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Research Article

QUERCETIN AMELIORATES THE STREPTOZOTOCIN-INDUCED DIABETIC RENAL INJURY BY INHIBITING APOPTOSIS

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Abstract: Diabetes mellitus is an important health problem worldwide due to its frequency and complications. In this study, the protective effect of quercetin on the apoptotic changes of rat kidney in the early stages of diabetes induced by streptozotocin (STZ) was evaluated.
Rats are divided into 3 groups as control, diabetic and diabetic+quercetin groups. STZ was applied as a single dose of 50 mg/kg intraperitoneal (i.p.) to diabetic and diabetic+quercetin groups. Quercetin was given at 30 mg/kg i.p. once a day for 15 days, 48 hours after induction of diabetes. At the end of quercetin treatment, all animals were sacrificed and kidneys were harvested and weighed. The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay (TUNEL) for apoptosis was performed and evaluated renal histopathology.
The induction of diabetes via STZ caused a significant increase in blood glucose levels, the index of glomerulosclerosis, the histopathologic score, the number of TUNEL positive tubular and glomerular cells. Quercetin treatment lowered blood glucose levels, prevented renal cell apoptotic changes and histopathological alterations in diabetic rat kidney.
The findings of the study suggested that quercetin may be useful in preventing diabetic nephropathy by regulating renal apoptotic changes that occur in the early stages of diabetes.

Özet: Diabetes mellitus, sıklığı ve komplikasyonları nedeniyle dünya çapında önemli bir sağlık sorunudur. Bu çalışmada, streptozotosin (STZ) ile oluşturulan diyabetin erken evrelerinde böbrek dokusunda meydana gelen apoptotik değişiklikler üzerine quercetinin koruyucu etkileri değerlendirildi.
Deneklerden kontrol, diyabetik ve diyabetik + quercetin grupları olarak 3 grup oluşturuldu. Diyabetik ve diyabetik+quercetin gruplarına tek doz 50 mg/kg STZ intraperitoneal (i.p.) olarak uygulandı. Quercetin, diyabet indüksiyonundan 48 saat sonra 15 gün boyunca günde bir kez 30 mg/kg i.p. olarak verildi.
Quercetin tedavisinin sonunda tüm hayvanlar sakrifiye edildi ve böbrekler çıkartılarak, tartıldı. Böbrek dokularında TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) yöntemi ile hücre apoptozu ve ayrıca histopatolojik değişiklikler değerlendirildi.
STZ yoluyla diyabet indüksiyonu, kan glukoz seviyelerinde, glomerülosklerozu indeksinde, histopatolojik skorda, TUNEL pozitif tübüler ve glomerüler hücre sayısında önemli bir artışa neden oldu. Quercetin tedavisi kan glukoz seviyelerini düşürdü, renal hücre apoptozunu ve histopatolojik değişiklikleri azalttı.
Bu çalışmanın bulguları, quercetinin, diyabetin erken evrelerinde meydana gelen böbrekteki apoptotik değişiklikleri düzenleyerek diyabetik nefropati gelişimini önlemede faydalı olabileceğini göstermektedir.

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Introduction

Diabetes mellitus (DM) is a metabolic disease, which affects 8.3% of the world population on average and results from the insufficiency and absence of insulin hormone released from pancreatic beta (β) cells or the unresponsiveness of insulin receptors (Cheisson *et al.* 2018, American Diabetes Association 2019). Retinopathy, nephropathy, neuropathy, and cardiomyopathy are the major complications associated with DM (American Diabetes Association 2019).

Diabetic nephropathy (DN) is a chronic and complex process in which the glomeruli are affected in their early stage and is characterized by thickened glomerular basement membrane (GBM), microalbuminuria, hypertrophy, and subsequent development of

glomerulosclerosis, tubular atrophy, and interstitial fibrosis (Ichinose *et al.* 2007). Hyperglycemia, insulin resistance, inflammation, oxidative stress, apoptosis, and the activation of the renin-angiotensin system (RAS) are very important in the pathogenesis of DN (Mori *et al.* 2014). It has been demonstrated that hyperglycemia induces oxidative stress and facilitates tissue damage by increasing the number of reactive oxygen species (ROS) and reducing the protective antioxidant capacity (Vural *et al.* 2001, Bhathena & Velasquez 2002). It has been reported that high glucose concentration is very important in the development of tubular atrophy and glomerular damage by causing both podocyte and renal tubule cell apoptosis (Gilbert & Cooper 1999, Susztak *et al.* 2006, Chuang *et al.* 2007). However, in the early or late stages of DN, it has been shown that proliferative changes occur in the endothelial, mesangial, and interstitial cells of the kidney and that these affect different fibrotic processes (Li *et al.* 2003).

Quercetin has antioxidant, anti-apoptotic and anti-inflammatory effects. It is a flavonoid that is found in various vegetables and fruits (Anjaneyulu & Chopra 2004, Harwood *et al.* 2007, Al-Rasheed *et al.* 2017). Previous studies have reported that quercetin plays a protective role in experimentally induced-DN by inhibiting oxidative damage (Anjaneyulu & Chopra 2004, Gomes *et al.* 2014, Lin *et al.* 2016). However, there are limited number of studies evaluating the relationship of quercetin with apoptosis, which is an important factor in DN (Zhou *et al.* 2012, Gomes *et al.* 2014, Lin *et al.* 2016, Tunçdemir *et al*. 2018). For this reason, in this study, we evaluated the effect of quercetin on apoptotic changes of rat kidney in the early stages of diabetes induced by streptozotocin (STZ).

Materials and Methods

Ethical approval and animals

The design of the study was approved by the Ethical Committee of Trakya University (TUHADYEK 2017/15). Twenty-four male Wistar albino rats (3-4 months old, weighing 300-370 g) were used in the study. Rats were kept in special conditions (22 ± 1°C temperature, 12 h light:12 h dark cycle, access to free food and water) in Experimental Animal Center of Trakya University. Subjects were divided into three groups as control, diabetic and diabetic+quercetin, each containing 8 rats.

Experimental protocol

Baseline fasting blood glucose (FBG) levels of the subjects were measured after 12 hours of fasting. This measurement was made weekly on blood samples taken from the tail using a glucometer (IME-DC, Hof, Germany). At the beginning of the study, the body weights (Bw) and FBG levels of animals were recorded. For the diabetes induction, the diabetic and diabetic+quercetin groups were given intraperitoneally (i.p.) a single dose of 50 mg/kg STZ (Sigma Aldrich, Taufkirchen, Germany) dissolved in a 0.1 M citrate buffer (Ali *et al.* 2017). Fourthy-eight hours after administration of STZ, diabetes was confirmed by measuring FBG levels > 250 mg/dl (Kushwaha & Jena 2012).

After 48 hours of diabetes induction, 30 mg/kg i.p. quercetin (Alfa Aesar, Ward Hill, Massachusetts, USA) dissolved in dimethylsulfoxide (DMSO) (Merck Millipore, USA) were given to animals of quercetin-treated group daily for 15 days. Control animals were treated with 1 ml/kg DMSO (vehicle of quercetin) in the same way. The doses of quercetin were selected based on previous studies (Yang & Kang 2018). At the termination of the quercetin treatment, after recording final body weights and blood glucose levels, all animals were sacrificed under xylazine/ketamine anesthesia, and their kidneys were harvested and weighed. The right and left kidneys were weighed and the mean kidney weight (Kw) for each rat was calculated. To reveal the profile of renal hypertrophy, the ratio between kidney and body weight (Kw/Bw) was calculated (Liu *et al.* 2003).

Light microscopy

Kidneys fixed in 10% formalin were processed with the standard paraffin embedding method. Sections of 5 μm thickness were stained with hematoxylin-eosin (H&E) and Periodic Acid Schiff (PAS).

Light microscopic analyses were done in randomly selected areas from the medulla and cortex in each kidney section. The evaluations were carried out by blind observers at 200× magnifications. Acute kidney injury including interstitial fibrosis, tubular, and glomerular alterations were semiquantitatively graded (0: normal, 1: mild, 2: moderate and 3: severe). Additionally, 100 glomeruli for each subject were evaluated for sclerosis under 400× magnification on the sections stained with PAS. Glomerulosclerotic injury was graded on a scale of 0 to 4 and the sclerosis index was calculated (Saito et al. 1987).

For 30 glomeruli in PAS-stained section of each rat, the greatest and the smallest diameters of each glomeruli were measured using a micrometric ocular and the average diameters of the glomeruli were calculated.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Renal cell apoptosis was evaluated by the TUNEL method using the ApopTag Plus Peroxidase *in situ* Apoptosis Detection Kit (S7101, Merck Millipore, Massachusetts, USA) as described by the manufacturer. To determine the apoptosis, tubular epithelial and glomerular cells on the randomly selected 30 glomeruli were counted in 20 fields/section using a light microscope by blinded observation at 200×. The TUNEL positive tubular and glomerular cells, whose nuclei were stained dark brown, were counted.

Statistical analysis

The results were expressed as means ± SD or median (minimum – maximum). Whether the numerical variables were normally distributed was evaluated using the One-sample Kolmogorov-Smirnov test. For the control, diabetic

and diabetic+quercetin group comparisons, the One-way ANOVA test was used if the numeric variables that were normally distributed and the Kruskal Wallis test if the variable that were not normally distributed. $p<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 20.0 program (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

Blood glucose levels

After 48 h of STZ injection, as indication of diabetes, FBG levels in all animals significantly increased compared with the control animals ($p<0.05$). At the end of the study, FBG levels in the quercetin-treated animals were significantly lowered than the untreated diabetic animals ($p<0.05$) (Fig. 1).

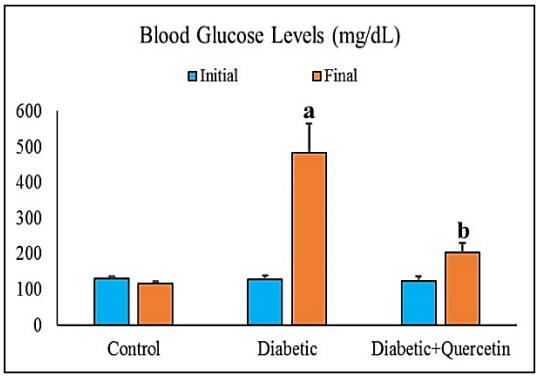

Fig. 1. The mean blood glucose levels (mg/dL) of all groups. [a] $p<0.05$ Compared to the control group, [b] $p<0.05$ Compared to the diabetic group.

Body and kidney weights

The changes in initial and final Bw for all groups are shown in Table 1. Diabetes caused a reduction in body weight. The initial body weights were similar in all groups, whereas, at the end of the experimental period, there was a marked weight loss in the diabetic and diabetic+quercetin groups. Weight loss was more pronounced, especially in the diabetic groups. Although the body weight loss of the quercetin treated group was less compared to the diabetes group, there was no statistically significant difference between the two groups ($p=0.074$).

There was no significant difference in the mean Kw among the groups (Table 1). To determine kidney hypertrophy, we calculated the Kw/Bw ratio (Table 1). All diabetic subjects had significantly higher Kw/Bw ratios compared to control. The highest ratio was determined in the diabetic group (Table 1).

Histopathological findings

The kidney morphologies of the control group were seen as normal (Fig. 2A, D). STZ-mediated diabetes caused severe glomerular and tubular alterations. Hypertrophic glomeruli causing narrowing in Bowman space were observed in the kidney of the untreated diabetic group (Figs 2B, E). The sclerotic injuries including GBM thickening, glomerular capillary collapse and mesangial matrix enlargement were determined in PAS-stained kidney section in this group (Fig. 2E). The tubular dilatation, thickening of the basement membrane, epithelial desquamation, and microvilli loss were observed in the kidney sections of this group (Figs 2B, E and inset). Additionally, the tubular vacuolation characterized by glycogen accumulation (PAS-positive) in the cytoplasm was detected in diabetic rat kidney (Figs 2B, E, and inset). In light of these findings, the kidney damage score, glomerular size, and the sclerosis index of the diabetic group were significantly higher (Table 2). In the quercetin-treated group, STZ-mediated renal structural alterations were reduced (Figs 2C, F), and in parallel, the tissue damage score, sclerosis index and glomerular size in this group was also significantly decreased (Table 2).

TUNEL assay

In the kidneys of the control groups rats, very few apoptotic cells were observed (Figs 3A, B). Diabetes induction with STZ resulted in a significant increase in both tubular epithelial and glomerular cell apoptosis (Table 2). TUNEL positive cells were detected mainly in dilated and damaged tubuli and some glomeruli. Glomerular apoptotic cells were observed to be podocytes due to their localization and size (Figs 3C, D). In the quercetin-treated group, the apoptotic tubular and glomerular cell numbers were significantly decreased compared with the untreated diabetic group (Figs 3E, F, Table 2).

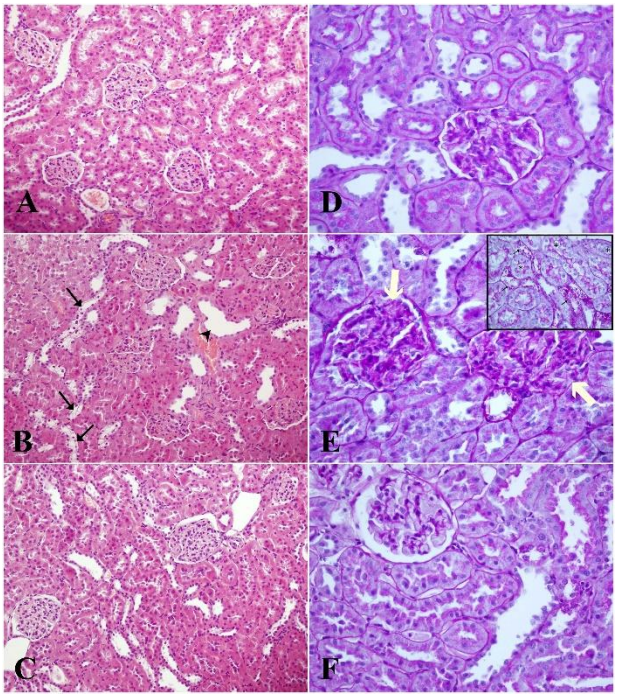

Fig 2. Photomicrographs illustrate morphological changes in rat kidney sections stained with hematoxylin eosin (A, B, C, 200×) and Periodic Acid Schiff (D, E and Inset, F, 400×): Control group (A, D), Diabetic group (B, E), Diabetic+quercetin group (C, F). Arrowheads: tubular dilatation, black arrows: glycogenic vacuolation (glycogen accumulation), asteriks: brush border loss, white arrows: degenere glomeruli enlarged mesangial matrix and narrowed Bowman space.

Table 1. Body weight and kidney weight of the subjects.

	Control	Diabetic Group	Diabetic+Quercetin Group	*p*
Initial Bw	336.17 ± 22.13	330 ± 25.93	327.50 ± 18.91	0.793
Final Bw	352.33 ± 19.98	231.50 ± 38.91 [a]	269.67 ± 25.97 [b]	<0.001
Bw difference	16.50 ± 23.90	-98.50 ± 28.36 [a]	-57.83 ± 35.19[b]	<0.001
Mean Kw	1.35 ± 0.19	1.46 ± 0.18	1.37 ± 0.15	0.552
Kw/Bw (x10^{-3})	3.50 ± 0.84	6.00 ± 0.89 [b]	4.67 ± 0.52[c]	0.002

Data were shown as mean ± S.D. Bw: body weight, Kw: kidney weight. [a] p<0.001 Compared to the control group, [b] p<0.01 Compared to the control group, [c] p<0.05 Compared to the diabetic group.

Table 2. The kidney damage score, sclerosis index, glomerular size and the number of TUNEL positive renal cells of all groups.

	Control	Diabetic Group	Diabetic+Quercetin Group	*p*
Kidney damage score	1 (0-2)	5.5 (4-6) [a]	3.0 (3-4) [ab]	0.001
Sclerosis index	0.2 (0.1-0.3)	1.4 (1.3-1.6) [a]	0.95 (0.9-1.0) [ab]	<0.001
Glomerular size (µm)	115.11 ± 1.25	135.53 ± 1.60[a]	126.00 ± 0.76 [ab]	<0.001
TUNEL positive tubular cells	0.30 (0.1-0.5)	47.5 (42.5-65.0) [a]	8.75 (6.5-17.5) [ab]	<0.001
TUNEL positive glomerular cells	0.5 (0.3-0.5)	2.3 (1.6-2.8) [a]	0.6 (0.6-0.7) [ab]	<0.001

Data were shown as mean ± S.D. or median (minimum – maximum). [a] p<0.05 compared to the control group, [b] p<0.05 compared to the diabetic group.

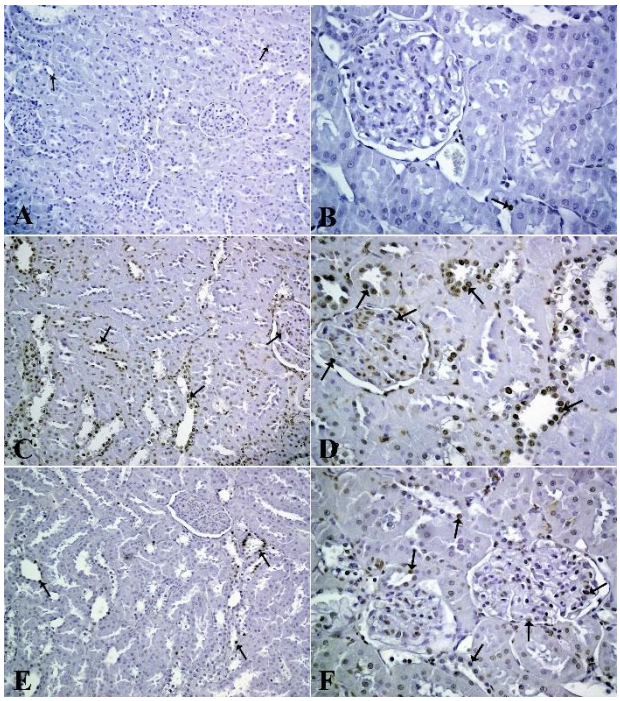

Fig. 3. Detection of apoptotic changes in diabetic rat kidney by TUNEL method. Control group (A, B), Diabetic group (C, D), Diabetic+quercetin group (E, F). Brown staining (arrows) indicates TUNEL positive cells. (A, C, E 200×; B, D, F 400×).

Discussion

In the pathogenesis of DN, hyperglycemia, insulin resistance, inflammation, oxidative stress, apoptosis, and RAS activation play an important role, besides genetic, metabolic, and hemodynamic factors (Ichinose *et al.* 2007, Mori *et al.* 2014).

Our findings show that quercetin treatment decreases blood glucose level and the kidney damage (histopathological score and glomerulosclerosis index) by regulating the renal cell apoptosis accompanying STZ-mediated diabetic nephropathy. Although hyperglycemia was observed in all diabetic animals from the 48th hour following STZ injection until the end of the experiment, quercetin treatment caused a significant decrease in high blood glucose values. This effect of quercetin on blood glucose levels is supported by previous studies (Vessal *et al.* 2003, Gomes *et al.* 2014, Elbe *et al.* 2015, Roslan *et al.* 2017, Tunçdemir *et al.* 2018, Senyigit *et al.* 2019). Vessal *et al.* (2003) reported that quercetin increased insulin release by regenerating β cells of Langerhans islets damaged by STZ in diabetic rats.

It is known that reduction of body weight occurs in diabetic individuals (Andallu & Varadacharyulu 2003). As shown in many studies (Kelly *et al.* 2002, Tunçdemir & Ozturk 2008, Elbe *et al.* 2015), the body weights in diabetic animals decreased significantly in our study. However, literature information on the renal weight in diabetes is controversial. Some studies reported that kidney weights of diabetic animals increased together with a glomerular enlargement (New *et al.* 1996, Tunçdemir & Ozturk 2008). However, it has been shown that diabetes does not alter kidney weight (Kelly *et al.* 2002, Offor *et al.* 2019) or even decreases (Coldiron *et al.* 2002). Despite the increase in glomerular diameters in our study, there was no statistically significant difference between the groups in terms of kidney weights. Renal hypertrophy is considered to be an indicator of structural damage that occurs in diabetic nephropathy (Ziyadeh & Goldfarb 1991). The ratio of "renal weight/body weight" of the diabetic group increased as an indicator of

hypertrophic changes in the kidney. As shown in other DN studies (Vessal *et al.* 2003, Elbe *et al.* 2015), we detected quercetin treatment prevented weight loss due to diabetes, and significantly decreased the kidney weight/body weight ratio and glomerular diameters.

DN is a chronic and complex process in which glomeruli are affected at the early stage and is characterized by tubular atrophy and interstitial fibrosis, leading to a gradual reduction of renal function with the development of glomerulosclerosis (Gilbert & Cooper 1999, Ichinose *et al.* 2007). It is known that hyperglycemia causes oxidative stress and increased oxidative stress in the diabetic kidney promotes apoptosis (Vincent *et al.* 2004, Armagan *et al.* 2006). It has been indicated that genes controlling apoptosis are affected in hyperglycemic conditions and it contributes to the development of diabetic nephropathy (Ortiz *et al.* 1997, Bamri-Ezzine *et al.* 2003). It has been shown the role of podocyte apoptosis in the glomerular injury, which is critical at the beginning of DN, and also, importance of the tubular cell apoptosis the in the tubular atrophy and the progression of the disease (Gilbert & Cooper 1999, Susztak *et al.* 2006, Chuang *et al.* 2007). Additionally, it is known that high levels of ROS under hyperglycemic conditions induce extracellular matrix (ECM) production by activation of the TGF-β Smad signaling pathway in various cells such as tubular epithelial cells, mesaengial cells, vascular endothelial cells (Chen *et al.* 2001, Yasuda *et al.*2001, Li *et al.*2003). The most prominent histological change in diabetic kidneys is the enlargement in mesangium as a result of excessive production and deposition of ECM proteins such as collagen type IV, fibronectin, and laminin (Kolset *et al.* 2012). Similar to other studies (Ichinose *et al.* 2007, Tunçdemir & Ozturk 2008, Offor *et al.* 2019) GBM thickening, mesangial matrix increase, Bowman distance constriction which is mediated by glomerular enlargement with collapsed luminal glomerular capillaries, were also detected in the kidneys of the diabetes group in the present study. An increase in the glomerular size and sclerotic index of the diabetic group were observed as a result of these changes. The histopathological findings obtained were consistent with the results of other studies (Sanai *et al.* 2000, Geoffroy *et al.* 2005, Giribabu *et al.* 2017). Previous studies indicated that glomerulosclerotic changes occurred in the early stages of DN and increased over time (Tucker *et al.* 1991, Sanai *et al.* 2000). Although ECM accumulation and GBM thickening are important mechanisms in the pathogenesis of DN, studies show that podocyte apoptosis caused by hyperglycemia also plays an important role in the development of DN (Susztak *et al.* 2006, Zhou *et al.* 2012). The decrease in the number of podocytes is one of the main reasons for the onset pathogenesis of DN (Wang *et al.* 2018). It has been indicated that renal damage could be prevented by reducing podocyte apoptosis in the early stage of DN (Zhou *et al.* 2012). As a result of oxidative stress in DN, the formation of advanced glycation end products (AGE) is accelerated and apoptosis in podocytes is thought to occur with the accumulation of AGE. The contribution of AGEs and their specific receptors (RAGE) to the development of diabetic nephropathy is known. RAGE is usually localized in podocytes and was shown to increase in diabetes. RAGE activation increases the production of ROS that mediates podocyte apoptosis due to hyperglycemia in early-stage DN (Tan *et al.* 2007, Chuang *et al.* 2007). Chuang *et al.* (2007) reported that AGE and its receptors induce podocyte apoptosis. In our study, the TUNEL analysis revealed an increased podocyte apoptosis. Similarly, recent studies reported increased podocyte apoptosis in the kidney of STZ-induced diabetic rats (Zhou *et al.* 2012, Giribabu *et al.* 2017).

It has been stated that hyperglycemia and AGE accumulated in cells play an important role in the pathogenesis of the changes observed in the tubules in DN (Tan *et al.* 2007). Bleyer *et al.* (1994) showed that tubular cells can be directly damaged by hyperglycemia. The excess glucose in the glomerular filtrate is reabsorbed in the proximal tubules and further increases the effects of hyperglycemia in the proximal tube. Exposure to high glucose stimulates collagen synthesis by increasing TGF-β release in tubular cells, thus leading to thickening of the basement membrane (Nessar 2005). Similar to other studies (New *et al.* 1996, Gilbert & Cooper 1999, Tunçdemir & Ozturk 2008, Offor *et al.* 2019), our study showed that tubular changes associated with diabetes are basal membrane thickening, glycogenic vacuolization in epithelial cells, microvilli loss, and dilatation. We also observed a significant increase in the number of TUNEL-positive tubular cells in diabetic kidneys. It is known that increased oxidative stress in the diabetic kidney due to the formation of free radicals induced by hyperglycemia promotes apoptosis and that apoptosis mediates the development of DN (Allen *et al.* 2003). Bamri-Ezzine *et al.* (2003) reported that glycogen accumulated in tubules triggers apoptosis of epithelial cells which leads to tubular atrophy. Consistent with the findings of the present study, an increase in tubular epithelial cell apoptosis was reported in diabetic kidney by the TUNEL method (Tunçdemir & Ozturk 2008, Ji *et al.* 2019).

The flavonoid quercetin we used in our study is known to exhibit antioxidant, anti-inflammatory, and antiapoptotic properties (Anjaneyulu & Chopra 2004, Harwood *et al*. 2007, Roslan *et al.* 2017, Al-Rasheed *et al.* 2017). Quercetin is also an antidiabetic compound that targets hyperglycemia (Vessal *et al.* 2003). Senyigit *et al.* (2019) reported that quercetin treatment significantly reduces the progression of STZ-induced hyperglycemia and oxidative stress in rats. During the course of diabetes, it is known that excessive formation of AGEs together with ROS increased by hyperglycemia mediates the development of DN and AGE formation decreases as a result of the use of flavonoid-containing antioxidants (Kaur *et al*. 2017). Li *et al.* (2014) stated that quercetin can capture methylglyoxal, a glucose metabolite, thus preventing the formation of AGE.

Previous studies reported that quercetin plays a protective role in experimentally induced-DN by inhibiting oxidative damage (Anjaneyulu & Chopra 2004, Gomes *et al.* 2014, Lin *et al*. 2016). According to our current knowledge, there is a limited number of studies assessing the effect of quercetin on renal apoptotic changes induced by DN (Zhou *et al.* 2012, Gomes *et al.* 2014, Lin *et al.* 2016, Tunçdemir *et al.* 2018). Zhou *et al*. (2012) demonstrated that pretreatment with the total flavone glycosides of Flos Abelmoschus manihot could prevent renal damage and podocyte apoptosis and thus decrease urinary albumin excretion in early-stage DN. Gomes *et al.* (2014) reported that quercetin treatment had beneficial effects on renal function and structural changes and also decreased oxidative stress and apoptosis in the kidney of STZ-induced DN mice. A recent study indicated that antiapoptotic effects of quercetin might be useful in reducing STZ-mediated DN (Tunçdemir *et al.* 2018). Our findings show that quercetin treatment decreases kidney damage (histopathological score and sclerosis index) in STZ-mediated diabetic rats by regulating renal tubular and glomerular cell apoptosis and lowering blood glucose levels. We think that this study may contribute to the literature by emphasizing the effects of quercetin on apoptotic changes and outlines a novel therapeutic strategy for this flavonoid in the treatment of DN.

Conclusion

In conclusion, the results obtained in this study indicated that quercetin of which antioxidant and antidiabetic effects are known was found to be useful in preventing the development of DN by regulating apoptotic changes that occur in the early stages of diabetes.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Trakya University by the number TUHADYEK 2017/15.

Author Contributions: Concept: E.C.S., Y.T.T, Desing: E.C.S., Y.T.T, Execution: E.C.S., Y.T.T, Material supplying: E.C.S., Y.T.T, Data acquisition: E.C.S., Y.T.T, Data analysis/interpretation: E.C.S., Y.T.T, Writing: E.C.S., Y.T.T, Critical review: E.C.S., Y.T.T.

Conflict of Interest: The authors have no conflicts of interest to declare.

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