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Effects of Silymarin on Blood Glucose Concentration, Hepatic Histopathological Changes and *FOXA2* and *FOXA3* Gene Expression in Streptozotocin-Induced Diabetic Male Wistar Rats

Sajad Nikkhah[1], Rahman Jafari Hafshejani[1], Farshid Gheibi Hajivar[1], Khalil Khashei Varnamkhasti [2]*, Sara Afzali[3]

[1] *Department of Biology, Science Faculty, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran*
[2] *Department of Genetics, School of Medicine, University of Islamic Azad, Kazerun Branch, Kazerun, Iran*
[3] *Division of Cytogenetic, Medical Genetic Laboratory of Dr. Keshavarz, Guilan, Iran*

Abstract

Since the liver is among the primary organs susceptible to the effects of hyperglycaemia, diabetes mellitus (DM) could be a risk factor for the development and progression of liver damage. In present study, since no side-effects from the herbal medicine have been reported, the effect of silymarin on blood glucose concentration, hepatic histopathological changes and *FOXA2* and *FOXA3* gene expression, which are key genes in liver regeneration, was investigated. In this fundamental with experimental approach study, 40 male Wistar rats weighing 180-220 g were used. Rats were kept under the standard conditions of temperature of 20-22°C and humidity of 50% and consecutive 12-hour periods of light and darkness. Rats were randomly divided into five different groups (n=8 each), including healthy control rats, diabetic control rats, diabetic rats receiving silymarin (50, 100 and 150 mg/kg). Diabetes was induced by injecting streptozotocin (50 mg/kg B.W., i.p.). For 4 weeks silymarin groups received the drug once every three days through gavage and fasting blood glucose concentration measured once every 10 days. At the end of a month experiment, livers were harvested for hepatic histopathological and *FOXA2* and *FOXA3* gene expression changes analysis. In the diabetic rats treated with silymarin (50, 100 and 150 mg/kg), by comparison with the diabetic control group ($p < 0.05$), glucose levels decreased significantly. Moreover, *FOXA2* and *FOXA3* expression in diabetic groups treated with silymarin significantly increased compared to diabetic control group ($p < 0.05$). Hepatic histopathological changes were improved in the treated groups. The present study indicates that silymarin significantly decreased blood glucose concentration and increased the *FOXA2* and *FOXA3* gene products level. Hence, silymarin is able to improve some of the symptoms associated with diabetes and possesses hepatoprotective effects in streptozotocin-induced diabetic rats.

Introduction

Diabetes mellitus (DM) is a complex chronic metabolic illness that has affected an estimated 451 million people worldwide in 2017 and is expected statistics increase to 693 million people by 2045 (Fernández et al., 2020; Chen et al., 2019). In such a metabolic progressive condition, impaired insulin production and/or insulin secretion or action, results in raised blood glucose level (hyperglycemia) (Chaudhury et al., 2017). The hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of multiple organs, such as kidney, liver, and cardiovascular. The liver is a major metabolic hub, and the diabetes metabolic changes is associated with an increased risk for developing spectrum of liver diseases such as fatty liver disorders, cirrhosis, and hepatocellular carcinoma (Bedi et al., 2019). Diabetes mellitus has been recognized as non-communicable diseases that leading to death, hence demand a global effort to address its prevalence and associated complications (Oguntibeju, 2019). Despite our growing understanding of the pathophysiology of diabetes, currently available therapies produce modest and transient efficacy and cannot be to completely prevent of this complication. Therefore, search for anti-diabetic compounds that can protect the patients against diabetes and diabetic complications is of great interest (Ghorbani, 2017). At present, significant attentions are getting directed toward assessing the effect of herbal medicines. Growing lines of evidence suggest that flavonoids from medicinal plants have a positive impact on diabetes by improving glycemic control, lipid profile, and antioxidant status. Silymarin (a polyphenolic flavonoid mixture extracted directly from dried fruits (seeds) or whole plants of *Silybum Marianum* (milk thistle)), including four major flavonolignans, namely, silybin, isosilybin, silychristin and silydianin has been used to treat of different diseases for many years and has been reported as a safe, non-toxic agent to treatment

various diseases (El-Far et al., 2016). The measurement of new medicine candidate capability in expression changes of genes involved in regeneration of diabetic damaged organs like liver, offer effectiveness of new approach in recover from injuries. Liver expression profile is controlled by a gene regulatory network. The major components of the liver gene regulatory network are the three members of the *Foxa* gene family (include *Foxa1*, *Foxa2*, *Foxa3*), which through the regulation of multiple target genes in the liver, play key roles in controlling metabolism and homeostasis (Wangensteen et al., 2015; Warren et al., 2020). Reports indicated that downregulation of Foxa gene family occur in liver diseases and low Foxa levels exacerbate the injury to the liver and enormous reduction in the expression of crucial liver genes (Bochkis et al., 2008; Reizel et al., 2020). Thus, development of therapeutic approaches that able to trigger regenerative genes expression while lowering blood glucose levels may be the most effective way to prevent the complications in diabetes mellitus. The present study was designed to investigate the effects of silymarin on blood glucose concentration, hepatic histopathological changes and *FOXA2* and *FOXA3* gene expression.

Material and methods

Animals and experimental design

A total of 40, 2-month-old male wistar rats that weighed approximately 180-220 g (from Pasteur Institute, Tehran, Iran) were used. Rats 1 week prior to the experiments were housed in standardized rat cages, in a temperature (20–22 ◦C) with 50% humidity and light–dark cycle (12/12 h) controlled room. Animals were provided with standard diet and drinking water ad libitum. Next, rats were then sorted randomly into five groups of 8 rats each: healthy control group (C), diabetic control group (D), diabetic group receiving 50 mg/kg silymarin (D+50 mg/kg S), diabetic group receiving100 mg/kg silymarin

(D+100 mg/kg S), diabetic group receiving 150 mg/kg silymarin (D+150 mg/kg S).

Induction of diabetes in rats

Diabetes was induced by intraperitoneal injection of freshly prepared solution of streptozotocin (STZ) (sigma) (50 mg/kg body weight in 0.1 M citrate buffer, pH 4.5) to fasted rats (12 h). 72 hour after STZ-injection, diabetes was confirmed by the demonstration of hyperglycemia (blood glucose $\geq$ 250 mg/dl).

Treatment schedule

Silymarin was purchased from Sigma-Aldrich Corporation (Sigma-Aldrich, USA) and for 4 weeks, once every three days, silymarin groups, received the drug (silymarin dissolved in distilled water) through gavage.

Estimation of glucose

The blood glucose concentration (1 ml blood from retro-orbital venous plexus), was monitored before STZ-injection (day 0) and 3, 10, 20 and 30 days after STZ -injection by enzymatic glucose kits (Sigma-Aldrich, USA), after 12 h fasting.

Surgery

On day 30st of STZ administration, all the rats of both control and experimental groups following 12 h fasting were anesthetized by intramuscular injections of ketamine hydrochloride (50 mg/kg) in conjunction with diazepam (50 mg/kg). Liver tissues were immediately collected and were treated with RNAlater™ (Sigma-Aldrich, USA) and 10% buffered formalin for gene expression analysis and histological study respectively.

RNA isolation and cDNA synthesis

Total RNA from rat liver tissues was extracted using Trizol reagent according to the manufacturer's instructions (Takara, China) and then treated with DNase Kit (Qiagen) to remove DNA contamination. Nano Drop spectrophotometry (Eppendorf, Germany) (at a wavelength of 260 nm) was used to calculated concentration of extracted RNA. To detect the purity of RNA, its optical density (OD) ratio at 260/280 nm was determined and samples with a ratio of >1.8 were used for cDNA synthesis. Reverse transcription was carried out using a cDNA synthesis kit (RevertAID First Standard cDNA Syn Kit), using 1 µg of RNA and random hexamer, based on manufacturer's protocol (Termo Scientific, Lithuania). cDNAs were stored at -20°C until used in the real-time polymerase chain reaction (PCR). To evaluate the expression levels of *FOXA2* and *FOXA3* in the liver, analysis was performed using real-time assay in triplicates using Rotor-Gene 6000 machine from Corbett Research, Australia. Reactions were performed using 20µl mixtures containing 1µl cDNA, 0.8µl of each primer (forward and reverse), 10 µl SYBR Green and 7.4µl RNase-free water. Relative expression levels of *FOXA2* and *FOXA3* transcripts were compared to rat *GAPDH* as the housekeeping gene. Sequences of forward and reverse primers of *GAPDH*, *FOXA2* and *FOXA3* genes were designed by using Oligo7 software and then blasted with NBCI to ensure their integrity and were finally synthesized by Macro gen Inc (Table 1). Then, the reaction mixture was placed under time-temperature condition (Table 2).

Table 1. The primer set used for RT- Real Time PCR

1	*FOXA2*	F	TTTCCGCCCTCCTTCTACTCA	21
		R	CTCTGTCCCCAACTCCTACCC	21
2	*FOXA3*	F	ACTACCAGAGCCTCTATTCCC	21
		R	ATCTGCCTTCACAATACCCCAA	22
3	*GAPDH*	F	TGGTGAAGGTCGGTGTGAACGGAT	24
		R	TCCATGGTGGTGAAGACGCCAGTA	24

Table 1. Time-temperature condition of RT- Real Time PCR

1	First denaturation	94^{0}c	4 Min	1 cycle
2	denaturation	94 ^{0}c	15 sec	
3	Annealing *FOXA2* gene	59 ^{0}c	20 sec	40 cycle
4	Annealing *FOXA3* gene	58 ^{0}c	20 sec	
5	Annealing *GAPDH* gene	60 ^{0}c	20 sec	
6	Extension	72^{0}c	20 sec	

Histopathological examinations

Histological processing of liver was performed after 24 h tissue fixation in 10% buffered formalin, by dehydrated of samples in a series of alcohols and embedded in paraffin blocks. Blocks were prepared in 5-μm and stained with hematoxylin and eosin, and further examined by the light microscope (Olympus, Japan) connected to digital camera. Photographs from all groups were compared for histological changes.

Statistical analysis

Data analyses were done using statistical processor system support (SPSS) 18.0 software. The data were presented as mean ± standard deviation. Ct method ($2^{-\Delta\Delta ct}$ formula) and REST software were used to examine relative changes in gene expression and comparisons groups after fold change calculated. The level of significance for all tests was set at P<0.05.

Results and Discussion

Blood glucose levels

At the day 0 (before STZ-injection) blood glucose level was within normal level in all groups. After the 3 days of STZ injection, the level of blood glucose within all diabetics showed significantly increased as compared with normal control. After 10, 20 and 30 days of treatment with silymarin the level of blood glucose decreased in the diabetic, drug receiving groups, compared to diabetic and normal control group. Decreased blood glucose level (in 10, 20 and 30 days after treatment) was more notable in diabetic receiving100 mg/kg silymarin group compared to control (Figure 1).
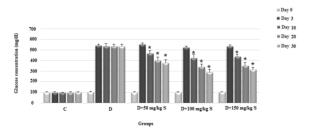

Figure 1. The effect of silymarin on glucose level (mg/dl) in streptozotocin- induce diabetic rats. Sign (*) denote significant differences (P<0.05).

Effect of silymarin on liver expression of FOXA2 and FOXA3 genes

Silymarin induced a significant increment in *FOXA2* and *FOXA3* gene expression in a dose and time-dependent manner within all diabetic, drug receiving groups in comparison to untreated diabetic group. As seen in figure 2 in the case of *FOXA2*, after treatment with silymarin the level of expression showed significantly increased values reached to 1.2443 ± 0.0366 in D+50 mg/kg S group, 1.6763 ± 0.0493 in D+100 mg/kg S group and 2.4967 ± 0.0122 in D+150 mg/kg S group as compared with diabetic control (1.0022 ± 0.0932). Similarly a significant increases in level of *FOXA3* expression was also shown in figure 3, after treatment with silymarin the level of expression values reached to 2.2265 ± 0.0222 in D+50 mg/kg S group, 3.6567 ± 0.1433 in D+100 mg/kg S group and 4.6451 ± 0.2048 in D+150 mg/kg S group as compared with diabetic control (1.0069 ± 0.1670).
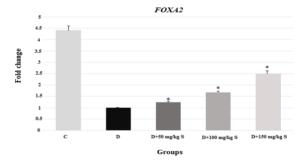

Figure 2. The liver *FOXA2* gene expression in different groups. Data were presented as mean ± SD. Sign (*) denote significant differences (P<0.05).
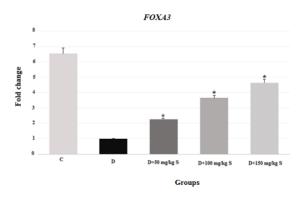

Figure 3. The liver *FOXA3* gene expression in different groups. Data were presented as mean ± SD. Sign (*) denote significant differences (P<0.05).

Effect of silymarin on histopathological changes of liver

The liver of healthy control group (C) showed normal connective tissue in shape with minimal or no inflammatory infiltration cells. Hepatocytes were arranged in the form of branching cords, separated by blood sinusoids, and radiated from the central vein. In contrast, the liver tissue in diabetic control group (D) showed hepatonecrosis and severe hyperemia in the area surrounding the central veins. Liver tissues from diabetic, drug receiving groups showed regenaration of liver. As seen in figure 4, diabetic group that received 50 mg/kg silymarin; show a bit decreases of hyperemia and necrosis, diabetic group that received 100 mg/kg silymarin; show nearly normal hepatocyte with mild hyperemia and diabetic group that received 150 mg/kg silymarin; show increases of liver regeneration with no hyperemia and necrosis.
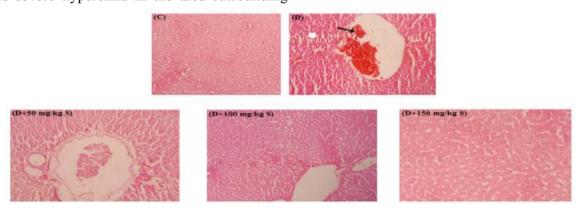

Figure 4. Light micrographs of midzone of sections of Rats Liver tissues (H and E, ×10). Observe: intact hepatocyte with typical features in healthy control group (C); hepatonecrosis (white arrow) and severe hyperemia (black arrow) in Diabetic control group (D); a bit decreases of hyperemia and necrosis in D+50 mg/kg S group; nearly normal hepatocyte with mild hyperemia in D+100mg/kg S group; regeneration of liver with no hyperemia and necrosis in D+100mg/kg S group.

Since despite the available treatments, the mortality and morbidity associated with diabetes remain high, evaluation of alternative medicine products instead of conventional drugs is necessary to treatment of diabetic and its complications (Stolf et al., 2017). *Silybum marianum*, since the time of ancient Greece until now widely used as herbal remedy for a variety of disorders. It contains mainly silibin A, silibin B, isosilibin A, isosilibin B, silichristin A, silidianin, and other compounds in smaller concentrations and has mainly been used to treat liver and gallbladder diseases. In addition recently also due to its hypoglycemic properties gained attention (Voroneanu et al., 2016; Kazazis et al., 2014). Therefore in the present study, the hypoglycemic and hepatoprotective effect of the silymarin in STZ-induced diabetic male wistar rats was evaluated. In this effort, diabetes was found to reduce as silymarin decreased the blood glucose levels of STZ-induced diabetic rats displayed elevated fasting blood glucose. In accordance with our findings, reduction of blood glucose levels was observed in 25 diabetic patients treated with 200 mg silimarin four months, three times a day before meals (Huseini et al., 2006). Other reviewed study show that silibin nanoparticles have hypoglycemic activity in STZ-induced diabetic mice (Das et al., 2014). It was also reported that glucose level decreases in hereditary hypertriglyceridemic rats, which treated by micronized form of silybin (MacDonald et al., 2021). Liver differentiation, development, metabolism and regeneration controlled by regulatory transcription factors which form a gene regulatory network (GRN). An important component of the hepatic GRN, established in rodent and humans, is the *Foxa* family. Findings suggest that downregulation of

Foxa gene family occurs in liver diseases and low *Foxa* levels exacerbate the injury to the liver (Warren et al., 2020; Bochkis et al., 2008). Thus nominate new medicine with capability to reprogramming members of this gene family can reduce injury to the liver. In this study attempted to evaluate capability of silimarin to reprogramming *Foxa* family. Quantitative real time PCR data showed that levels of *Foxa2* and *Foxa3* were increased within all diabetic, drug receiving groups compared to diabetic control group. This comes in accordance with previous studies; for example, the results of a study showed that silymarin led to liver regeneration by increasing expressions of HGF, TGFα, and TGFβ1 (Wu et al., 2015). Also recent studies have shown that silymarin able to alter gene expression and protein synthesis by interacts with transcription factors (Kheiripour et al., 2019). Additionally, our histopathological results confirmed other findings of study. Histopathological data showed that silymarin could restore liver cells and reverse the hepatocytes damage caused by diabetes. These results were in accordance with previous studies that demonstrated; silymarin is highly effective in preventing and improvement of hepatic histophatological alteration induced by isotretinoin (Kumaş et al., 2018). Currently also found that silymarin modulates enzymes associated with the development of cellular damage (Gillessen et al., 2020).

Conclusion

In conclusion, silymarin a polyphenolic flavonoid, as a safe and nontoxic agent is well-known for the treatment of various diseases including diabetes. This histopathological study supported by genetic investigation proved its hepatoprotective effect against STZ induced diabetic changes in male wistar rat livers and created a better understanding of its importance in diabete treatment. Further studies concerning molecular mechanism responsible for this silymarin effect may provide more details on its therapeutic potentials.

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