

The Effect of Silymarin on the Expression of Urotensin–II and Urotensin–II Receptor Genes in the Liver Tissue of Type 2 Diabetic Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Studies have shown that the increase in urotensin – II is associated with diabetes disorders. Considering that using herbal medicines for the treatment of diseases leads to fewer complications compared to most chemical drugs, the present study was conducted to investigate the effect of silymarin on glucose, and insulin levels and the expression of urotensin – II (U–II) and urotensin – II receptor (U–II R) genes in the liver tissue of type 2 diabetic male rats.

METHODS: In this experimental study, 36 male albino Wistar rats were randomly divided into 6 groups (n=6): 1. Control group; 2 and 3. Control groups treated with 60 and 120 mg / kg / day silymarin; 4. Type 2 diabetic group which received an intraperitoneal (i.p.) injection of 60 mg / kg streptozotocin and 120 mg / kg nicotinamide; 5 and 6. Diabetic rats treated with 60 and 120 mg/kg/day silymarin. After 60 days of treatment, serum and liver tissue samples were collected. Glucose, insulin, HOMA-IR index and liver enzymes were evaluated by spectrophotometry and ELISA methods, while gene expression in liver tissue was analyzed by Real-time PCR method.

FINDINGS: Insulin levels increased significantly in diabetic groups treated with silymarin (60 and 120 mg/kg) (9.6 ± 1.11 and 9.8 ± 0.96 , respectively) in comparison with the diabetic control group (7.10 ± 1.06) ($p < 0.05$). Moreover, glucose level, HOMA-IR, liver enzymes, U–II and U – II R expression in diabetic group treated with silymarin significantly decreased compared to diabetic control group ($p < 0.05$).

CONCLUSION: The results of this study showed that administration of silymarin improves liver function in diabetic rats.

KEY WORDS: Diabetes Mellitus, Silymarin, Urotensin – II, Urotensin – II Receptor, Liver.

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Introduction

Diabetes mellitus is one of the most important metabolic disorders. This disease occurs due to various causes such as reduced insulin secretion or resistance to the function of this hormone (1). In 2017, the global prevalence of diabetes was 382 million people, which is predicted to reach 592 million people by 2035. Its prevalence is increasing dramatically, especially in developing countries like Iran (2). Diabetes is considered as one of the major causes of the incidence of hepatic disorders (3).

Urotensin – II gene (U – II) with 11 amino acids has been identified as the strongest factor in vascular contraction in mammals. In humans, the U – II mRNA has been discovered in the heart, liver, kidneys, adrenal glands, placenta, spleen, and thymus glands tissues (4). The U-II molecule performs its actions through its receptor, which is G protein-coupled receptor. The U-II and U-II R systems are effective in insulin resistance, impaired glucose tolerance, impaired glucose transport to muscles via GLUT4, impaired pancreatic beta-cell function, and induction of type 2 diabetes (5). Therefore, U – II and U – II R systems are potential therapeutic goals in the treatment of diabetes-related disorders.

Silymarin is the most important active ingredient in milk thistle (*Silybum marianum*), which consists of a group of chemicals called Flavonolignans (6,7). For centuries, the seeds of this plant have been used as a very useful drug for the treatment of liver disease, and they have been commercially manufactured and used since 1970 (3).

Considering the importance of U – II and U – II R, and the fact that they have recently been considered as a new therapeutic goal in metabolic diseases, finding compounds, particularly with herbal origin, that decrease the expression of this genes is very important for diabetes treatment. Therefore, the present study was conducted to investigate the effect of silymarin on glucose, insulin, HOMA-IR, liver enzymes and expression of U – II and U – II R genes in liver tissue of type 2 diabetic male rats.

Methods

This experimental study was carried out on 36 male Wistar rats (220±10 g). Animals were kept in animal house condition with free access to water and food and 12/12h light-dark cycle. Then, the animals were randomly divided into 6 groups (n=6): control group with normal nutrition, control groups treated with 60

mg/kg and 120 mg/kg silymarin respectively, type 2 diabetic group, and type 2 diabetic groups treated with 60 mg/kg and 120 mg/kg silymarin respectively for 60 days. To induce of type 2 diabetes, Fifteen minutes after injection of 60 mg/kg streptozotocin, nicotinamide 120 mg/kg was injected intraperitoneally to the rats. To confirm diabetes, after seven days blood glucose were measured by glucometer. Rats with a fasting blood glucose above 150 were included in the study. Then, treatment started with silymarin (Livergol tablet as suspension in normal saline and once a day for 60 days by oral gavage) (8). All procedures on the animals were based on the Ethical Guidelines for the Use of Animals in Research and approval at the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1395.205).

At the end of the study, the rats were anesthetized with ketamine (50 mg/kg) after overnight fast and serum samples were collected and kept for measuring liver enzymes and insulin. The liver tissue was kept at a temperature of -80 °C until performing gene expression. **Measurement of fasting blood glucose, insulin and HOMA-IR levels:** The fasting blood glucose levels were measured by ACCU-CHEK Aviva Blood Glucose Meter (Germany) after collecting blood from tail vein. Insulin was measured using ELISA Kit (Mercodia, Sweden). The Insulin resistance index (HOMA-IR) was evaluated using the following formula (4):

$$\text{HOMA-IR} = \frac{\text{Fasting insulin } (\mu\text{U/ml}) \times \text{Fasting glucose (mg/dl)}}{405}$$

Measurement of Liver Enzymes: Serum levels of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Alkaline Aminotransferase (ALP) were measured through colorimetric by spectrophotometer in fasting condition.

Evaluation of U – II and U – II R genes: To investigate the expression of U – II and U – II R genes, RNA extraction was performed by RNX-Plus solution (Sinaclon, Iran). The quality and quantity of the extracted RNA were determined by electrophoresis and NanoDrop (Fig 1). Then, cDNA synthesis was performed by Takara Kit. Then, 1 µg of synthesized cDNA entered the Real Time PCR reaction, which was carried out using SYBR Green Kit (Takara Co.). The primers required for the reaction were designed by Primer 3 software (Table 1). β-Actin was used as the housekeeping gene. Finally, the results were analyzed by $2^{-\Delta\Delta C_t}$ method (9).

Statistical analysis: All parameters were determined as the mean and standard deviation value and were analyzed with SPSS-16 (Chicago, IL). One-way ANOVA followed by the

Tukey's post hoc test was used to analyze the normal value that was evaluated by Kolmogorov–Smirnov test. $P<0.05$ was considered significant.

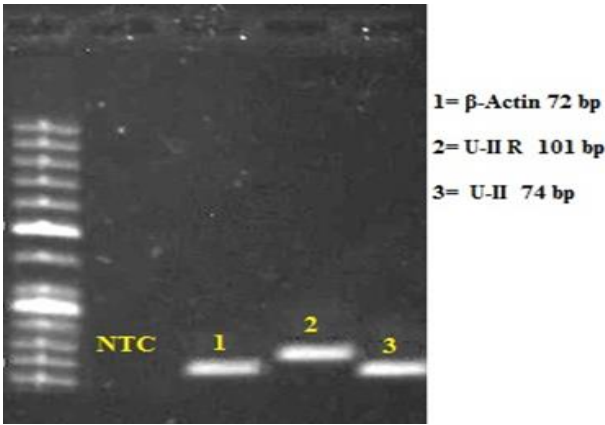


Figure 1. Agarose 1% gel electrophoresis of U-II, U-II R, β -Actin genes and Ladder 100bp.

Table 1. The specifications of used primers

Gene name		Primer sequence (5'->3')	Primer length (bp)	Product length (bp)
Urotensin II	Forward	5'GTCGTCATGGACAGGGTG3'	18	74
	Reverse	5'GAGGGTTTATTTCTCATAGTGG3'	22	
Urotensin II receptor	Forward	5'GCACGCCAGCATCTTCAC3'	18	101
	Reverse	5'TGGTCCCAAAGAGCAACG3'	18	
β -Actin	Forward	5'CCCGCGAGTACAACCTTCT 3'	19	72
	Reverse	5'CGTCATCCATGGCGAACT 3'	18	

Results

The STZ/NIC injection increased blood glucose in the diabetic group (299.3 ± 27.0) compared to the healthy control group (83.3 ± 10.9) ($p<0.01$). In diabetic groups treated with 60 and 120 mg/kg silymarin (202.6 ± 45.1 and 182.5 ± 35.8), blood glucose levels showed a significant decrease compared to the diabetic group ($p<0.01$).

Moreover, the induction of diabetes reduced serum insulin levels in the diabetic group (7.10 ± 1.06) compared to the control group (11.46 ± 0.47) ($p<0.01$) (Table 2). In diabetic groups treated with doses of 60 and 120 mg/kg silymarin (9.6 ± 1.11 and 9.80 ± 0.96), there was a significant increase in insulin levels compared to diabetic group ($p<0.01$). Furthermore, induction of diabetes increased the HOMA-IR levels in the diabetic group (5.79 ± 0.90) compared to the control group (2.35 ± 0.27) ($p<0.01$). The diabetic groups treated with 60 and 120 mg/kg silymarin (4.71 ± 0.64 and 4.35 ± 0.56) showed a significant decrease in HOMA-IR levels compared to diabetic group ($p<0.05$). The activity

of ALT (160.66 ± 7.11), AST (239.16 ± 14.17), and ALP (730.16 ± 41.88) in the diabetic group increased significantly compared to the healthy control group (53.00 ± 6.10 , 108.33 ± 6.59 , and 250.50 ± 19.59 , respectively) ($p<0.01$) (Table 2). In the diabetic group treated with doses of 60 mg/kg (73.50 ± 2.07 , 152.00 ± 6.71 , and 396.50 ± 34.56) and 120 mg/kg (70.50 ± 3.04 , 140.50 ± 10.44 , and 431.83 ± 24.92), significant decrease was observed in the activity of ALT, AST and ALP enzymes compared to the diabetic group ($p<0.01$). The expression of U-II and U-II R genes in the diabetic group (10.02 ± 2.47 and 3.80 ± 0.85) increased significantly compared to the control group (1 ± 0.00 and 1 ± 0.00) ($p<0.01$). Treatment with silymarin significantly reduced the expression of U – II (3.01 ± 0.37 at the dose of 60 mg/kg, and 2.40 ± 0.53 at the dose of 120 mg/kg) and U – II R (2.09 ± 0.83 at the dose of 60 mg/kg, and 1.71 ± 0.47 at the dose of 120 mg/kg) genes compared with the diabetic group ($p<0.01$) (Fig 2).

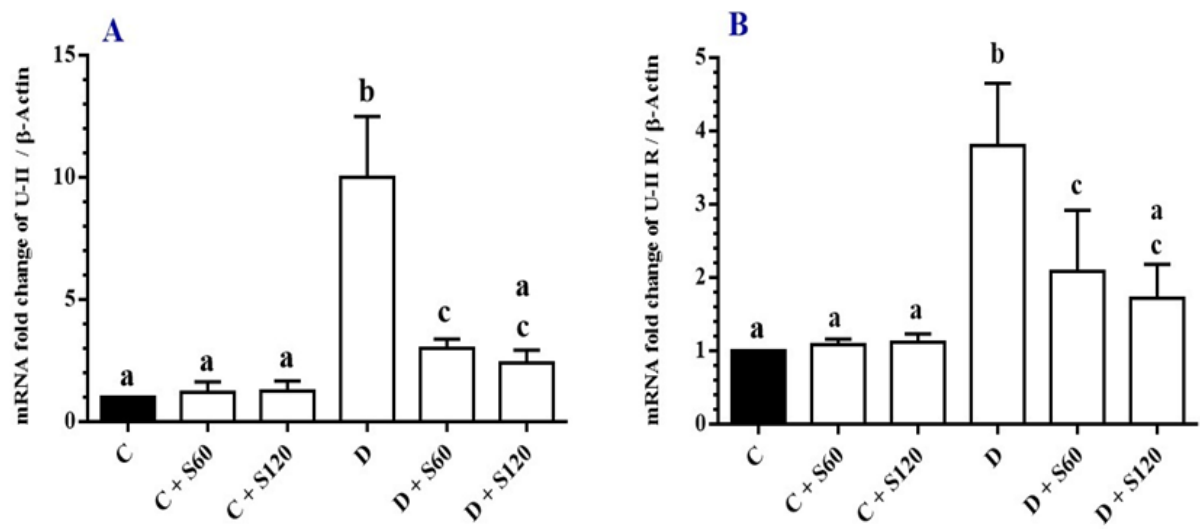


Figure 2. The expression of (A) U-II and (B) U-II R genes in the liver tissue of the study groups (n=6) after 60 days of treatment with silymarin (60 and 120 mg/kg). Data are shown as mean $\pm$ SD. C: control group, S: Silymarin, D: Diabetic. Non-similar letters indicate significant difference. P<0.05 was considered significant

Table 2. The level of glucose, insulin, insulin resistance index and liver enzymes in the study groups (n=6) after 60 days of treatment with silymarin (60 and 120 mg/kg)

Groups	Variables	Fasting glucose (mg/dl)	Insulin (μ U/ml)	Insulin resistance index (HOMA-IR)	Alkaline phosphatase (U/L)	Aspartate transaminase (U/L)	Alanine Transaminase (U/L)
Control		83.3 $\pm$ 10.9 a	11.46 $\pm$ 0.47a	2.35 $\pm$ 0.27 a	250.50 $\pm$ 19.59 a	108.33 $\pm$ 6.59 a	53.00 $\pm$ 6.10 a
Control + Silymarin 60 mg/kg		76.5 $\pm$ 12.2 a	11.96 $\pm$ 0.67a	2.24 $\pm$ 0.26 a	267.16 $\pm$ 20.74 a	119.16 $\pm$ 11.16a	47.16 $\pm$ 5.26 a
Control+Silymarin 120 mg/kg		79.5 $\pm$ 13.8 a	11.65 $\pm$ 1.64a	2.28 $\pm$ 0.41 a	270.33 $\pm$ 21.44 a	115.66 $\pm$ 9.79 a	55.00 $\pm$ 5.51 a
Diabetic		229.3 $\pm$ 27 b	7.10 $\pm$ 1.06 b	5.79 $\pm$ 0.90 b	730.16 $\pm$ 41.88b	239.16 $\pm$ 14.17b	160.66 $\pm$ 7.11 b
Diabetic+Silymarin 60 mg/kg		202.6 $\pm$ 45.1c	9.60 $\pm$ 1.11 c	4.71 $\pm$ 0.64 c	396.50 $\pm$ 34.56 c	152.00 $\pm$ 6.71 c	73.50 $\pm$ 2.07 a
Diabetic+Silymarin 120 mg/kg		182.5 $\pm$ 35.8c	9.80 $\pm$ 0.96 c	4.35 $\pm$ 0.56 c	431.83 $\pm$ 24.92 c	140.50 $\pm$ 10.44c	70.50 $\pm$ 3.04 a

Data are shown as mean $\pm$ SD. Non-similar letters indicate significant difference. P<0.05 was considered significant

Discussion

In this study, induction of type 2 diabetes in rats reduced insulin, increased glucose, HOMA-IR and the expression of U-II and U-II R genes compared to healthy control group. Moreover, treatment of diabetic animals with silymarin improved this parameters compared to control diabetic rats. Research has shown that in diabetes, the transport of glucose to the muscles is decreased, resulting in increased blood glucose levels (10). STZ is also introduced into a pancreatic beta cell via a glucose transporter (GLUT2) and causes DNA alkylation and this process induces poly ADP-ribosylation. The poly ADP-ribosylation process reduces the levels of ATP and NAD in the cell. The exacerbation of ATP dephosphorylation, after the effect

of STZ, creates a substrate for the xanthine oxidase enzyme, which leads to the formation of superoxide radicals and DNA damage, which causes the necrosis of the pancreatic beta cells, and this decreases serum insulin levels and increases serum glucose levels (11). In diabetic groups treated with 60 mg/kg and 120 mg/kg silymarin, blood glucose, insulin level and HOMA-IR improved compared with the diabetic group. In a study by Soto et al., it was reported that silymarin improves pancreatic beta cells by increasing the expression of pancreatic duodenal homeobox (Pdx1), increases insulin production, and thus decreases blood glucose (12). Hyperglycemia and dyslipidemia, which are the main characteristics of type 2 diabetes, impair the

function of pancreatic beta cells by decreasing the expression of Pdx1. Pdx1 also increases the expression of insulin, and proliferation and survival of pancreatic beta cells (13, 14).

Furthermore, Pferschy-Wenzig et al., reported that the active ingredient of Isosilybin A in silymarin has PPAR- γ agonist properties. The PPAR- γ molecule is a target for thiazolidinedione's, such as pioglitazone; these drugs increase the sensitivity of insulin to decrease blood glucose levels in type 2 diabetic patients (15). Similarly, in the studies of Alabdan, Tuorkey and Feng, treatment of diabetic rats with silymarin has been shown to improve blood glucose, protect the pancreatic beta cells, increase insulin secretion and improve HOMA-IR (16–18).

It has been claimed that flavonoids in the silymarin extract, such as silybin and syldianin may decrease insulin resistance and improve glucose metabolism in diabetic patients (19). In type 2 diabetes mellitus, insulin resistance, dyslipidemia and increased blood glucose may damage different tissues, including the liver tissue (20). Increase in the activity of AST, ALT, and ALP enzymes is an appropriate marker to measure liver cell damage (21).

In the early stages of liver damage, cytoplasmic enzymes leak into the blood stream due to increased penetrability of the hepatocyte membrane (22). Moreover, increased catabolism of proteins along with gluconeogenesis and the formation of urea that is observed in diabetes is likely to be responsible for increasing these transaminases in the blood (23). On the other hand, since insulin suppresses the gluconeogenic enzyme genes, and ALT is also a gluconeogenic enzyme, ALT production increases in the diabetes (24). In the present study, the results indicated an increase in liver aminotransferase enzyme in the diabetic group compared to healthy controls. It was also shown that

treatment with silymarin significantly decreased liver damage markers in serum. Silymarin is a common treatment for hepatic diseases. Over 33% of people with chronic hepatitis C virus infection and cirrhosis have been reported to use silymarin for treatment (25, 26). Silymarin is prescribed in traditional medicine to improve liver disorders due to its antioxidant properties, increasing cellular glutathione and cell membrane stability, and it decreases liver damage markers through this mechanism (27).

The result of the present study showed a treatment with silymarin significantly reduced the expression of U – II and U – II R genes in the liver tissue of treated diabetic groups compared to control diabetic group. Consistent with the results of the present study, the study of Rahimi et al., showed the effect of silymarin on reducing the expression of U – II and U – II R genes in the heart tissue of diabetic rats (4). In addition, total flavones of *Rhododendron simsii* Planch flower (TFR) was used in rats in the study of Cheng et al. This plant contains flavonoids that have antioxidant, anti-inflammatory and anti-fibrosis properties. This study showed that TFR protected the heart of rats against heart attack by reducing the expression of U – II and U – II R genes (28). The results of the present study showed that silymarin has potential hypoglycemic properties, increases insulin levels and sensitivity. Silymarin reduced the expression of U – II and U – II R genes in the liver tissue of type 2 diabetic rats, which may as well reduce insulin resistance.

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