Study of histopathological and biochemical effect of *Punica granatum L.* extract on streptozotocin -induced diabetes in rabbits

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Abstract

This study was undertaken to determine the antidiabetic effects of oral administration of *Punica granatum L.* extract on serum and tissues of streptozotocin induced diabetic rabbits at 100 mg/kg. The present study was carried out at the Faculty of veterinary Medicine, Tikrit University, from February to August 2017 for 10 weeks. For this purpose, 30 rabbits were randomly separated into three groups, each containing 10 animals: Group 1, healthy control rabbits; Group 2, diabetic rabbits received streptozotocin (STZ, 65 mg/kg); Group 3, diabetic rabbits treated with PS extract (the 100 mg PS+1 ml DW) for 21 days. At the end of experiment, blood samples were taken for measuring serum biochemical parameters. For histopathological evaluation, sections of kidneys were fixed in 10% buffered formalin and 5micron thick sections with H&E stain were prepared using routine histopathological techniques. The treatment revealed that PSE extract significant decreased serum glucose thrombospondin-1, nitric oxide, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase alkaline phosphatase, and C-reactive protein in diabetic treated rabbits as compared to diabetic rabbits. Histopathology of kidney showed lesions similar to human glomeruloscleroses, glomerular membrane thickening, arteriolar hyalinization and tubular necrosis. From the above one can conclude that PSE extract possess nephroprotective effect in experimentally induced diabetic rabbits.

Keywords: Histopathology, *Punica granatum* L., Extract, Rabbits
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دراسة التأثيرات المرضية النسيجية والبايوكيميائية لمستخلص بذور الرمان على داء السكري المستحدث في الأرانب بواسطة عقارالستريبتوزوسين

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الخلاصة

أجريت هذه الدراسة لتقييم مدى فاعلية مستخلص بذور الرمان على مصل وأنسجة الأرانب المستحدث بها السكري بالستريبتوزوسين عند ١٠٠ ملغم/ كغ. أجريت الدراسة الحالية في كلية الطب البيطري، جامعة تكريت، من فبراير إلى أغسطس ٢٠١٧ لمدة ١٠ أسابيع تم توزيع ٣٠ ذكر من الأرانب عشوائيا إلى ثلاث مجموعات، كل منها يحتوي على ١٠ حيوانات: المجموعة ١، الأرانب سليمة. تم استحداث الإصابة بداء السكري في الحيوانات في المجموعة ٢ عن طريق حقنهم بمادة الستريبتوزوسين بجرعة مقدارها ٦٥ غم/كغم من وزن الجسم. أما المجموعة ٣ فقد كانت حيوانات محدثة بها السكري وتم إعطاؤها مستخلص بذور الرمان (100 PS ملغم PS+1 مل DW) لمدة ٢١ يومًا. في نهاية التجربة، تم أخذ عينات الدم لقياس الاختبارات البيوكيميائية. لتقييم الأنسجة، تم حفظ الكلى في الفورمالين ١٠ ٪ واخذ أجزاء بسمك ٥ ميكرون لكل عينة وتصبيغها بصبغة الهيماتوكسلين والايوسين تم ترتيبها باستخدام تقنيات الأنسجة الروتينية. بينت النتائج ان مستخلص بذور الرمان أدى إلى انخفاض كبير في مستويات سكر الدم، ثرومبوسبوندين-١، وأكسيد النيتريك، ناقلة أمين الالنين، وناقلة

أمين الاسباراتات، نازعة هيدروجين اللاكتات، الفوسفاتاز القلوي، وبروتين سي التفاعلي، عامل نخر الورم نوع الفا في الأرانب المعالجة مقارنة بالأرانب المصابة بالسكري. وأظهرت التشريح المرضي للكلى آفات تشبه تصلب الكبيبات، وسماكة الغشاء الكبيبي، والتهييج الشرياني، والنخر الأنبوبي. من أعلاه يمكننا الاستنتاج ان مستخلص بذور الرمان يمتلك تأثيراً كلوياً واقياً في الأرانب المستحدثة بها السكري تجريبيا.

Introduction

In diabetes mellitus (DM), chronic hyperglycaemia produces multiple biochemical sequelae, characterized by hyperglycemia and hyperlipidemia, which have a severe impact on public health (1). It is well documented that chronic hyperglycaemia of diabetes is associated with long-term damage dysfunction and eventually the failure of organs especially the eyes, kidneys, nerves, heart and blood vessels (2). Many plant species are used in folk medicine for their hypoglycemic properties and therefore potentially used for treatment of DM, in this study we have selected Punica granatum L. to evaluate its anti-diabetic activity. *Punica granatum L.* (Punicaceae) (*P. granatum*) is commonly known as pomegranate contains diverse groups of polyphenols, including ellagitanins, gallotannins, and ellegic acid, as well as flavonoids, such as anthocyanins. However, its antioxidant activity is mainly due to hydrolysable tannin, including punicalagins, anthocyanins and ellagic acid (3,4). Biological actions of pomegranate fruit that lead it to be considered as a healthy fruit are due to its potent phytochemicals contents that scavenge wide spectrum of free radicals (5). This great action of pomegranate is ascribed to a large number of phytochemicals therein including hydrolysable tannins and phenolic compounds found in different parts of this miracle fruit (6,7). Numerous studies were carried out to investigate the effects of natural products on diabetes induced by streptozotocin. The pomegranate has been considered beneficial to health because of its high antioxidant content (8). The aim of the present study was to investigate the potential ameliorative effect of PSE in serum and tissues of streptozotocin induced diabetic rabbits.

Materials and methods

Preparation of plant extract

The pomegranates fresh fruits *(P. granatum L.*) were washed and peeled. The peels and seeds were separated and air dried in an oven (40°C, 24 h). After that, using a blender the dried materials turned into a powder. Thereafter, 500 g of pomegranate seed and pomegranate peel powders were separately extracted in methanol (Merck, Germany) (1:10 w/v) at 25°C for 24 and 96 h, respectively. The mixture of each was then filtered throw 0.45 µ pore size filters. The methanol was completely evaporated (rotary vacuum evaporator, Heidolph, Germany) at 40°C. The PSE was stored in a deep freezer (-70°C) until use (9).

Induction of diabetes mellitus

Diabetes was induced by a single intra-peritoneal injection of streptozotocin (Sigma, St. Louis, Mo, USA) at a dose of 65mg/kg bodyweight. STZ was extemporaneously dissolved in 0.1M cold sodium citrate buffer, pH4.5. After 18 h, animals with fasting blood glucose levels greater than 16.5mmol/L were considered diabetic and then included in this study (10). Fasting blood glucose was estimated by using one touch glucometer (Accu-Chek sensor) of Roche Diagnostics, Germany.

Animal stock

Thirty rabbits (all male) weighing 1.5 to 2.45 Kg were purchased from the college of veterinary medicine / Tikrit University, during February to August 2017. All the animals were housed in a cross-ventilated room (22 ± 2.5°C), 12h light 12h dark cycle) and were fed with standard diet (Grower's mash) and water ad-libitum. The animals were divided into three groups and were assigned randomly into each group that was made up of ten rabbits each housed in cages assigned to them. Group 1: was administered with normal saline (5 m1/kg PO), and served as the (standard control). Group 2: contained streptozotocin induced diabetic rabbits, which were left untreated (single injection of 65 mg/kg streptozotocin, IP). Groups 3: received the100 mg PSE + 1 ml DW per rabbits for 21 days.

The blood samples were collected and centrifuged (5000 rpm) and clear sera was separated and collected for the following investigations.

Thrombospondin-1, tumor necrosis factor-α and C-reactive protein were measured by ELISA technique. The level of serum glucose, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and lactate dehydrogenase determined using a fully automated clinical chemistry analyzer (Abbott Architect C8000). Serum nitric oxide was estimated by the method of Smarason *et al.* (11).

Statistical analysis

Data were calculated as mean with standard deviation. One-way ANOVA was employed for statistical analysis with a significant level if P is < 0.05, $< 0.001 < 0.0001$.

Results

The results depicted in Table 1 showed that there is significant increase in the TSP-1, NO, ALT, LDH, ALP, GGTP, TNF-α, C-RP levels of diabetic group with respect to the control group. Whereas treatment with PSE extract led to a significant increment in the levels of glucose TSP-1, NO, ALT, LDH, ALP, TNF-α, C-RP compared with diabetic group.

Table 1: Effects of *Punica granatum* L. Extract on the serum biochemical parameters in rabbit

Parameters	NC (G:1)	DC(G:2)	PSE (G:3)
TSP-1 (ng/ml)	1.791 ± 0.1028 b	11.060± 1.526 a	3.800 ±0.894 b
NO (µmol/L)	10.01 ± 1.766 c	44.86 ±5.26 a	17.108 ± 2.131 b
AST (IU/L)	86.33 ± 8.15 c	131.02 ± 13.10 a	103.23 ± 7.84 b
ALT (IU/L)	47.4 ± 2.052 c	197.70 ± 8.01 a	83.27 b 4.76
LDH (U/L)	326.57 ± 21.25 c	597.60 ± 26.29 a	417.00 ± 17.38 b
ALP (IU/L)	125.18± 2.369 c	249.53 ± 9.13 a	137.7 ± 3.23 b
TNF-α (pg/mL)	21.05 ± 1.569 c	38.427 ±1.945 a	28.37 ±1.579 b
C-RP (mg/liter)	1.12 ± 0.2446 c	2.66±0.2451 a	1.5570 ± 0.2285 b

Rabbits in control group showed normal kidney architecture and histology (Figure 1). Sections of the kidney in diabetic rabbits showed, the hypertrophy of glomerular (Figure 2), arterioles appear constricted, the epithelial cells of the proximal and distal convoluted tubules are degenerated (Figure 3).

Sections of the kidney in diabetic rabbits treated with pomegranate showed, arterioles constricted, having thick wall, the epithelial cells of the proximal convoluted tubules are vacuolated (Figure 4), some of epithelial cells of the distal convoluted tubules degenerated, and some of the glomeruli are collapsed but most of them nearly return to normal number and diameter (Figure 5, 6).

Figure 1: Demonstrating the shape of glomerulus (A), surrounded by a great number of proximal and distal convoluted tubules in control group (G:1) (H&E ×20).

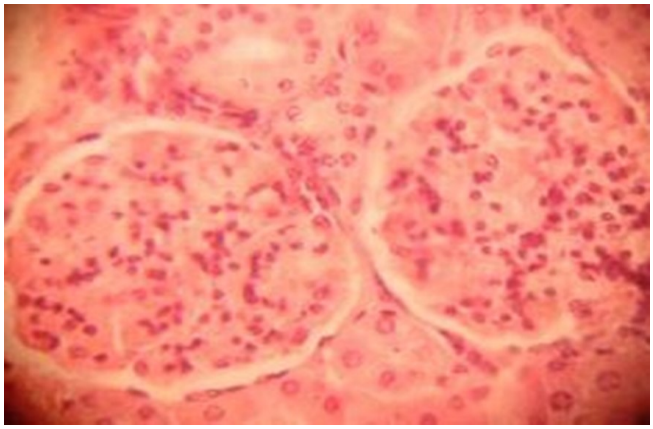

Figure 2: Hypertrophy of glomeruli in diabetic group (G:2) (H&E, 40X).

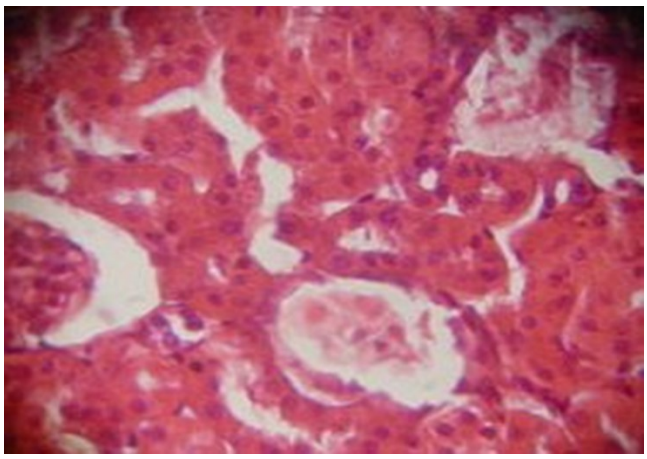

Figure 3: Degeneration of distal and proximal convoluted tubules (arrow) in diabetic group (G:2) (H&E, 40X).

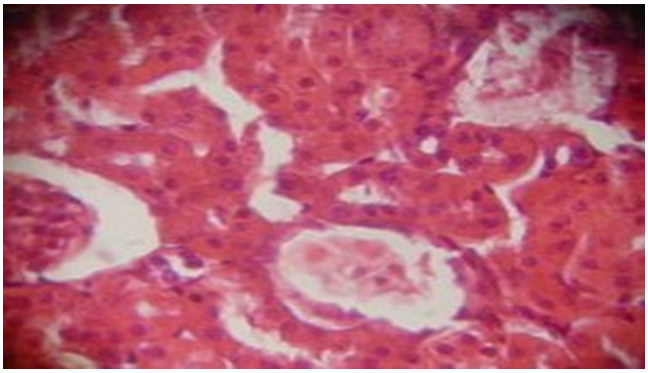

Figure 4: Vacuolation of the proximal convoluted tubules in treated group (G:3) (arrow) (H&E, 75X).

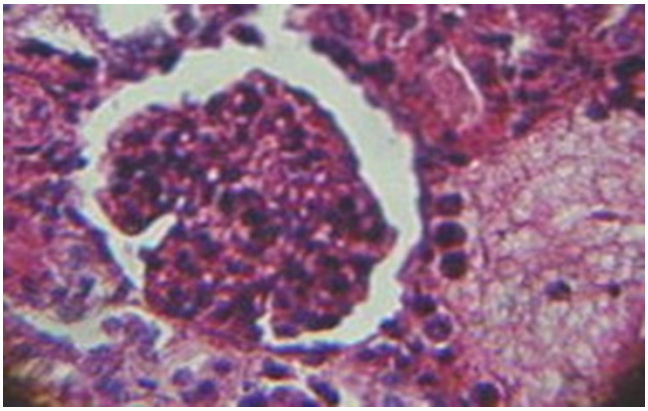

Figure 5: Vacuolation of the proximal convoluted tubules in treated group (arrow) (G:3) (H&E, 75X).

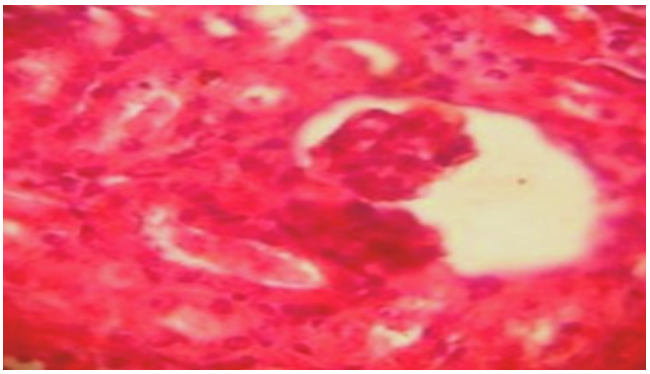

Figure 6: Shrinkage of glomerulus in treated group (G:3) (H&E, 40X).

Discussion

The STZ-induced diabetic rats exhibited a significant hyperglycemia compared to non-diabetic control rabbits. This hyperglycemia resulted from selective cytotoxic effect of STZ on pancreatic β-cells which resulted in DM in experimental rabbits. It is proposed that, the cytotoxic effect of STZ be closely related to free radical generation in pancreatic - cells which interfered with the cellular metabolic oxidative mechanisms (12,13)**.**

The hypoglycemic effect of the tested extracts may be due to the active principles present in these extracts such as polyphenols and flavonoids (14) which possess the properties of regenerating pancreatic β cell, increasing insulin secretion, enhancing glucose uptake by adipose or muscle tissues, inhibiting glucose absorption from intestine and glucose production from the liver and resolving the problem of insulin deficiency. these findings are in accordance with those of other study (15).

Thrombospondin-1 (TSP1) is a matricellular protein, first discovered in platelets. It is widely expressed in diverse tissues such as endothelial cells, monocytes and macrophages, smooth muscle cells, fibroblasts and adipocytes .TSP1 is readily measurable in human plasma and has a half-life of 9h (16,17) . Under physiological and pathological conditions TSP-1 is an endogenous inhibitor of angiogenesis It might be due to suppresses endothelial cell proliferation, migration, and tube-formation and induces endothelial apoptosis (18). TSP-1 controls homeostasis and tissue perfusion via regulation of nitric oxide signaling. Thus, the roles of TSP-1 in the regulation of angiogenesis are extremely complex and involve effects on stromal cells and the extracellular matrix (19).

Vascular smooth muscle cell (VSMC) from patients with diabetes exhibit increased proliferation, adhesion, and migration. Stimulating VSMCs through increased levels of TSP-1 in the diabetic vessel wall may explain the enhanced proliferation of VSMCs**.** Two mechanisms have been suggested for the effect of higher plasma TSP-1 level on atherosclerosis in patients with DM (20). First, dysfunction of endothelial cells (ECs) in patients with DM is well known (21) and TSP-1 certainly contributes to this dysfunction because of its antiproliferative and apoptotic effects on ECs (22). Second, although TSP-1 in diabetic vessels may affect metabolism of the luminal EC monolayer and VSMCs, the large amounts of TSP-1 present in the adventitia ultimately results in compromised growth and remodeling of the vasa vasorum.

Oxidative stress and overexpression of nitric oxide synthase (NOS) consider as interrelated contributing factors in pancreatic β-cell dysfunction (23). The marked decrease in NO level noticed in this study in the diabetic group, is in accordance with Chu (24), who reported that hyper glycaemia play a role in the decreased NO production in DM, because high glucose per se inhibited endothelial NOS activity in the glomeruli, through a protein kinase C associated mechanism (24). The decrease in production of NO due to reduced production of NO by NOS and inactivation of NO by ROS produced either by glycosylated

proteins or directly from vascular endothelium as high level of HbA1c was observed in DM (25).

PSE is known as a powerful antioxidant and could improve diabetes-induced endothelial dysfunction**.** PSE can exert beneficial effects on the evolution of clinical vascular complications, coronary heart disease, and atherogenesis in humans by enhancing the endothelial NOS (NOSIII) bioactivity because the pomegranate juice reverts the potent down-regulation of the expression of NOSIII induced by oxidized low-density lipoprotein (oxLDL) in human coronary endothelial cells (26). PSE its capacity to protect NO against oxidative destruction and enhance the biological actions of nitric oxide. The results demonstrate that pomegranate juice was found to be a potent inhibitor of superoxide anion-mediated disappearance of nitric oxide (27).

In the present study, the mean CRP reductions were more pronounced in subjects supplemented with PS in comparison with the control group. C-reactive protein also appears to progressively increase when glucose metabolism deteriorates, as evident in subjects with Type2 DM (28).

The mechanisms of the anti-inflammatory properties of PSE are not clear. However, it has been suggested that PS inhibits the enzymes related to inflammation, such as peroxisome proliferator active receptors (PPARs), nuclear transcription factor kappa B (NF-κB), and NSAID activated gene-1 (NAG-1), which reduces pro-inflammatory cytokine secretion through the inhibition of MAP kinases (29). Similar finding was observed by (30).

In the present study, the treatment of G3 with the PSE elicited significant decrease in serum ALT, AST, ALP, LDH, and GGT. The increase in aminotransferase levels may be attributed to cellular damage in the liver induced by diabetes. Cell damage results to increased permeability leading to the leakage of cytosolic enzymes into the blood (31). However, PSE produced a marked significant decrease of the elevated AST and ALT activities. This decrease may be attributed to the hepatoprotective and antioxidant activity of a number of flavonoids in the PSE.

This agrees with the report of Ghada (32). where it was reported that the effect of STZ-diabetes on ALP activity revealed a significant increase in ALP activity of STZ induced untreated rats. A marked decrease of serum and liver ALP activity of STZ-induced rats after treatment with JS indicates its protective effect over liver and improvement in liver function efficiency.

Histological examinations indicated that the kidney of diabetic control rats exhibited, decreased blood flow through the glomerular capillary system because of the thickening of the arteriole and arteriolar walls, and the consequent reduction in the Lumina of this vessels, produces chronic ischemia of the tubular system and reduces glomerular filtration if prolonged, this led to tissue shrinkage of the compounds of the glomerulus and atrophy of the tubules.

The mechanism of kidney destruction because of the oxidative stress involves the secretion of cytokines, mainly tumor necrosis factor TNF-α, interleukin IL-1, and IFN-c. These alterations might be caused of abnormal production of cytokines and growth factors, which facilitate the synthesis of extracellular matrix proteins and the depositions in the glomerular level that finally lead to glomerular shrinkage, and glomerular basement thickening (33).

Treatment of STZ -induced diabetic rabbits with PSE extracts drastically improved kidney function as a result of its antithrombogenic action, which in turn controls the arachidonic acid cascade system (34). High level of antioxidant in pomegranate could boost to quenching of some free radicals inside cells, as well as have the capability to protect kidney and liver tissue from oxidative stress damage.

Conclusion

This study concluded that the PSE extracts may be capable of improving blood glucose TSP-1, ALT, LDH, ALP, TNF-α, C-RP levels has nephroprotective activity in streptozotocin-induced diabetic rabbits. Many questions related to antioxidant effect of PSE extract remain unanswered. Much more work is clearly needed before phytotherapy for diabetic nephropathy can be advanced to clinic.

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