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ORIGINAL ARTICLE

Protective Effects of Ursolic Acid in the Kidneys of Diabetic Rats

Diyabetik Sıçanların Böbreklerinde Ursolik Asidin Koruyucu Etkileri

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ABSTRACT

Objectives: Diabetes, a heteregenous metabolic and chronic disease, is a growing health problem in most countries. It has been claimed that diabetes is associated with the increased formation of free radicals and decreased in antioxidant potential. Oxidative stress formed in diabetes may cause DNA damage in the tissues. Ursolic acid, a well-known pentacylic triterpene, is commonly used in traditional Chinese medicine due to its beneficial health effects such as antioxidant, anticancer, and antiulcer properties. The aim of this study was to investigate the effects of ursolic acid in the kidneys of Wistar albino rats with streptozotocin-induced diabetes.

Materials and Methods: DNA damage was evaluated in the kidney cells of rats using alkaline comet assays. Oxidative stress parameters such as CAT, SOD, GR, and GSH-Px enzyme activities and total GSH and MDA levels were also evaluated.

Results: Ursolic acid treatment was found to significantly decrease DNA damage, GR enzyme activities, and MDA levels, and significantly increase GSH levels and CAT, SOD and GSH-Px enzyme activities in diabetic rats.

Conclusion: According to our results, it seems that ursolic acid may be beneficial against diabetes-induced renal damage.

Key words: Diabetes, DNA damage, oxidative stress, kidney

ÖZ

Amaç: Metabolik ve kronik bir hastalık olan diyabetin birçok ülkede önemli sağlık sorunu olma durumu artış göstermektedir. Diyabetin artmış serbest radikallerin üretimi ve azalmış antioksidan kapasiteyle ilişkili olduğu iddia edilmektedir. Diyabette oluşan oksiatif stres dokularda DNA hasarına neden olabilir. Antioksidan, antikanser, antiülser özellikler gibi sağlığa yararlı etkileri nedeniyle geleneksel Çin halk tıbbında yaygın olarak kullanılan ursolik asit çok iyi tanımlanmış pentasiklik triterpendir. Bu çalışmanın amacı, streptozotosin ile diyabet oluşturulmuş Wistar albino sıçanların böbreklerine ursolik asidin etkisini değerlendirmektir.

Gereç ve Yöntemler: Bu amaçla, böbrek hücrelerindeki DNA hasarı alkali comet yöntemiyle araştırılmıştır. KAT, SOD, GR ve GSH-Px enzim aktiviteleri ve toplam GSH ve MDA düzeyleri gibi oksidatif stres parametreleri de ölçülmüştür.

Bulgular: Ursolik asit tedavisinin, DNA hasarını, GR enzim aktivitelerini ve MDA seviyelerini önemli ölçüde azalttığı ve diyabetik sıçanlarda GSH düzeylerini ve CAT, SOD ve GSH-Px enzim aktivitelerini önemli ölçüde artırdığı bulundu.

Sonuç: Sonuçlarımıza göre, ursolik asidin diyabetin neden olduğu böbrek hasarında yararlı olabileceği görülmüştür.

Anahtar kelimeler: Diyabet, DNA hasarı, oksidatif stres, böbrek

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INTRODUCTION

Diabetes mellitus, a heteregenous metabolic and chronic disease, is caused by an absolute or relative lack of insulin. It is a growing health problem in most countries, especially in developing countries.[1] Around 3% of the world's population has diabetes and it is concluded that it may exceed 5.4% by the year 2025.[2] In Turkey, 7.4% of the population has diabetes, and it is also estimated that the number of patients will increase to 9.6% of the population by 2030.[3]

Studies on diabetes therapy have gained interest due to its unwanted effects on human life e.g. changing lifestyles lead to reduced physical activity, and increased obesity.[3] Drugs used in diabetes therapy have higher costs, limited efficacy and/or significant adverse effects.[4,5] As a result of these factors, patients with diabetes often use alternative therapy such as herbal medicines.[6] Epidemiologic studies have associated diets rich in isoflavones with a lower risk of diabetes and diabetes-related complications.[7,8] However, pharmacologic and toxicologic evidence validating the safety and efficacy of these medicinal plants is not readily available.[9]

For experimental diabetes model in animals, streptozotocin (STZ) and alloxan are the most frequently used drugs. According to the administered dose of these agents, syndromes similar to either type 1, type 2 diabetes mellitus or glucose intolerance can be induced. STZ enters the pancreatic β-cell via a glucose transporter 2 and causes alkylation of deoxyribonucleic acid (DNA).[10] The potential problem with STZ is that its toxic effects are not restricted to pancreatic β-cells because it may cause renal injury and oxidative stress.[11,12]

Ursolic acid (3β-hydroxy-12-urs-12-en-28-oic acid), commonly used in traditional Chinese medicine, is a well-known pentacylic triterpene. *Malus pumila*, *Ocimum basilicum*, *Vaccinium* spp., *Vaccinium* macrocarpon, *Olea europaea*, *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia,* and *Thymus* plants are the main sources of ursolic acid.[13] It has various biologic effects including anticancer, antiulcer, antidiabetic properties due to its antioxidant activity. Ursolic acid has been suggested to increase insulin levels with the preservation of pancreatic β-cells and modulate blood glucose level in diabetic mice.[14]

In this paper, oxidative DNA damage in the kidney cells of STZ-induced diabetic rats were evaluated using alkaline comet assays. Oxidative stress parameters such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) activities, and total GSH and malondialdehyde (MDA) levels in the kidney tissues were also measured to investigate the effects of ursolic acid on diabetes-induced oxidative damage.

MATERIALS AND METHODS

The study was approved by Ankara University Animal Ethics Committee (2015-12-138).

Chemicals

The chemicals used in the study were purchased from the following suppliers: normal-melting agarose and low-melting-point agarose from Boehringer Manheim (Mannheim, Germany); sodium chloride, sodium hydroxide, and potassium chloride from Merck Chemicals (Darmstadt, Germany); dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), Triton-X-100, phosphate-buffered saline (PBS) tablets, STZ and ursolic acid from Sigma-Aldrich Chemicals (St Louis, Missouri, USA); ethylendiaminetetraacetic acid disodium salt dihydrate (EDTA-Na2), natrium lauroy lsarcosinate, and Tris from ICN Biomedicals Inc. (Aurora, Ohio, USA), SOD assay kit, CAT assay kit, GR assay kit, GSH-Px assay kit and GSH assay kit from Cayman Chemicals Co. (Ann Arbor, MI, USA), ketamin hydrochloride from Eczacıbaşı (İstanbul, Turkey).

Animals

Wistar albino rats (180-250 g, n=24) were used in all experiments. The animals were housed in plastic cages with stainless steel grid tops. The rats were maintained on a 12-h light-dark cycle, at controlled temperature (23±2°C) and humidity (50%). The animals were fed with standard laboratory chow and allowed to access feed and drinking water ad libitum. The blood glucose levels of the animals were measured before the experiments. The rats were treated humanely according to the Helsinki Declaration of 2008 and with regard for the alleviation of suffering.

STZ-induced diabetes mellitus model

Wistar rats were subjected to type 1 diabetes through STZ injection (60 mg/kg in freshly prepared PBS) as previously described.[15] Two days later, blood was taken from the tails of the rats using a lancet to measure the blood glucose levels with a glucometer (Plusmed). Rats with a blood glucose level higher than 250 mg/dL were considered to be diabetic.

Experimental design

The rats were divided into four groups:

Group 1: Sham group (n=6). This group consisted of animals treated with oral saline alone.

Group 2: Diabetic group (n=6). This group consisted of animals in which only diabetes was induced and the animals were treated with oral saline.

Group 3: Ursolic acid-treated group (n=6). This group consisted of animals treated with a dose of 50 mg/kg b.w. per oral ursolic acid (in saline) for 28 days.

Group 4: Ursolic acid-treated diabetic group (n=6). This group consisted of animals treated with a dose of 50 mg/kg b.w. per oral ursolic acid (in saline) for 28 days following the induction of diabetes.

Ursolic acid dose (50 mg/kg b.w. per oral) was selected according to our unpublished studies. At the end of the experimental period, all animals were decapitated under anesthesia (90 mg/kg ketamine hydrochloride, i.p.). The kidneys were removed. The organs were examined for changes in size, color, and texture. The samples were kept in the dark at 4°C and processed within 4 hours for comet assays. The kidney homogenates were kept at -80°C for the determination of oxidative stress parameters.

Determination of oxidative stress parameters

Oxidative stress parameters were assayed in the plasma samples and in the liver homogenates. The liver tissues were weighed and extracted following the homogenization and sonication procedure.[16]

The determination of CAT, SOD, GR, and GSH-Px enzyme activities and GSH and MDA levels in the kidney tissues were performed spectrophotometrically using CAT, SOD, GR, GSH-Px, GSH, and MDA assay kits (Cayman Chemicals Co., Ann Arbor, MI, USA) at 540, 440, 340, 340, 420, and 535 nm, respectively. The results are expressed as mmol/min/mg for enzyme activities, μM for GSH levels, and nmol/g for MDA levels.

Determination of DNA damage

Kidney homogenates were used for the comet assays. The kidney tissues were carefully dissected from their attachments and totally excised. Preparation of a single-cell suspension from the organs was performed according to standard procedures.[17,18] In brief, approximately 0.2 g of each organ was placed in 1 mL chilled mincing solution [Hanks' balanced salt solution (HBSS) with 20 mM EDTA and 10% DMSO] in a Petri dish and chopped into pieces using a pair of scissors. The pieces were allowed to settle and the supernatant containing the single-cell suspension was taken. The concentrations of renal and hepatic tissue cells in the supernatant were adjusted to approximately 2 x 106 cells/mL in HBSS containing 20 mM EDTA / 10% DMSO.

The alkaline comet assay technique of Singh et al.[19], as further described by Aydın et al.[20] and Bacanlı et al.[21] was followed.

The dried microscopic slides were stained with EtBr (20 μg/mL in distilled water, 60 μL/slide), covered with a cover-glass prior to analysis using a Leica® fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd, Liverpool, UK) to determine the extent of DNA damage after the electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, 100 nuclei per slide were examined at 40x magnification. The results are expressed as percent of DNA in tail ('tail intensity').

Statistical analysis

Statistical analysis was performed using the SPSS for Windows 20.0 computer program. Differences between the means of data were compared using the One-way variance analysis (ANOVA) test, and post-hoc analysis of group differences was performed using the least significant difference test. The Kruskal-Wallis K test followed by the Mann-Whitney U test was used to compare parameters displaying abnormal distribution between the groups. The results are given as mean ± standard deviation. P values of less than 0.05 were considered as statistically significant.

RESULTS

Assessment of oxidative stress parameters

The CAT, SOD, GR and GSH-Px enzyme activities and GSH and MDA levels in the kidney tissues were shown in the Table 1.

CAT, SOD, and GSH-Px enzyme activities and GSH levels were found to be significantly lower in the diabetic group compared with the sham group ($p<0.05$). CAT, SOD, and GSH-Px enzyme activities and GSH levels were significantly increased in the ursolic acid-treated diabetic group compared with the diabetic group ($p<0.05$). There were no significant differences between the sham and ursolic acid-treated diabetic group.

Renal GR enzyme activities and MDA levels were found to significantly increased in the diabetic group compared with the sham group ($p<0.05$). However, the levels were found to significantly decreased in the ursolic acid-treated diabetic group compared with the diabetic group ($p<0.05$). There was no significant difference between the sham and ursolic acid-treated diabetic group in terms of MDA levels ($p<0.05$).

Ursolic acid alone caused no significant changes in all studied oxidative stress parameters compared with the sham group.

Table 1. Oxidative stress parameters in the kidneys of the experimental groups

	Group 1	Group 2	Group 3	Group 4
CAT activity (nmol/min/mL)	15.130±2.189[b]	10.463±4.711[a]	167.390±8.200[b]	136.386±29.559[a,b]
SOD activity (U/mL)	0.451±0.304[b]	0.241±0.147[a]	0.921±0.348[b]	0.738±0.129[b]
GSH-Px activity (nmol/min/mL)	124.126±2.563[b]	56.514±5.466[a]	138.145±5.795[b]	125.657±7.120[b]
GR activity (nmol/min/mL)	3.737±1.281[b]	11.382±6.265[a]	4.976±1.239[b]	5.956±0.423[b]
GSH levels (μM)	9.938±3.628[b]	5.419±0.584[a]	8.396±2.125[b]	7.965±1.458[b]
MDA levels (nmol/g)	13.452±5.128[b]	24.367±4.785[a]	14.108±3.025[b]	15.203±7.520[b]

The values are expressed as mean ± standard deviation; a: Statistically different from the sham group ($p<0.05$), [b]: Statistically different from the diabetes group ($p<0.05$).
Group 1: Sham, Group 2: Diabetic rats, Group 3: Ursolic acid (50 mg/kg)-treated rats, Group 4: Ursolic acid (50 mg/kg)-treated diabetic rats.
CAT: Catalase, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, GR: Glutathione reductase, GSH: Glutathione, MDA: Malondialdehyde

Assessment of DNA damage

The DNA damage expressed as tail intensity in the kidney cells of rats in comet assays is shown in the Figure 1.

In all samples studied, there were no statistically significant differences in tail intensity between the sham group and the ursolic acid-treated groups ($p>0.05$). The DNA damage was found significantly higher in the diabetic group compared with the sham group ($p<0.05$). Ursolic acid treatment in the diabetic group was found to decrease the DNA damage significantly ($p<0.05$).

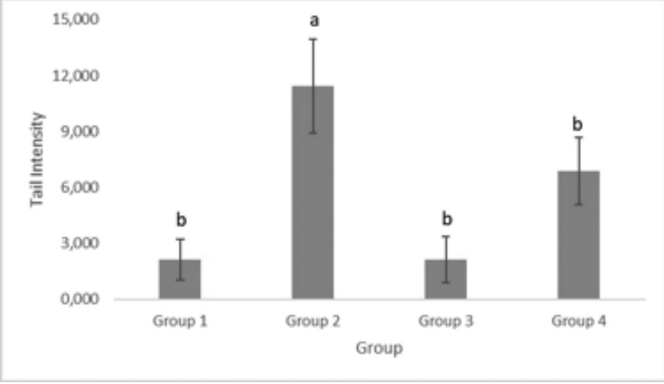

Figure 1. DNA damage of the experimental groups of the rats

The values are expressed as mean ± standard deviation a: Statistically different from the sham group ($p<0.05$); b: Statistically different from the diabetic group ($p<0.05$)

Group 1: Sham, Group 2: Diabetic rats, Group 3: Ursolic acid (50 mg/kg)-treated rats, Group 4: Ursolic acid (50 mg/kg)-treated diabetic rats

DISCUSSION

In the present study, we evaluated the protective effects of ursolic acid against the renal effects of STZ-induced diabetes in rats. STZ is a pancreatic β-cell toxin that causes irreversible necrosis of β-cells.[16] When injected into animals at different doses, it causes mild-to-severe types of diabetes according to the dose used.[22]

Multiple factors can cause oxidative stress in diabetes. The most important factor is glucose autoxidation, which leads to the production of free radicals. Other factors include cellular oxidation/reduction imbalances and reduction in antioxidant defenses including decreased cellular antioxidant levels and a reduction in the activity of enzymes that dispose of free radicals.[23]

It has been shown that oxidative stress exists in patients with diabetes, as evidenced by increased total antioxidant capacity in the saliva and blood of patients.[24] MDA has been identified to bind and damage DNA. It is a direct product of lipid peroxidation. In different studies, it has been demonstrated that MDA levels were significantly increased in different tissues such as liver and kidney tissues.[25,26] Increased MDA levels were shown in patients with diabetes[27] and diabetic rats.[28]

Our findings are consistent with the data of some recent studies that showed changes in oxidative stress parameters in diabetes. In the present study, we observed that GR enzyme activities and MDA levels were significantly increased, and GSH levels and CAT, SOD, and GSH-Px enzyme activities were significantly decreased in diabetic rats.

Recently, there has been a growing interest in replacing synthetic diabetic drugs with natural antioxidants from plant materials.[29] Phytochemicals with antioxidant effects include cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins, and triterpenes.[30] Wang et al.[31] demonstrated that oleanolic acid (0.1 and 0.2%) and ursolic acid (0.1 and 0.2%) markedly suppressed renal aldose reductase activity and enhanced glyoxalase I activity, which contributed to decrease renal advanced glycation end products formation and improvedd renal functions. The impact of these two triterpenes on mRNA expression of renal aldose reductase and glyoxalase I revealed that the effects of these agents occurred at the transcription level. Low-dose ursolic acid (0.01% in food) administration in STZ-induced diabetic mice for three months, glomerular hypertrophy and type IV collagen accumulation in the kidneys were found to be markedly ameliorated.[32] In a 16-week study on rats with STZ-induced diabetes, ursolic acid treatment prevented biochemical and histopathologic changes in the kidneys associated with diabetes such as alterations in renal function and increases in oxidative stress, NF-κB activity, and P-selectin expression in the kidneys.[33] In our study, we found that ursolic acid regulated all of the alterations of oxidative stress parameters in diabetes. Ursolic acid treatment was found to significantly decrease GR enzyme activities and MDA levels and significantly increase GSH levels and CAT, SOD, and GSH-Px enzyme activities in diabetic rats.

Kushwaha et al.[34] determined the DNA damage in lung, liver, aorta, heart, kidney, pancreas, and blood samples of experimentally-induced diabetic rats using comet and endonuclease III and formamidopyrimidine (fpg) modified comet assays. The authors found that DNA damage was significantly greater in the diabetic group compared with the non-diabetic group. In our previous study with ursolic acid, we reported the antigenotoxic effect of this phytochemical against hydrogen peroxide induced oxidative DNA damage in the human lymphocytes and Chinese hamster fibroblast cells.[35] In the present study, DNA damage in diabetic rats was found to be greater compared with the sham group. Ursolic acid treatment was found to significantly decrease oxidative DNA damage in diabetic rats.

CONCLUSIONS

Our results are in accordance with previous reports about diabetes, its complications and the effects of natural antioxidants against diabetes. We conclude that ursolic acid treatment may be preventive against diabetes and its effects. Nevertheless, further *in vitro* and *in vivo* studies are needed to elucidate their efficacy, mechanism, and toxicity on diabetes treatment.

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