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Potential Nephroprotective Effect of Dorsomorphin Homolog 1 (DMH1) in a rat model of diabetic nephropathy

A. ESMAT[1,2], A.M. ALZAHRANI[1], B.T. ALHARTHY[1], W.S. RAMADAN[3,4,5], M.A.A. SATTAR AHMAD[1,6]

[1]Department of Pharmacology, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt
[3]Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia
[4]Embryonic Stem Cell Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia
[5]Department of Anatomy, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[6]Department of Pharmacology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Abstract. – OBJECTIVE: Diabetic nephropathy (DN) represents the most common cause of end-stage renal disease. On the other hand, Bone Morphogenetic Protein signaling pathway (BMP/Smad) is one of the most interesting prophylactic targets, since inhibition of this pathway may preserve kidney functions. Therefore, a BMP pharmacological inhibitor, Dorsomorphin Homolog 1 (DMH1) was used to assess the potential nephroprotective effect in an animal model of DN.

MATERIALS AND METHODS: STZ-induced diabetic rat was the selected model to assess the nephroprotective effect of DMH1(5 mg/kg) for eight weeks. Rats were divided into normal control (C=10), diabetic control (DC=10), diabetic+vehicle (DV=10) and diabetic DMH1-treated rats (DT=10). Fasting blood glucose (FBG) level was measured on a weekly basis. Then, glycated hemoglobin (HbA1c), serum Creatinine (sCr), Cystatin-C (Cys-C) and Blood Urea Nitrogen (BUN) were measured by the end of the experiment. Furthermore, Tumor Necrosis Factor (TNF-α), Interleukin-6 (IL-6) and Malondialdehyde (MDA) levels were determined in kidney tissues. The histopathological study was also performed using Hematoxylin and Eosin (H&E), Periodic acid Schiff (PAS) and Masson's trichrome stains.

RESULTS: DMH1 treatment has significantly reduced HbA1c along with sCr, Cys-C and BUN *vs.* the diabetic non-treated groups ($p < 0.001$). Furthermore, TNF-α, IL-6 and MDA levels were also significantly decreased in the DT group compared to the diabetic non-treated groups ($p < 0.001$). This improvement was further confirmed and found in correspondence with histopathological findings.

CONCLUSIONS: The present findings revealed a nephroprotective activity of DMH1 against STZ-induced DN in rats. DMH1 also showed anti-inflammatory and antioxidant activities, which may explain part of the nephroprotective mechanism. This can shed light on the importance of DMH1 and BMP/Smad pathway for further experimental studies.

Key Words:
Dorsomorphin homolog 1, DMH1, Diabetic nephropathy, Bone morphogenetic protein, BMP/Smad pathway.

Introduction

Kidneys are fundamental for maintaining overall health. In contrast, failure of this critical organ would result in a devastating negative impact on health, mortality and quality of life[1,2]. Unfortunately, the initial stages of chronic kidney disease (CKD) have no apparent symptoms, which added further challenges by dealing with a "silent disease". According to the Centers for Disease Control and Prevention (CDC), about 15% of adults in the United States (US) are estimated to have CKD, and almost 90% of them are unaware of their disease[3]. On the other hand, diabetic nephropathy (DN) is standing as the leading cause of renal failure in the US, besides being a well-known major complication of diabetes[4]. DN can be described as the clinical syndrome of diabetes and persistent albuminuria or the remarkable decline in the glomerular filtration rate or both (GFR)[5]. Moreover, almost ⅓ of type 1 diabetes mellitus patients and

Corresponding Author: Abdulaziz M. Alzahrani, MSc; e-mail: aalzahrani3093@stu.kau.edu.sa; pharmazizt@gmail.com

about 40% of patients with type 2 diabetes end up developing DN[6].

Sadly, there is no cure for DN yet, and the current recommendations emphasize adjusting the main modifiable risk factors. However, these recommendations essentially aimed to delay, and may not prevent, the disease[7]. In a cohort study of type 2 diabetic patients, adequate glycemic and blood pressure control failed to lower the risk of end-stage renal disease ESRD in older patients[8]. Moreover, other risk factors, such as genetic predisposition or advanced age, cannot be adjusted. Lately, the renin-angiotensin-aldosterone system (RAAS) may be commonly thought of as the main clinical target, however, the renal benefit of blocking this pathway showed some limitations. The outcomes of three randomized trials of the DIRECT study (Diabetic Retinopathy Candesartan Trials) were indicating the limited efficacy by reporting failure of candesartan to prevent microalbuminuria in normotensive patients with either type 1 or type 2 diabetes[9]. Furthermore, using RAAS blockers for nephroprotection may be complicated by their main side effects (e.g., hyperkalemia and hypotension). Therefore, attempts to find new drugs with better safety and efficacy to prevent the initiation and progression of DN are critically needed.

On the other hand, bone morphogenetic protein (BMP) is a protein family that is involved in many diseases, including DN. Specifically, BMP4 and its downstream signaling protein (Smad1) were implicated in performing a key role in DN pathogenesis. Furthermore, BMP4-neutralizing antibody resulted in about 40% reduction in the mesangial expansion (one of the characteristic morphological features of DN) with a concurrent reduction in Smad1 phosphorylation[10]. Recently, mesangial matrix expansion and glomerular basement membrane (GBM) thickening were noted to accompany the overexpression of BMP4, BMP4 receptor (activin receptor-like kinase 3 or shorty ALK3) and collagens in the diabetic condition. In contrast, silencing bmp4-gene in high glucose cell culture using siRNA (small interfering RNA) demonstrated a significant decrease in BMP4, ALK3 and collagens[11]. Therefore, the previous reports can strengthen each other to unveil BMP4 inhibition as an attractive and promising target for DN prevention.

Formerly, a structure-activity relationship study for small molecules revealed Dorsomorphin Homolog 1 (DMH1) as the preferred inhibiting molecule, which almost exclusively targets BMPs with no off-target effects[12]. Furthermore, DMH1 has shown several promising results[13-15] with low toxicity and effective inhibition of the BMP/Smad pathway in many experiments. The prior literature results have displayed DMH1 as an attractive selection to be tested for BMP inhibition. Therefore, the current study aimed to investigate the potential nephroprotective effect of DMH1 using an animal model of DN.

Materials and Methods

Chemicals and Kits

Streptozocin (STZ) (Cat. No. 1621) was purchased from Tocris Bioscience® (Bristol, UK) and supplied as crystalline solid to be dissolved in 0.1 M sodium citrate buffer (pH 4.5) just prior use. DMH1 (Dorsomorphin Homolog 1) (Cat. No. 4126) was also purchased from Tocris Bioscience® (Bristol, UK) and supplied as a yellow crystalline solid. (2-Hydroxypropyl)-β-cyclodextrin powder (Cat. No. OH05393) was acquired from Biosynth Carbosynth® (Compton, UK) and was freshly prepared as a 12.5% solution to be used as a vehicle for DMH1. Rat glycated hemoglobin A1c (HbA1c) ELISA kit (Cat. No. MBS2033689), rat creatinine (CREA) ELISA kit (Cat. No. MBS749827), rat cystatin-C ELISA kit (Cat. No. MBS749827) and rat urea ELISA kit (Cat. No. MBS2600001) were all purchased from MyBioSource (San Diego, CA, USA). Hematoxylin and Eosin (H&E) staining kit (Cat. No. ab245880) was bought from Abcam® (Cambridge, UK), while Periodic acid-Schiff's (PAS) staining kit (Cat. No. 395B) and Masson's trichrome staining kit (Cat. No.HT15) were obtained from Sigma-Aldrich® (Munich, Germany).

Induction of Diabetes and Experimental Design

Male adult Wistar rats (n = 40), weighing approximately (180-220) g, were purchased from the pharmaceutical consultation and research unit, Faculty of Pharmacy, King Abdulaziz University (KAU), Jeddah, Saudi Arabia. The animal experiments were approved by the Research Ethics Committee (REC) at King Abdulaziz University (KAU) (Reference No. 546-19) and carried out based on the Good Clinical Practice (GCP) Guidelines. Standard animal room temperature (29-30ºC) and 12 hours of light/dark cycle were maintained during the entire study period. Feed and tap water *ad libitum* were provided. After one week of acclimating to the facilities, ten random rats were marked as control (C=10), and the remaining 30

rats were injected intraperitoneally (i.p.) with 60 mg/kg of streptozotocin (STZ) to induce diabetes[16]. Three days later, rats with blood glucose levels of ≥ 300 mg/dl were considered diabetic rats and randomly divided into:

- Diabetic non-treated group (DC, n = 10) supplemented by regular food and water.
- Diabetic non-treated group + vehicle (DV, n = 10) injected (i.p.) with equivalent amount of vehicle (12.5% 2-hydroxypropyl-b-cyclodextrin) for eight weeks. The vehicle was selected based on its property of enhancing drug solubility and bioavailability[17], besides being previously used as a vehicle for DMH1[14].
- Diabetic treated group (DT, n = 10) injected (i.p.) with 5 mg/kg DMH1 each other day (q.o.d) for eight weeks[14,18].

Body weights and fasting blood glucose (FBG) were determined at the baseline and weekly thereafter. ACCU-CHEK® Active (Roche Diagnostics GmbH, Mannheim, Germany) was used for the measurement of FBG. Blood samples, for serum creatinine (sCr) and blood urea nitrogen (BUN), were withdrawn by the end of the 4th week for early assessment. Then, at the end of the experiment, body weights and FBG for all rats were documented, whereas blood samples were withdrawn. Next, blood samples were centrifuged at 3000 g for 10 min to get clear sera which were used for assessment of sCr, BUN and cystatin-C (Cys-C) using the proper enzyme-linked immunosorbent assay (rat ELISA kit). The rats were anesthetized with diethyl ether, and then, sacrificed. Both kidneys were harvested. The right kidneys were weighted, and kidney weight/body weight ratios (KW/BW) were calculated as g/kg. Additionally, appropriate rat ELISA was applied following the manufacturer's instructions for homogenate analysis. Homogenate content of malondialdehyde (MDA) was determined as the oxidative marker, while tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) contents were the inflammatory markers.

Histopathological Examination of the Renal Tissues

Left kidneys were fixed in 4% paraformaldehyde for 48 hrs. Then, the tissue was embedded in paraffin and cut into slices with a thickness of 4 μm. After dehydration, hematoxylin and eosin (H&E), Periodic acid-Schiff's (PAS) and Masson's trichrome stains were used, individually, to assess the histopathological findings.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to examine the gathered results using SPSS statistics software package, version 23 (SPSS Corp., Armonk, NY, USA) followed by Tukey's HSD multiple comparison post-hoc test. Differences if p-values < 0.05 were considered statistically significant.

Results

During the experiment, nine rats had died; three rats were in the DC group, two rats were in DV group, and the other four rats were in the treated group (DT).

Effect of DMH1 on Body Weights, Kidney Weights and KW/BW Ratios

The body weights of the control group (C) were markedly higher ($p < 0.001$) than the weights of all diabetic groups (DC, DV, DT). Nevertheless, the difference in the body weights between diabetic groups (DC, DV, DT) was not significant ($p > 0.05$). Moreover, no significant change was found in the kidney weights of all tested groups. However, KW/BW ratios for the DC, DV and DT groups were significantly increased ($p < 0.001$) *vs.* the control group. This may suggest an increase in kidney weights occurred irrelevant to the body weights. By comparing the DT group to DC group, DMH1 showed no significant effect in the rats' weights, kidneys' weights or KW/BW ratios ($p > 0.05$) (Table I).

Effect of DMH1 on FBG and HbA1c Levels

Fasting blood glucose (FBG) levels were steady and significantly high during the eight weeks for all diabetic groups (DC, DV& DT) when compared to the control group ($p < 0.001$). However, comparing FBG levels between the three diabetic groups (DC, DV & DT) revealed no significant difference (Table II). Unexpectedly, the results of glycated hemoglobin (HbA1c) were not consistent with FBG results; while diabetic non-treated groups (DC & DV) have significantly higher levels *vs.* the control group ($p < 0.001$), the results of the treated group were significantly lower than DC or DV ($p < 0.001$), they even approximated levels of the control rats ($p > 0.05$) (Figure 1A).

Effect of DMH1 on renal biomarkers (sCr, BUN and Cys-C)

The measurements of serum creatinine (sCr) and blood urea nitrogen (BUN) were early as-

Table I. Body weights, kidney weights & kidney weight /body weight ratios of all rats after eight weeks of the study.

Parameters	Groups Control	DC	DV	DT (5 mg/kg)
Initial BW (g)	185 ± 11	181.3 ±30	176.9 ± 30	180.5 ± 25
BW (g) After 8 weeks	329.3 ± 34.3	197.7 ± 45.7***	194.1 ± 49.4***	219.5 ± 62.7***
KW (mg)	917.4 ± 70.9	892.9 ± 149.6	881.3 ± 121.7	965 ± 153.6
KW/BW ratio (g/kg)	2.8 ± 0.23	4.62 ± 0.77***	4.69 ± 0.79***	4.65 ± 1.23***

Data were expressed as mean ± SD. BW body weight (gram), KW kidney weight (milligram). Control rats (n=10), DC= Diabetic Control group (n=7), DV= Diabetic + Vehicle group (n=8), DT= Diabetic DMH1-treated group [5 mg/kg q.o.d] (n=6). *** very highly significant ($p < 0.001$) against control group.

sessed in the 4th and later by the end of the 8th week of the study. At the end of the 4th week, sCr levels revealed a non-significant difference ($p > 0.05$) between the four groups, while BUN demonstrated a significant elevation in DC and DV levels *vs.* the control group ($p < 0.001$) (Figure 1B & 1C). Additionally, at the end of the experiment, sCr and Cys-C (Figure 1D) levels were observed significantly higher for both DC & DV groups when they matched to the C group ($p < 0.001$). Nevertheless, sCr and Cys-C for the DT group were almost within a similar range to the control group. BUN readings for the DT group showed less resemblance to the control group than findings of sCr & CysC. However, BUN levels for the DT group were remained significantly decreased compared to both DC & DV groups ($p < 0.001$), with simultaneous significant higher levels ($p < 0.05$) *vs.* the control group.

Effect of DMH1 on inflammatory markers (TNF-α and IL-6) and oxidative stress marker (MDA)

Inflammatory markers (TNF-α & IL-6) were considerably elevated ($p < 0.001$) in both diabetic non-treated groups (DC & DV) in comparison to the control group. Treatment with DMH1 in the DT group resulted in a very significant suppression in levels of these markers ($p < 0.001$) and almost bore some similarity to the values of the control group (Figure 1E). Additionally, kidney tissues of diabetic non-treated groups (DC & DV) have also significantly higher levels of Malondialdehyde (MDA) compared to the control group ($p < 0.001$). However, DMH1-treated group (DT) showed significantly lower levels of MDA *vs.* DC or DV groups ($p < 0.001$). Moreover, no statistical difference was found when comparing DT group with the control group ($p > 0.05$) (Figure 1F).

Assessment of Histopathological Examination of Renal Tissue for the Tested Groups of Rats After Eight Weeks

The evaluation of the renal cortex (Figure 2) using hematoxylin and eosin (H & E) stain revealed the normal structure of the control group; a looked-healthy glomerular tuft of capillaries and bowman's space, besides normal cuboidal cells lining the proximal and distal convoluted tubules. By contrast, the DC group showed some shrunken glomeruli with a widening of the subcapsular space. Congestion of the intertubular capillaries was also noticeable. DV group, on the other hand, showed increased mesangial cellularity of the glomerulus and obliteration of the bowman space. Vacuolation of the cuboidal cells lining both proximal and distal convoluted tubules was also apparent. Nevertheless, the DT group displayed better conserved integrity of glomerulus tuft of capil-

Table II. Averages of weekly FBG levels (mg/dl) for all tested groups of rats during the eight weeks.

	Groups Weeks							
	1st	2nd	3rd	4th	5th	6th	7th	8th
C	95 ± 10	100 ± 15	106 ± 11	105 ± 20	96 ± 15	106 ± 19	92 ± 14	98 ± 12
DC	488 ± 65 ***	455 ± 49 ***	445 ± 42 ***	496 ± 53 ***	500 ± 60 ***	432 ± 75 ***	420 ± 82 ***	332 ± 80 ***
DV	516 ± 83 ***	480 ± 152 ***	490 ± 64 ***	507 ± 132 ***	467 ± 61 ***	446 ± 195 ***	381 ± 70 ***	356 ± 86 ***
DT	539 ± 70 ***	485 ± 92 ***	444 ± 44 ***	519 ± 122 ***	444 ± 56 ***	477 ± 156 ***	361 ± 90 ***	295 ± 99 ***

Data were expressed as mean ± SD. C = Control rats (n=10), DC= diabetic control group (n=7), DV= diabetic+vehicle group (n=8), DT= diabetic DMH1-treated group [5 mg/kg q.o.d] (n=6). *** very highly significant ($p < 0.001$) against control group.

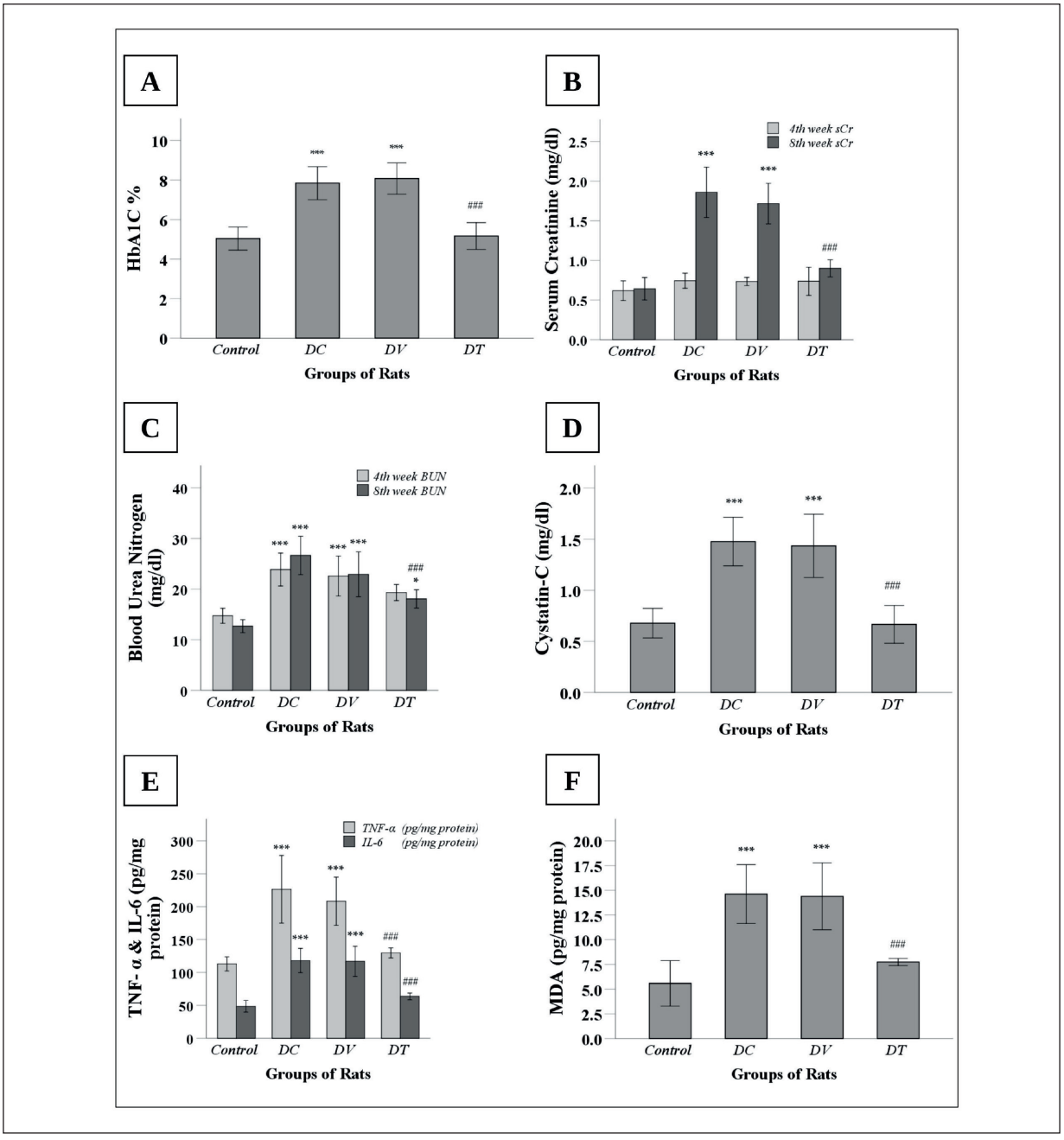

Figure 1. A, Mean HbA1c readings for all tested groups of rats after eight weeks of the study. **B**, Mean sCr levels for all groups of rats at the 4th week versus 8th week of the study. **C**, Mean BUN levels for the different groups of rats at the 4th week versus 8th week of the study. **D**, Mean Cys-C levels for all groups of rats after eight weeks of the study. **E**, Mean TNF- α and IL-6 levels in kidney tissues of rats after eight weeks of the study. **F**, Mean MDA levels in kidney tissue of rats after eight weeks of the study. Data were presented as Mean ± SD. C = Control rats (n=10), DC= diabetic control group (n=7), DV= diabetic+vehicle group (n=8), DT= diabetic DMH1-treated group [5 mg/kg q.o.d] (n=6).* significant ($p < 0.05$), *** very highly significant ($p < 0.001$), against control. ### very highly significant ($p < 0.001$) compared to DC or DV group.

laries and epithelial lining of both proximal and distal convoluted tubules. Additionally, examining the renal medulla (Figure 3) of the DV and DC groups revealed distended congested intertubular capillaries and dilated collecting ducts. Differently, non-engorged intertubular capillaries were observed between apparently normal collecting ducts in the DT group. Furthermore, Periodic acid-Schiff (PAS) stain (Figure 4) was similarly demonstrating the normal structure of the glomeruli and normal

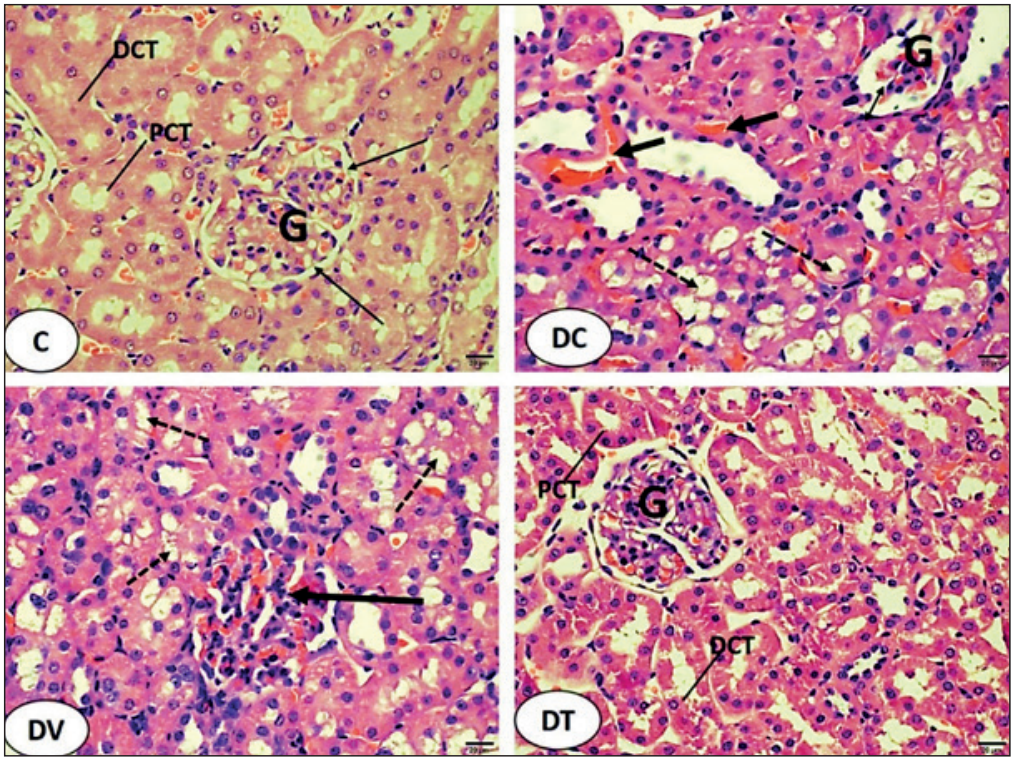

Figure 2. Photomicrographs of H&E-stained sections of renal cortex of rats after eight weeks (H&E x 200). **C**, Control rats: a normal glomerular tuft of capillaries (**G**) and bowman's space (thin arrow), in addition to apparently normal cuboidal cells lining PCT and DCT. **DC**, Diabetic control rats: shrunken glomeruli (**G**) with a widening of the subcapsular space (double head arrow), congestion of the intertubular capillaries (thick arrows) and vacuolation of PCT and DCT epithelial cells (dashed arrows). ***DV***, Diabetic rats which received vehicle: increased mesangial cellularity of the glomerulus (arrow), obliteration of the bowman space, vacuolation of the cuboidal cells lining the PCT and DCT (dashed arrows). **DT**, Diabetic DMH1-treated rats (5 mg/kg q.o.d): better preserved integrity of the glomerulus tuft of capillaries (**G**), the epithelial lining of PCT and DCT. G, glomeruli, PCT, proximal convoluted tubule, DCT, distal convoluted tubule.

GBM in the control group. DC group, on the other hand, was displaying lobulations of a glomerulus and expansion of the mesangium. Likewise, DV group was exhibiting a sclerotic nodule with intense deposition of glycogen and apparent thickening of the GBM. Conversely, DT rats were showing a preserved integrity of GBM. Masson's trichrome stain (Figure 5) was further used to explore the discrepancy of collagen accumulations between groups. More intense collagen accumulations were displayed in DC and DV groups compared to either control or DT groups, however, the deposition was mainly around blood vessels.

Discussion

Diabetic patients constitute a considerable part of the population, while its complication, particularly DN, is forming a challenging complication and majorly involved in the progression of patients to ESRD[19]. Therefore, efforts to target DN for prevention and treatment should attract more attention and encouragement since no cure has been found yet for the ESRD. In the current study, the prophylactic effects of Dorsomorphin Homolog 1 (DMH1) on renal functions were investigated in STZ-induced diabetic rats. Moreover, underlying mechanisms were further explored by determining levels of inflammatory and oxidative stress markers, besides the different histopathological studies.

By the end of the experiment, the inhibitory effect of diabetes was clear on rats' ability to gain weight. A previous study already revealed that higher doses of STZ were associated with more weight loss. However, the researchers have addressed 60 mg/kg as the optimum dose to induce diabetes[20]. On the other hand, DMH1 did not demonstrate any significant effect on body weights, which is affirmative to the previous finding in the literature[18]. Furthermore, kidney weights were also showing no significant differ-

ence between the four groups, yet KW/BW ratios of all diabetic groups showed a significant increase in the relative kidney weights which may propose an overgrowth of the residual renal tissue in response to the damage that happened to other renal tissue[21]. In fact, kidneys in type 1 diabetic patients were found to be ~30% larger than normal kidneys. Also, the larger size of the diabetic kidney can be a predictive factor for the progression to chronic kidney disease (CKD)[22,23]. Nevertheless, DMH1 did not reveal any significant effect in body weights, kidney weights or KW/BW ratios.

In the present study, DMH1 perceptible renal protective effect does not rely on blood glucose levels, since the weekly measures of FBG revealed persistent hyperglycemias for all rats in the treated group with no significant difference to the other diabetic groups (DC & DV). Additionally, the inability of rats to gain weight, biomarkers results, and the histopathological findings were all indicating the successful achievement of the selected model. Moreover, there was remarkable-observed polyuria for all the diabetic groups, besides a noticeable increase in daily consumption of chew and water, which might have led to severe dehydration and death for the nine rats.

Unexpectedly, glycated hemoglobin (HbA1c) levels for the DT group were within a comparable range to the control group. However, concerns regarding the discordance between glycemic blood levels and HbA1c have emerged many years ago. In 2008, researchers described certain genetic, environmental, glycation factors and other technical aspects of the performed assay that can alter HbA1c level and obscure its appropriate interpretation[24]. In 2009, American Diabetes Association (ADA) concluded that the use of HbA1c can lead to a false diagnosis of diabetes among special populations. They discouraged interpretation of HbA1c solely for the diagnosis and assessing glycemia without consideration of other factors (e.g., hemoglobinopathy, testing for anemia and testing for renal impairment)[25]. Furthermore, many drugs were reported to cause false alterations in HbA1c, e.g., dapsone[26], regular aspirin ingestion and high doses of vitamin C & E. Oxidative stress is also an established condition to affect glycation rate and,

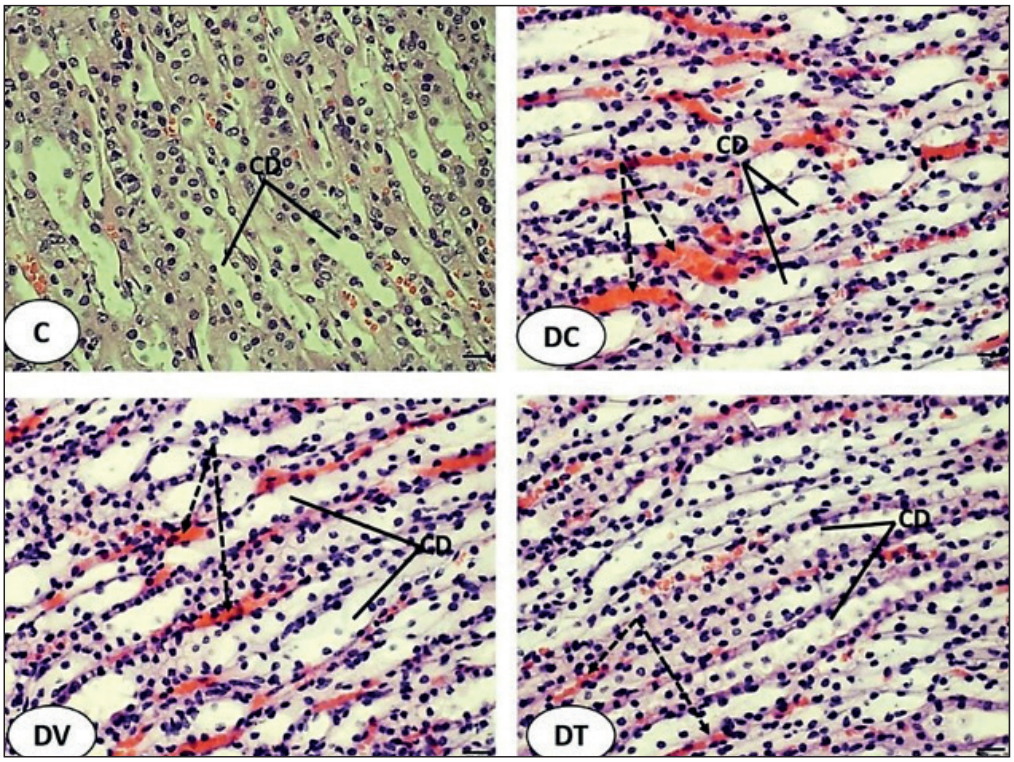

Figure 3. Photomicrographs of H&E-stained sections of renal medulla of rats after eight weeks (H&E x 200). **C**, Control group: rows of CD lined with simple cuboidal epithelium. **DC**, Diabetic control rats & **DV**, diabetic rats which received vehicle: dilated congested intertubular capillaries (dashed arrows) and dilated CD in both non-treated groups. **DT**, diabetic DMH1-treated rats (5 mg/kg q.o.d): non-engorged intertubular capillaries were observed between apparently normal CD. **CD**, collecting ducts.

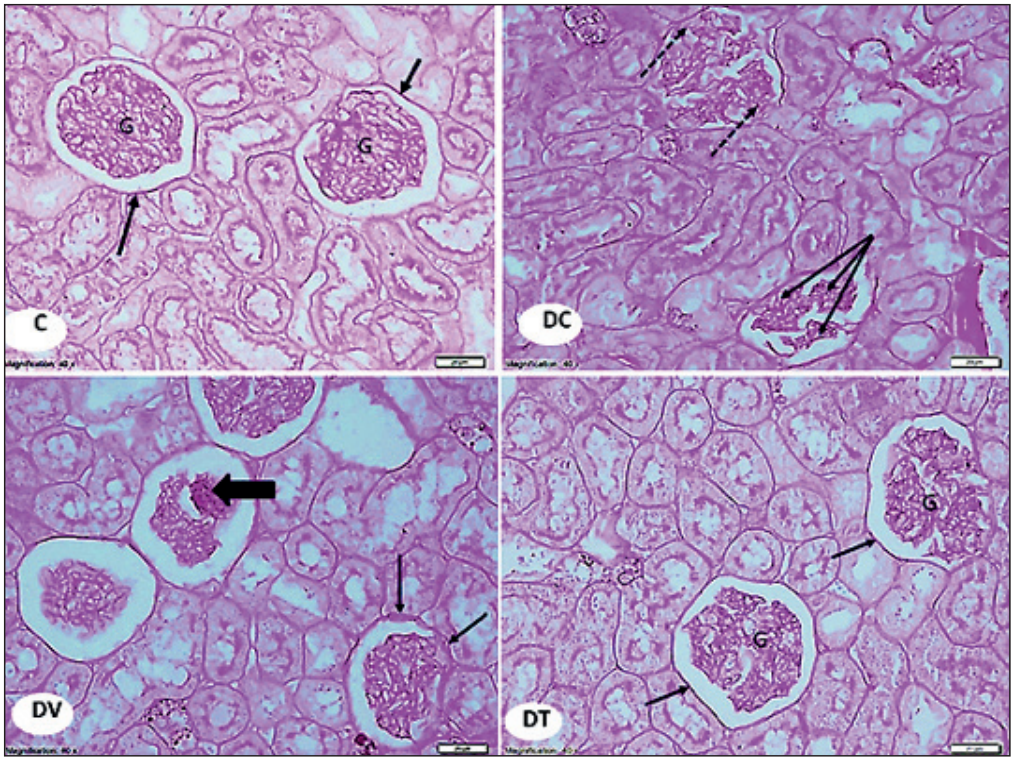

Figure 4. Photomicrographs of PAS-stained sections of renal cortex of rats after eight weeks (PAS x400). **C**, Control rats: normal structure of the glomerular tuft of capillaries (**G**) and normal thickness of the glomerular basement membrane (arrow). **DC**, Diabetic control rats: lobulations of a glomerulus (thin arrows) and expansion of the mesangium (dashed arrows). **DV**, Diabetic rats which received vehicle: sclerotic nodule with intense deposition of glycogen (thick arrow) and apparent thickening of the glomerular basement membrane (thin arrows). **DT**, Diabetic DMH1-treated rats (5 mg/kg q.o.d): integrity of glomerular basement membrane was maintained (arrows). **G**, Glomeruli.

consequently, HbA1c readings[27]. Other drugs like ribavirin, antiretrovirals, hydroxyurea and sulfonamides were also reported to, incorrectly, lower HbA1c results[28-31]. In the current study, DMH1 was probably implicated in revealing false negative values of HbA1c in DT groups. Although the exact mechanism behind this influence is not clear yet, however, it is conceivable to hypothesize that DMH1, either directly or indirectly, affected one or more of the following processes: inhibition of glycation rate; increasing erythropoiesis; or increasing erythrocytes loss.

In the present investigation, there was a time-dependent decline in renal function parameters in DC and DV groups. The results of sCr at the 4th week were not statistically significant, however, BUN levels for DC and DV groups were significantly higher than the control group. The 4th-week results can support the concept of an earlier increase of urea than sCr in renal disease[32]. Both sCr and Cys-C are endogenous markers used to estimate GFR, which is fundamental for assessing renal function. While muscle wasting can affect the computed creatinine-based estimation of GFR, Cys-C, on the other hand, is less influenced by muscle mass than creatinine. Multiple reports found Cys-C reflecting a better assessment of renal functions in muscle-wasting conditions, vegetarians and those with chronic diseases. However, the Cys-C test has also some flaws; it may not be recommended alone for routine GFR estimation due to possible influence by other factors (e.g., inflammation). Therefore, Cys-C was used as a supporting test to combine sCr and give a more accurate and reliable estimation of GFR than either one alone[33]. Likewise, BUN solely is a weak predictor of renal function[32], and rationality is to aid BUN interpretation with concurrent sCr level measurement. In this study, the increase in BUN in DC and DV groups was parallel with a simultaneous increase in sCr and Cys-C. Collectively, these results can strengthen each other to represent more reliable signs of deteriorating GFR or decline in renal excretory function in those groups. Moreover, histopathological findings stand next to the results of renal biomarkers to validate the DN

model. It may be also suggested that eight weeks of persistent hyperglycemia was enough time for Wistar rats to precipitate kidney dysfunction and can be used as a model of DN. On the other hand, treatment with DMH1, despite the long-lasted hyperglycemia, kept the renal biomarker results within normal ranges and close to that of the control group.

Low-grade inflammation is believed to associate diabetes marked by the elevation of various inflammatory parameters. The majorly involved inflammatory cytokines are TNF- and IL-6, which can be produced by renal cells. The renal hypertrophy in DN was the expected outcome of TNF- overactivity. TNF- can trigger renal cell injury by either direct cytotoxicity, alteration of intraglomerular blood flow or even by promoting local reactive oxygen species (ROS) production. Likewise, IL-6 was found elevated in patients with DN. Angiotensin-converting enzyme (ACE) inhibitors have displayed a remarkable reduction in both TNF- and IL-6 activities, which may explain part of their renal protective properties[34]. In the present study, DMH1-treated group (DT) has revealed a significant reduction in both TNF- and IL-6. Accordingly, DMH1 may have an outstanding anti-inflammatory activity since the levels of inflammatory parameters for the DT group were close to the non-diseased levels. The current results support the established literature evidence of the anti-inflammatory effect of DMH1[35]. Concurrently, DMH1 treatment exhibited a remarkable suppression of MDA levels, which may indicate a substantial antioxidant property. Furthermore, inflammation can trigger oxidative stress, while oxidative stress, *per se*, is also known to stimulate inflammation through the pro-inflammatory mediators. Hence, the interrelationship between oxidative stress and inflammation should get more consideration in DN management[36]. DMH1 has shown beneficial results for both oxidative stress and inflammation. Thus, DMH1 appeared a promising therapeutic choice for further investigation in other oxidative stress and inflammatory diseases.

Interestingly, the evidence does exist demonstrating that a considerable percentage (33-50%) of patients may not develop albuminuria while having a significant reduction of GFR or renal impairment[37,38]. Also, a couple of published reports were describing albuminuria as "not correlated" with the degree of underlying glomerular lesions or mesangial expansion in DN of both humans

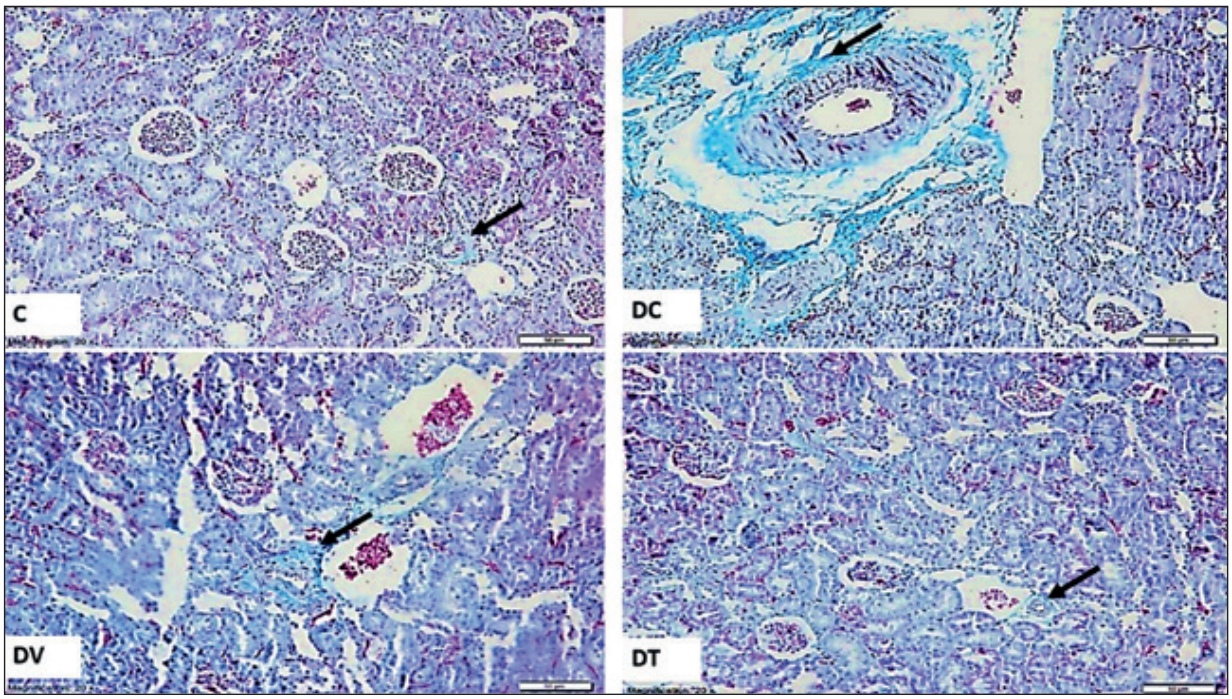

Figure 5. Photomicrograph of Masson's trichrome-stained sections of renal cortex of rats after eight weeks (Masson's trichrome x20). **C**, Control rats. **DC**, Diabetic control rats. **DV**, Diabetic rats which received the vehicle. **DT**, Diabetic DMH1-treated rats (5 mg/kg q.o.d). The blue-colored collagen accumulation (arrow) was more intense in DC and DV groups compared to the control and DT groups.

and animals[39,40]. In this study, albuminuria was not studied due to the previously mentioned concerns. Besides the many disfavored features of the metabolic cage, such as incomplete separation of urine and feces, raised emotional stress, oxidative stress and increased overall metabolism condition. Hence, it cannot be a representative method for normal physiology[41].

So, the biochemical analyses have been reinforced with histopathological studies, which were also consistent. Usually, the early morphological change in DN is the thickening of GBM. Then, further structural changes progressed along with disease course to "Kimmelstil-Wilson nodules", which are characteristic nodules for the advanced DN. Ultimately, the kidney tissues undergo a complete obliteration and sclerosis in late stages [42]. Some recent publications[11,43] have explained the involvement of BMP4 in the pathogenesis of diabetic nephropathy. In the current experiment, the histopathological study revealed more of the GBMs were either thickened or deformed with glomerular lobulation in DC & DV groups, while DMH1 treatment showed less extent of GBM thickening and better structural integrity. The thickening of GBM in DN may be explained by glycation or accumulation of Advanced Glycation End-products (AGEs)[42], which was also observed in PAS-stained tissues of DV & DC groups with a larger extent than control or DT groups. Mesangial matrix production, which is mainly stimulated by hyperglycemia[42], was also less marked in the treated group than diabetic non-treated groups. It may indicate that DMH1, despite existing hyperglycemia, has partially blocked the mesangial matrix expansion, and possibly AGEs accumulation, leading to a noticeable preserving effect of GBM and mesangium. Moreover, Masson's trichrome staining revealed a lesser extent of collagen accumulation with DMH1 treatment. The increased collagen accumulation is also evident as a prominent part of extracellular matrix (ECM) changes in DN, while in the advanced stages of DN, massive mesangial accumulation of ECM, glomerulosclerosis and arteriolosclerosis are more common[44].

DMH1 was identified to be a selective inhibitor of ALK2 and ALK3, which both are type I BMP receptors[12]. Other studies[14,45] found that DMH1 can effectively inhibit BMP4/Smad signaling pathway. Accordingly, it is reasonable to attribute the current renal protective activity of DMH1 to, at least, partial BMP4/Smad inhibition and consequent suppression of structural alterations and collagen overaccumulation in renal tissues. However, the present study was conducted using one dosage of DMH1 on a practically small sample size. Nevertheless, the current results can encourage and pave the way for further research on DMH1 to clarify its specific mechanism, downstream signaling and the involved transcriptional genes. Furthermore, additional studies to address DMH1 pharmacokinetics and pharmacodynamics properties are critically needed. Also, it remains an essential requirement to perform comprehensive toxicological studies in multiple species before the trial in humans.

Conclusions

In the present investigation, DMH1 has revealed a renal protective effect against persistent hyperglycemia and diabetic nephropathy, which was clearly observed in renal biomarkers and histopathological studies. The characteristic feature of DMH1 to, specifically, block BMP4/Smad signaling pathway may be the suggested molecular mechanism for this preserving effect. DMH1 has also an anti-inflammatory effect against elevated TNF- and IL-6 and antioxidant property, which may explain part of the nephroprotective benefit. The definite mechanism of DMH1 in diabetic nephropathy has not been fully elucidated here and might be the suggestive spotlight for future research.

Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

This work was carried out in collaboration between all authors. All authors reviewed the results and approved the final version of the manuscript.

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