

Suppression of Diabetes-Induced Renal Inflammatory Cell Infiltration by Metformin Associated with the Amelioration of Renal Injury Biomarkers

Supresión de la Infiltración de Células Inflamatorias Renales de Diabetes-Inducida, por Metformina Asociada con la Mejora de los Biomarcadores de Lesión Renal

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SUMMARY: Diabetes and hypertension account for the majority of chronic kidney injury cases that can lead to renal failure. The link between the leukocytes common antigen (CD45) and diabetic kidney disease (DKD) with and without metformin incorporation in an animal model has not been investigated before. Therefore, we sought to assess the extent of leukocytes infiltration into kidney tissues 10 weeks following the induction of diabetes in rats treated with metformin. In addition, we monitored blood and urine parameters associated with diabetes. The model group of rats received streptozotocin (STZ; 50 mg/kg) injection after being fed for 14 days on a high-fat diet (HFD) and continuously fed a HFD until they were culled, at week 12. The protective group was treated in the same way except that these animals were put from day 1 on metformin (200 mg/kg) until being culled, on week 12. Kidneys were immunostained with CD45 as a marker of leukocytes infiltration and examined by light microscopy. Urine samples were tested for urine albumin and collected blood was analyzed for sugar, urea, creatinine, and oxidative stress and antioxidants biomarkers. Kidney injury secondary to diabetes was developed as demonstrated by (i) increased blood glucose, urea, and malondialdehyde (MDA) as a marker of lipid peroxidation; and (ii) kidney tissue damage and marked increase in kidney tissues expressing CD45 positive cells. The above markers were inhibited ($p \leq 0.0006$) by metformin. Also, a significant correlation was observed between CD45 score and glycemia, urea, MDA, and the antioxidant superoxide dismutase (SOD). Thus, our data demonstrate an association between the infiltration of CD45+ inflammatory cells into kidney tissues and biomarkers of kidney damage in a rat model of DKD, which was effectively protected by metformin.

KEY WORDS: Diabetes; Kidney injury; CD45; Oxidative stress; Metformin; Rat model.

INTRODUCTION

Chronic kidney disease secondary to diabetes develops in approximately 20 - 50 % of patients with diabetes, and nearly 44 % of new cases of end-stage renal failure in USA are DKD (Dalla Vestra *et al.*, 2000). Excessive consumption of unhealthy food and reduced physical activity are the main causes of the observed rapid rise in obesity, which claims the life of millions globally per annum (Lustig *et al.*, 2012). Obesity induces inflammatory and oxidative stress markers and mediators such as TNF- α and reactive oxygen species (ROS) which induce insulin resistance via the activation of NADPH oxidase by the accumulation of lipids in the adipocytes, which can eventually cause vascular injury (Shoelson *et al.*, 2006). Obesity is also associated with hypertension, and renal dysfunction contributes to diabetic

kidney disease, glomerulopathy and renal failure (Iseki *et al.*, 2004; Shen *et al.*, 2010; Bayliss *et al.*, 2012).

Diabetic kidney disease (DKD) is characterized by a drop in the rate of glomerular filtration, an increase in proteinuria, hypertension, and cardiovascular disease (Russell, 2006; Lim, 2014). Numerous factors contribute to the development of DKD, including oxidative stress, inflammatory cytokines, recruitment of leukocytes to the injury site, as well as activation of the renin-angiotensin system that increases the glomerular capillary pressure (Galkina & Ley, 2006; Jefferson *et al.*, 2008). Recruitment of leukocytes into the diabetic kidney and vascular injury are well-documented in DKD (Galkina & Ley, 2006).

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Macrophages, T-lymphocytes, and neutrophils infiltration into diabetic renal tissues caused renal vascular inflammation and injury that participate in the development of DKD (Galkina & Ley, 2006). Indeed, in experimental model of glomerulonephritis, macrophages induced mesangial expansion and proteinuria (Ikezumi *et al.*, 2003).

Treating patients with DKD using the prescribed medications was recognized to be effective in delaying the prognosis of this serious disease; for example, (i) inhibiting the rennin-angiotensin-aldosterone system by the drug captopril in diabetic nephropathy patients substantially slowed down the deterioration in renal function and decreased the risk of reaching the end-stage renal failure by 50 % (Chawla *et al.*, 2010); and (ii) angiotensin II type I receptor inhibitors ameliorated kidney injury in hypertensive patients with diabetes (Ozaki *et al.*, 2010). In addition, several types of kidney diseases such as injury to renal podocyte and renal toxicity induced by the antibiotic gentamicin in rats were inhibited by the incorporation of the hypoglycemic drug metformin (Amini *et al.*, 2012). As a result, our aim herein was to investigate the degree of inflammatory cell infiltration into kidney tissue in diabetic rats as well as whether the anti-diabetic drug metformin was able to ameliorate leukocytes infiltration and other investigated biomarkers of diabetes-induced kidney injury.

MATERIAL AND METHOD

Animals. All rat (Albino rats; 170-200 g) studies were executed in accordance with King Khalid University guidelines for care and handling of animal work that ensured minimizing pain and stress to animals. Rats were kept in a clean facility with a controlled humidity and a temperature of 22 °C. They held in cages with a 12-h light/dark cycles and permitted unrestricted access to water and food.

Experimental design. 24 rats were separated equally in a random way into three groups after a one week acclimatization. Type 2 diabetes mellitus (T2DM) was induced as previously reported (Collino *et al.*, 2010). Briefly, one group of rats were fed for 14 days with a high carbohydrate and fat diet (HCFD) then received once streptozotocin (STZ; 50 mg/kg, i.p.), and fed on a HCFD for an extra 10 weeks. To investigate the effects of metformin on diabetes-induced DKD, a second cohort of rats (Met+T2DM) were treated in the same way except that these animals were placed on 200 mg/kg metformin from the first day until being culled, on week 12. The control group (Control) non-diabetic rats were placed on a standard laboratory chow for the whole experimental period. Rats were then

anesthetized and blood was collected. Animals were sacrificed and kidneys were collected.

Immunostaining and assessment of kidney injury.

Immunostaining was performed as described (Al-Ani *et al.*, 2021). Briefly, kidneys were fixed in formalin (10 %) for 12 hours prior to dehydration with alcohols and embedding in paraffin using standard methods. 5 mm paraffin kidney sections were de-paraffinized, rehydrated, and antigen retrieval was performed. Sections were incubated overnight at 4 °C with anti-CD45 (Cat # ab10558, Abcam, Cambridge, UK) antibody. Sections were incubated at room temperature for 30 minutes with the secondary antibody, and counter-stained with Meyer's hematoxylin. Quantification of CD45 staining was performed using image analyzer.

Determination of blood glucose, urea, MDA, and SOD.

Randox reagent kit (Sigma-Aldrich) was used to evaluate levels of glucose in the blood. Glucose blood levels were evaluated using a Randox reagent kit (Sigma-Aldrich). Blood urea was assessed using colorimetric methods according to manufacturer's instruction (BioAssay System, USA). Malondialdehyde (MDA) for lipid peroxidation (Cayman Chemical, MI, USA) and superoxide dismutase assay kit (SOD; Cayman Chemical, MI, USA) were used according to the instructions set by the manufacturer.

Statistical analysis. All numbers were expressed as the mean \pm SD. Data were analyzed using the SPSS version 10.0. One-way ANOVA was performed followed by Tukey's post hoc test to analyze the differences between the control and the experimental groups. For the detection of a probable significance between two different parameters, Pearson correlation statistical analysis was completed. Results are significant if $p \leq 0.05$.

RESULTS

HFD and STZ induce diabetic kidney disease (DKD) in rats.

We first modelled DKD secondary to diabetes in rats in order to test our working hypothesis mentioned above. After feeding rats with HFD for 14 days and administration of STZ, 87.5 % of rats developed diabetes (≥ 210 mg/dL) one week after STZ injection. Elevated blood levels of glucose and urea (Figs. 1A and 1B) confirmed DKD in the model group at the end of the experiment. Also, hematoxylin and eosin (H&E) stained images of glomeruli from the model group further confirms kidney injury development secondary to diabetes as demonstrated by pyknotic nuclei, dilated capsular space, damaged glomeruli, and congested blood vessels (Figs. 1C and 1D).

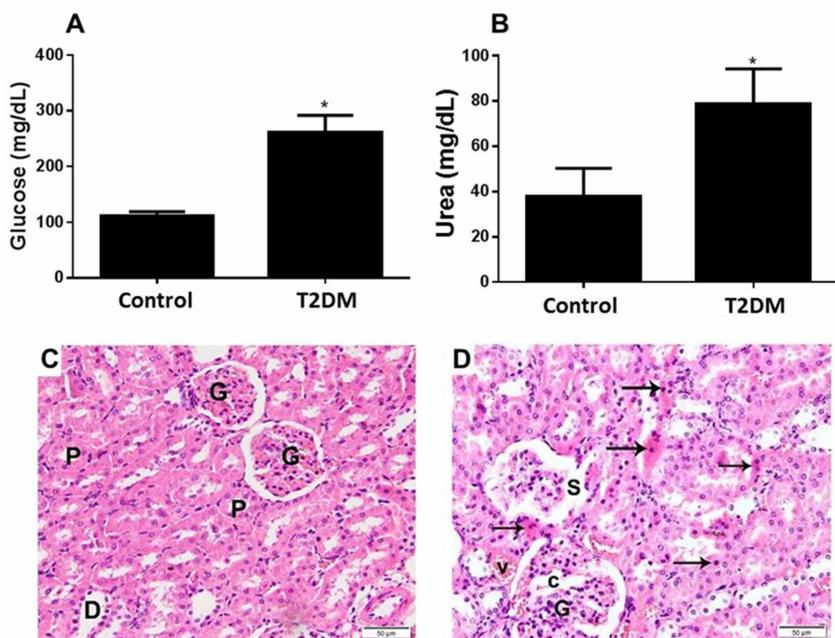


Fig. 1. Diabetes induces kidney injury in rats. Glucose (A) and urea (B) concentrations were assessed in the blood harvested at week 12 from two groups; the control and the model (T2DM) groups. * $p < 0.0001$ versus control. (C and D) Kidney tissue images (x200) stained by H&E obtained from the control (C) and the model (D) groups are depicted using light microscopy. Note the pyknotic nuclei (arrows) in (D). Abbreviations: G: glomerulus; P: proximal convoluted tubules; D: distal convoluted tubules; v: congested blood vessels; C: glomerular capillaries.

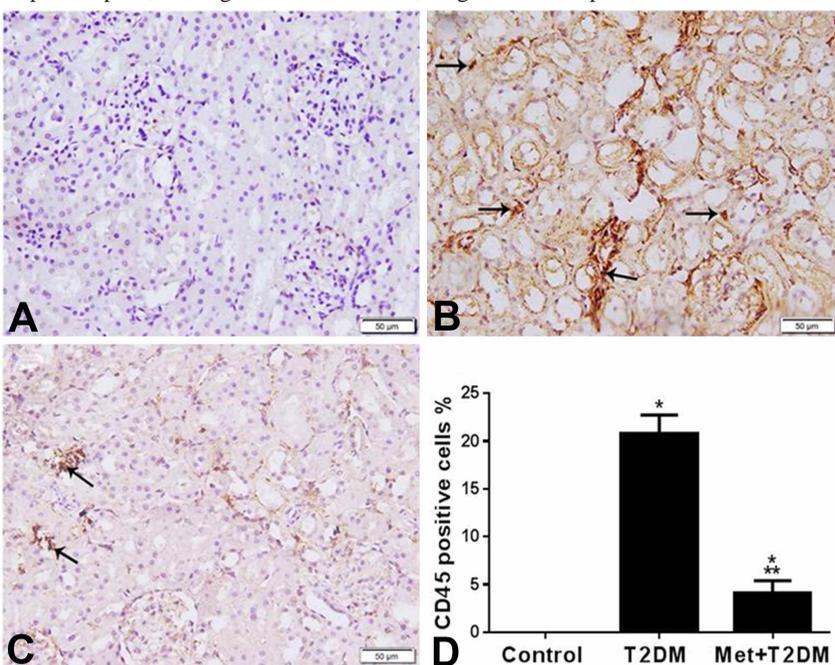


Fig. 2. Inhibition of diabetes-induced kidney infiltration of leukocytes by metformin. CD45 positive immunostained kidney cells (x200) are shown in all three groups of rats; control group (A), diabetic group (B), and treated group (C). A quantitative analysis of CD45 positive cells % deduced from the above immunostained kidney sections groups is shown in (D). * $p < 0.0001$ versus control, ** $p < 0.0001$ T2DM. CD45: leukocyte common antigen; T2DM: type 2 diabetes mellitus; Met: metformin.

Metformin inhibits leukocytes infiltration caused by diabetes in kidneys.

Recruitment of leukocytes into diabetic kidneys is reported (Vartak *et al.*, 2021). To determine whether diabetes induces the expression of CD45+ cells in kidneys and whether metformin can effectively decrease infiltration of leukocytes into diabetic kidney, we assessed the protein expression of CD45 levels in rats' groups (Fig. 2). Immunohistochemical staining of kidney samples derived from the diabetic group (T2DM) showed enhanced leukocytes recruitment (CD45+ immunostaining) (Fig. 2B) compared to a negative staining in the control group (Fig. 2A). Metformin treatment markedly ameliorated CD45+ immunostaining tissue (Figs. 2C and 2D). Furthermore, quantification of CD45 expression demonstrated an effective ($p < 0.0001$) inhibition of leukocytes infiltration by metformin.

Inhibition of biomarkers of DKD by metformin in rats.

Blood parameters associated with DKD were assessed. Diabetic rats fed on a HFD for 12 weeks showed a marked modulation in glucose (Fig. 3A), urea (Fig. 3B), MDA (Fig. 3C), and SOD (Fig. 3D), which were ($p \leq 0.0006$) protected by metformin (Fig. 3). The inhibition was incomparable to controls for blood glucose and MDA.

Correlation between leukocytes infiltration score and DKD markers.

We acquired the correlation between CD45 score and biomarkers of DKD; glucose, urea, MDA, and SOD. This links leukocyte infiltration into diabetic kidney with biomarkers of DKD and further show the therapeutic value of metformin in DKD. CD45 score presented positive correlation with glucose ($r = 0.926$; $p < 0.0001$) (Fig. 4A), urea ($r = 0.800$; $p < 0.0001$) (Fig. 4B), and MDA ($r = 0.950$; $p < 0.0001$) (Fig. 4C). Whereas, SOD displayed a negative correlation with CD45 score ($r = -0.888$; $p < 0.0001$) (Fig. 4D).

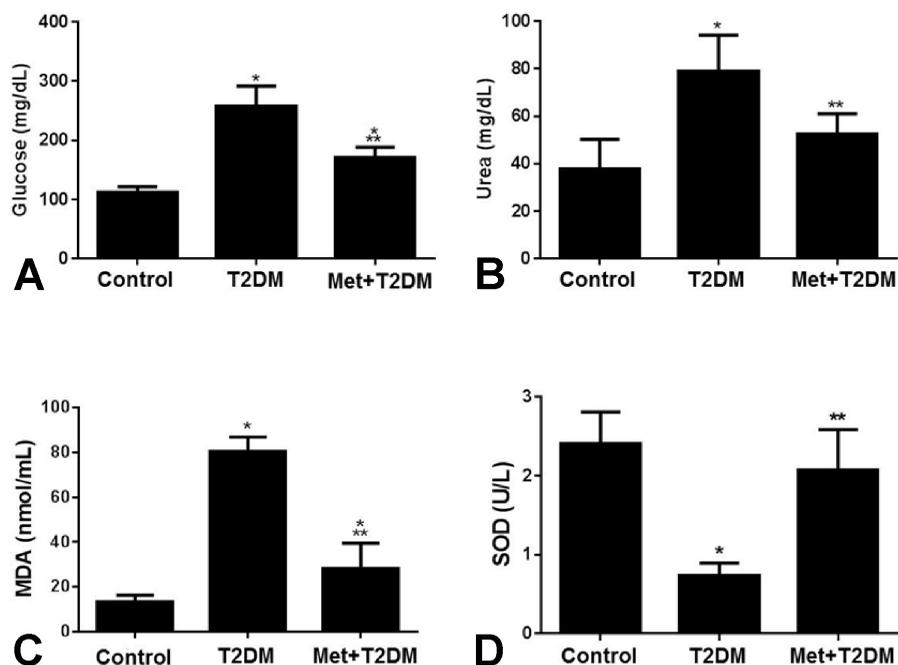


Fig. 3. Metformin inhibits biomarkers of diabetic kidney disease (DKD). Glucose (A), urea (B), MDA (C), and SOD (D) concentrations were assessed in the blood harvested at week 12 from the three groups; the control, the model (T2DM), and the treated (Met+T2DM) animal groups. * $p \leq 0.029$ versus control, ** $p \leq 0.0006$ versus T2DM. T2DM: type 2 diabetes mellitus; Met: metformin; MDA: malondialdehyde; SOD: superoxide dismutase.

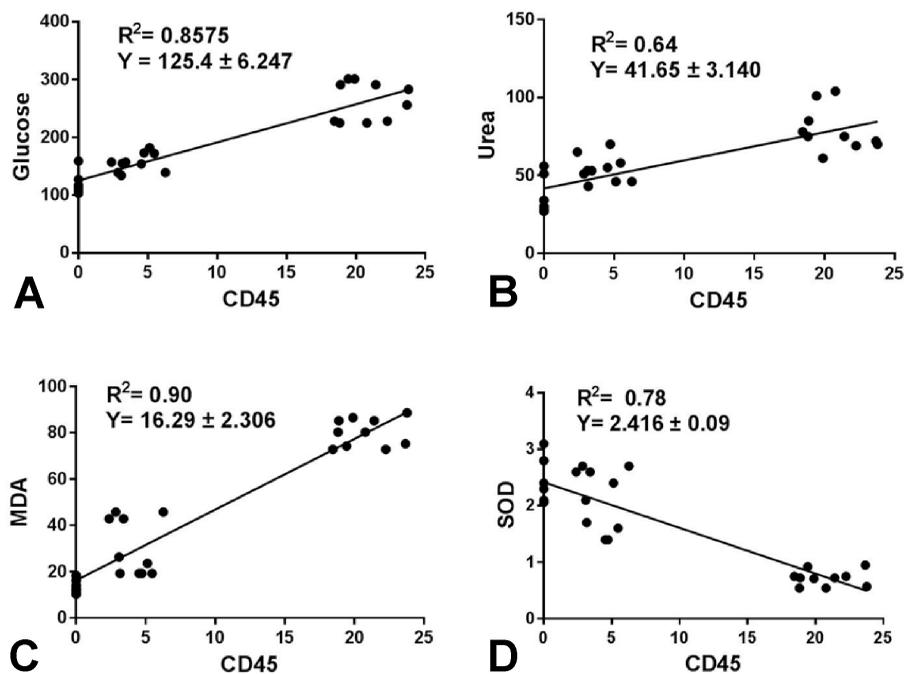


Fig. 4. The CD45 score correlates with biomarkers of diabetic kidney disease. After the completion of the experiment, the degree of CD45 tissue expression was evaluated in all groups of rats and a significant positive correlation was detected between CD45 versus glucose (A), Urea (B), and MDA (C). Whereas, a ($p < 0.0001$) significant negative correlation is shown between CD45 versus the antioxidant SOD (D). MDA: malondialdehyde; SOD: superoxide dismutase.

DISCUSSION

In this study, we induced DKD in rats, which developed 10 weeks post diabetic induction. We were required to model this disease in order to test the hypothesis that diabetes can induce leukocytes infiltration into kidneys measured as an increase in CD45 tissue expression associated with the augmentation of lipid peroxidation and biomarkers of kidney injury, and the antidiabetic and antioxidant drug metformin can protect against these changes. Here, we report that induction of diabetes by a combination of HFD and STZ caused after 10 weeks a strong CD45+ leukocytes immunostaining in kidney tissues harvested from the model group of rats and upregulated oxidative and nitrosative stress, and biomarkers of DKD, which were inhibited by metformin (Fig. 5). Also, an important correlation was observed between CD45 score and DKD biomarkers (Fig. 4) using data obtained from the three animal groups, which further confirm that metformin is a beneficial drug to treat DKD. Our data support our working hypothesis mentioned above.

The kidney is a well-known target of diabetes in humans and animals that lead to the development of DKD (Glastras *et al.*, 2016; Alicic *et al.*, 2017) represented by proteinuria, elevated levels of serum creatinine, increased macrophage infiltration, and augmentation of oxidative stress (Jefferson *et al.*, 2008; Glastras *et al.*, 2016; Alicic *et al.*, 2017). These reports agreed with our study showing augmentation of leukocytes infiltration, albuminuria, oxidative stress, and blood creatinine. Similarly, our data that point to metformin inhibition of leukocytes infiltration (CD45+ expressing cells) and other parameters are in agreement with previous studies showed (i) metformin decreased the infiltration of immune cells into the kidney in mice with unilateral ureteral obstruction (Christensen *et al.*, 2019); (ii) metformin ameliorated macrophage and neutrophils infiltration into lungs and atherosclerotic

regions in animals (Feng *et al.*, 2021); (iii) metformin given post diabetic induction demonstrated reno-protective effects in diabetic rats after 13 weeks such as reduced blood urea and creatinine (Zhang *et al.*, 2017); and (iv) metformin was partially protected against diabetic nephropathy in diabetic patients treated for 5 years before they developed the disease (Lachin *et al.*, 2011).

In summary, our data demonstrated in a rat model of DKD the activation of leukocytes infiltration into kidneys measured as CD45 positive protein expression associated with proteinuria and oxidative and nitrosative stress upregulation, which showed significant inhibition by metformin for a period of 10 weeks. In addition, we demonstrated an association between the infiltration of inflammatory cells and biomarkers of DKD. Future study will monitor these parameters in DKD induced secondary to insulin dependent diabetes.

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RESUMEN: La diabetes y la hipertensión representan la mayoría de los casos de lesión renal crónica que pueden provocar insuficiencia renal. El vínculo entre el antígeno común de los leucocitos (CD45) y la enfermedad renal diabética (DKD) con y sin incorporación de metformina en un modelo animal no se había anteriormente investigado. El objetivo fue evaluar el grado de infiltración de leucocitos en los tejidos renales 10 semanas después de la inducción de diabetes en ratas tratadas con metformina. Además, monitoreamos los parámetros de sangre y orina asociados con la diabetes. El grupo modelo de ratas recibió una inyección de estreptozotocina (STZ; 50 mg/kg) después de ser alimentadas durante 14 días con una dieta alta en grasas (HFD) y continuamente alimentadas con un HFD hasta que fueron sacrificadas, en la semana 12. El grupo protector fue tratado de la misma manera excepto que estos animales fueron recibidos desde el día 1 metformina (200 mg/kg) hasta ser sacrificados, en la semana 12. Los riñones fueron inmunoteñidos con CD45 como marcador de infiltración de leucocitos y examinados por microscopía óptica. Las muestras de orina se analizaron en busca de albúmina y la sangre recolectada se analizó en busca de glucosa, urea, creatinina y biomarcadores de estrés oxidativo y antioxidantes. La lesión renal secundaria a la diabetes se desarrolló como lo demuestra (i) el aumento de la glucosa en sangre, la urea y el malondialdehído (MDA) como marcador de la peroxidación lipídica; y (ii) daño del tejido renal y marcado aumento en los tejidos renales que expre-



Fig. 5. Proposed model for leukocytes infiltration into kidneys in a rat model of diabetic nephropathy appears inhibited by metformin. CD45: leukocyte common antigen; ROS: reactive oxidative stress; T2DM: type 2 diabetes mellitus; Met: metformin.

san células positivas para CD45. Los marcadores anteriores fueron inhibidos ($p \leq 0.0006$) por metformina. Además, se observó una correlación significativa entre la puntuación de CD45 y la glucemia, la urea, la MDA y la superóxido dismutasa antioxidante (SOD). Por lo tanto, nuestros datos demuestran una asociación entre la infiltración de células inflamatorias CD45+ en los tejidos renales y biomarcadores de daño renal en un modelo de rata con DKD, que fue protegido de manera efectiva por metformina.

PALABRAS CLAVE: Diabetes; Lesión renal; CD45; Estrés oxidativo; Metformina; Modelo de rata.

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