Bull. Egypt. Soc. Physiol. Sci. *Vol. (44), Issue (3)*, 185-195

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Targeting autophagy explaining therapeutic potential of silymarin against streptozotocin-induced type 2 diabetic nephropathy in a rat model: A histological and immunohistochemical study.

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Submit Date : 15 May 2024
Accept Date : 24 May 2024

Keywords

- Diabetic nephropathy
- Autophagy
- Silymarin
- Oxidative stress

Abstract

Background: Diabetic nephropathy (DN), the leading cause of end-stage renal disease, is the most significant microvascular complication of diabetes and poses a severe public health concern. Autophagy is a lysosomal process that degrades damaged proteins and organelles to preserve cellular homeostasis. The present study was designed to evaluate the nephroprotective effects of silymarin (SM) on the kidney of adult male diabetic rats. **Methods:** Forty male Wistar rats, weighing between 120 and 150 g were used and subdivided into four groups; control, control received silymarin, type II DM and type IIDM treated with silymarin. For all groups, the volume of urine was recorded, and the samples were analyzed to determine the 24-hour urine protein levels. blood samples were collected via cardiac puncture for further analysis of creatinine levels, renal oxidative stress markers malonaldehyde (MDA), glutathione (GPX) and superoxide dismutase (SOD) activity levels using ELISA kits stained sections with hematoxylin and eosin (H&E) for histopathological evaluation. Immunohistochemical staining for alpha smooth muscle actin, autophagy markers LC3 and P62 were done**. Results:** diabetic nephropathy was associated with significant proteinuria, increased serum creatinine, significant decrease in the levels of antioxidant enzymes (SOD, GPX) and significant elevation in MDA. also, histological examination revealed damaged renal tubules, glomerular congestion, fibrosis, decreased autophagy but treatment with silymarin showed significant improvement in laboratory and histopathological features of the kidney.

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Introduction

Diabetes mellitus (DM), identified as a major risk factor for the onset and advancement of nephropathy, has been implicated in kidney damage through the induction of reactive oxygen metabolites and the suppression of antioxidative mechanisms **(1)**. The hallmark features of diabetic nephropathy (DN) encompass both ultrastructural and morphological changes within the kidney, such as glomerular mesangial expansion, basement membrane thickening, and tubular hypertrophy **(2)**. The pathogenesis of DN is multifaceted, with oxidative stress widely recognized as a contributing factor to its progression **(3)**. This is attributed to the excessive production of reactive oxygen species (ROS), including mitochondrial ROS, which incites oxidative stress, potentially resulting in mitochondrial impairment and cellular demise **(4)**. Hence, effective prevention and management of DN remain imperative.

A growing body of evidence has indicated that autophagy presents a promising therapeutic target for diabetic nephropathy (DN) **(5,6)**. Autophagy, an evolutionarily conserved cellular process, facilitates the degradation of damaged organelles and surplus proteins to sustain normal cellular homeostasis in response to stress and injury **(7)**. Central to this process is the unobstructed autophagic flux, crucial for the degradation of organelles and proteins. Mitophagy, a specialized form of autophagy, involves the selective degradation of dysfunctional mitochondria by lysosomes. Nonetheless, impairment of autophagy has been implicated in various diseases, including Alzheimer's disease **(8)**, cardiovascular disease, and inflammatory bowel disease **(9)**.

In traditional medicine, numerous medicinal plants renowned for their hypoglycemic properties have been advocated for managing diabetes mellitus **(10)**. Silymarin, a flavonoid compound derived from Silybum marianum (Milk thistle), holds a prominent place in traditional herbal remedies for a spectrum of ailments **(11)**, primarily focusing on liver disorders **(12)**. Furthermore, its noteworthy antidiabetic potential has been attributed to its antioxidant and anti-inflammatory attributes **(13)**. Studies have demonstrated silymarin's efficacy in ameliorating glucose and lipid profiles, scavenging reactive oxygen species (ROS), and suppressing the production of proinflammatory cytokines in diabetic rodent models **(14–16)**. Consequently, silymarin emerges as a promising agent for mitigating diabetic complications **(15)**. While prior investigations have explored silymarin's effects on vascular leakage in human retinal endothelial cells **(17)**, its potential protective effects on renal tissues in diabetic animal models remain unexplored. Thus, this study was undertaken to evaluate the beneficial impact of silymarin on renal damage in diabetes and elucidate possible protective mechanisms.

2. Material and methods

2.1 Experimental Animals

Forty male Wistar rats, weighing between 120 and 150 g, were obtained from the Faculty of Medicine, Kafrelsheikh University. Following procurement, the rats underwent a 7-day acclimatization period in plastic cages, during which they were provided with a standard laboratory diet and water ad libitum. Subsequently, they were divided into four groups, each

comprising 10 rats per group. All experimental procedures and techniques were approved by Kafrelsheikh faculty of Medicine (KFS-IACUC/187/2024).

2.2 Experimental Design

Four groups of rats, each containing 10 animals, were used in this study: Group 1, or the healthy control, in which normoglycemic animals were receiving 1 mL of normal saline per day; Group 2 healthy silymarin, in which normoglycemic rats were receiving 120 mg/kg silymarindissolved in corn oil and taken intraperitoneal injection per day for 60 days (18); Group 3, or diabetic controls, in which diabetic rats were receiving 1 mL of vehicle per day; Group 4, DM+ silymarin, in which diabetic rats were receiving 120 mg/kg silymarin in 1 mL vehicle per day. Diabetes was induced by a single intraperitoneal (IP) dose of 60 mg/kg STZ. Three days after STZ injection, blood glucose (BS) was determined by a glucometer (Accu-Chek ®, Roche Diagnostics, Basel, Switzerland) using a drop of blood from the caudal vein. Rats with a BS level of more than 250 mg/dL were considered diabetic **(19)**.

2.3 Specimen Retrieval and Biochemical Analyses

Following 60 days of silymarin treatment, the animals were housed in metabolic cages for 24 hours to collect urine samples. The volume of urine was recorded, and the samples were analyzed to determine the 24-hour urine protein levels. Subsequently, blood samples were collected via cardiac puncture for further analysis of creatinine levels.

2.4 Assessment of Renal Tissue Oxidative Damage

Renal tissue was collected and placed in 1.5 ml centrifuge tubes. To prepare a 10% tissue homogenate, kidney tissues were submerged in saline and centrifuged at 1,000 x g for 10 minutes at 4°C. The resulting supernatant was collected for the evaluation of MDA, GPX levels, and SOD activity levels using ELISA kits, following the manufacturer's protocols.

2.5 Hematoxylin and Eosin (H&E) Stain

The cortical tissues from the right kidney were fixed in 4% formalin and allowed to sit at room temperature for 24 hours. Subsequently, the tissues were embedded in paraffin. Sections measuring 1x1x1 cm were prepared, dewaxed, and stained with H&E (1% hematoxylin for 5 minutes followed by dyeing with 0.5% eosin solution for 1-3 minutes).

2.6 Immunohistochemistry

The kidney tissue, embedded in paraffin wax, was sectioned to a thickness of 5 μm. Following dewaxing and rehydration of the paraffin sections, microwave antigen retrieval was performed in citrate buffer for 15 minutes, as previously described for immunohistochemistry. After cooling, slides were treated with 4% hydrogen peroxide for 10 minutes at room temperature, followed by blocking with 5% BSA (MilliporeSigma) for 1 hour at room temperature. Subsequently, the slides were incubated overnight at 4°C with primary antibodies against LC3 (Cat# PA1-46286), P62 (Cat# MA5-42726), and alpha smooth muscle actin (Cat# 14-9760-82), each at a dilution of 1:100. After washing with PBS, the slides were incubated with secondary antibodies (FITC-labeled mouse IgG; cat. no. A0568,

Beyotime Institute of Biotechnology) for 1 hour at room temperature. Following another round of washing, the slides were stained with DAPI at room temperature for 3 minutes to visualize the nuclei.

2.7 Statistical analysis

The continuous variables were presented as mean ± standard error. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the Tukey multiple comparison test, utilizing GraphPad Instat software (version 8.01, GraphPad Software Inc, La Jolla, USA). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Ameliorative effect of silymarin against DM-induced renal function deterioration and oxidative stress

Table 1 shows a significant $p < 0.05$ elevation of serum creatinine and 24-hours urinary protein in comparison to control group. Furthermore, renal homogenate of the diabetic rats showed a significant $p < 0.05$ decrease in the level of antioxidant enzymes SOD, and GPX, at the same times showed an elevation in the lipid peroxidation marker MDA. On the controverse, treatment of diabetic rats with silymarin significantly $p < 0.05$ improved renal function tests, as well as improved antioxidant enzymes level with a decrease in MDA.

3.2. Light microscopic examination

HE examination of kidney sections of control and silymarin groups revealed normal structure of glomeruli and tubules (Fig. 1A, B). Meanwhile. diabetic rat's renal sections showed a marked destructive damage in the form of swelling in tubular epithelial cells, destruction of brush borders, necrosis of tubular epithelium, and glomerular congestion (Fig. 1C). Additionally, diabetic rats that received silymarin showed improvement in the picture of renal tubules (Fig. 1D).

3.3. Immunohistochemical examination

Immunohistochemical investigation of diabetic rat's kidney sections showed a significant $p < 0.05$ increase in the immunoexpression of fibrosis marker alpha smooth muscle actine and decreased LC3 and increased p62 an indicators of autophagy process (Fig. 2C, 3C, 4C) in relation to control rats. on the contrary, diabetic rats that received silymarin markedly decrease alpha smooth muscle actin and p62 with elevation in LC3 immunoexpression compared to diabetic rats (Fig. 2D, 3D, 4D). from aforementioned finding we founded that silymarin treatment combats DM-induced renal histological changes and enhanced renal autophagy.

Table (1) Effect of silymarin on Creatinine, 24 h urinary protein, SOD, GPX, MDA of diabetic rats.

Groups	Creatinine mg/dl	24-hour urine protein (mg)	SOD (IU/gm tissue)	GPX (µm/ gm tissue)	MDA (nmol/gm tissue)
Control	0.82±0.17	2.08±0.18	135.8±3.84	10.15±0.90^{f}	13.80±1.85^{g}
Silymarin	0.89±0.16	2.22±0.19	149.5±5.76	12.38±1.55^{f}	15.58±2.79^{g}
DM	1.80±0.21	11.38±1.55	89.56±3.01	2.08±0.31^{f}	74.54±5.25^{g}
DM+silymarin	1.44±0.17	6.84±1.51	106.4±4.84	8.36±1.39^{f}	38.16±6.56^{g}

All values expressed in Mean±Standared deviation; fp < 0.05 significant vs control group; gp < 0.05 significant vs silymarin group.

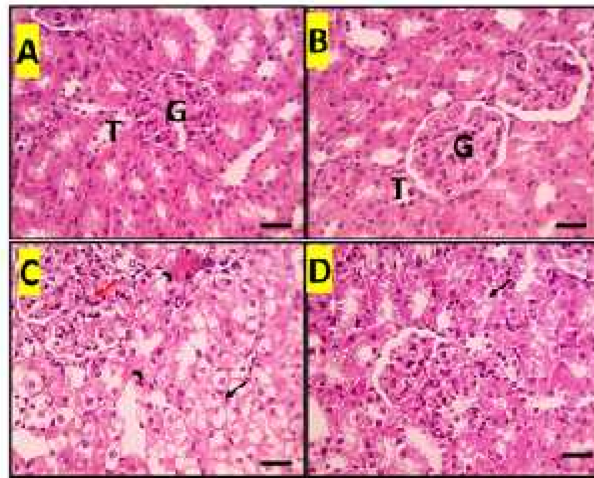

Fig. 1: Microscopic picture of HE stained renal sections of (A, B) control and silymarin groups showing (A) normal glomeruli (G) and tubules (T); (C) renal sections from the DM group demonstrate marked pathological changes: tubular cell swelling (black arrows), loss of brush-border membranes, necrosis (arrowheads), and congested glomeruli (red arrows). (D) Renal sections from the DM+silymarin group reveal very mild epithelial cells degeneration in a few tubules (arrows) (H&E, X: 400, Bar 50).

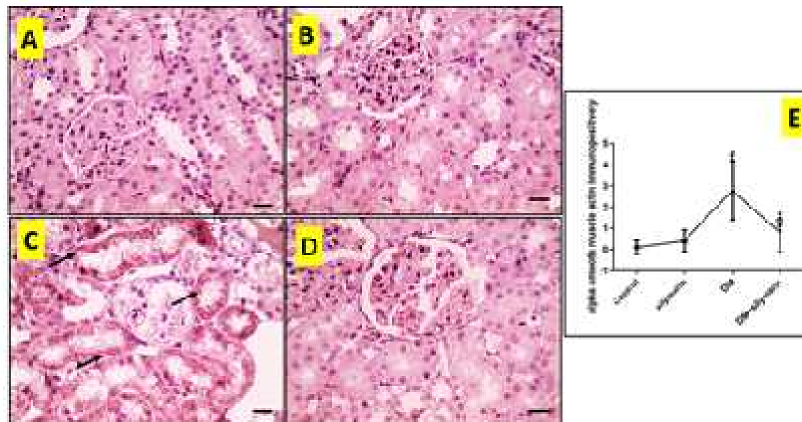

Fig. 2: Microscopic pictures of immunostained renal sections against alpha smooth muscle actin demonstrates a negative tubular reaction in (A, B) control and silymarin groups. (C) a strong positive brown reaction in the tubular epithelium in DM group (Black arrows). (D) A mild brown reaction in DM+silymarin group. (E) The histogram of immunopositivity. values are presented as mean ± SD, fp < 0.05 significant vs control group gp < 0.05 significant vs DM group (X:400, Bar 50).

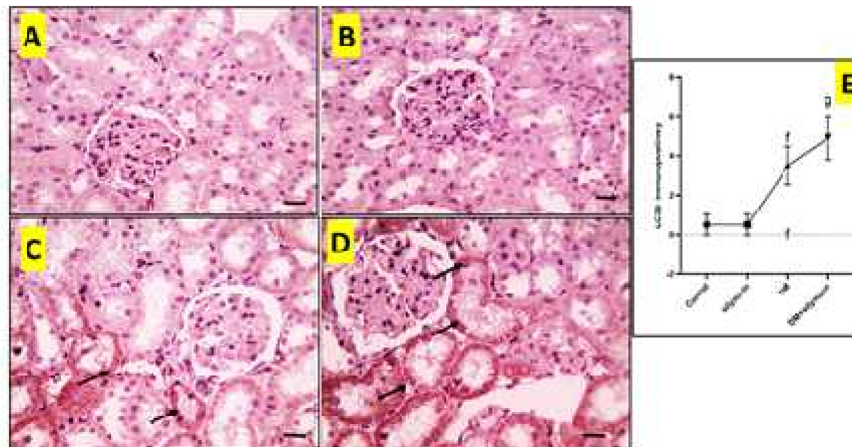

Fig. 3: Microscopic pictures of immunostained renal sections against LC3B demonstrates a negative tubular reaction in (A, B) control and silymarin groups. (C) A moderate brown reaction in the tubular epithelium in DM group (Black arrows). (D) A strong positive brown reaction in DM+silymarin group. (E) The histogram of immunopositivity. values are presented as mean ± SD, fp < 0.05 significant vs control group gp < 0.05 significant vs DM group (X:400, Bar 50).

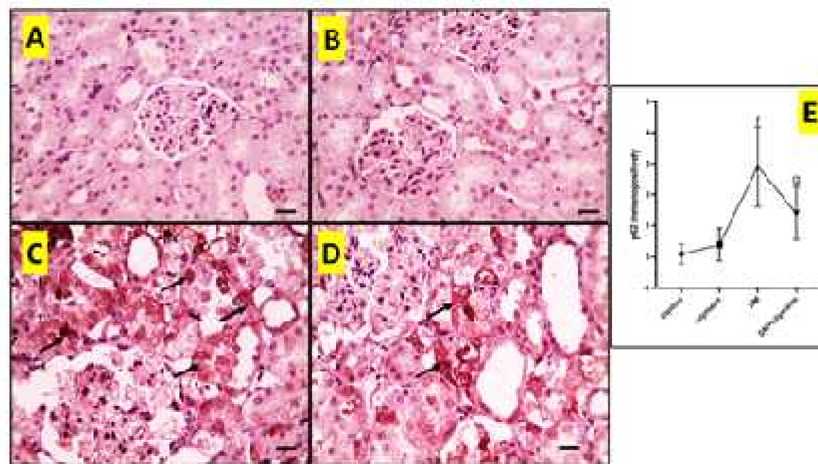

Fig. 4: Microscopic pictures of immunostained renal sections against p62. (A, B) control and silymarin groups showing a negative tubular reaction. (C) DM group showing a strong positive brown reaction in the tubular epithelium in (Black arrows). (D) DM+silymarin group showing a moderate brown reaction. (E) The histogram of immunopositivity. values are presented as mean ± SD, fp < 0.05 significant vs control group gp < 0.05 significant vs DM group (X:400, Bar 50).

4. Discussion

In The present study, STZ induced rat model of type II diabetes was used to examine the protective effect of Silymarin on the kidney of diabetic rats. Silymarin decreased 24-hour urinary total protein, serum creatinine compared to diabetic group. Diabetic nephropathy (DN) is characterized by albuminuria and reduced renal function evidenced by a decreased glomerular filtration rate (GFR) and increased serum creatinine and blood urea nitrogen concentration(**20, 21**).

Podocytes play a major role in maintaining the integrity and permeability of the glomerular filtration barrier. (**23**). Moreover, in diabetic nephropathy (DN), a reduction in podocyte number, the effacement of podocyte foot processes, and the loss of slit diaphragm proteins such as nephrin and podocin result in a leakage of albumin and proteinuria (**24**). Therefore, renal function can be improved by the reduction of kidney damage and glomerular hyperpermeability. Silymarin prevented kidney damage and the elevation of serum Creatinine, 24h proteinuria, in STZ-induced diabetic rats via its antioxidant and anti-inflammatory properties. SM can also elevate protein and nucleic acid synthesis and contribute to the regeneration of the renal cells (**25**).

In this study, lipid peroxidation product MDA increased significantly, and SOD antioxidant activity decreased in diabetic rats, which indicated there was an oxidation effect. Administration of silymarin improved oxidative stress parameters significantly compared to diabetic group.

Oxidative stress and inflammatory response are inseparable because each reaction will produce and amplify another. Redox homeostasis and redox signaling are pivotal components of maintenance for a normal physiological steady state (**26**). The oxidative stress process is one of the threat agents in early diabetic and later development. The increased glucose during diabetes involves in the advanced glycation end-products (AGE) generation and reactive oxygen species release that leads to renal dysfunction including tubular atrophy, glomerular hypertrophy, podocytes dysfunction, thickening of

glomerular basement membranes, interstitial fibrosis **(27)**. It has been reported that AGEs induce extracellular matrix expansion and epithelial mesenchymal transition and inflammation **(23)**. Previous study investigated renoprotective impact of silymarin (SM) in rats with alloxan-caused DM**(28)**. The outcomes of their study illustrated that SM elevates the renal activity and expression of antioxidant enzymes (SOD, GPX and CAT) and restitutes renal morphology. These findings came also in accordance with **(29)** who found that Silymarin nanoliposomes ameliorate STZ-induced kidney injury by improving oxidative stress, renal fibrosis in diabetic rats.

In this study, histological examination of kidney sections diabetic rats renal sections showed marked destructive damage in the form of swelling in tubular epithelial cells, destruction of brush borders, necrosis of tubular epithelium and glomerular congestion. These findings came in accordance with **(30)** who reported morphologic injury to renal tubular cells in the form of (vacuolization, flattening, degeneration, and necrosis) in STZ induced diabetic rats. Also **(31)** stated that, DN is featured by glomerular mesangial expansion, increased extracellular matrix deposition, thickened glomerular and tubular basement membranes, renal inflammation, and fibrosis.

In this study, diabetic rats kidney showed significant increase in immunoexpression of fibrosis marker alpha smooth actin (SMA), while in silymarin treated group there was marked decrease in it.

-SMA, as a common marker for smooth muscle cells and myofibroblasts, is highly expressed in kidneys, and the over production of -SMA partially results from tubular epithelial–myofibroblast transdifferentiation, which plays an important role in renal interstitial fibrosis **(32)**.

This came in accordance with chen et al., **(29)** who found that Silymarin nanoliposomes ameliorate STZ-induced kidney injury by improving oxidative stress and renal fibrosis.

In this study, immunohistochemical study in diabetic kidney, showed significant decrease in LC3 expression and increase in p62 which are indicators for autophagy process. Administration of silymarin markedly decreased p62 and increased LC3. It has been known that the impaired autophagy is evidenced by the increased collection of p62 and the decreased expression of autophagy-related proteins in diabetic kidney tissues and cells **(33)**.

Autophagy plays a crucial role in maintaining normal islet structure. However, in a state of high glucose, autophagy is inhibited, resulting in impaired islet function, insulin resistance, and complications. Studies have shown that modulating autophagy through activation or inhibition can have a positive impact on the treatment of T2DM and its complications **(34)**.

Impairment of autophagy is implicated in various inflammatory diseases, and particularly in the pathogenesis of diabetic kidney disease. Hyperglycemia-induced alterations in intracellular metabolism and cellular events, including accumulation of advanced glycation end-products (AGEs), increased oxidative stress, endoplasmic reticulum stress, and activation of the renin angiotensin system **(35)**.

It was also found that autophagy stimulation was associated with the degradation of

NLRP3 leading to alleviation of inflammation as well as renal interstitial fibrosis. Based on these findings, there is a complex interaction among ROS, NLRP3 inflammasome, and autophagy in the development of renal fibrosis **(36)**. Alterations in autophagy have been observed in diabetic podocytes **(37)** indicating that regulating autophagy to maintain homeostasis could be a potential target for treating diabetic nephropathy.

oxidative stress affects autophagy in the development of DN as a two-edged sword and antioxidant therapy may protect the kidney against diabetes through activating autophagy. This notion has been supported by some evidence that antioxidant compounds derived from plants effectively attenuate the kidney injury induced by diabetes or poisons by promoting autophagy **(38)**.

Autophagy plays a protective role in diabetic nephropathy. However, it is not clear whether autophagy is active or inactive in DKD. It is acknowledged that inflammation is the vital factor in DKD pathogenesis. Importantly, autophagy may play a protective role against kidney inflammation in DKD, which may help us to explore the mechanism of DKD. Conversely, it is also suggested that autophagy can be induced under hyperglycemia due to the production of ROS or direct cytotoxicity of hyperglycemia. In future, we still need to be determined whether over-nutrition status and hyperinsulinemia could suppress autophagy in kidney **(39)**.The present study possesses several limitations. First, we wanted to do electron microscopy for more detection of renal tissue, but it was not available. Second, we wanted to detect role of apoptosis in diabetic nephropathy, but Kittswas notavailable. Third, weneed to examine role of inflammatory markers as tumor necrosis factor and TGF beta, but we could not.various signaling pathways are involved in the regulation of metabolic disorders, can lead to more recent studies to investigate the role ofautophagy and TGFb1/SMAD signaling cascades have been considered as the useful therapeutic strategies in the overcome DM.

5. Conclusion:

We have demonstrated that silymarin attenuated laboratory and histopathological injury in the kidney of DN rats. This may be due to its antioxidant, ant inflammatory and its stimulation for autophagy.Therapeutic strategies aiming to regulate autophagy present promising remedies in the treatment of diabetes and its complications.

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