IJMS
Vol 47, No 4, July 2022

Original Article

Silymarin Administration Attenuates Cirrhotic-induced Cardiac Abnormality in the Rats: A Possible Role of β_1-adrenergic Receptors and L-type Voltage-Dependent Calcium Channels

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Received: 12 May 2021
Revised: 10 July 2021
Accepted: 17 September 2021

What's Known

- Cirrhosis is a progressive liver disease with a wide range of extrahepatic complications. Secondary cirrhosis-induced cardiac abnormality is known as cirrhotic cardiomyopathy. Several mechanisms have been considered for the development of this type of cardiac disease.
- The cardioprotective effect of silymarin has not been reported in the experimental biliary cirrhosis

What's New

- Silymarin treatment in higher dose significantly attenuated cirrhosis-associated cardiac remodeling and reduced cirrhosis-induced mechanical dysfunctions.
- Silymarin's cardioprotective effect was confirmed by the restoration of cardiac contractility parameters, correction of ventricular β_1-AR and L-VGCC expression.

Abstract

Background: Cirrhotic cardiomyopathy is a well-recognized cardiac dysfunction in cirrhotic patients. Studies have confirmed the protective effects of silymarin in different types of cardiac injury. This study aimed to examine the effectiveness and molecular mechanism of silymarin against myocardial dysfunction and hypertrophy in a rat model of cirrhosis.

Methods: The experiment was performed at Alborz University of Medical Sciences (Karaj, Iran) during 2020-2021. Thirty-two male Wistar rats were randomly divided into four groups of Sham-operated (control group for surgical procedures), Bile Duct Ligated (BDL), and two Silymarin extract (SE)-treated groups of 300 and 600 mg/Kg/day. After 28 days, serum levels of AST, ALT, GGT, and ALP, liver histopathological status, as well as cardiac mechanical function, were assessed. Cardiac β_1-adrenergic receptors (β_1-AR), L-type voltage-dependent calcium channels (L-VDCC), and GATA4 mRNA expression were also determined using real-time RT-PCR. Data analysis was performed using the one-way ANOVA followed by Duncan's multiple range test. Histological data has been analyzed with Kruskal-Wallis nonparametric test. The analysis was performed at $P \leq 0.05$.

Results: BDL was associated with a significant elevation in serum AST, ALT, GGT, and ALP, development of necrosis and fibrosis of the liver texture, increased Heart Weight and Heart Weight to Body Weight ratio, enhanced cardiac mechanical function as well as a significant up-regulation of ventricular β_1-AR and L-VDCC. Administration of SE600, but not SE300, significantly reduced the serum levels of the enzymes and alleviated signs of liver necrosis and fibrosis. Cirrhotic-induced cardiac dysfunction was also restored by SE600, but not by the lower dose. In addition, cardiac expression of the β_1-AR and L-VDCC was down-regulated toward normal values by either higher or lower doses of the SE.

Conclusion: Silymarin treatment in higher dose attenuated cirrhosis-associated cardiac remodeling and reduced cardiac mechanical dysfunctions.

Please cite this article as: Bayat GR, Mazloom R, Hashemi SA, Pourkhalili K, Fallah P, Shams AR, Esmaeili P, Khalili A. Silymarin Administration Attenuates Cirrhotic-induced Cardiac Abnormality in the Rats: A Possible Role of β1-adrenergic Receptors and L-type Voltage-Dependent Calcium Channels. Iran J Med Sci. 2022;47(4):367-378. doi: 10.30476/IJMS.2021.90750.2172.

Keywords ● Silymarin ● Liver cirrhosis ● Cardiomyopathies ● Receptors, Adrenergic, beta ● Calcium channels, L-type

Introduction

Cirrhosis is a common liver disease that can be associated with several complications including cardiac and hemodynamic dysfunctions. Cirrhotic cardiomyopathy (CCM) is a clinical syndrome in patients with liver cirrhosis and was originally demonstrated in alcoholic cirrhotic patients.[1] It is characterized by a typical hyperdynamic status with increased cardiac output and heart rate with a concomitant reduction in blood pressure due to peripheral vasodilation.[1] Moreover, ventricular hypertrophy, systolic and diastolic dysfunction as well as electrophysiological abnormalities, especially QT prolongation, are also other clinical manifestations of CCM.[2] The observed diastolic dysfunction and elevated diastolic pressure may be induced by myocardial hypertrophy and tissue fibrosis.[3]

Despite introducing the CCM for more than four decades, the exact molecular mechanisms involved in its secondary induced cardiac disease are not completely understood. Various neural, humoral, and structural alterations have been implicated in the pathogenesis of cirrhotic cardiomyopathy. One of the most important parameters of these changes is the overactivity of the sympathetic nervous system in order to counteract cirrhosis-induced systemic vasodilation.[4] The β_1-adrenergic receptors (β_1-AR) and their downstream signaling pathways play a crucial role in modulating cardiac contractility and structural remodeling. These receptors belong to a family of G protein-linked receptors that are coupled to G_s protein and activate adenylyl cyclase.[5] L-type voltage-dependent calcium channel (L-VDCC) is another fundamental component that determines the efficacy of cardiac contractility. Activation of L-VDCC triggers the calcium release from intracellular sources, the phenomenon known as Calcium (Ca^{+2})-entry Ca^{+2}-release. Making beats, maintaining the plateau phase of the cardiac action potential, and excitation-contraction coupling are some essential roles of L-VDCC. Functionally, there is a relationship between L-VDCC and β_1-AR.[6]

Since the β_1-AR signaling alter in pathologic condition,[7] this pathway can be considered as an effective diagnostic and therapeutic target for cirrhotic-induced cardiac abnormality. However, the obtained results from different models of heart failure on the β_1-AR number and their downstream signaling are not the same. While some researchers have shown the upregulation of β_1-AR,[8] the others indicated the downregulation of the receptors in the human failing heart.[9] Studies on both experimental and clinical models have shown that both the density and the function of the β_1-AR decrease in the early stages of CCM.[10] However, it is not clear whether this reduction is the cause of CCM or the effect. Downregulation of L-VDCC, as well as diminished calcium entry and release, have also been reported in isolated cardiomyocytes in the rat model of biliary cirrhosis.[11]

Despite advances in the early diagnosis of cirrhotic cardiomyopathy, no specific treatment has yet been found for it. Milk thistle (*Silybum marianum)* is one of the potent medicinal herbs that is promising in the treatment of mild to severe hepatic diseases. The Milk thistle extract known as silymarin is composed of several active ingredients such as silibinin, isosilibinin, silychristin, isosilychristin, and silydianin.[12] The hepatoprotective properties of the silymarin and its active ingredients were confirmed in several experimental models of liver disease.[13, 14] Moreover, new evidence has also confirmed the cardioprotective effects of silymarin against ischemia/reperfusion injury,[15] chemical-induced cardiotoxicity,[16] diabetic-induced cardiomyopathy,[17] and cardiac hypertrophy.[18] However, the underlying mechanisms of silymarin-induced cardioprotection are not well-understood and require further investigations.

Therefore, in the present study, we aimed to investigate the cardioprotective effects of silymarin in an experimental model of cirrhotic-induced cardiac abnormality. The cardiac mechanical function, cardiac hypertrophy, β_1-AR, L-VDCC, GATA4 mRNA expression along with the biochemical and histopathological evaluation were assessed as the study endpoints.

Materials and Methods

Chemicals

Ketamine and Xylazine were obtained from Alfasan (Woerden, Holland). Milk thistle seeds were obtained from Tehran botanical market and authenticated by the Herbarium Department of School of Traditional Medicine, Shahid Beheshti University of Medical Sciences (voucher No. *HMS-516*).

Extract Preparation

The extraction protocol was performed according to Ziai and others.[19] Milk thistle seeds were milled and extracted with methanol 99.99% via the percolation method. The extract was concentrated by a vacuum rotary evaporator (Heidolph, Germany) to eliminate methanol. Then, the extract was floated in and mixed with petroleum ether for three hours to remove the hydrophobic part. The extract was then filtered and left to dry in a desiccator. The extraction

yield (w/w) of the herbal extract was calculated as the weight of dry extract/weight of dry starting material×100.

Quantification of Active Ingredients

The main active component of milk thistle extract was determined using High-performance liquid chromatography (HPLC: Knauer, Germany). HPLC instrument was equipped with an Agilent Knauer- UV K2501diode array detector, Knauer- K1001 pump, Agilent Eclipse-XDB-C18 analytical column (125 mm, 4.6 mm, 5 µm). The aqueous mobile phase A: phosphoric acid, methanol, water (0.5:35:65 V/V/V), B: phosphoric acid, methanol, water (0.5:50:50 V/V/V), mobile phase flow rate 0.8 $mLmin^{-1}$, injection volume 20 µL.

Animals

Thirty-two male Wistar rats weighing 230-250 g were obtained from Royan Animal Breeding Center (Karaj, Iran). Animals were kept in the standard living conditions including controlled room temperature (22±2 ºC) with a 12 hour light/dark cycle and relative humidity (40%-60%). During the study, animals had free access to food and water. The animal care and experimentation were performed according to the national guidelines, and protocols were approved by the Research Ethics Committee of Alborz University of Medical Sciences in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (IR.Abzums.Rec.1396.208).

Experimental Design

Animals were randomly divided into four experimental groups (n=8 in each) including a sham-operated group (received 0.3% Carboxymethyl Cellulose (CMC), as a vehicle), a bile duct ligation (BDL) group without any treatment, and two BDL groups, which were assigned to receive silymarin extract (SE) at 300 and 600 mg/Kg/day by oral gavage, respectively. The SE solutions were freshly made using 0.3% CMC just prior to daily administration and were given once a day at the same time.

Experimental Protocol

The surgical procedure was performed in clean conditions using surgical loupes and microsurgical instruments. Animals underwent deep surgical anesthesia using a single intraperitoneal injection of ketamine (60 mg/Kg) and xylazine (8 mg/Kg) (Alfasan, Netherlands). After diminution of the animals' reflexes, we ligated the bile duct according to the methods described in Yang and others.[20] Briefly, after the middle line incision, the bile duct was separated from surrounding tissue. Then, the exposed bile duct was ligated with double ligature using 4-0 silk (SUPA Medical Devices Co., Iran) sutures. The first ligature was made near the junction of the hepatic ducts, and the second ligature was made above the entrance of the pancreatic duct. To prevent the recanalization, the duct was transected between the ligatures. The sham-operated group underwent a middle incision and isolation of the bile duct without ligation. The animals' body temperature was monitored during the surgical procedure and post-recovery period (37±0.5 °C). The abdominal wall and skin incisions were then sutured using absorbable (3-0 catgut) (SUPA Medical Devices Co., Iran) and non-absorbable (3-0 silk) (SUPA Medical Devices Co., Iran) suture materials, respectively. Thereafter, animals were transferred into separated cages. For the first week of the procedure, the wounds were kept clean with topical tetracycline ointment 3% (Iran-Najo. Co., Iran). The study was conducted in 28 days. The animals were observed during the experiment once daily for any sign of morbidity and/or mortality.

Biochemical Analysis

At the end of the study, animals were given an intraperitoneal injection of Ketamine (60 mg/Kg) (Alfasan, Netherlands) and Xylazine (8 mg/Kg) (Alfasan, Netherlands). Then, under deep anesthesia, blood samples were collected. Biochemical analysis was performed to determine the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and Gamma-glutamyltransferase (GGT) using Pars Azmun commercial kits (Pars Azmun Co, INC, Karaj, Iran) according to the manufacturer's guidelines.[21]

Isolated Heart Study (Langendorff)

After blood sampling, the isolated heart study was performed according to the protocol.[22] Briefly, after bilateral thoracotomy, the heart of the animals heart were quickly removed and mounted via aorta on a Langendorff perfusion system and perfused retrogradely with Krebs-Henseleit buffer with a pH of 7.4 and the following composition in mmol/L: NaCl 118.0; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.0; and glucose 11.0. The constant temperature of 37 ºC, bubbling of 95% oxygen and 5% carbon dioxide was maintained during the protocol. For the continuous recording of the ventricular pressure, a latex balloon was placed in the left ventricle, and the balloon's catheter was connected to a Power Lab data acquisition system via a pressure transducer (PowerLab

4/35, four channel recorder, Data Acquisition Systems). Left ventricular end-diastolic pressure was kept on 5-10 mmHg. The hemodynamic function in the present isolated heart study was adjusted at a constant-pressure mode (70 mmHg). In order to stabilize the cardiac condition for 30 minutes, the measurement of some cardiac hemodynamic parameters, including the left ventricular systolic pressure (LVSP), left ventricular developed pressure (LVDP), rate of increase (+dp/dt) and decrease (–dp/dt) ventricular pressure (indices of contraction and relaxation function), heart rate (HR), rate-pressure product (RPP), and coronary flow (CF) was performed. CF was quantitated by collecting the one-minute sample of coronary artery effluent after the 30-min period of stability.

Heart and Body Weights

At the end of the isolated heart study, the animal's absolute Heart Weight (HW) was recorded and the Heart Weight to Body Weight (HW/BW) ratio was measured as an index of cardiac hypertrophy.[23]

Histopathological Examination

After cardiac removal, the livers were dissected out. The largest right lobe of each liver was cut and immediately fixed in a 10% formalin solution (Neutron Pharmaceutical Co. Iran). Tissue staining of Hematoxylin and Eosin (H&E), Masson's Trichrome, and Reticulin were done for detecting any pathological signs of injury, fibrotic scars, or necrotic lesion, respectively.

Real Time RT PCR Assessment

To identify the exact mRNA expression of cardiac β_1-AR, L-VDCC, and GATA4, real-time RT PCR was performed according to the protocol.[24] Briefly, about 50 mg of the left ventricle tissue was homogenized using a polytron tissue homogenizer (DAIHAN-brand Homogenizing Stirrer, HS-30E; Korea). The RNA then was extracted using Trizol (Qiagen) based on the manufacturer's instructions. Then, the cDNA synthesis was performed using a reverse transcriptase cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran), based on the manufacturer's protocol. Expression of the aforementioned genes was measured by Real-Time PCR using SYBR green (Yekta Tajhiz Azma Co. Iran). Experiments were performed in duplicates as follows: denaturation at 95 °C for 10 minutes subsequently followed by 45 cycles at 95 °C for 10 seconds, 60 °C for 10 seconds and 72 °C for 10 seconds. The expression level was normalized to the GAPDH and expressed as Fold-change. The exact nucleotide sequences of the genes and GAPDH primers were shown in table 1.

Statistical Analysis

Data, except for the histological reports, was presented as mean±SEM. Between-group analysis was conducted using the One-way analysis of variance (ANOVA) and in the case of any significant difference, it was followed by Duncan's multiple ranges as a *post hoc* test to measure specific differences between the means. Histological data has been analyzed with Kruskal-Wallis nonparametric test and reported as the median. A P value of less than 0.05 was considered statistically significant. Graphs were drawn using GraphPad Prism version 8.0.2 (GraphPad software; San Diego, California, USA).

Results

All values of the Sham-operated/vehicle group were statistically similar to those of the control group. To avoid duplication of similar data, the Sham-operated group has been considered as the control group.

Yield of Extract and Analytical Quantification

The extraction yield of the total process was 8.2% (123 g/1500 g ×100). Standardization of the SE sample was performed using the HPLC method based on silibinin content, and the exact amount in the sample was 50.2%. Analytical quantification results of the SE content have previously been reported in our other research project.[25]

Biochemical Findings

Table 2 shows the effects of BDL and BDL+SE treatment on serum levels of the liver enzymes The serum levels of AST (P<0.001), ALT

Table 1: Forward and reverse sequence of all cardiac genes used in the study

Target gene	Foreword sequence	Reverse sequence
L-VDCC	TACACCAGCCGCCCATCCGA	TCATCCTCCTGGGCTGCGCT
β_1-AR	GGAAGGCTTTGTGAACTGTC	AGTCTGGTTAGTGTCCTGTC
GATA4	GCTATCCATCTCCTGTCACTC	GCCCCAGCCTTTTACTTTG
GAPDH	GCCTTCTCTTGTGACAAAGTG	CTTCCCATTCTCAGCCTTG

Table abbreviations: β_1-AR: β_1-Adrenergic receptor; L-VDCC: L-type voltage-dependent calcium channel; and GATA4: GATA binding protein 4

Table 2: Effects of BDL and/or Silymarin extract on serum levels of liver enzymes

	Sham	BDL	P value	BDL+SE300	P value	BDL+SE600	P value
AST	137.5±14.5	426.4±43.5	<0.001	370.3±31.2	<0.001	269.8±29.8$^{\dagger\dagger\Delta}$	0.020
ALT	109.8±7.4	173.0±15.3	0.005	154.4±12.7	0.020	132.6±6.3†	0.037
GGT	6.66±1.50	52.8±11.70	0.003	45.20±12.0	0.010	16.5±3.1$^{\dagger\Delta}$	0.018
ALP	834.1±79.10	1616±120.0	<0.001	1467.0±112.0	<0.001	1001.0±50.0$^{\dagger\dagger\dagger\Delta\Delta}$	<0.001

Data were presented as mean±SEM. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase (ALT); GGT: Gamma-glutamyltransferase; ALP: Alkaline phosphatase; BDL: Bile duct ligation; SE300 and SE600: Silymarin Extract at 300 and 600mg/Kg/day. †Denotes a significant difference from the BDL groups (††P<0.01; †††P<0.001). $^{\Delta}$Denotes a significant difference from the BDL+SE300 groups ($^{\Delta}$P<0.05; $^{\Delta\Delta}$P<0.01)

(P=0.005), GGT (P=0.003), and ALP (P<0.001) were significantly higher in the BDL group than those of the sham-operated group. Similarly, compared to those of sham-operated, the values of AST (P<0.001), ALT (P=0.020), GGT (P=0.010), and ALP (P<0.001) were still higher despite administration of SE300. There were no significant differences in the serum levels of the liver enzymes between the BDL and BDL+SE300 groups. Although treatment with the higher dose, was effective in reducing the serum levels of the enzymes, the levels of AST (P=0.020), ALT (P=0.037), and GGT (P=0.018) in the SE600-treated group significantly remained higher than those of the sham-operated group. The serum levels of the AST (P=0.005), ALT (P=0.037), GGT (P=0.020), and ALP (P<0.001) in the SE600 were also significantly lower than the BDL group. The was a significant reduction in the serum levels of AST (P=0.040), GGT (P=0.041), and ALP (P=0.006) in the SE600-treated group in comparison with those in the SE300 group.

Isolated Heart Study Findings

The hemodynamic parameters of cardiac function have been summarized in figure 1. The values of the LVSP (P=0.002), LVDP (P<0.001), +dp/dt (P=0.014), and RPP (P=0.003) in the BDL group were significantly higher than those of the Sham-operated group (figures 1 a-c, f). In comparison to the sham-operated group, the LVSP (P=0.021), LVDP (P=0.006), and +dp/dt (P=0.020) were still higher in the BDL+SE300 group, so that there was no significant difference between the values and the BDL ones. All aforementioned parameters have been restored to the normal level in the BDL+SE600 group. The same pattern was also observed for the LVSP (P=0.052), LVDP (P=0.006), and +dp/dt (P=0.020) of SE600, when compared to those of BDL (figures 1a-c). Compared to the BDL group, the RPP was significantly lower (P<0.001) in both SE receiving groups (figure 1f). The dose-related comparison showed that the +dp/dt in the BDL+SE600 group was significantly lower than that of the BDL+SE300 (P=0.023). Compared to that of the sham-operated group, a significant negative

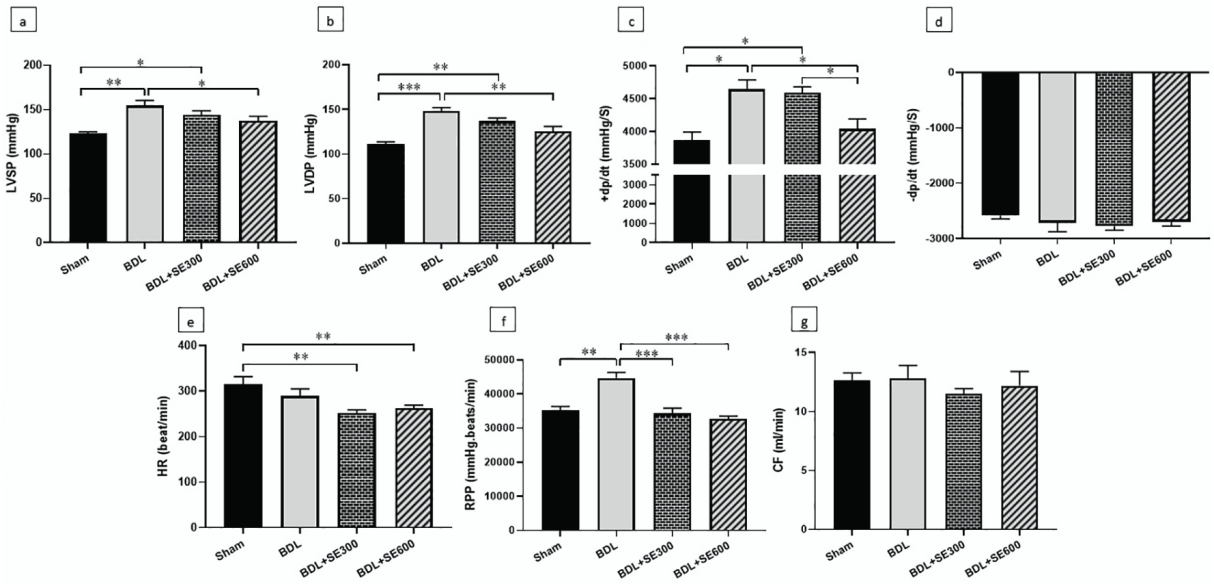

Figure 1: The figure illustrates the effect of bile duct ligation and/or Silymarin extract on the cardiac hemodynamic parameters. BDL: bile duct ligation; SE300: Silymarin Extract 300 mg/Kg/day; SE600: Silymarin Extract 600 mg/Kg/day (a) LVSP: left ventricular systolic pressure; (b) LVDP: left ventricular developed pressure; (c and d): ±dp/dt: derivative of pressure over time, (e) HR: heart rate; (f) RPP: rate-pressure product and (g) CF: coronary flow. Data were expressed as mean±SEM. *Denotes a significant difference from the Sham-operated groups (*P<0.05; **P<0.01; ***P<0.001)

chronotropic effect (P=0.002) was observed in both SE-receiving groups (figure 1e). Ligation of the bile duct and/or administration of SE did not lead to a significant change in the –dp/dt or CF parameters (figures 1d and g).

Heart and Body Weights Findings

The initial BWs among the experimental groups were not different, so the values were not shown in table 3. There was no significant difference in the initial and the final BWs of each group. Moreover, no significant difference in the final BWs was observed among the experimental groups (table 3). The absolute HW (P<0.001) and HW/BW (P=0.018) ratio in the BDL group were significantly higher than those of the sham-operated group. Compared to the sham-operated group, administration of SE, did not change the BDL-induced cardiac hypertrophy; so, the HW (P<0.001; P=0.002) and the HW/BW (P=0.013; P=0.016) ratio were significantly higher in BDL+SE-300 and BDL+SE-600, respectively. No significant difference was observed in HW and HW/BW ratio among BDL, SE300, and SE-600-treated groups.

Histopathological Findings

Based on our H&E findings and compared to those of the sham-operated group, four-weeks ligation of the bile duct led to the significant liver necrosis and fibrosis associated with marked infiltration of the inflammatory cells, bile duct proliferation, Kupffer cell hyperplasia, apoptosis as well as clear signs of regeneration (table 4; figure 2). The development of established fibrotic bundles was also confirmed in Masson's trichrome stained samples of the BDL group. In addition, Reticulin staining showed a significant thickening of the Reticulin fibers in this group. Despite the obvious distorted hepatic structure, no cirrhotic nodules were apparently observed in the BDL group. Treatment with SE300 did not alter the abnormal liver texture compared to those of the BDL ones, but administration of SE600 significantly improved the liver signs of structural damage such as fibrosis and necrosis. Details are given in the table 4.

Gene Expression Findings

Table 3: Effects of BDL and/or Silymarin extract on Bodyweight, Absolute heart weight, and Heart weight/body weight ratio (HW/BW)

	Sham	BDL	P value	BDL+SE300	P value	BDL+SE600	P value
Final BW (g)	292.1±7.4	297.7±10.3	>0.05	299.6±10.0	>0.05	291.2±8.9	>0.05
Absolute HW (mg)	1051±21.4	1271±31.5	0.001	1269±38.7	0.001	1230±37.6	0.005
HW/BW (mg/g)	3.60±0.06	4.280±0.11	0.015	4.245±0.07	0.013	4.239±0.15	0.011

Data were presented as mean±SEM. *Denotes significant difference from the Sham groups (*P<0.05; **P<0.01; ***P<0.001)

Table 4: Histopathological assessment of liver samples following bile duct ligation and/or Silymarin extract treatment

	Sham	BDL	P value	BDL+SE300	P value	BDL+SE600	P value
H&E staining							
Glycogen depletion	0	2.5 (1.5)	0.002	2 (1)	0.206	0 (1)$^{\#}$	>0.999
Hemorrhage	0	0	-	0	-	0	-
Congestion	0	0 (0.5)	>0.999	0 (0.5)	>0.999	1 (1)	0.089
Sinusoidal dilation	0	2 (2.12)	0.010	2 (1.25)	0.011	1.5 (1.25)	0.117
Edema	0	0	-	0	-	0	-
Inflammatory infiltration	0	2 (2)	0.002	1.5 (2.5)	0.016	1 (0.37)	0.096
Vesicular fat	0	0	-	0	-	0	-
Plasma cells	0	1 (0.25)	0.006	0 (0.25)	>0.999	0$^{\#\#}$	>0.999
Bile stasis	0	0	-	0	-	0	-
Bile plugs	0	0	-	0	-	0	-
Bile duct proliferation	0	3 (1.25)	0.012	4 (1.25)	0.001	3 (0.25)	0.090
Kupffer cell hyperplasia	0	3 (1.25)	0.005	3 (0.5)	0.002	2.5 (1)	0.058
Pyknosis	0	1 (1.5)	0.007	1 (0.25)	0.069	1 (0.25)	0.069
Karyolysis/Apoptosis	0	1.5 (1.25)	<0.001	1 (0.25)	0.101	1 (0.25)	0.101
Regeneration	-	2 (0.25)	0.626	3.5$^{\#\#}$(1)	0.002	4 (1)	<0.001
Cirrhotic nodule formation	0	0	-	0	-	0	-
Masson's trichrome staining							
Fibrotic bundles	0	2 (2)	0.003	1.5 (1.25)	0.067	0 (0.25)$^{\#}$	>0.999
Reticulin staining							
Necrosis	0	2 (0.63)	0.007	1.5 (2)	0.163	0 (1.25)	>0.999

Data were presented as Median with (IQR). BDL: bile duct ligation; SE300 and SE600: Silymarin Extract at 300 and 600mg/Kg/day; 0: no abnormality detected; 1: damage/active changes up to 25%; 2: damage/active changes up to 50%; 3: damage/active changes up to 75%; 4: damage/active changes >75%. $^{\#}$Denotes a significant difference from the BDL groups ($^{\#}$P<0.05; $^{\#\#}$P<0.01)

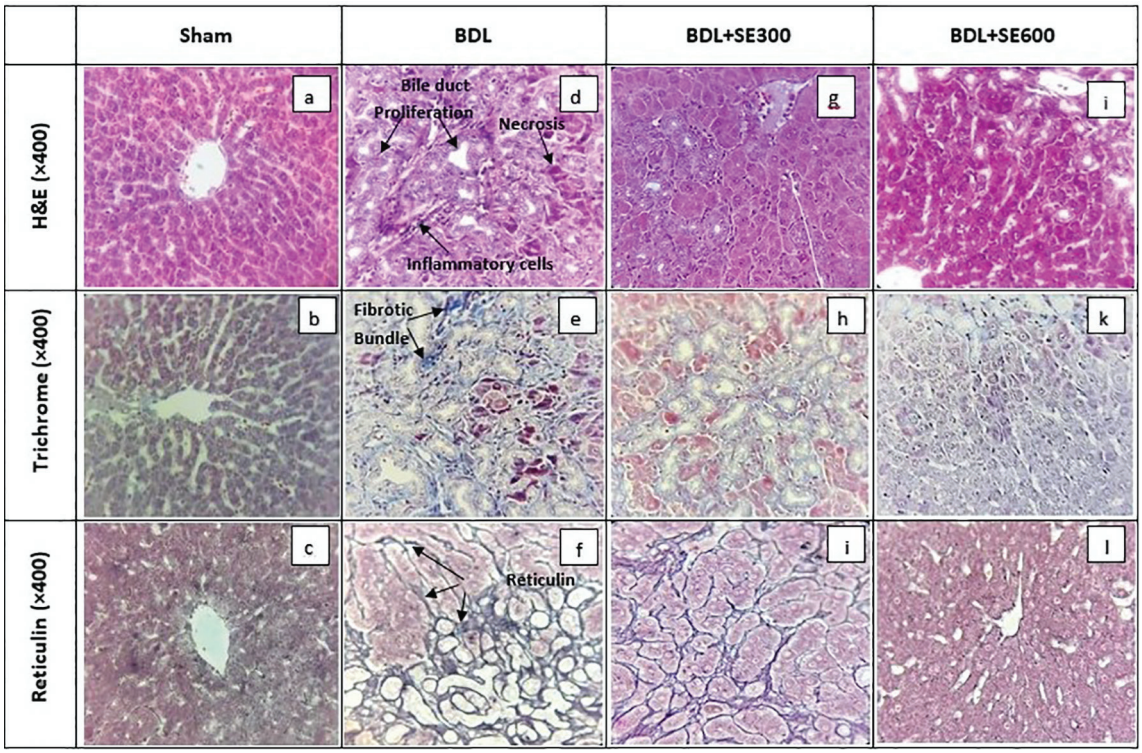

Figure 2: Microphotographs show sections of the rat liver stained by H&E, Masson's trichrome, and Reticulin. Massive distortions of hepatic architecture and its radial arrangement induced by BDL (d), compared to that of sham-operated ones (a, b, c). The obvious proliferation of bile duct along with clear focal inflammation and necrosis (d), well-defined fibrotic bundles in Masson's trichrome slides (e), and noted thickening of reticulin fibers induced by BDL (f). No defined changes following administration of SE300 in favor of hepatocellular protection from BDL-induced damages (g). Presence of Bile duct proliferation and necrosis in spite of SE-300 treatment (g). Clear detection of fibrotic bundles (h) and reticulin content in SE-300 treated ones (i). Clear normalization of aforementioned liver damages following administration of SE600, characterized by a well-rearrangement of Liver radial architecture and suppression of inflammation (j). Fading the BDL-induced fibrotic bundles (k) and thickening of the reticulin fibers (l).

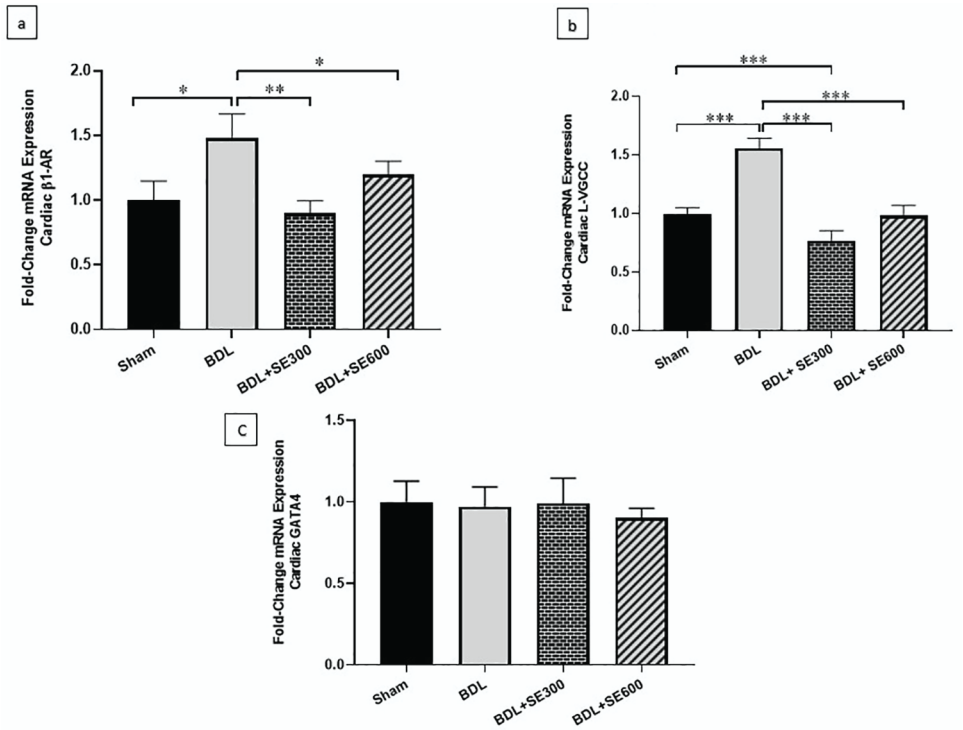

Figure 3: The figure illustrates the effect of bile duct ligation and/or Silymarin extract on the expression of ventricular targeted genes. β_1-AR: β_1-adrenergic receptors; L-VDCC: L-type voltage-dependent calcium channels Data were expressed as mean±SEM. * Denotes a significant difference from the Sham-operated groups (*P<0.05; **P<0.01; ***P<0.001)

The effects of BDL and BDL+SE treatment on the expression of cardiac genes are shown in figure 3. The expression of ventricular β_1-AR significantly increased in the BDL group (1.0 *vs.* 1.48 fold, P=0.026) compared to that of the sham-operated group (figure 3a). The same upregulation was also observed in the expression of ventricular L-VDCC (1.0 *vs.* 1.56 fold, P<0.001) in the BDL group (figure 3b). The expression of GATA4 mRNA did not change in the BDL group (figure 3c).

Four weeks of administration of SE was associated with significant changes in the expression of cardiac target genes. Compared to the BDL group, a significant reduction was observed in the expression of the ventricular β_1-AR (1.0 *vs.* 0.636 fold, P=0.009) and L-VDCC (1.0 *vs.* 0.493 fold, P<0.001) genes in the BDL+SE300 group (figures 3a and b). In a similar pattern, the expression of the β_1-AR (1.0 *vs.* 0.746 fold, P=0.027) and L-VDCC (1.0 *vs.* 0.629 fold, P<0.001) in the BDL+SE600 were significantly lower than that of the BDL one (figures 3a and b). Comparison between the two SE-treated groups did not show a statistically significant difference in the expression of these cardiac genes. Similar to those of the BDL group, the expression of GATA4 did not alter due to SE administration (figure 3c).

Discussion

The main objective of the present study was to investigate the cardiac effects of SE in secondary cardiac disease due to liver cirrhosis. Induction of biliary cirrhosis via bile duct ligation was associated with a significant cardiac abnormality characterized by cardiac hypertrophy, irregularity of cardiac hemodynamic parameters along with a significant dysregulation in the expression of the ventricular β_1-AR and L-VDCC genes. Surprisingly, the findings of this study show a prominent cardioprotective effect of the silymarin extract, at higher doses, in the four weeks BDL rats.

Cirrhosis is a common liver disease that can be associated with several complications, including cardiac and hemodynamic dysfunctions. One of the serious complications of cirrhosis is cardiac mechanical and electrical abnormality.[2, 26] Depending on the time-course of cardiomyopathy, there are different kinds of cirrhotic-induced cardiac mechanical dysfunction. Of course in this regard, the issue of “time” is very decisive. The process is started with an initial compensatory sympathetic overactivity followed by a non-compensatory phase and could be accompanied by either blunted systolic or diastolic function. The present findings revealed that four weeks of ligation of the bile duct, induced a significant increase in the indices of cardiac contraction including LVSP, LVDP, +dp/dt, and RPP along with remarkable cardiac hypertrophy. In this regard, the elevation of the maximum rate of pressure rise (+dp/dt) was not proportional to its fall (-dp/dt). It can be partially concluded that in spite of no clear change in the absolute -dp/dt value, the diastolic function suffers from an abnormality in the BDL ones. Diastolic dysfunction is an early marker of cardiac dysfunction occurring before systolic dysfunction.[27] Our data was inconsistent with the others who reported a diminished cirrhotic-induced cardiac contractility.[28]

Histopathological analysis using three types of staining showed that four weeks ligation of the bile duct was associated with severe liver structural damages. Massive bile duct proliferation and hepatocyte regeneration were related to focal inflammatory infiltration, glycogen depletion, pyknosis, apoptosis, necrosis, and well-defined fibrosis were hallmarks of severe liver injury in this study. Such massive hepatocellular damage and necrosis were also confirmed by a significant elevation of AST and ALT. Moreover, a significant rise in serum levels of GGT and ALP confirmed the induction of cholestasis and bile duct necrosis. In spite of this extensive architectural distortion, there was doubt about the development of cirrhotic nodules. Treatment with the higher, but not the lower dose of SE, significantly decrease BDL-induced focal inflammation, necrosis, and fibrosis scores, which was biochemically confirmed by a remarkable decrease in serum levels of liver enzymes. Based on our histological findings, treatment with SE600 greatly repaired the large-scale structural abnormalities and protected the cells from BDL-induced hepatocellular necrosis and fibrosis.

The molecular findings were also consistent with those of the isolated heart study. In this way, increased cardiac contractility could be explained by significant upregulation of the ventricular β_1-AR and L-VDCC in the BDL group. Although the observed findings were not in agreement with the others in this regard, they could elucidate a pathological status in the cardiac function. As Engelhadt and others showed, the overexpression of the β_1-AR in the cardiomyocytes of transgenic mice was associated with a short-term improvement of cardiac function followed by a marked deterioration.[29] In addition, the development of dilated cardiomyopathy accompanied by heart failure manifestation in young age animals

was a consequence of the receptor gene overexpression.[29] Continuous activity of these receptors was also associated with calcium overload and cardiomyocytes toxicity.[30] Despite mentioned explanations, there are several experimental reports indicating that the impaired β_1-AR density as well as its blunted signaling pathways and subsequently dysregulation of the L-VDCC have an essential role in the development of the negative inotropic and/or chronotropic properties of the failing hearts.[7]

In the present study, the development of cardiac abnormality was secondary to liver cirrhosis, so it is expected that restoration of the liver function may improve these secondary cardiac complications. Therefore, the present study focused on the cardioprotective role of silymarin as a potent hepatoprotective agent. Despite the numerous studies on the hepatoprotective activity of silymarin,[31] there are few reports on its cardioprotective properties. Thus, silymarin and/or its active ingredient are prominent against ischemia-reperfusion-induced cardiac damage, due to the restoration of endogenous antioxidant enzymes, inhibition of neutrophil infiltration, and its cytoprotective activity.[32] Similar results have been reported regarding the cardioprotective effects of silymarin on post-myocardial infarction remodeling,[33] cisplatin,[34] and doxorubicin-induced cardiotoxicity.[35] There are also some reports on the cardioprotective properties of the silymarin or silibinin against several cardiotoxic chemicals such as arsenic,[36] isoproterenol,[37] and acrolein as an environmental pollutant.[38] As reported by Yang and others, silymarin can also reduce cardiac apoptotic cells, collagen accumulation, TGF-β down-regulation, and BNP reduction in CCL4-induced cirrhotic cardiomyopathy.[39] While, there are no defined experimental findings about the cardiac effects of silymarin on BDL-cirrhosis, in consistence with the findings of the other experimental models of cardiac disease, our findings confirm clear cardioprotective properties for the SE, at the higher dose. The observed cardioprotective effect of the SE600 was confirmed by considerable recovery of the cardiac hemodynamic parameters including LVSP, LVDP, +dp/dt, and RPP. Improved hemodynamic parameters were also supported by other molecular findings. Significant downregulation of β_1-AR and L-VDCC expression, which were upregulated due to BDL, was other evidence for the cardioprotective activity of silymarin. The observed restoration of LVSP, LVDP, +dp/dt, and RPP are partly due to significant downregulation of the ventricular β_1-AR and L-VDCC mRNA expression. Although the latter effect was also seen by the lower dose of the SE, it could not effectively improve the cardiac hemodynamic parameters.

The GATA4, another indicator of cardiac hypertrophy, did not change due to either BDL and/or silymarin administration. A similar unchanged level of GATA4 was also shown in the Hautala and others study, which indicated that regardless of an increase in DNA-binding activity, the expression level of the GATA4, was not affected by hypertrophic stimulation induced by pressure overload.[40] On the other hand, the cardiac hypertrophy has been confirmed by the higher absolute and relative heart weights of the BDL ones.

For unknown reasons, administration of the extract at both doses was associated with marked bradycardia. The observed bradycardia might be suggested to be due to the effects of the SE on the channels and/or signaling pathways involved in the activity of S.A nude pacemaker. Further molecular investigations need to clarify the exact mechanism of the extract-induced bradycardia. Cardiomyopathy, especially in the progressive stages, is associated with the development of life-threatening arrhythmia, so if silymarin could also work as an anti-arrhythmic agent, it could be considered a valuable prophylactic and/or therapeutic target in this situation.

According to the present findings, the authors suggest that silymarin could affect cardiac function either directly or indirectly. The higher dose of SE exhibited a significant hepatoprotective effect characterized by decreasing the level of liver enzymes and also alleviation of hepatic necrosis and fibrosis rejoins in histopathological findings. Restoration of the cardiac hemodynamic parameters and correction of ventricular β_1-AR and L-VDCC expression elucidated the cardioprotective effects of SE at the higher dose. It could be resulted from an indirect effect (alleviation of the symptoms of BDL-induced liver injury) or a direct cardiac effect. The latter hypothesis comes from the observations, which showed that the lower dose could downregulate the cardiac genes without obvious effects on liver enzymes and/or histology. The SE pharmacological effects in this experiment showed a dose-dependent pattern.

Besides its advantages, the main limitation of the present study was the short duration of the follow-up. A longer experimental period in this situation could be helpful to determine the more realistic effects of silymarin on secondary cardiac abnormality due to biliary cirrhosis. Besides, the addition of a stressor agent, such as isoproterenol, would be beneficial to realize the cardiac response of the silymarin. This issue needs to be considered for future experiments.

Conclusion

Taken together, the isolated heart study along with the molecular findings showed that the higher dose of silymarin had a cardioprotective effect against BDL-induced cardiac abnormality. Silymarin's cardioprotective effect was confirmed by the restoration of cardiac contractility parameters and correction of ventricular β_1-AR and L-VDCC expression. The observed cardiac effects could be mediated directly and/or indirectly in the higher dose.

Acknowledgment

The authors would like to thank Alborz University of Medical Sciences for providing the laboratory facilities and financial support for this project.

Authors' Contribution

G.B and A.Kh. conceived and designed the study. G.B, R.M, A.Kh, P.E, S.A.H and A.Sh performed the experimental animal procedure. A.Kh, G.B and Kh.P analyzed data and write the manuscript. P.F and G.B designed and conducted the real time RT-PCR parts. All the authors contributed to drafting or revising the work. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest: None declared.

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