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Original Article

Renoprotective effect of grape seed extract in diabetic nephropathy by attenuating hyperglycemia-facilitated oxidative stress

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Abstract: Diabetic nephropathy is associated with microvascular complications and results from an excessive reactive free radicals and oxidative stress, both of which are common in patients with diabetes mellitus. The current study aimed to investigate the potentially beneficial effect of dietary grape seed extract (GSE) supplementation on renal function, oxidative stress and nitric oxide bioavailability in experimental diabetes. Male albino rats (Rattus norvegicus) were divided into four groups: 1) normal rats, 2) alloxan-induced diabetic rats, 3) normal rats who have been given GSE and 4) diabetic rats who administered GSE. Alloxan injection produced severe diabetic renal damage, as revealed by significantly greater malondialdehyde (MDA) levels and superoxide dismutase (SOD) activity in the renal tissues compared to the normal rats, accompanied with a fall in nitric oxide (NO), reduced glutathione (GSH), vitamin C, glutathione peroxidase (GPx) and catalase (CAT) activity. GSE orally to diabetic rats for a 12 week period correlated with a significant improvement in glucose, urea, uric acid, creatinine levels and of renal aspartate transaminase, alanine transaminase and alkaline phosphatase activities compared to diabetic group. Moreover, GSE supplementation decreased MDA levels and increased NO, GSH, GPx and CAT activity when compared to the activity of diabetic rats who had not been given GSE. In conclusion, these results suggested that GSE extract was able to improve the antioxidant defense and reduce diabetic oxidative stress and protect the kidneys against diabetic nephropathy and slow its early progression so having potential protective role.

Keywords: Diabetes, nephropathy, oxidative stress, antioxidants, nitric oxide, GSE

Introduction

Diabetes mellitus type 1 (DMT1) is the leading cause of renal failure, causing about 45% of new cases each year. Even when diabetes is controlled and blood sugar levels are maintained, the kidneys may be damaged or may fail. Diabetic nephropathy affects approximately one third of diabetes patients and is the leading cause of end-stage renal disease in many countries [1].

The severity of the diabetic nephropathy determines the patient's prognosis. Hyperglycemia causes diabetic nephropathy by producing oxidative stress and increasing the severity of the glycation reaction [2]. Oxidative stress induces the production of highly reactive oxygen radicals that are toxic to tissues [3].

The mechanism of hyperglycemia that generates free radicals occurs via the promotion of glycosylation of circulating and cellular protein. This initiates a series of auto-oxidative reactions that end with the development and accumulation of advanced glycosylation end-products (AGE) in tissue proteins. AGEs have oxidizing potential and can promote tissue damage by free radicals [2]. The activation of hyperglycemia-induced secondary mediators, such as protein kinase C (PKC) and mitogen-activated protein kinase, and cytokine production are responsible for oxidative stress-induced renal injury in diabetic patients [4].

Hyperglycemia is induced in renal diabetic pathways, which are linked to each other. This results in a common pathway in which free radicals is overproduced in the mitochondrial electron transport chain [5].

Lipid peroxidation is a complicated process including the effect of oxygen-derived reactive species with unsaturated fatty acids, producing a diversity of highly reactive electrophilic MDA. As diabetic nephropathy became understood, research starting a association between this form of oxidative damage, and disease a wealth of knowledge to the scientific community have been provided. There is an increasing number of evidence that oxidative stress injuries the function of the kidney [6].

Currently, various plant materials are being studied to find a novel type of antioxidant. Many plant extracts and plant products contain significant levels of antioxidant activity. Polyphenolic flavonoids, which are commonly found in plants, are recognized for their interesting clinical properties. Many flavonoids, including silymarin, catechin, and quercetin, play defending roles against the effects of diabetes; they do this by increasing antioxidant enzyme activity [7, 8].

Recently, GSEx has been found to have several valuable pharmacological properties, including chemoprotective effects against reactive oxygen species and oxidative stress, GSPE proanthocyanidin is known to be an effective natural polyphenol capable of removing free radicals in vivo [9, 10]. With this in mind, the present study aimed to induce a model of diabetic nephropathy with which to evaluate the protective role GSEx can play against diabetic nephropathy. This study also assessed the mechanism behind GSEx action in diabetic rats.

Materials and methods

Experimental animals

Forty male albino rats (*Rattus norvegicus*) weighing 100-180 g Bwt, 10 weeks old were used in this investigation. Balanced ration with water ad libitum were provided to rats for 2 weeks before starting the experiment for acclimatization.

Alloxan (diabetogenic agent)

Alloxan monohydrate (2,4,5,6-tetraoxohexahydropyrimidine) was purchased from (Sigma Company), with melting point of 250°C and dissolved immediately before use.

Grape seed extract (GSE)

It is marketed as (Gervital®) by (Arab Co. for Medicinal Plants & Pharmaceuticals MEPACO-Egypt). It was obtainable as pills of 150 mg and suspended in distilled water.

Experimental diabetes

Overnight fasted rats (18 hr) were intraperitoneally (I.P) injection with alloxan monohydrate 150 mg kg^{-1} BW for induction of diabetes [11], Then, after 10 days of alloxan injection, rats were screened for blood glucose levels. Rats with postprandial serum glucose level of 180-300 mg/dl were considered as mildly diabetic and were included in the experiment (n = 40 rats) cited by Abdel-Reheim [12].

Animal grouping

The experimental animals were divided in to four groups, each group comprises of ten rats detailed as follows: Group 1, served as normal control rats; group 2, was considered as diabetic (alloxan induced) keep without treatment for 10 weeks; group 3, alloxan diabetic rats was received GSE (250 mg/kg Bwt/day) in aqueous suspension orally for 10 successive weeks by gastric intubation and group 4, normal animals were treated with GSE at dose level of (250 mg/kg bwt/day) for 10 successive weeks by oral administration.

Sampling and preparation

At the end of the experimental period, blood samples (fasting and postprandial) were collected from the rats in each group to measure glucose levels and other biochemical parameters. The rats' kidneys were removed, rinsed with ice-cold saline, and homogenized. The tissue homogenate and sera of each rat were kept at -20°C until further analysis.

Biochemical assay

Fasting and post-prandial serum glucose level was estimated spectrophotometrically using reagent kits from Reactivos Spinreact Company (Spain) according to method of trinder [13]. Serum insulin was assayed by radioimmunoassay kits of DPC (Diagnostic Products Corporation, Los Angeles, USA) via the method of Marschner *et al.* [14]. Uric acid, Urea and cre-

Table 1. Changes in FBS, PPBS, Insulin levels and kidney function inserum of different groups

	Normal	Diabetic control	Diabetic+GSE	Normal with GSE
FBS (mg/dl)	85 ± 5.54[a]	220 ± 7.30[b]	143 ± 1.81[c]	81 ± 3.51[a]
PPBS (mg/dl)	147 ± 2.9[a]	243.5 ± 7.28[b]	199.3 ± 4.04[c]	108.9 ± 6.02[d]
Insulin (mIU/ml)	0.45 ± 0.04[a]	0.12 ± 0.04[b]	0.78 ± 0.08[a]	0.34 ± 0.07[a]

These values represent means and standard errors, the different superscript letters (a, b, c, d) indicate a significant difference at P < 0.05.

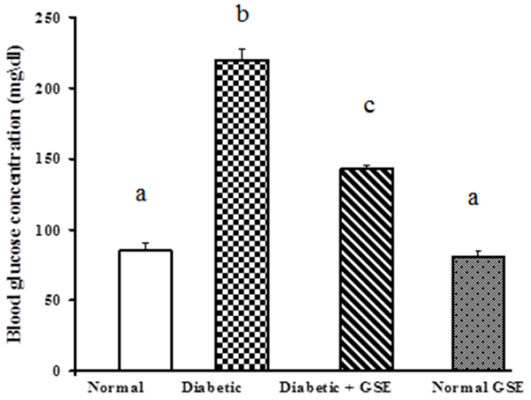

Figure 1. Effect of GSE on FBS, in serum of different groups. The different letters indicated a significant difference between groups at P < 0.05.

atinine concentration were determined in serum according to the method of Fossati *et al.* [15], Patton and Crouch [16] and Jaffe [17], respectively.

The lipid peroxidation products were estimated by measurement of malondialdehyde (MDA) reactive product at 532 nm according to the chemical method of Preuss *et al.* [18]. Superoxide dismutase (SOD EC 1.15.1.1), Catalase (CAT, EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9) activity, reduced glutathione (GSH), Vitamine C (Ascorbic acid), nitric oxide concentration, Alanine Aminotransferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) activity in kidney homogenates were determined according to the chemical method of Marklund and Marklund [19], Cohen *et al.* [20], Paglia and Valentine [21], Beutler *et al.* [22], Harris and Ray [23], Montgomery and Dymock, [24] respectively.

Statistical analysis

The data were analyzed using one-ways analysis of variance (ANOVA) (PC-STAT) followed by least significant difference (LSD) analysis to compare various groups with each other. Results were expressed as mean ± standard error and values of $P < 0.05$ were considered statistically significant. Statistical analysis was achieved by GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effect of GSE on serum glucose and insulin concentration

The treatment of diabetic rats with GSE induced a highly significant decrease on the elevated fasting and post-prandial serum glucose concentration in comparison with diabetic rats (**Table 1** and **Figure 1**). Alloxan treatment produced a significant decrease in the serum insulin level with respect to the control group. The administration of GSE significantly elevated the level of serum insulin compared to diabetic group (**Table 1**).

Effect of GSE on renal function biomarkers (serum uric acid, urea and creatinine concentration)

Alloxan produced a significant increase in serum uric acid, urea and creatinine of diabetic rats, the treatment of diabetic rats with GSE normalize the elevated value of serum uric acid, urea and creatinine as compared to control rats. The results are shown in (**Table 2**).

Effect of GSE on kidney lipid peroxidation products (MDA) and various antioxidants

Alloxan produced a significant increase in brain MDA level of diabetic rats. The administration of GSE ameliorated the alloxan induced elevation in lipid peroxidation moreover, the treatment of diabetic rats with GSE normalize the value of MDA production as compared to control rats. The results are shown in **Table 3**.

The results of antioxidant enzymes (SOD, GPx, CAT) activities in kidney homogenate are illustrated in **Table 3**, respectively. A significant increase in SOD activity was detected in diabetic

Table 2. Effect of grape seed extract (GSE) on various renal functions variables in serum of diabetic rats

	Normal	Diabetic control	Diabetic+GSE	Normal with GSE
Uric acid (mg/dl)	3.50 ± 0.098[a]	4.57 ± 0.22[b]	3.25 ± 0.15[a]	3.39 ± 0.18[a]
Urea (mg/dl)	34.18 ± 1.23[b]	61.37 ± 5.94[a]	44.23 ± 6.96[b]	34.92 ± 1.15[b]
Creatinine (mg/dl)	0.55 ± 0.080[a]	1.31 ± 0.065[c]	0.65 ± 0.062[a]	0.26 ± 0.032[b]
Serum albumin (g/dl)	4.94 ± 0.2[a]	2.83 ± 0.12[b]	3.51 ± 0.10[c]	4.28 ± 0.06[d]

These values represent means and standard errors, the different superscript letters (a, b, c, d) indicate a significant difference at $P < 0.05$.

Table 3. Changes in LPO and various antioxidants in kidney of different groups

	Normal	Diabetic control	Diabetic+GSE	Normal with GSE
MDA (nmolMDA/g/hr) ×100	2.80 ± 0.12[b]	5.50 ± 0.14[a]	1.90 ± 0.06[c]	2.30 ± 0.14[c]
Reduced Glutathione (nmol/100 mg) ×100	99.66 ± 9.48[c]	74.16 ± 10.36[c]	264.16 ± 11.30[a]	204.50 ± 27.54[b]
SOD (U/g) ×100	155.00 ± 2.38[b]	189.83 ± 0.87[a]	148.00 ± 5.93[b]	146.83 ± 6.43[b]
GPx (mU/100 mg)	132.30 ± 0.39[b]	93.07 ± 2.23[c]	153.8 ± 2.41[a]	149.06 ± 2.33[a]
Catalase (k.10^2) ×100	11.90 ± 0.37[a]	7.86 ± 0.05[b]	9.15 ± 0.49[c]	17.21 ± 0.49[d]
(Vit C) (mg/L)	125.90 ± 2.94[a]	94.00 ± 3.75[b]	108.34 ± 1.52[a,b]	157.23 ± 13.88[c]

Data are expressed as Mean ± SE. Values, having the same superscript symbol (s) (a, b, c, d) are not significantly different.

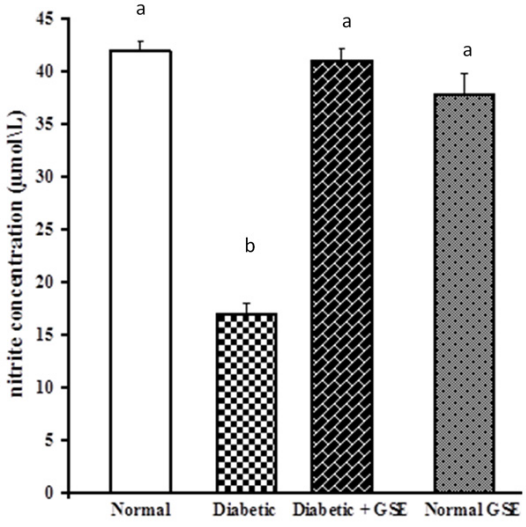

Figure 2. Effect of GSE on nitrite (nitric oxide) concentration, in serum of different groups.

animals while significant decrease ($P < 0.001$) in the activity of GPx and CAT activity, on the other hand, the data represented in **Table 3** illustrate that diabetic animals showed a non-significant ($P < 0.001$) change in kidney glutathione while a significant decrease in ascorbic acid concentration when compared with normal animals.

The increment in kidney SOD activity was modulated by the oral administration GSE to diabetic rats, while CAT and GPx activity in kidney were significantly increased by oral administration of GSE as compared to diabetic control group. The use of GSE showed a highly significant increase ($P < 0.001$) in glutathione level while a non-significant increase ($P < 0.001$) in ascorbic acid concentration was reported in diabetic treated rats in comparison with diabetic group.

Effect of GSE on kidney nitrite level

In kidney of diabetic rats a significant decrease in nitrite level as compared to normal one. The treatment of diabetic rats with GSE produced a significant decrease in the kidney nitrite level as compared with diabetic group (**Figure 2**).

Effect of GSE on activity of ALT, AST and ALP in kidney

The diabetic rats showed an increase in kidney ALT, AST and ALP activity as compared to normal rats. The treatment of diabetic animals with grape seed extract (GSE) induced a significant regulation of elevated ALT and ALP activity, while a non-significant decrease in AST activity of kidney as compared to diabetic control group as presented in **Figure 3A-C**.

Compared with the diabetic group, the urinary protein/24 h, levels of blood urea nitrogen

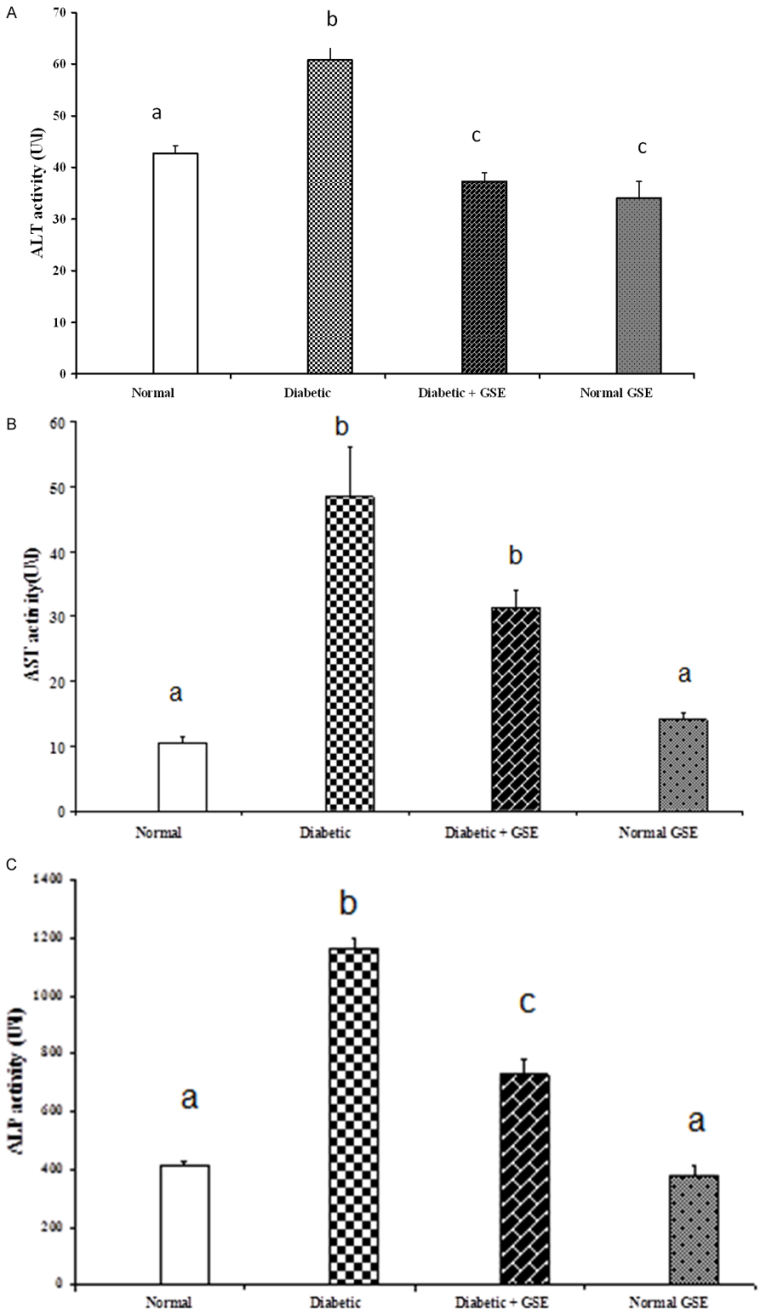

Figure 3. A. Effect of GSE on liver function biomarkers ALT activities of different rat groups; B. Effect of GSE on liver function biomarkers AST activities of different rat groups; C. Effect of GSE on liver function biomarkers ALP activities of different rat groups. The different letters indicated a significant difference between groups at $P < 0.05$.

(BUN) and serum creatinine (SCr), creatinine clearance rate (CCr) and the ratio of kidney weight/body weight were decreased, the SOD activity in kidney was raised while MDA content was fall in the GSPE group (high dose), and the differences were all significant. The NO content in the kidney and NOS activity in kidney and serum decreased in the GSPE (low dose) group, and there was significant difference when compared with diabetic group ($P < 0.05$).

Discussion

Diabetic nephropathy is one of the most serious microvascular complications, and it is one of the leading causes of end-stage renal disease. The pathogenesis of diabetic nephropathy is multifactorial, though chronic hyperglycemia plays a crucial role [25].

During the diabetic milieu, supraphysiological glucose is involved in the formation of AGEs and the mitochondrial production of free radicals. Consequently, it is involved in cell death and renal dysfunction. Thus, oxidative stress plays a decisive role in the development of diabetic nephropathy, which is characterized by the thickening of glomerular basement membranes, expansion of mesangial cells, glomerular hypertrophy, and loss of podocytes, as well as by the expansion of tubular basement membranes, tubular atrophy, interstitial fibrosis, and arteriosclerosis.

The results of the present study obtained from the fasting and postprandial serum glucose concentrations of alloxan-diabetic rats showed that these rats had high glucose levels and massive reductions in insulin release compared to the non-diabetic rats. These results are in accordance with the results of several other authors who have used alloxan-diabetic animals in their studies [26-28]. The pathophysiological alterations and deterio-

rated renal functions caused by chronic hyperglycemia-mediated oxidative stress in these experimental rats closely resemble the effects of human diabetic nephropathy [25]. The development of renal hypertrophy, glomerular injury, and renal dysfunction in the diabetic rates were indicated by elevated blood glucose; increased kidney weight; altered intraperitoneal insulin tolerance; elevation of uric acid; elevation of urea and creatinine concentration; and increased activity of renal AST, ALT, and ALP. These results are consistent with those of Abo-Salem *et al.* [29, 30, 25].

The treatment of diabetic rats with GSE can potentially decrease elevations in uric acid, urea, and creatinine as a renal function biomarkers. GSE treatment can regulate the altered levels of ALT and ALP, bringing them to almost normal levels. However, GSE treatment had an insignificant effect on AST. These results are consistent with the findings of Benzer *et al.* [31], which demonstrated that GSE treatment efficiently attenuated acute nephrotoxicity induced by a single induction of ciptaline (CP) in diabetic rabbits. In addition, pretreatment with red GSE protected against gentamicin-induced acute kidney injury [32].

Diabetes is associated with chronic hyperglycemia, and one of the foremost consequences of hyperglycemia is an increased rate of oxidative stress. Chronic hyperglycemia is often associated with a significant decline in intracellular antioxidants and an elevation in the formation of pro-oxidants such as reactive free radicals and electrophilic substances. This eventually results in renal dysfunction and deterioration. There are a number of enzymatic and non-enzymatic sources of reactive free radicals in the diabetic kidney. These ROS as marker of oxidative stress, include the autoxidation of glucose, the transition of metal-catalyzed Fenton reactions, AGEs, polyol pathway flux, mitochondrial respiratory chain deficiencies, xanthine oxidase activity, peroxidases, nitric oxide synthase, and NAD(P)H oxidase [33].

The results of this study showed that excessive ROS production occurred in the kidney MDA levels and SOD activity, while reduction occurred in the CAT, GPx activity, GSH, and vitamin C concentration in untreated diabetic rats.

In the present study, the GSE, being an antioxidant, is thought to suppress the over-generation of ROS. It is therefore thought to eliminate the intracellular ROS level of the kidney tissue in the experimental rats. GSE treatment following alloxan exposure has been found to be effective in reducing the oxidative stress of alloxan-induced kidneys under hyperglycemic conditions. Our results are in agreement with the findings of Chis *et al*. [34], who found that long-term administration of GSE offers the potential for the enhancement of antioxidants and protection of tissue lipid peroxidation and protein oxidation.

Diabetic animals treated with GSE also showed significant amelioration in elevated brain SOD activity and improvements in brain CAT, GPx activity, and GSH and ascorbic acid (vitamin C) concentrations. The data reveal that GSE offers the potential for the enhancement of antioxidants and protection against tissue lipid peroxidation. One possible explanation for this effect is that the antioxidant activity of the grapeseed polyphenols and their redox properties allow the properties to act as reducing agents by donating hydrogen, quenching singlet oxygen, or acting as metal chelators [34].

GSE is a rich source of one of the most beneficial groups of plant flavonoids: procyanidin oligomers. These flavonoids have many beneficial health effects, including the ability to increase intracellular vitamin C levels, decrease capillary permeability and fragility, and scavenge oxidants and free radicals [31, 35]. In contrast to these findings, however, Alía *et al*. [36] reported that antioxidant enzymes such as SOD, catalase, and glutathione content did not change following GSE consumption, but that glutathione peroxidase activity increased after consumption of grape seeds and grape skins.

NO is a multifunctional molecule that plays an important role in the regulation of vascular tone. It inhibits vascular smooth muscle cell proliferation, blood cell adhesion, and lipid peroxidation [37, 38]. Constitutively NO produced by eNOS is therefore necessary to maintain endothelial function. Endothelial dysfunction is caused by a reduction in vascular NO bioactivity; this occurrence has recently been observed during the early stages of experimental DM [39, 40] (Satoh *et al*., 2005; Komers *et al*., 2006). An increase in NO breakdown and a decrease in NO synthesis are the key features of DM that lead to abnormal NO bioavailability [41].

In our study, nitrite levels were reduced in the kidneys of diabetic rats when compared to the nitrate levels in the kidneys of the control rats. This indicates that while DM develops and progresses, tissue nitrite becomes susceptible for modification before it begins to decrease, reflecting NO bioavailability. These diabetic conditions occurring in the kidneys may decrease nitrite levels through the consumption of endogenous NO [41].

Concerning the mechanism of DM through NO level, Excessive reactive oxygen species (ROS) in DM is associated with subsequent impaired nitric oxide (NO) bioavailability. NO is synthesized by endothelial cells and has an important effect on vascular contraction regulation, playing an anticoagulant and anti-inflammatory role under certain physiological conditions [37, 38]. Endogenous NO synthesis is impaired in DM due to decreased availability of L-arginine and increased consumption of NADPH, which is an essential cofactor for NO synthase (NOS) activity [42].

Prolonged hyperglycemia also results in increased NO breakdown through ROS. This results in the consequent development of peroxynitrite, which is a powerful oxidant that attacks several kinds of biological molecules [43, 44]. Moreover, in Altered redox state in DM, the tetrahydrobiopterin (an essential cofactor for NOS) availability is also affected. This causes the NOS to uncouple, and it increases production of O_2^- rather than NO [39]. These ROS-mediated complex metabolic alterations interact with the endogenous NO and cause impaired NO bioavailability in patients with DM [42].

GSPE has the effect in protecting kidney of diabetic rats, the mechanism might be related with its action in increasing the renal antioxidant ability, decreasing the content of NO and the activity of NOS in kidney and serum [45]. Wild grape seeds procyanidins WGP exerts potent anti-inflammatory activity through the inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions by regulating NF-κB and p38 mitogen-activated protein kinase (MAPK) pathway. Also, WGP significantly reduced LPS-stimulated expression of proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-(IL-) 1β [46].

Specific mechanism have been explored by researchers indicated that, GSPE significantly improved renal function parameters, reduced the expression of tissue inhibitor of metalloproteinase-1 and also increased the activity of matrix metalloproteinase-9. GSPE ameliorates renal injury in type 2 diabetic rats through its antioxidative activity (via rising serum antioxidant enzymes activities) and anti-inflammatory effects (reduced c-reactive proteins level expression of tumor necrosis factor-α, monocyte chemoattractant protein-1 and intercellular adhesion molecule-1) particularly at a dose of 500 mg/kg bwt [10]. Furthermore, Lan *et al.*, [6] conclude that the anti-hypertensive and anti-oxidative stress beneficial effects of GSPE on renal injury in rats with DOCA-salt hypertension occur via the attenuation of JNK and p38 kinases activity.

These findings indicate that there is a pathogenic role played by oxidative damage to the kidneys during the early stages of DM. The data from the current study indicated that oxidative stress and NO increase while antioxidant defenses are compromised in patients with DM. These derangements are of a higher magnitude in DM patients with nephropathy. From these results, we concluded that GSE ameliorates renal injury in experimental diabetic rats through its anti-oxidative activity, NO, and anti-inflammatory role. The results of the present study demonstrate the renoprotective potential of GSE against hyperglycemia-mediated oxidative stress in alloxan-induced diabetic nephropathy. We concluded that GSE attenuate renal injury in experimental diabetic rats through its antioxidant activity, NO and anti-inflammatory effects.

Disclosure of conflict of interest

None.

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