Research Article

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Evaluation of Renal Effects of Dapagliflozin in Diabetic Rats With Subacute Exposure

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ABSTRACT

Dapagliflozin (DAPA), a sodium glucose co-transporter 2 (SGLT2) inhibitor, is used for the treatment of type 2 diabetes. Although several studies have demonstrated its protective effects on the kidney, the FDA warns about the risk of DAPA-induced nephrotoxicity. SGLT2 inhibitors may induce oxidative stress and inflammation in the kidney due to their mechanism of action. In the present study, it was aimed to clarify the molecular effects of DAPA on the kidney. Diabetes was induced by single injection of streptozotocin (STZ) (35 mg/kg b.w.) after the rats were fed with high-fat diet for two-weeks. Diabetic rats were administered with DAPA at 10 mg/kg by oral gavage for 28 days. The oxidative stress, inflammation and apoptosis induction potentials of DAPA were evaluated. The morphological changes and apoptosis were investigated by histological examinations. The findings showed that DAPA treatment reduced oxidative parameters and slightly inhibited inflammatory mediator levels. According to the histological examinations, DAPA ameliorated the diabetes-induced changes and apoptosis. As a result, DAPA showed a protective effect on the kidney by alleviating oxidative stress and inhibiting inflammation and apoptosis. However, further studies are needed to determine the long-term effects of DAPA on the kidney in diabetic patients.

Keywords: Dapagliflozin, SGLT2 inhibitors, nephrotoxicity, diabetes, renoprotective effect

1. Introduction

Sodium glucose co-transporter 2 (SGLT2) inhibitors are a novel therapy with non-insulin dependent action for the treatment of type 2 diabetes mellitus (T2DM). Dapagliflozin (DAPA), a SGLT2 inhibitor, was approved in 2014 for the treatment of T2DM, and it is used as a monotherapy along with diet and exercise or in combination with other antidiabetic drugs, such as metformin or saxagliptin [1,2]. DAPA was recently approved by the FDA for reducing hospitalization related to heart failure in patients who have cardiovascular diseases or cardiovascular diseases risk factors in T2DM and decreasing the risk of sustained eGFR decline, end stage kidney disease cardiovascular death and hospitalization for heart failure in adults with chronic kidney disease at risk of progression [3,4]. However, the FDA warns health professionals and patients about DAPA-induced nephrotoxicity, and one of the main concerns is acute kidney injury [5-7]. Since SGLT2 inhibitors decrease glucose by urinary excretion, they have been stated to be associated with acute nephrotoxic effect due to higher uric acid levels in kidney tubules, which may induce inflammation and oxidative stress. Also, SGLT2 inhibition could make diabetic patients vulnerable to acute kidney injury because of shifting oxygenation from medulla to cortex [8]. Some reports have recently been published on DAPA-induced kidney damage [6,7]. However, DECLARE–TIMI 58 trial reported that DAPA decreased acute kidney injury and risk of death due to kidney diseases [9] and another clinical trial called DAPA-CKD showed DAPA reduced kidney failure and can be used in patients with chronic kidney disease to reduce cardiac complications [10]. Although nephrotoxicity has been reported after DAPA treatment, clinical studies have shown that DAPA reduces kidney failure and death from renal causes. In the present study, it was aimed to investigate the effects of DAPA on the kidney focusing on oxidative stress, inflammation which play important role in diabetic nephropathy and nephrotoxicity in diabetic male rats.

2. Material and Methods

2.1. Animal and study design

A total of twenty-one (10-12 weeks old) male Sprague-Dawley rats were obtained from the Acibadem University Laboratory Animal Application and Research Centre. Rats were housed in polystyrene standard cages with 4-5 animals each, and they were maintained at 22-24°C and 55±10% humidity at Istanbul University Faculty of Pharmacy Animal Facility Unit (EDEHAB). The study was conducted in accordance with and the guidelines approved for animal experimental procedures by the Istanbul University Local Ethics Committee of Animal Experiments (IUHADYEK, approval number 2018/24).

After 2 weeks of dietary manipulation (60% fat, 20% protein, and 20% carbohydrate), diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (35 mg/kg b.w. in citrate buffer), and the healthy control group received a single i.p. injection of citrate buffer and fed with standard pellet chow diet during the experiment [11]. The blood glucose level was measured with an automated glucose analyzer device after one week following STZ injection. Animals with blood glucose level >270 mg/dL were considered diabetic and enrolled in the study [11,12].

Rats were randomly allocated into three groups. DAPA was suspended in 0.5% methyl cellulose, and the diabetic DAPA-treatment group was treated with 10 mg/kg DAPA for 28 days. DAPA-treated healthy animal group could not be included in the study due to lack of ethical approval by the local ethic committee, since it can cause hypoglycemia risk in healthy animals.

- *Healthy control group (n:5)*

  35 mg/kg citrate buffer injection (i.p.),

  1 ml/kg water (p.o.)

- *Diabetic control group (n:8)*

  35 mg/kg STZ injection (i.p.),

  1 ml/kg 0.5% methyl cellulose (p.o.).

- *Diabetic DAPA-treated (n:8)*

  35 mg/kg STZ injection (i.p.),

  1 ml/kg b. DAPA (p.o.)

2.2. Changes in body weights, relative kidney weights and blood glucose levels

Rats were weighed at the beginning, three times a week and at the end of the experiments (Sartorius, Mettler H20, Germany). All animals were observed for feed consumption and clinical signs, including locomotor activity, mortality and posture. At the

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end of 28th day, the rats were sacrificed by removing a large volume of blood from orbital veins under inhalation anesthesia. Kidneys were surgically dissected and weighed (Precisa XB220A, Switzerland), and the relative kidney weights were calculated. The blood glucose levels were monitored in tail blood samples using an automated glucose analyser device for one week after STZ injection (Vivacheck, Biotech Inc., China).

2.3. Determination of oxidative stress potential

Malondialdehyde (MDA), a marker of lipid peroxidation, and reduced glutathione (GSH) and total oxidant status (TOS) levels were evaluated in kidney homogenates using commercial kits according to the manufacturer's instructions. The kidney tissues were homogenized in phosphate-buffered saline (PBS) (1:10, w/v) and kept at -80°C (Daihan-Scientific Wisecry, South Korea). A thiobarbituric acid reactive substances (TBARS) assay (AssayGenie, Ireland) was used to measure lipid peroxidation levels, and TOS levels were evaluated using commercial kits according to manufacturer's instructions (Sunred, China).

2.4. Determination of inflammatory cytokines

Blood samples were centrifuged at 3000 g for 10 min at -4°C (Hettich Universal 32R, Germany). Plasma was separated and stored at -80°C (Daihan-Scientific-Wisecry, South Korea).

Plasma interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 18 (IL-18), TNF alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) levels were evaluated with the LEGENDplex™ Rat Inflammation Panel (BioLegend, USA) according to the manufacturer's instructions using a FACSCalibur flow cytometer (BD Biosciences, USA). The results were analysed by LEGENDplex v8.0 software (BioLegend, USA).

2.5. Determination of gene and protein expression levels

Gene expression levels were evaluated using EvoScript RNA Probes Master and Catalogue Assays (Roche Life Sciences, Switzerland) on a Light Cycler 480 (Roche Life Sciences, Germany) by real-time polymerase chain reaction (RT-PCR). RNA isolation was performed using a High Pure RNA Tissue Kit (Roche Life Sciences, Germany) in a benchtop laminar flow hood according to manufacturer's instructions. Gene expression levels were determined with 5 µL of RNA in a final 20 µL reaction mix. cDNA synthesis and quantification were performed simultaneously in the reaction mix. mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method after housekeeping gene normalization [13]. β-actin was used as the housekeeping gene in the assays, and the analysis was performed in triplicate for each sample. The expression levels of), c-Jun N terminal kinase (JNK, Assay ID: 05583055001), nuclear factor kappa beta (NFκB, Assay ID: 05583055001), Kelch like ECH- associated protein 1 (Keap1, Assay ID: 05583055001), were examined.

Kidney samples were homogenized in radioimmunoprecipitation (RIPA) lysis buffer according to the manufacturer's instruction (Santa Cruz Biotechnology, USA). After homogenization, homogenates were centrifuged at 14000 rpm for 15 min at +4°C, and the supernatant was collected and stored at -80°C. Sample protein concentrations were determined using a Pierce™ BCA Protein Assay Kit (23225 Thermo Fisher Scientific, USA). Proteins were separated by 7% SDS/PAGE and transferred on to 0.22 µm nitrocellulose membranes (Bio-Rad, Germany). Membranes were blocked in 5% skim milk at room temperature for 1 h and then incubated with primary antibodies against NFκB (Abcam-ab16502, UK, 1:1000 dilution), JNK (Abcam-ab199380, UK, 1:1000 dilution), and β-actin (Santa Cruz Biotechnology, USA, 1:5000 dilutions) at +4°C overnight. Membranes were washed three times with TBS-Tween20 and incubated with HRP-conjugated goat anti-rabbit (Thermo 31460-Thermo Fisher Scientific, USA, 1:20000 dilution) or HRP-conjugated rabbit anti-mouse (Abcam-ab6728, UK, 1:20000 dilution) secondary antibodies for 1 h at room temperature. β-actin was used as the loading control. Imaging analysis was performed using the Pierce™ ECL Western Blotting Substrate (32106 Thermo Fisher Scientific, USA) in Fusion Fx–Vilber Lourmat (France).

2.6. Histological examinations

Kidney samples were fixed in 10% neutral buffered formalin and then examined with routine histological processing. Approximately 4-µm-thick paraffin sections were stained with haematoxylin and eosin. Sections were examined and photographed using a light microscope (BX51; Olympus Corp., Japan) attached to a digital camera (DP72; Olympus Corp., Japan).

The following components were evaluated in each section: glomerular structure; Bowman's capsule; proximal and distal tubules; and interstitial and glomerular vascular congestion.

2.7. Detection of apoptosis induction

The terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method was used according to the manufacturer's instructions (Millipore, USA). Paraffin sections were incubated with proteinase K, washed with distilled water and incubated with 3% hydrogen peroxide in PBS. Sections were then washed with PBS, placed in balance buffer and incubated with recombinant terminal transferase TdT enzyme at 36-37°C. Sections were then rinsed in washing buffer, washed in PBS, incubated with anti-digoxigenin conjugate and washed again with PBS. Slides were incubated in DAB, washed with distilled water and counterstained with Mayer's haematoxylin. Slides were then dehydrated with an alcohol series, cleared with toluene and mounted with Entellan. $TUNEL^+$ cells were evaluated in at least 20 similar areas in each section.

2.8. Statistical analysis

The significant differences between the experimental groups were analysed by one-way analysis of variances (ANOVA) using SPSS v.20 (Chicago, IL, USA). Significant differences were determined by Tukey's test, and the results are presented as mean ± standard error of the means (SEM). Changes of body weights and blood glucose levels were analyzed using paired sample t test.

3. Results and Discussion

SGLT2 inhibitors may cause nephrotoxic effects by inducing oxidative stress and inflammation [7,14]. DAPA is a new SGLT2 inhibitor for the treatment of T2DM and, it is unclear whether DAPA has a toxic or a protective effect on the kidney. DAPA is used at 5 mg or 10 mg once daily in humans. FDA warns patients and health professionals about DAPA-induced nephrotoxicity in 2016 [5], and it has been reported DAPA induced acute kidney injury within one month after starting of the treatment in the literature [6,7]. However, the results of DAPA-CKD clinical trial showed end-stage kidney disease, death from renal or cardiovascular causes is significantly lower in DAPA group in patients with chronic kidney disease, regardless of the presence or absence of diabetes [15]. Besides, DAPA showed reno-protective effects at 0.1 mg/kg and 1 mg/kg doses in experimental models [16,17]. The antidiabetic activity of DAPA was found at 0.1-10 mg/kg dose in rats and urinary glucose excretion increased in a dose dependent manner [18]. No observed adverse effect level (NOAEL) value of DAPA was 50 mg/kg/day in rats for 4-week study [19]. It was aimed to clarify the effects of DAPA on oxidative stress, inflammation, and apoptosis in diabetic rats at 10 mg/kg b.w, at which under area of plasma concentration-time curve (AUC) in Sprague Dawley rats is 130 times higher relative to humans at the same dose in the study [20].

In the last day of the experiment compared to the first day, there was a significant decrease in the body weight of the animals in the diabetic control; however, the body weight increased significantly (p=0.02) in the healthy control group. The body weight of DAPA-treated groups slightly increased (p>0.05). Relative kidney weights (≥37.17%; p<0.05) were higher in diabetic groups compared to healthy controls. STZ (35 mg/kg b.w.) i.p. induced diabetes in the diabetic control and diabetic DAPA-treated groups (Table 1). Similarly, it has been reported body weight reduces after inducing T2DM with STZ (30 and 35 mg/kg) injection in animals fed high fat diet [21,22]. Relative kidney weight increased in diabetic groups in our study. Kidney weight has also been demonstrated to increase in diabetic animal model [23].

It is well known that chronic hyperglycemia causes production of reactive oxygen species, and oxidative stress is one of the underlying mechanisms for the onset of diabetic nephropathy and nephrotoxicity [24,25]. In the present study, the levels of MDA (≥74.35%; p<0.05) and TOS (≥45.21%; p<0.05) levels were significantly increased in the diabetic groups. Conversely, plasma GSH levels of diabetic animals reduced (p>0.05). As shown in Figure 1, the increase of MDA (p=0.02) significantly alleviated by DAPA treatment compared to diabetic control group. The total oxidative status was insignificantly reduced in the diabetic DAPA-treated group compared to the diabetic control group.

The Keap1 signalling pathway is important for oxidative stress response. Keap1 gene expression is induced with increasing oxidative stress [26], and it is also up-regulated in diabetes (27). Accordingly, our results showed that Keap1 (≥4-fold; p<0.05) in-

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Table 1. Body weights, relative kidney weights and blood glucose levels of the animals.

Groups	Healthy control group (n:5)	Diabetic control group (n:8)	Diabetic DAPA-treated group (n:8)
Weights (g)			
Body weights (Day 1)	355.84 ± 14.75	314.52 ± 10.25	318.10 ± 15.57
Body weights (Day 28)	377.00 ± 15.18	296.85 ± 16,27*	330.55 ± 15.94
Relative kidney weights (mg/g)	0.35 ± 0.01	0.51 ± 0.02*	0.48 ± 0.01*
Blood glucose levels (mg/dL)			
Day 1	133.00 ± 5.02	127.67 ± 8.11	122.50 ± 6.82
One-week after STZ injection	-	296.25 ± 27.46*	278.2 ± 5.62*
Day 28	136.40 ± 5.50	398.75 ± 38.92*	143.38 ± 1.74*#

Data are shown as the mean ±SEM. *p<0.05 healthy control group versus diabetic groups, #p<0.05 diabetic control versus diabetic DAPA-treated group.

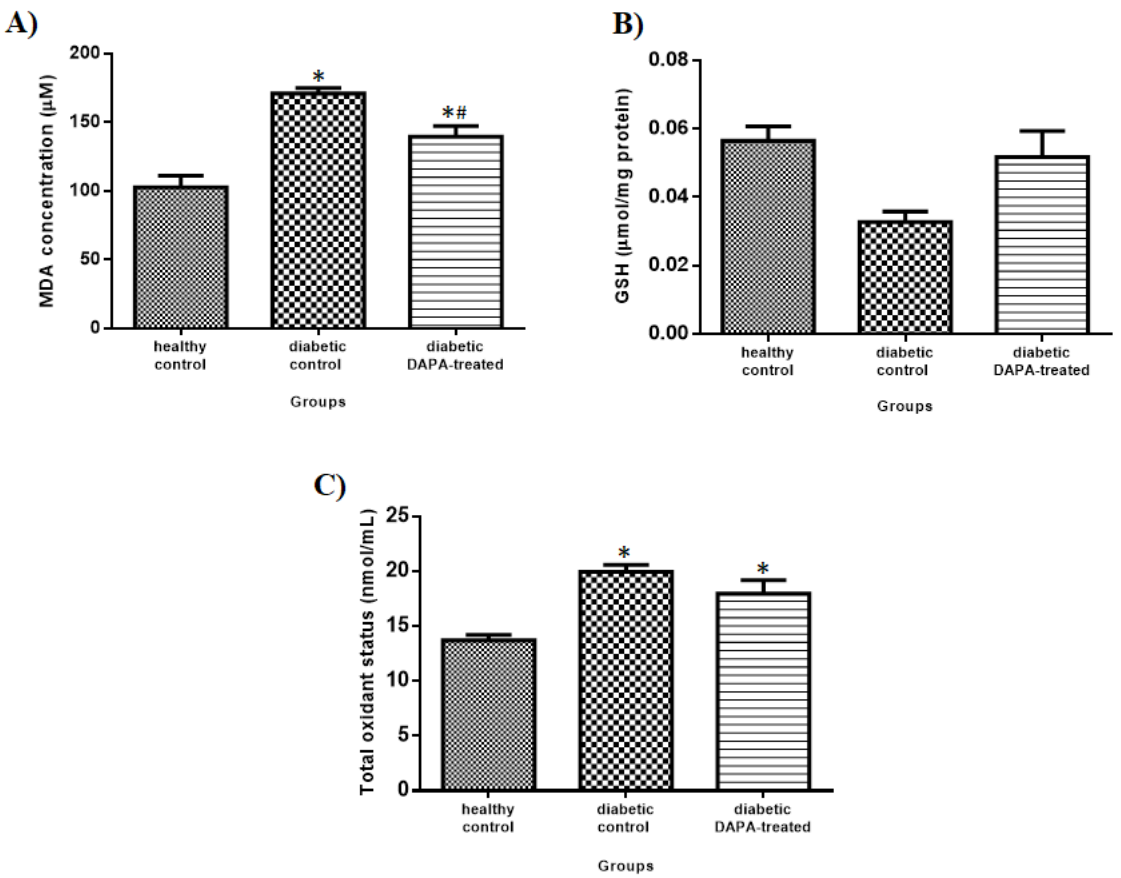

Figure 1. Effects of DAPA on A) MDA, B) GSH and C) Total antioxidant status on the kidney. Data are shown as the mean ±SEM. *p<0.05 healthy control group versus diabetic groups #p<0.05 diabetic control versus diabetic DAPA-treated group. MDA, malondialdehyde; GSH, reduced glutathione. Healthy control group (n:5), diabetic control group (n:8) and diabetic DAPA-treated group (n:8).

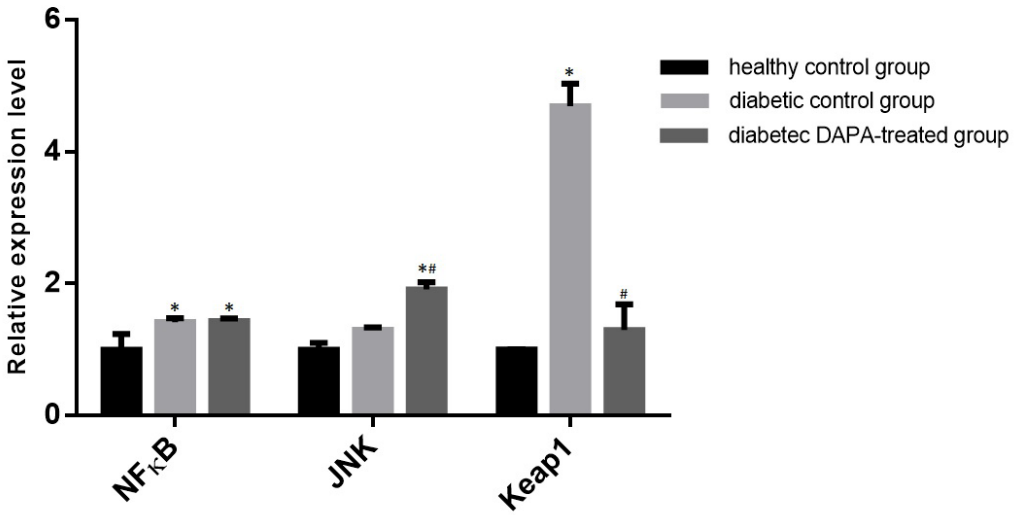

Figure 2. Effects of DAPA on gene expression levels in the kidney homogenates. Data are shown as the mean ± SEM. *p<0.05 healthy control group versus diabetic groups #p<0.05 diabetic control versus diabetic DAPA-treated group. *NFκB*, nuclear factor kappa beta; *JNK*, c-Jun N terminal kinase; *Keap1*, Kelch like ECH associated protein 1; β-actin was used as the housekeeping gene. Healthy control group (n:5), diabetic control group (n:8) and diabetic DAPA-treated group (n:8).

creased significantly in the diabetic control group compared to the healthy control group. DAPA treatment significantly reduced the increase in Keap1 gene expression levels (≥3-fold; p<0.05) (Figure 2).

Inflammation is one of the possible mechanisms for the onset of kidney toxicity. MCP-1 IL-1, IL-6, IL-18 and TNF-α are important inflammatory mediators for the development and progression of diabetic nephropathy and kidney toxicity [28-30]. Plasma MCP-1 levels increased significantly in all diabetic groups (≥2.3-fold; p<0.05). TNF-α, IL-6 and IL-18 were elevated nearly 2-fold (p<0.05) in the diabetic control group compared to the healthy control group. MCP-1 and IL-18 levels decreased significantly (p<0.05) in the diabetic DAPA-treated group compared to the diabetic control group. Plasma IL-1 levels showed a non-significant increase in the diabetic groups (Figure 3).

NFκB is a key transcription factor for inflammatory responses regulated by cytokines and chemokines [29]. It has been known that NFκB is up-regulated in diabetic conditions [31]. Similarly, NFκB expression was significantly increased (≥0.5-fold; p=0.03) at the transcription level in the diabetic control and diabetic DAPA-treated groups compared to the healthy controls (Figure 2). When it was examined NFκB protein expression level, we found that NFκB protein expression was significantly higher (60%; p<0.05) in the diabetic control group. This increase was slightly reduced (27.64%; p>0.05) by DAPA treatment compared to the diabetic control group (Figure 4). Translation of mRNA to protein is complex because protein expression is affected by many factors [32]. Therefore, mRNA and protein expression could be different. Additionally, NFκB which is stimulated by TNF-α, IL-1 and IL-18, and also induces the release of TNF-α, IL-1 and IL-6 [29,33]. Therefore, the increase in the NFκB protein level may have resulted from elevated TNF-α and IL-18 levels or vice versa. Also, DAPA treatment may decrease the NFκB protein level by reducing TNF-α and IL-18, or decreased TNF-α and IL-18 levels, which is a result of reduced NFκB level, and DAPA may protect against kidney toxicity by inhibiting inflammatory responses. Similarly, previous experimental studies demonstrated that NFκB, TNF-α, IL-1, and IL-18 levels decreased after 4 weeks of 1 mg/kg DAPA-treatment [17,34].

JNK is a transcription factor that has roles in the regulation of cellular responses, such as inflammation and apoptosis [35]. JNK is found to be activated in many tissues in diabetic conditions [36]. It has been reported that there is a correlation between JNK activation and loss of renal function in patients with diabetic nephropathy. Furthermore, IL-1, IL-6 and TNF-α all activate JNK signalling, and this activa-

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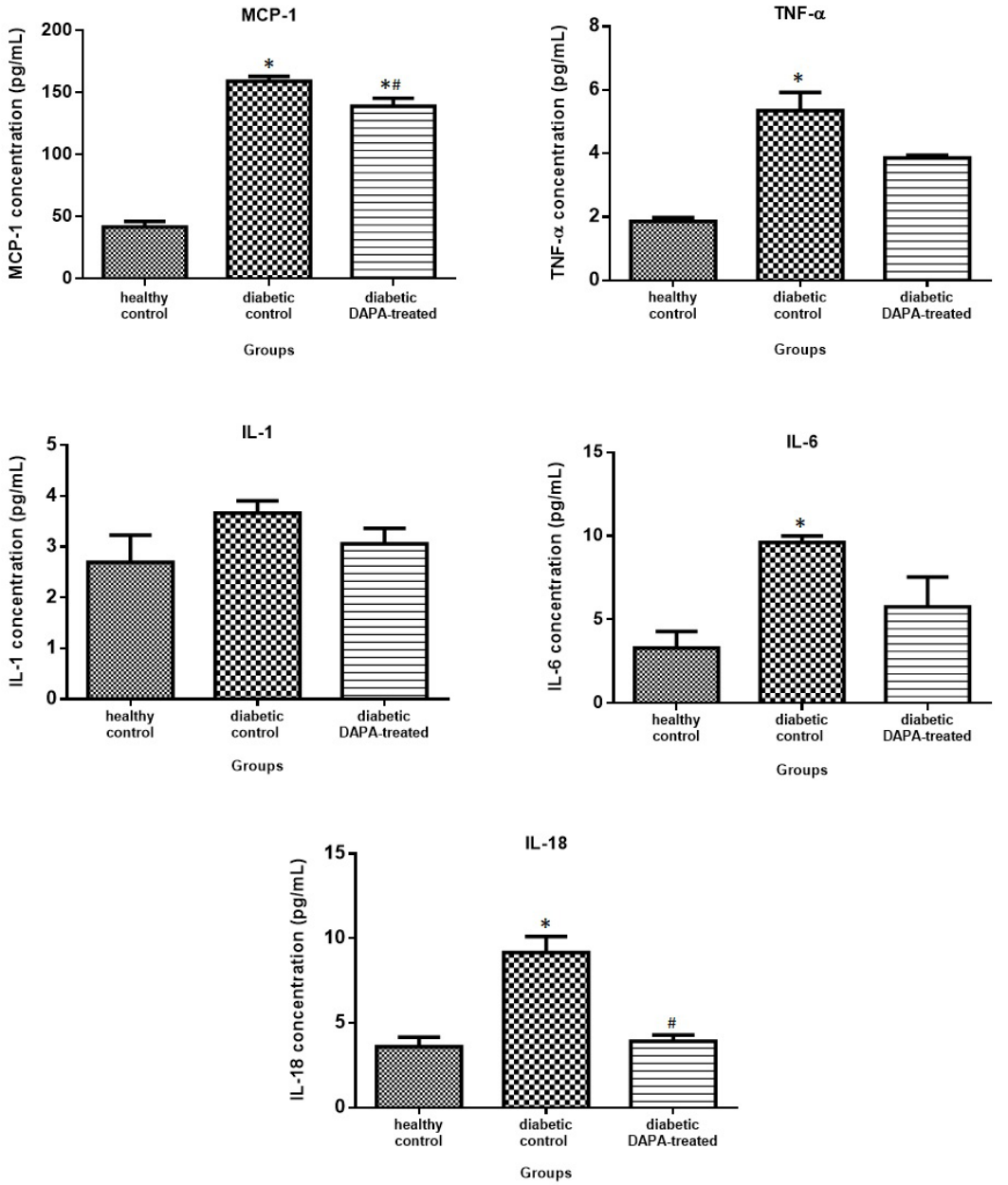

Figure 3. Effects of DAPA on plasma inflammatory mediators. Data are shown as the mean ±SEM. *p<0.05 healthy control group versus diabetic groups, #p<0.05 diabetic control versus diabetic DAPA-treated group. MCP-1, Monocyte chemoattractant protein-1; TNF-α, tumour necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6; and IL-18, interleukin-18. Healthy control group (n:5), diabetic control group (n:8) and diabetic DAPA-treated group (n:8).

tion may result in apoptosis. In this study, JNK gene (≥0.5-fold; p<0.05) and protein expression (0.5 fold, p>0.05) levels increased in the diabetic groups (Figure 2 and Figure 4). This increase was ameliorated by DAPA treatment at the protein level, but the changes were not statistically significant (Figure 4). This result may be associated with the duration of treatment because sub-acute exposure may not be enough to change protein level of JNK.

According to the histological examinations, regular morphology of the interstitial space, Bowman's space, Bowman's glomeruli, proximal tubules and distal tubules was observed in the healthy control group (Figure 5A). Interstitial congestion, glomeru-

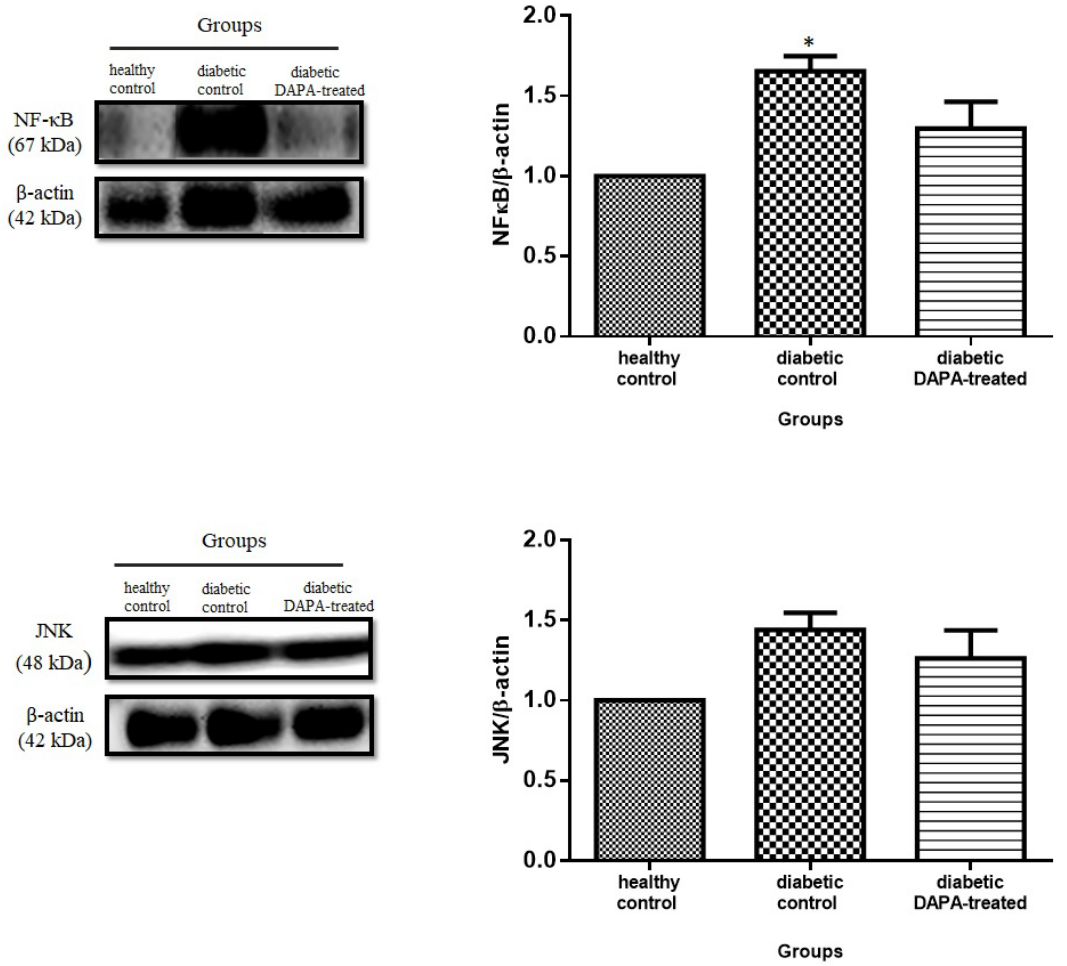

Figure 4. Effects of DAPA on protein expression levels in the kidney homogenates. Data are shown as the mean ± SEM. **p*<0.05 healthy control group versus diabetic groups, NFκB, nuclear factor kappa beta; JNK, c-Jun N terminal kinase. β-actin was used as the loading control. Healthy control group (n:5), diabetic control group (n:8) and diabetic DAPA-treated group (n:8).

lar congestion, significant dilation of Bowman's space with cellular debris, and tubular degeneration with luminal debris were observed in the diabetic control group (Figure 5B). Regressions in the dilation of Bowman's space, tubular degeneration, mild glomerular congestion and interstitial vascular congestion were observed in the diabetic DAPA-treated groups (Figure 5C). The histological findings also suggested that DAPA ameliorated diabetes-induced morphological damage.

Apoptosis is known to be associated with renal dysfunction leading to renal cell loss [37]. Tubular apoptosis has been reported to be high in patients with type II diabetic nephropathy [38]. In the present study, apoptosis was evaluated with TUNEL assay. Brown coloured TUNEL$^+$ cells were observed in each group. Only a few TUNEL$^+$ cells were observed in the healthy control group. The number of TUNEL$^+$ cells was increased in both tubular and glomerular cells in the diabetic control group. A decreased number of TUNEL$^+$ cells was observed in the diabetic DAPA-treated group ($p<0.05$) compared to diabetic control group (Figure 6). Our findings showed that DAPA treatment significantly decreased apoptotic cell number in the kidney. JNK and TNF-α are also known to have role in apoptosis [27,39]. Thus, slight decrease in JNK protein and TNF-α plasma level in diabetic DAPA-treated group could be a sign of inhibition of apoptosis in the kidney. In addition, DAPA also ameliorated diabetes-induced morphological

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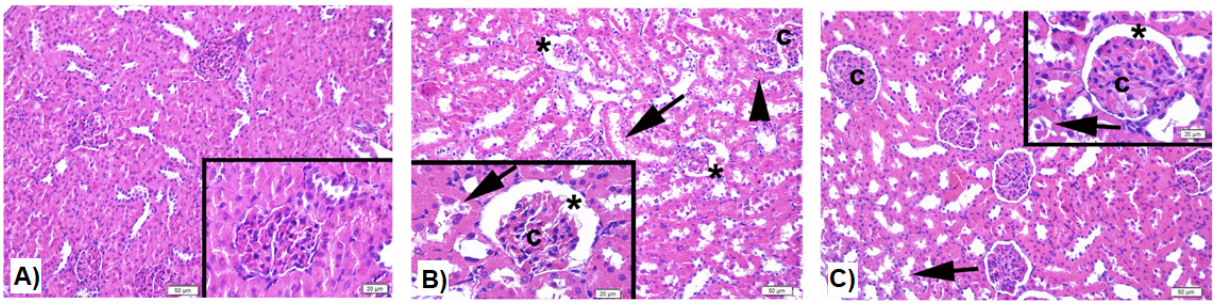

Figure 5. Representative photomicrographs of kidney in the experimental groups. Regular kidney morphology with renal corpuscles and tubular structures were observed in the healthy control group (**A**). Interstitial (arrowhead) congestion, glomerular congestion (c), dilation of Bowman's space (*), and degenerated tubules with luminal debris (arrow) were observed in the diabetic control (**B**). Bowman's capsule with regular morphology (*), mild glomerular congestion (*) and several degenerated tubules (arrow) were observed in the diabetic DAPA-treated group (**C**). H&E staining. Scale bars: 50 µm (x20), inset: 20 µm (x40). n:5 for health control group, n:8 for diabetic control and diabetic DAPA-treated groups were used for the experiments.

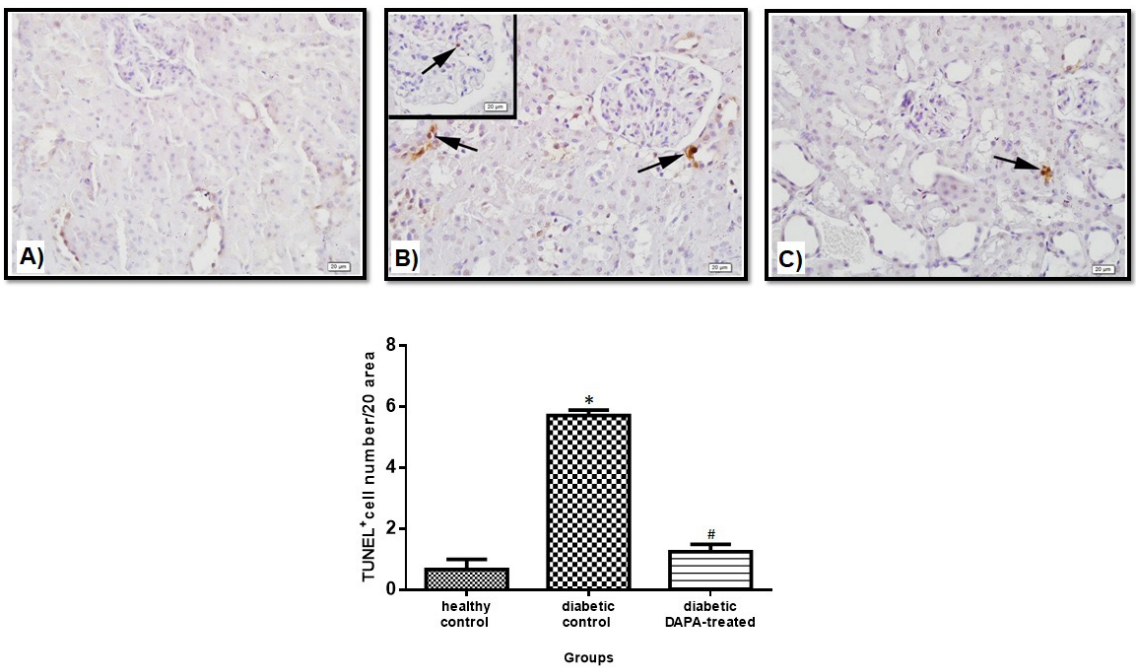

Figure 6. Representative photomicrographs of TUNEL– stained kidney tissues in the experimental groups. There was an absence of TUNEL$^+$ cells in the healthy control group (A). An increased number of TUNEL$^+$ cells (arrow) in both tubules and glomeruli were observed in the diabetic control (B) groups. A decreased number of TUNEL$^+$ cells (arrow) were observed in the diabetic DAPA-treated group (C). Scale bars: 20 µm (x40). TUNEL$^+$ cell number in experimental groups. Data are shown as the mean ±SEM. *p<0.05 healthy control group versus diabetic groups, $^{\#}p$<0.05 diabetic control versus diabetic DAPA-treated group. n:5 for health control group, n:8 for diabetic control and diabetic DAPA-treated groups were used for the experiments.

damage. Similarly, DAPA has been found to improve gentamicin-induced nephrotoxic effects at 10 mg/kg dose by reducing apoptosis and oxidative stress following 14-days treatment [40].

4. Conclusions

We conclude that DAPA does not show nephrotoxic effects at 10 mg/kg dose on the kidney, and may protect against diabetes-induced nephropathy through

alleviating oxidative stress and inflammation and inhibiting apoptosis. The FDA approval of DAPA in patients with chronic kidney disease also supports our findings. However, further sub-chronic and chronic studies are needed to clarify the renal effects of DAPA.

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Conflict of Interest

The authors have no conflicts of interest, financial or otherwise, to declare.

Statement of Contribution of Researchers

Concept – TB, GÖ; Design – TB, BUK, GO; Supervision – GÖ; Resource – GÖ; Materials -GÖ; Data Collection and/or Processing – TB, BUK, AKK; Analysis and/or Interpretation – TB, BUK, AKK, FE, GO; Literature Search –TB, BUK, AKK, FE, GÖ; Writing - TB, BUK, AKK, FE, GÖ; Critical Reviews - TB, BUK, AKK, FE, GÖ.

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