Biochemical Effect of Silymarin Treatment on Blood and Tissue Parameters in Experimental non Alcoholic Steatohepatitis in Rats

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Abstract

Non alcoholic steatohepatitis (NASH) is a pathological condition characterized by accumulation of lipids in the liver of non alcoholic individuals and consequent oxidative stress leading to cirrhosis of liver in the long run. Silymarin is a unique flavonoid complex extract isolated from seeds of the milk thistle plant (Silybum marianum) and has strong antioxidant and radical scavenging properties. The present research aimed to evaluate the therapeutic effects of silymarin (Slym) as natural antioxidant and anti-inflammatory on liver tissue of male rats exposed to experimental model of non alcoholic steatohepatitis (NASH) induced by supplementation of high fat diet (HFD) for 3 months, Through evaluation of serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP) and Gamma Glutamyl-Transferase (γ-GT) activities and Albumin, Total Protein, Total Bilirubin, Total Cholesterol and triglycerides concentrations. Levels of reduced glutathione (GSH) and activities of Superoxide Dismutase (SOD) and Catalase (CAT), were determined in liver tissues. Extent of oxidative stress was also assessed by hepatic lipid peroxides (MDA). HFD supplementation induced a significant increase in 1) serum ALT, AST, ALP and γ-GT activities, in addition to Total Bilirubin, Total Cholesterol and triglycerides concentrations. 2) Liver MDA concentration. On contrast, it exhibited a significant decrease in serum Albumin and Total Protein, also marked depletion in liver GSH, CAT and SOD, were observed after HFD supplementation. Silymarin treatment was able to mitigate and ameliorate hepatic NASH induced by HFD and showed pronounced curative effect against lipid peroxidation and deviated serum enzymatic variables as well as maintained glutathione status and antioxidant enzymes toward control levels. Silymarin treatment was highly effective against HFD induced NASH. The results of the present study suggest that silymarin has the potential to exert curative effects against liver NASH.

Keywords: Silymarin, Non alcoholic steatohepatitis, NAFLD, Oxidative stress.

1. Introduction

Liver is one of the most important organs which plays a crucial role in the daily functions of our body. It is the main site for carbohydrates, proteins and lipid metabolism, synthesis of essential materials, and detoxifying harmful substances. Furthermore, expulsion of waste metabolites, detoxification, blood coagulation, homeostatic activities, storage of vitamins, finally excretion of bile, hormones, and drugs are other significant functions of liver [1].

Nonalcoholic fatty liver disease (NAFLD) is a fatty infiltration of the liver in absence of other causes of steatosis, such as alcohol consumption. It is characterized by excessive fat accumulation in the liver (hepatic steatosis). NASH is a subgroup of NAFLD characterized by steatosis with additional findings of liver cell injury and inflammation. Hepatic steatosis and steatohepatitis can be distinguished only by liver biopsy and histology [2].

NAFLD is defined as accumulated lipid in hepatocytes, more than 5% of liver weight, in absence of virus infections and alcohol usage (more than 30 gm per day), including a wide spectrum of liver damage, on the one hand hepatic steatosis and the other hand NASH related to fibrosis, necrosis, inflammation and hepatocellular cancer [3].

According to a hypothesis called "two-hit", the changing and progressing of the simple steatosis to hepatic steatosis and advanced fibrosis is caused by two hits, in which the first hit leads to insulin-resistant accumulated lipid in the liver and the second one due to the accumulated lipid in liver, causes oxidative stress and then facilitated inflammation, progressing steatosis and fibrosis [4].

The pathophysiology of NASH has been considered a "two hit" process [5]. The first hit is the development of hepatic steatosis via accumulation of triglycerides in hepatocytes, leading to insulin-resistance, while the second hit includes a variety of cellular stresses, such as oxidative stress, apoptosis, gut-derived stimulation [6] and then facilitated inflammation, progressing steatosis and fibrosis [4].

Nonalcoholic steatohepatitis (NASH) is histologically characterized by significant accumulation of hepatic lipid and predominantly lobular necroinflammation, with or with out centrilobular fibrosis. NASH is histologically similar to alcoholic liver disease, but without a history of ingesting significant amounts of ethanol [7].

Silymarin is a C25 containing flavonoid mixture, extracted from the *Silybum marianum* (milk thistle) plant. It has been used to treat liver diseases since the 16th century [8]. Today's standardized (according to its silibinin, often called silybin, content), its extract contains approximately 65% to 80% flavonolignans (silybin A and silybin

B, isosilybin A, isosilybin B, silychristin and silydianin), with small amounts of flavonoids, and approximately 20% to 35% of fatty acids and polyphenolic compounds possessing a range of metabolic regulatory effects [9].

It should be noted that silymarin can contribute to the antioxidant defenses in different ways. Firstly, by direct free radical scavenging. Secondly, by preventing free radical formation by inhibiting specific enzymes responsible for free radical production, or by maintaining the integrity of electron-transport chain of mitochondria in stress conditions. Thirdly, by participating in the maintenance of optimal redox status of the cell by activating a range of antioxidant enzymes and non-enzymatic antioxidants, mainly via transcription factors, including Nrf2 and NF-κB. Finally, by activating an array of vitagenes, responsible for the synthesis of protective molecules, including heat shock proteins (HSP), thioredoxin (Trx), sirtuins, *etc.*, and providing additional protection in stress conditions [9].

The pharmacological profile of silymarin has been well defined and hepatoprotective properties of silymarin were investigated both in vitro and in vivo. Experimental studies demonstrated antioxidant and free radical scavenging properties,

improvement of the antioxidative defense by prevention of glutathione depletion and anti-fibrotic activity [10].

A significant anti-inflammatory effect of silymarin has been described lately. Silymarin exhibits many effects such as inhibition of neutrophil migration, downregulation of Kupffer cells, decline of leukotriene production and utilization of prostaglandins [11]. The anti-inflammatory effect of silymarin is not well known up till now; it could be associated to the decrease of the production of nuclear factor-kappa (NF-kB), which controls the synthesis of several genes that plays an important role in the inflammatory progression [12].

2. Materials and methods
2.1 Composition of high fat diet
Formulated according to **[13].** Table (1)

2.2 Chemicals
Silymarine (purity~99%) was purchased from SIGMA Pharmaceuticals Co. First industrial zone, Quweisna, Egypt. It is characterized by high solubility in ethanol 225.2 mg/ml. [14]. All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

Table (1) Composition of high fat diet (For induction of NASH)

Composition	High fat diet (g/kg)	Composition	High fat diet (g/kg)
Casein	200	Corn starch	275
Saturated fat	200	Sucrose	80
Corn oil	150	Dextrin	50
Cholesterol	3	Fructose	5
Amino acids	5	NaCl	5
Vitamins	10	Cellulose	5
Moisture	12		

2.3 Experimental animals
A total number of 60 Male albino rats, 8-10 weeks old and average body weight 150-200 gm were used in the experimental investigation of this study, and obtained from the Laboratory Animals Research Center, Fac. Vet. Med., Benha University, Rats were housed in separated metal cages, exposed to good ventilation, humidity and to a 12-hr light-dark cycle, and provided with a constant supply of standard pellet diet and fresh, clean drinking water ad libitum.

2.4 Preparation and administration of dosage
Silymarin was freshly dissolved within ethanol then diluted by distilled water, and administered to rats at a dose of (100 mg/kg body weight) orally by intra-gastric tube, between 7 and 8 a.m. daily for 10 weeks as a treatment after induction of NASH for 12 weeks.

2.5 Experimental design
Rats were randomly divided into three main groups (n=20/group) , placed in individual cages and classified as following: (Group 1): served as control normal group (20 rats); (Group 2): served as induced NASH group (20 rats) fed on high fat diet for 3 months; (Group 3): served as NASH + Silymarin treated group (20 rats) fed on high fat diet for 3 months for induction of NASH followed by administration with silymarin (100 mg/kg body weight) orally by intra-gastric tube, between 7 and 8 a.m. daily for 10 weeks as a treatment.

2.6 Sampling
A. Blood samples
Blood samples were collected from retro-arbitral plexus, from all animal groups twice at 6th and 10th week of treatment period. Rats were fasted overnight and Blood samples were collected in dry, clean, screw capped tubes and allowed to clot for 30 min and serum was separated by centrifugation

at 3000 rpm for 15 min at 4 ºc. The serum was separated by automatic pipette and received in dry sterile tubes, kept in a deep freezer at -20 ºc until used for subsequent biochemical analysis.

B. Liver specimens

Rats were killed by decapitation. The liver specimens quickly removed, cleaned by rinsing with cold saline and stored at -20°C. Briefly, liver tissues were minced into small pieces, homogenized in normal saline 0.9%. The homogenates were centrifuged at 10,000 for 15 minute at 4°C. The supernatant was used for estimation of L-MDA, GSH concentration and CAT, SOD activity.

2.7 Determination of key liver function biochemical markers

ALT and AST [15], ALP [16], γ-GT [17], Total bilirubin [18], Albumin [19], Total Protein [20], Total cholesterol [21], Triglycerides [22].

2.8 Determination of key oxidative stress markers

GSH [23], MDA [24], CAT [25], SOD [26], were determined according to the methods described previously.

2.9 Statistical analysis

The results were expressed as mean (±S.E.) and statistical significance was evaluated by one way ANOVA using SPSS (version 10.0) program followed by the post hoc test, least significant difference (LSD). Values were considered statistically significant when $p < 0.05$.

3. Results

The obtained data in table (2) revealed a significant increase in ALT, AST, ALP and γ-GT activities, in addition to levels of Total Bilirubin, Total Cholesterol and triglycerides in HFD induced NASH group, accompanied with significant decrease in Albumin and Total protein levels, when compared with control normal group. Administration of silymarin as treatment to HFD induced NASH group, resulted in significant decreases in ALT, AST, ALP and γ-GT activities, in addition to levels of Total Bilirubin, Total Cholesterol and triglycerides, accompanied with significant increases in Albumin and Total protein levels, in comparison with NASH group.

The obtained data in table (3) revealed that, supplementation of HFD to normal rats exhibited a significant increase in L-MDA level and significant decreases in GSH level, CAT and SOD activities in liver of HFD induced NASH group, when compared with control normal group. Silymarin administration to NASH rats resulted in significant decrease in L-MDA level and significant increases in GSH level, CAT and SOD activities in its liver, when compared with NASH group.

Table (2) Effect of silymarin treatment on blood biochemical parameters in experimental model of NASH induced by HFD in male rats

	Animal groups	ALT U/L	AST U/L	ALP U/L	γ-GT U/L	Bilirubin (mg/dl)	Albumin (g/dl)	Protein (g/dl)	T. Chol (mg/dl)	TAG (mg/dl)
After 6th week	Control normal	40.57 ± 1.46[c]	119.28 ± 1.92[c]	194.85 ± 2.46[c]	2.01 ± 0.10[c]	0.39 ± 1.67[c]	3.78 ± 5.83[a]	7.27 ± 0.13[a]	72.14 ± 2.84[c]	59.03 ± 2.20[c]
	NASH	215.14 ± 5.85[a]	355.28 ± 8.41[a]	454.42 ± 8.36[a]	7.60 ± 0.28[a]	1.35 ± 5.24[a]	2.15 ± 7.36[c]	4.42 ± 0.12[c]	349.85 ± 5.97[a]	402.85 ± 6.15[a]
	NASH +Silymarin	108.29 ± 3.49[b]	168.57 ± 3.42[b]	273.57 ± 4.03[b]	4.24 ± 0.26[b]	0.67 ± 3.84[b]	3.24 ± 3.74[b]	6.19 ± 0.11[b]	166.43 ± 3.87[b]	135.14 ± 3.37[b]
After 10th week	Control normal	48.34 ± 1.54[c]	136.08 ± 1.79[c]	213.85 ± 2.97[c]	2.51 ± 0.15[c]	0.45 ± 2.26[c]	3.61 ± 3.48[a]	6.98 ± 0.12[a]	79.18 ± 2.61[c]	66.85 ± 2.09[c]
	NASH	257.00 ± 4.75[a]	377.42 ± 3.61[a]	467.42 ± 7.38[a]	8.66 ± 0.29[a]	1.62 ± 6.02[a]	1.92 ± 5.97[c]	3.99 ± 0.13[c]	377.57 ± 4.43[a]	443.42 ± 4.60[a]
	NASH +Silymarin	82.12 ± 4.04[b]	153.71 ± 2.91[b]	241.55 ± 3.68[b]	3.89 ± 0.24[b]	0.50 ± 3.65[b]	3.47 ± 3.31[b]	6.74 ± 0.12[b]	156.71 ± 4.18[b]	122.71 ± 3.08[b]

Data shown are mean ± standard deviation of number of observations within each treatment. Mean values with different superscript letters in the same column are significantly different at (P<0.05).Small letters are used for comparison between the means within the column.

Table (3) Effect of silymarin treatment on liver antioxidant parameters in experimental model of NASH induced by HFD in male rats

	L-MDA (nmol/g. tissue)		GSH (mg/g. tissue)		Catalase (U/g. tissue)		SOD (U/g. tissue)	
	6th week	10th week	6th week	10th week	6th week	10th week	6th week	10th week
Control normal	64.80 ± 2.75[c]	67.48 ± 2.35[c]	96.55 ± 3.46[a]	93.31 ± 3.44[a]	65.57 ± 3.07[a]	62.28 ± 1.97[c]	455.14 ± 9.82[a]	434.58 ± 7.79[a]
NASH	174.58 ± 3.42[a]	186.29 ± 3.36[a]	35.35 ± 2.13[c]	31.23 ± 2.07[c]	27.14 ± 1.79[c]	23.42 ± 1.67[a]	188.47 ± 4.05[c]	171.25 ± 3.91[c]
NASH +Silymarin	89.13 ± 3.35[b]	76.12 ± 2.85[b]	74.01 ± 3.37[b]	84.38 ± 3.15[b]	52.00 ± 2.76[b]	56.16 ± 2.54[b]	389.42 ± 6.60[b]	406.85 ± 5.57[b]

Data shown are mean ± standard deviation of number of observations within each treatment. Mean values with different superscript letters in the same column are significantly different at (P<0.05).Small letters are used for comparison between the means within the column.

Discussion

The major goal of this work was to evaluate the potential benefit of silymarin administration on NASH, at the biochemical levels for the liver as well as antioxidant parameters of male rats. This study investigated the ability of silymarin and to counteract NASH in rats.

NASH led to an alteration of liver functions and antioxidant capacities, decreased total protein and albumin levels, increased ALT, AST, ALP and γ-GT activities in addition to bilirubin, total cholesterol and triglycerides levels, as well as increased MDA level, while decreased GSH level, CAT and SOD activities. Concomitant administration of silymarin to NASH rats ameliorated most of the altered biochemical and antioxidant variables induced by HFD suggesting their therapeutic efficacy.

The biochemical markers used to evaluate liver function were ALT, AST, total protein and LDH. ALT and AST are the most sensitive biomarkers directly implicated in the extent of hepatic damage and toxicity [27].

Our results showed that serum ALT, AST, ALP and γ-GT activities were markedly elevated in HFD-induced NASH group when compared with control group. ALT, AST are important indicators of liver damage in clinical findings. These enzymes were secreted into the blood in hepatocellular injury and their levels increase. Changes in these enzyme levels might differ depending on exposure time and dose [10]. The significant rise in ALT, AST, ALP and γ-GT subsequent to HFD induced NASH reflected the rise in enzyme activities due to an increase in hepatic cell membrane fragility that led to enzyme release into circulation. These enzymes, being cytoplasmic in location, are released into the circulation after autolytic breakdown or cellular necrosis due to the damaged structural integrity of the liver [28]. ALP resides in cells lining biliary duct of livers, bone and placental tissue is elevated due to cholestasis and increased biliary pressure [29] and this coincides with our results and refer to liver damage caused by NASH.

Liver is the major source of most of the serum proteins, in which the parenchymal cells are responsible for synthesis of albumin, fibrinogen and other coagulation factors and most of the α and β globulins [10] Albumin, being the most abundant plasma protein, accounts for 60% of the total serum protein and is incorporated in many physiological processes. The observed decrease in albumin in NASH group, which is consequently reflected the decrease in total protein, could be a result of a decline in the number of cells responsible for albumin synthesis in the liver through necrosis on the same grounds [30]. This explains the decline in total protein in our study after HFD supplementation for 3 months.

Greenlee et al., [31]. and Tamayo and Diamond, [32] reported that silymarin have protective effects on the liver and greatly improve its function since it is typically used to treat liver cirrhosis and chronic hepatitis (liver inflammation). In addition, milk thistle extract prevent and repair damage from toxic chemicals and medications. The liver regenerating effect induced by silymarin results from stimulation of RNA polymerase enzyme in the nucleus of liver cells. This results in an increase of ribosomal protein synthesis which helps to regenerate hepatocytes [10].

The significant changes in serum total proteins induced by silymarin after the end of the 6th and 10th weeks of treatment coincide with [33], they reported that silymarin can enter into the nucleus and act on RNA polymerase I enzyme and the transcription of rRNA result in an increase of ribosomal formation. This in turn hastens protein and DNA synthesis which enhances the biosynthetic apparatus in the cytoplasm, thus leading to an increase in the synthesis rate of both structural and functional proteins.

This action has important therapeutic implication in the repair of damaged hepatocytes and restoration of normal functions of the liver. Moreover, Feher and Lengyel [34] found that silymarin protects liver in 3 ways: by enhancing DNA polymerase, stabilizing cell membrane and scavenging free radicals. Whereas, silymarin

stimulates DNA polymerase, increase synthesis of ribosomal RNA and stimulates liver cell regeneration and it stabilizes cellular membranes and increases the glutathione content of liver.

Silymarin administration exhibited a significant hypolipidemic activity reflected on the significant decline of cholesterol and triglycerides in comparison with zero time group. This action is due to the influence of increased cellular permeability which is closely associated with changes of membrane lipids [35]. This proposes that silymarin may stimulate lipoprotein secretion and uptake. Also, silymarin and silibinin decrease the synthesis and turnover of phospholipids in the liver. Furthermore, silibinin is able to inhibit phospholipid synthesis [36]. In addition, silibinin induces phosphatidyl choline synthesis and increases the activity of cholinephosphate cytidyl-transferase in rat liver in normal conditions. Silymarin, also stimulate fatty acid oxidation and may reduce triglyceride synthesis in the liver [37].

Lipid peroxidation is supposed to cause the destruction and damage to cell membranes, lead to changes in membrane permeability and fluidity and enhance the protein degradation in mice [38]. In the present study, the levels of lipid peroxidation were increased, indicating an increase in the generation of free radicals in NASH group and this level was decreased in the other group treated with silymarin. Consequently, the accumulated free radicals could further stimulate lipid peroxidation process and therefore increase malondialdehyde content [39].

Glutathione is a ubiquitous tripeptide present in all cell types in millimolar concentrations. The major roles of glutathione maintain the intracellular redox balance and eliminate xenobiotics and ROS. The observed decrease in GSH activity might be due to a decrease in availability of GSH produced during enhanced lipid peroxidation processes [39].

A wide variety of oxidizing molecules such as ROS and/or depleting agents can alter glutathione redox state, which is normally maintained by the activity of glutathione-depleting (GPX, GST) and glutathione-replenishing enzymes. So, it can be assumed that the decrease in glutathione concentration might cause the effectiveness of GST and GPX activity to be restricted, as evident by the intensification of lipid peroxidation [10].

The fall in catalase activity which proven in our results indicated a decrease in the antioxidative capacity as well. It has been shown that the decreased activity of SODs may be attributed to the consumption of these enzymes in ROS detoxification and also its increasing is due to increased lipid peroxidation [10]. It is also known that antioxidant enzymes can be inactivated by lipid peroxides and ROS. SOD is inhibited by hydrogen peroxide, while catalase is inhibited by an excess of superoxide radical on the same grounds as indicated in our study.

The superoxide formation may promote peroxynitrite generation and protein nitration that may further result into oxidative damage to proteins, DNA and lipids. In addition, occur depletion of mitochondrial glutathione content, decline in ATP content, and uncoupling of the mitochondrial respiratory chain combined with electron leakage and this explanation is greatly supported by our findings [40].

Silymarin is a flavonoid that is used clinically to treat chronic inflammatory liver disease and hepatic cirrhosis. The therapeutic effect of silymarin can be attributed to its antioxidant properties by scavengering free radicals and increasing intracellular concentration of glutathione. Silymarin also has anti-inflammatory properties that regulate inflammatory mediators such as TNF-α, nitrous oxide and interleukins [41].

Silymarin may inhibit lipid peroxidation by scavenging free radicals and increasing intracellular concentration of glutathione. Soto et al., [42] reported that the protective effect of silymarin on pancreatic damage induced by alloxan may be due to an increase in the activity of antioxidant enzymes. Oliveira et al., [43]. reported that silymarin protects liver against ischemia/reperfusion injury by induction of the antioxidant enzyme system.

Conclusion

In conclusion, the findings of the present study demonstrated that silymarin provided an effective therapeutic treatment against NASH induced by HFD in rats, since this compound was able to ameliorate serum biochemical parameters, enzymatic and non-enzymatic antioxidant defense system and to prevent the lipid peroxidation in liver tissue. We recommended that, we can take advantage of the great therapeutic effects of silymarin by its administration for patients suffering from NASH.

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