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Antioxidant Status and Anti-inflammatory Effects of Oleuropein in Streptozotocin-induced Diabetic Nephropathy in Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors FKC, OH and MFB designed the study and prepared the manuscript. Author FKC managed the literature searches. Author OH prepared rat application. Author MFB made histopathological examinations. All authors read and approved the final manuscript.

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ABSTRACT

The aim of the study was the evaluation of the potentially antioxidant and anti-inflammatory effects of oleuropein against streptozotocin (STZ)-induced diabetic nephropathy in rats. Animals were allocated into 4 groups of 8 rats each. The control group was fed standard rat feed and received no added treatment. In the oleuropein group, oleuropein was given to normal animals at a dosages of

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20 mg/kg intraperitoneally (i.p) for 28 days. In the diabetic group, STZ was injected into rats at a single dose of 50 mg/kg i.p. The last group, oleuropein was given to diabetic animals at dosage of 20 mg/kg i.p for 28 days. STZ induced significant increases of inflammation cytokines as serum TNF-α, IL-6, IL-10, IL-18, and IF-γ. Also, serum blood urea nitrosamine and creatinine levels as markers of nephropathy were increased in the diabetic group. STZ increased kidney total oxidant status, malondialdehyde, superoxide dismutase and catalase whereas it decreased total antioxidant capacity. In addition, kidneys were examined by histopathological and immunohistochemical methods; mononuclear cell infiltration, proliferations, tubular dilatations, vacuolar degenerations, and red-brown cytoplasmic and nuclear expansion were observed in STZ-induced diabetic rats. In contrast, oleuropein decreased amelioration of oxidant status and tissue damage in diabetic rats. In conclusion, oleuropein treatment shows an antioxidant and anti-inflammatory effects in diabetes by decreasing oxidative stress and regenerated kidney tissue.

Keywords: Oleuropein; diabetes; nephropathy; oxidative stress; inflammation; rat.

1. INTRODUCTION

The most common endocrine disorder is diabetes mellitus characterized by hyperglycemia. Its mechanism is an unclear, but much attention has been focused on the role of oxidative stress. Diabetics and experimental animal models exhibit high oxidative stress due to persistent or chronic hyperglycemia, thereby depleting the activity of the antioxidant defense system and in turn promoting free radical generation [1]. Recently, there has been a renewed interest in understanding the role of reactive oxygen species, which play a key intermediate role in the pathophysiology of diabetic nephropathy [2].

The cellular antioxidant status determines the susceptibility to oxidative damage and is usually altered in response to oxidative stress. Accordingly, many antioxidants have recently been used to prevent oxidative damage in diabetes with high oxidative stress. Antioxidants such as vitamins (C and E), enzymes (superoxide dismutase, catalase, glutathione peroxidase), and minerals (selenium, boron) have been shown to protect the cells against lipid peroxidation and DNA damage, which is the initial step in many pathological processes [3-6]. Reduced antioxidant levels as a result of increased free radical production in experimental diabetes have been reported by many authors [6,7].

Oleuropein, a phenolic constituent, is pharmacologically mainly active component of olive oil [8]. In many researches, oleuropein has been shown antimicrobial effect [9] against many pathogenic bacteria and beneficial health effect such as cardioprotective [10]. Also, it has been antioxidant [11], anti-inflammatory [12], anti-cancer [13], anti-atherogenic [14], and neuroprotective effects [15].

The aim of the present study was to determine the effect of oleuropein on antioxidant status and anti-inflammatory effects in STZ-induced diabetic rats. In addition, we explored whether oleuropein treatment improves kidney tissue cells against STZ damage in rats, using histopathological and immunohistochemical staining.

2. MATERIALS AND METHODS

2.1 Chemicals

Oleuropein and STZ were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co. St. Louis, MO, USA). TNF-α, IL-6, IL-10, IL-18, and IF-γ using a competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). TAS and TOS chemicals were purchased from Rel Assay Diagnostics.

2.2 Animals and Experimental Design

Apparaently healthy male Wistar rats, 60 days old and weighing 180–200 g, were purchased from the Animal Breeding Laboratories of the Experimental Animal Research and Application Center (Afyon, Turkey). The animals were kept at room temperature (25ºC) and relative humidity (50–55%) in a 12 h light/dark cycle with *ad libitum* access to a standard rodent diet and water. The rats were allowed to acclimatize to the animal facility for at least seven days before the experiment started. Prior to the experiment, rats were fed a standard rodent diet for one week in order to adapt to the laboratory conditions.

The animals fasted overnight and diabetes was induced by a single intraperitoneal injection (i.p) of a freshly prepared solution of STZ (50 mg/kg

body weight) in dissolved in 0.1 M citrate buffer (pH 4.5) [6]. The animals were allowed to drink 5% glucose solution overnight to overcome the drug-induced hypoglycemia. Control rats were injected with 0.9% saline only. Diabetes was confirmed in STZ rats by measuring the fasting blood glucose concentration using a glucometer, ACCU-CHEK (Bayer, Germany), 48 h after the injection of STZ. The rats with blood glucose level ≥200 mg/dl were considered to be diabetic and were used in the experiment. The treatment was started on the second day after the STZ injection, and this was considered as the first day of treatment.

The rats were divided into 4 groups consisting of 8 animals each. Normal diet and tap water were given to both the control and treatment groups for a period of 28 days. Any drug treatment was given to the diabetic group. The dose of oleuropein (20 mg/kg/day) was given to non diabetic rats by i.p to the oleuropein group for 28 days [11]. Oleuropein (20 mg/kg/day) was also given i.p to the oleuropein+diabetes group throughout the entire 28-day period.

2.2.1 Blood collection and serum preparation

Following the experimental procedures, the rats were sacrificed after an overnight fasting period (12 hours) under anesthesia with an injection of 65 mg/kg i.p ketamine and 7 mg/kg i.p xylazine.

After blood was collected it has to be centrifuged before serum will be collected.

Blood samples from the rats were collected were centrifuged after all samples were stored at -80℃ until use.

2.2.2 Measurement of BUN, Creatine, TNF-α, IL-6, IL-10, IL-18, and IF-γ levels in serum

Serum creatinine and blood urea nitrogen (BUN) levels were determined by an auto analyzer using the manufacturer's kits (Roche Diagnostics Systems, Istanbul, Turkey). The serum samples were examined for their concentration of TNF-α, IL-6, IL-10, IL-18, and IF-γ using a competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor, MI, USA).

2.3 Measurement of Tissue MDA, SOD, CAT, TAS, and TOS in Kidney

Malondialdehyde (MDA) level which is an lipid peroxidation (LPO) marker, was determined by the method of Ohkawa et al. [16] in tissue homogenates by reaction of thiobarbituric acid with MDA and spectrophotometric measurement at 532 nm. The concentration of MDA was expressed as nmol/g for tissue. The activities of superoxide dismutase (SOD) were measured according to Sun et al. [17] by spectrophotometry at 560 nm; activities were expressed in U/mg protein in tissue homogenate. Catalase (CAT) activities were determined according to Aebi [18]. The reaction mixture consisted of 2.90 mL 50 mmol/L phosphate buffer (pH 7.0), 5 mL 10 mmol/L H_2O_2, and 50 mL sample. The reduction of H_2O_2 was followed at 240 nm for 45 s at room temperature. CAT activities were expressed as nmol/mg protein in tissue homogenates. Total antioxidant status (TAS) levels were measured according to Erel's method [19], which represents the quantity of plasma antioxidants, and were determined using a kit from Rell Assay Diagnostics in Turkey. The results were expressed as millimol Trolox equivalents per liter (mmol Trolox equiv./L). Also, the total oxidant status (TOS) levels were determined using a novel automated measurement method developed by Erel [20]. The results were calibrated with hydrogen peroxide and expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmolH_2O_2 equiv./L).

2.4 Histopathological and Immunohisto-chemical Examination

The kidney was removed from each animal immediately after sacrificing and rinsed in ice-cold saline. The renal tissues were fixed in buffered formalin solution, for histopathological examination. After routine processing, tissues were embedded in paraffin wax. Using a microtome, 5-μm-thick sections were cut and mounted onto glass slides. The sections were then stained with two separate staining techniques: Hematoxylin and eosin (H&E), and Masson's trichrome.

Immunohistochemically, the tissue samples were fixed in paraformaldehyde, dehydrated in a graded series of ethanol, and embedded in paraffin wax before sectioning. Sections were dewaxed and rehydrated. After being washed in phosphate-buffered saline, sections were immersed in a solution of 3% H_2O_2 for 10 min. The sections were then pre-incubated with non-immune serum for 15 min and subsequently replaced with rabbit anti-caspase-3 antibody (1:20, ab4051, Abcam, USA) for incubation at room temperature for 2 h. Biotinylated anti-rabbit

immunoglobulin (IgG BA1000, Vector Laboratories Inc., CA, USA) was used as a secondary antibody. They were labelled with streptavidin peroxidase (Standard Vectastain Elite ABC Kit, PK-6100, Vector Laboratories Inc, CA, USA) following incubation with the secondary antibody at 37℃ for 30 min. The localization of the antigen was indicated by a red-brown color obtained with 3-amino-9-ethyl-carbazole (ImmPACT Amec Red Peroxidase Substrate, SK-4285, Vector Laboratories Inc, CA, USA) as a chromogenic substrate for peroxidase activity. The specificity of the immunohisto-chemical staining was checked by omission of the primary antibody or by using an inappropriate antibody (anti-gastrin). All sections were analyzed under a light microscope (Zeiss Axio Lab A1 model, Jena, Germany) equipped with camera (AxioCam ICc 5 model, Jena, Germany).

2.5 Statistical Analysis

Data obtained from experimental animals were expressed as means and standard deviation of means (±SD), and analysed using one-way analysis of variance (ANOVA), followed by Duncan post-hoc tests on the SPSS (20) computer software program. A difference in the mean values of P ≤0.05 was considered to be significant.

3. RESULTS

3.1 Effect of Oleuropein Treatment on Fasting Blood Glucose Levels

The blood glucose levels of animals are shown in Fig. 1. Animals induced by STZ consistently exhibited hyperglycemia (P <0.001) compared to initial glucose levels. Oleuropein treatment caused a decrease in the elevated serum glucose levels in STZ diabetic rats, as measured at the end of the study (p<0.001).

3.2 Effects of Oleuropein Treatment on BUN, Creatine, TNF-α, IL-6, IL-10, IL-18, and IF-γ Levels

Blood urea nitrogen (BUN) and creatine levels in groups are shown in Fig 2 and Fig 3, respectively. BUN and creatinine levels were found to be high (93.77±2.22 and 17.44±2.93 mg/dL, respectively) in the diabetic group compared to the control group (21.94±1.43 and 2.47±0.22 mg/dL, respectively) (P < 0.001). Oleuropein treatment decreased to these levels compared to the diabetic group and these levels were also found to be at 63.97±2.54 and 10.07±1.24 mg/dL in oleuropein plus diabetes, respectively (p<0.001). In the oleuropein group, BUN and creatinine levels were also found to be at 23.01±1.62 and 2.50±0.30 mg/dL, respectively. These results suggested that the administration of oleuropein alleviated the STZ-induced alteration of nephropathy compared to the diabetic group (P < 0.001). Cytokines, TNF-α, IL-6, IL-10, IL-18, and IF-γ, are also summarized in Table 1. There were a significant increases of their levels in the diabetic group (P <0.001) compared to the control group. However, administration of oleuropein at dose 20 mg/kg/day prior to the diabetes challenge was observed to reverse the STZ-induced alteration of inflammation parameters. Also, only oleuropein (20 mg/kg/day) treatment was not changed these parameters in the experiment.

3.3 Effects of Oleuropein Treatment on BUN, Creatine, TNF-α, IL-6, IL-10, IL-18, and IF-γ Levels

There were significant increases of MDA, SOD, CAT and TOS levels whereas decreases TAS level in the diabetic group compared to control group (Table 2). In addition, oleuropein treatment decreased to their levels in diabetic rats compared to diabetic groups (p<0.001). These results suggested that the administration of oleuropein alleviated the STZ-induced alteration of oxidative stress and antioxidant status compared to the diabetic group (P < 0.001).

3.4 Histopathological and Immunohisto-chemical Evaluation

Mononuclear cell infiltration, proliferations, tubular dilatations, and vacuolar degenerations were observed in kidney tissues of diabetic rats stained with hematoxylin-eosine (Fig. 4C). In trichome stained, significant increases of mesangial collagen were observed in kidney of diabetic rats (Fig. 4G). Immunohistochemical staining with caspase-3, there was mostly red-brown cytoplasmic and nuclear expansion (Fig. 4K) in kidney tissue of diabetic rats. Oleuropein treatment at dose of 20 mg/kg protected the majority of cells in kidney (Fig. 4D, Fig. 4H, and Fig. 4L, respectively). Also, the histology, trichome, and immunohistochemical staining of the kidney were normal in the control (Fig.4A, Fig.4E, and Fig.4I, respectively) and oleuropein groups (Fig. 4B, Fig. 4F, and Fig. 4J,

respectively). In addition, the results of a semi-quantitative analysis of immunohistochemical staining of kidney in control and diabetic groups are shown in Table 3.

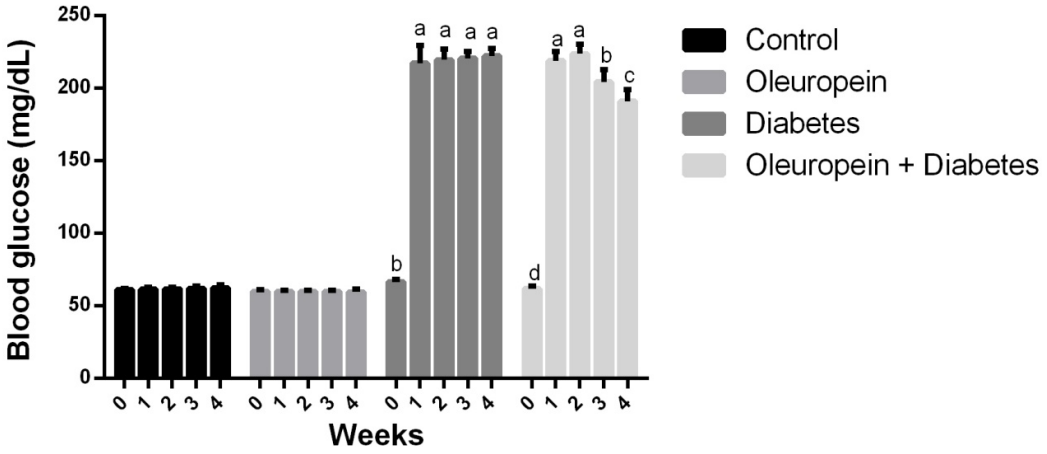

Fig. 1. The effect of diabetes alone and treated with oleuropein on blood glucose levels in rats. Oleuropein was administered at 20 mg/kg per day for 28 days and STZ was administered at 50 mg/kg. Blood glucose level of rats were measured at 0, 1, 2, 3, and 4 weeks

*Results are expressed as mean + SD of eight rats and * P <0.05*

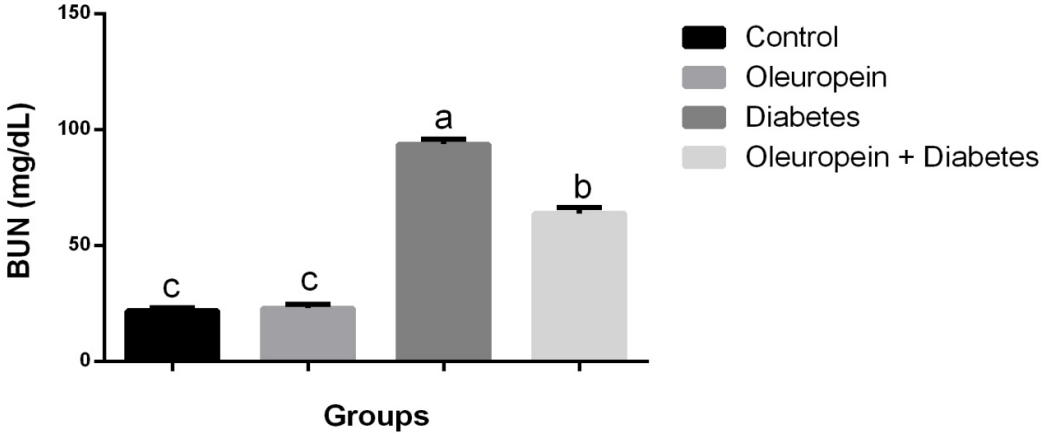

Fig. 2. The effect of diabetes alone and treated with oleuropein on blood urea nitrogen (BUN) in rats. Oleuropein was administered at 20 mg/kg per day for 28 days and STZ was administered at 50 mg/kg

Results are expressed as mean + SD of eight rats

Table 1. Effects of oleuropein at dose of 20 mg/kg and diabetes on serum TNF-α, IL-6, IL-10, IL-18, and IF-γ levels in rats

Groups	TNF-α (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-18 (pg/ml)	IF-γ (pg/ml)
Control	90.77±3.87^{c}	72.43±2.51^{c}	42.18±1.91^{c}	54.43±2.56^{c}	26.58±3.74^{c}
Oleuropein	89.64±6.94^{c}	75.82±4.62^{c}	46.18±3.79^{c}	53.07±2.61^{c}	29.22±3.19^{c}
Diabetes	515.31±12.41^{a}	687.70±11.37^{a}	329.67±22.58^{a}	677.02±17.87^{a}	155.87±4.67^{a}
Oleuropein + Diabetes	242.68±19.27^{b}	310.86±18.89^{b}	143.29±13.02^{b}	281.03±11.89^{b}	70.39±2.28^{b}

Values are mean ± S.D., n=6

^{a,b,c}: in the same column values with different letters show statistically significant differences (P <0.05)

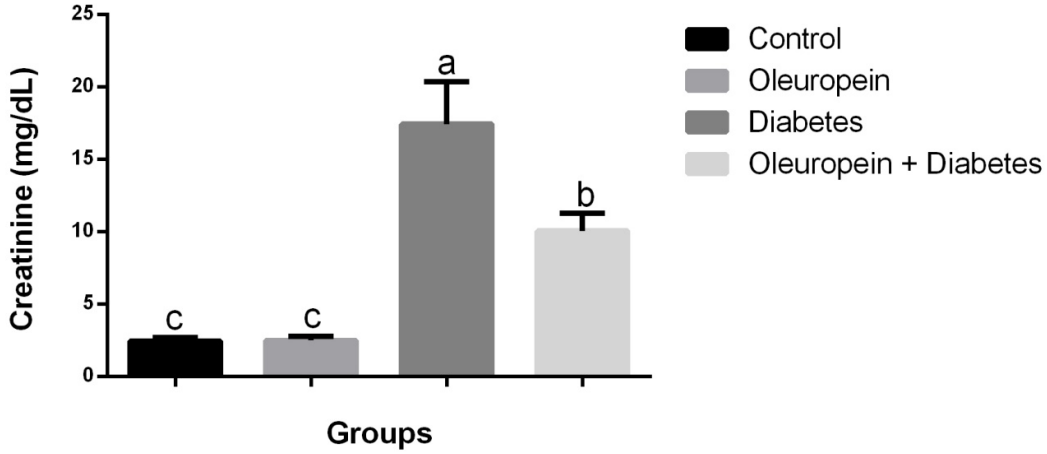

Fig. 3. The effect of diabetes alone and treated with oleuropein on serum creatinine in rats. Oleuropein was administered at 20 mg/kg per day for 28 days and STZ was administered at 50 mg/kg per day

Results are expressed as mean + SD of six rats

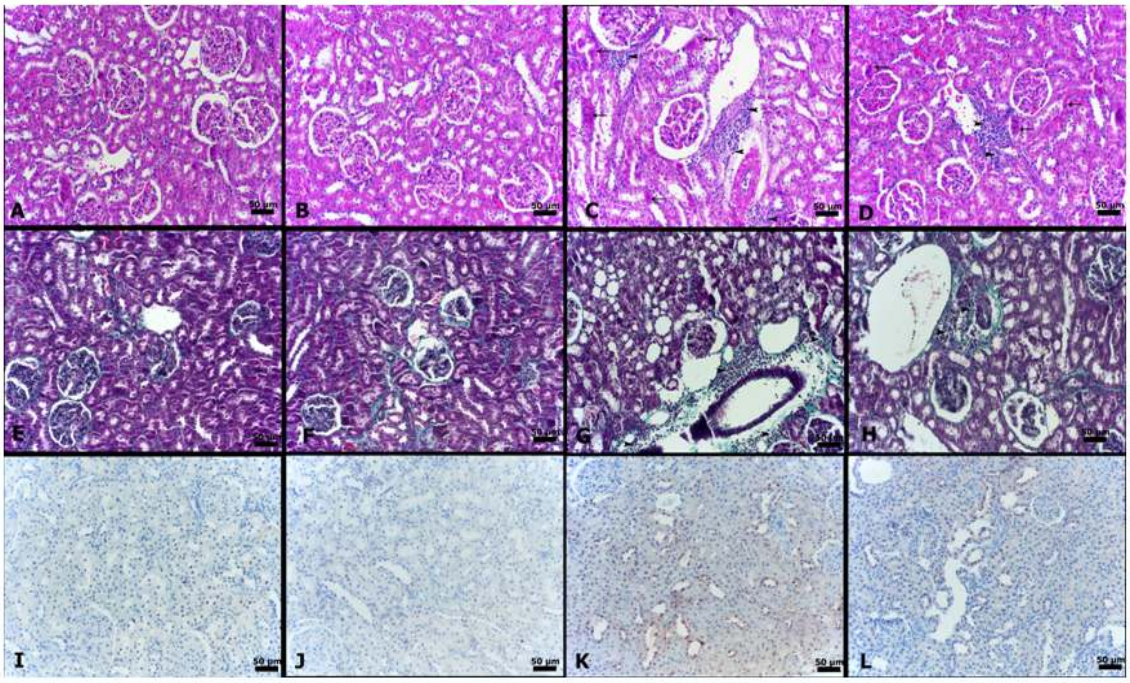

Fig. 4. The histopathological effect of oleuropein on kidney of rats induced-STZ. Representative figures were stained with hemotoxylene-eosine (H&E) (A-D), trichome (E-H), and immunohistochemically with caspase-3 (I-L). The original magnification was x 20 and the scale bars represent 20 µm. Arrows indicate degenerations and arrowheads indicate mononuclear cell infiltrations in H&E staining. In trichome staining, arrows indicate parenchyma degenerations and arrowheads indicate mononuclear cell infiltrations. Also, proliferations were shown to be green color. Immunohistochemically, red-brown cytoplasmic and nuclear expansion were observed. (A, E, and I) Control group, (B, F, and J) animals treated with 20 mg/kg per day oleuropein, (C, G, and K) animals treated with 50 mg/kg STZ, (D, H, and L) animals treated with 50 mg/kg STZ and 20 mg/kg per day oleuropein

Table 2. Effects of oleuropein at dose of 20 mg/kg and diabetes on malodialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), total oxidant status (TOS), and total antioxidant status (TAS) in kidney tissue homogenates of rats

Groups	MDA (nmol/g tissue)	SOD (U/mg protein)	CAT (nmol/mg protein)	TOS (μmol H_2O_2 equiv/L)	TAS (mmol trolox equiv/L)
Control	441.54±20.5^{c}	125.82±13.23^{c}	0.30±0.05^{c}	10.62±2.42^{c}	158.09±14.32^{a}
Oleuropein	455.88±36.35^{c}	126.62±11.56^{c}	0.32±0.06^{c}	10.59±2.41^{c}	155.34±11.22^{a}
Diabetes	910.80±39.34^{a}	321.79±37.89^{a}	0.57±0.05^{a}	37.94±2.82^{a}	72.59±12.32^{c}
Oleuropein + Diabetes	601.72±21.51^{b}	189.54±6.14^{b}	0.49±0.03^{b}	20.82±4.45^{b}	113.58±10.43^{b}

Values are mean ± S.D., n=6.

^{a,b,c}: in the same column values with different letters show statistically significant differences (P <0.05)

Table 3. Semi-quantitative analysis of immunohistochemical staining of kidney in control and diabetic groups

Groups / Animals	1	2	3	4	5	6
Control	-	-	-	-	-	-
Oleuropein	-	-	-	-	-	-
Diabetes	+++	+++	++	+++	++	+++
Oleuropein+ Diabetes	++	++	+	++	+	++

Data was expressed as weak (+), moderate (++), and strong (+++)

4. DISCUSSION

Diabetes induced by STZ to rats reduced body weight gain and increased blood glucose levels [21]. Hovewer, oleuropein administration was found to alleviate these parameters in animals. Similarly, Hazman and Ovalı [22] reported that STZ injection intended to form β-cell dysfunction led to a decrease in weight gain levels in diabetic study groups. Additionally, many researchers [22,23] have suggested that diabetes caused hyperglycemia and glucose was measured at a high level in the blood. This result is consistent with our results; glucose levels were found to be high in the blood. In contrast, glucose levels were found to be low in the oleuropein-treated groups compared to diabetic group.

These results may suggest that oleuropein exhibited its hypoglycemic effect due to its influences on energy substrate metabolism and can attenuate the rise in plasma glucose concentration [24].

Nephropathy is reported to be one of the most significant complications in experimental models with STZ injection-induced diabetes [25,26] and it has increased levels of BUN and creatinine as kidney function parameters [27].

After treatment with oleuropein, serum BUN and creatinine levels were significantly reduced. This suggests that oleuropein have nephro-protective function, hence reduced renal damage. It may be explained by the fact that oleuropein has protected kidney function, thus reduced renal damage.

Immunologic and inflammatory mechanisms play a significant role development and progression of diabetic nephropathy. Therefore, diverse cells, including leukocytes, monocytes, and macrophages, as well as other molecules, such as chemokines (IL and NfKB) are implicated in processes related to diabetic nephropathy [28]. Many research suggested that TNF-α, IL-1, IFN-γ, IL-6, and IL-18 are among the inflammatory cytokines that do affect the development of diabetic [27-30]. Hazman and Bozkurt [26] studied renal damage in diabetic rats and inflammation parameters (TNF-α, IL-1β, IFN-γ, and IL- 18) were found to be high levels in kidney tissue. Similarly, inflammation parameters (TNF-α, IL-6, IL-10, IL-18, and IF-γ) found to be at a high level in the serum of STZ-treated rats in this study. Conversely, oleuropein affects these levels, which decrease in oleuropein-administered rats compared to untreated rats in the diabetic group. The explanation for this result could be that oleuropein may alleviate the renal damage and suppressed inflammation.

Many diseases such as diabetes mellitus, atherosclerosis, and hemolytic and immune

diseases exhibit high oxidative stress due to persistent and chronic hyperglycemia, which thereby depletes the activity of the antioxidative defense system and thus promotes the generation of free radicals [6]. Several studies have found increased free radicals or oxidative stress in different animal models of diabetes [22,23]. Oxidative stress has recently been shown to be responsible for the kidney dysfunction caused by glucose toxicity and to have caused tissue damage [22].

MDA is the major peroxidation product and increased MDA content is an important indicator of lipid peroxidation [31]. In the study, diabetes caused lipid peroxidation but oleuropein inhibited its level. The reduced lipid peroxidation observed in the oleuropein-treated diabetic animals may be attributed to the important role of oleuropein as antioxidant. This activity may be attributed to its ability to decompose free radicals by quenching reactive oxygen species and by trapping radicals before reaching its cellular targets [32]. Moreover, the CAT and SOD activities were reestablished by oleuropein. It has been reported that diabetes lowered the activities of antioxidant enzymes, which is possibly due to their increased implication in fighting excessive oxidative stress in diabetic rats [6]. The increase of renal antioxidant enzyme activities observed in oleuropein-supplemented rats may be due to the removal toxic reactive species resulting from the diabetes.

Determination of TOS may indicate the concentration of all free oxidant radicals caused by diabetes-related oxidative damage. Likewise, the number of different antioxidants in all biological samples makes it difficult to measure each antioxidant separately. Thus, TAS may be an important factor in providing protection from cell damage caused by diabetes-related oxidative stress [19,20,22,23]. In this study, we evaluated oxidative stress using TAS and TOS parameters. We found increased TOS and decreased TAS levels in the serum of diabetic rats compared to control rats. These increased oxidants may be due to overproduction or decreased excretion of oxidant substances. Because of an increase in these oxidants and a decrease in total antioxidants, the oxidant/antioxidant balance shifted toward oxidative stress in the diabetic rats. Treatment of the diabetic rats with oleuropein significantly reduced TOS and increased TAS levels compared to the untreated diabetic rats. This situation showed that the oxidant/antioxidant balance shifted toward antioxidant status with the oleuropein treatment of the diabetic rats. Consistently with our results, many researchers suggested that TAS and TOS levels were increased in diabetic rats due to oxidative stress [22,23].

Many researches showed that STZ resulted histopathological changes in kidney tissue.

Muruganandan et al. [33] reported that tubular degeneration in kidneys of rats treated with STZ alone. Also, Sharma et al. [34] observed glomerular thickening, interstitial fibrosis and arteriolopathy in diabetic rats. In this study, STZ caused mononuclear cell infiltration, proliferations, tubular dilatations, vacuolar degenerations, significant increases of mesangial collagen and red-brown cytoplasmic and nuclear expansion in the kidney.

5. CONCLUSION

In conclusion, the results of this study demonstrated that oleuropein decreased amelioration of oxidant status, inflammation, and tissue damage in diabetic rats. The results of this study showed that the antioxidant and anti-inflammatory effects of oleuropein may be due to its ability to increase the antioxidant defense system and also protect cell proliferation in diabetic rats.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experimental protocols were approved by the Animal Care and Use Committee at Afyon Kocatepe University (2015/372-14) and are in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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