by away of concentration-dependent (1).

From present results can be postulated the important pharmacological value of silymarin not only as an antioxidant but also as an inducer of endogenous enzymatic and non-enzymatic antioxidants.

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