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Upregulation of KIM-1 and Inflammatory Mediators is Associated with Kidney Tubular Injury in Diabetic Rat Model

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Abstract: Chronic diabetes mellitus (DM) leads to diabetic nephropathy (DN) that contributes to the Global Burden of Disease. Sprague Dawley male rats (3 months old, 200 grams) were divided into four groups: control (n=6) and DM groups that received an intraperitoneal injection of Streptozotocin (60 mg/kg BW) for 1 month (DM1, n=6), 2 months (DM2, n=6), and 4 months (DM4, n=6). The rats were sacrificed, and retro-orbital blood was withdrawn to assess creatinine serum levels. The kidneys were harvested to assess tubular injury by Periodic-Acid Schiff (PAS), kidney fibrosis by Sirius Red staining, and mRNA expression of KIM-1, E-Cadherin, and Vimentin by reverse transcriptase PCR (RT-PCR). Long-term diabetes mellitus enhanced creatinine serum level (p<0.001), tubular injury (p<0.05), and kidney fibrosis (p<0.001) in the DM2 and DM4 groups compared to the control group. mRNA expression of E-Cadherin significantly decreased in the DM4 group (p<0.05) while mRNA expression increased both in vimentin DM4 group (p<0.001) and KIM-1 DM4 group (p<0.05) compared to the control group. The inflammatory mediators consisting of NFκB, MCP1, and CD68 significantly increased in DM4 groups compared to the control group (p<0.05). Chronic diabetes mellitus elevates tubular injury and fibrosis and is associated with upregulation of KIM-1 mRNA expression.

Keywords: diabetes mellitus; tubular injury; KIM-1; EMT; inflammation.

1. Introduction

Diabetes mellitus with malfunction of insulin production or failure to process insulin effectively becomes one of the world's health problems. As many as 1.5 million people died because of diabetes mellitus globally in 2012, and there were 422 million adults with diabetes mellitus in 2014 [1]. Uncontrolled hyperglycemic conditions in DM lead to a reduction of the quality of life and increasing mortality due to systemic microvascular and macroscopic complications resulting in multiple organ failure, including kidneys, nerves, and blood vessels. One of the most common complications is diabetic nephropathy (DN), occurring in 30% and

40% of patients with type 1 and type 2 diabetes mellitus, respectively [2]. Diabetic nephropathy, which can be classified as Chronic Kidney Disease (CKD), ultimately contributes to the Global Burden of Disease, although receiving less attention [3].

Chronic hyperglycemia induces morphological and functional injury in the kidney, such as nephron damage. It is characterized by increased vessel permeability and impaired glomerular filtration barrier [4,5], thus inducing further albumin excretion in urine. Due to microalbuminuria, reactive oxygen species (ROS) and high protein levels in tubular ultrafiltrate will activate various inflammatory mediators, which may induce tubular injury of the kidney and eventually end-stage renal damage [6]. Nephron damage in diabetic nephropathy is associated with both systemic and local inflammation with the involvement of inflammatory cells, cytokines, and signaling pathways, for example, nuclear factor-κB (NF-κB), Janus N-terminal kinase/signal transducers and activators of transcription (JAK/STAT), and macrophages [7]. The NF-κB is a nuclear transcription factor found in all types of cells, which has various roles in cellular response towards stress, inflammation, free radicals, and viral and bacterial antigens [8]. Hyperglycemic conditions induce advanced glycation end-products (AGE) formation [9] and ROS overproduction through polyol flux pathways and protein kinase-C activation [10]. AGE and ROS initiate pro-inflammatory response and endothelial dysfunction through NF-κB activation. During hyperglycemic conditions, NF-κB increases significantly and causes the release of various chemokines, cytokines such as transforming growth factor-β (TGF-β) and vascular cell adhesion molecule-1 (VCAM-1) [11].

NF-kB is expressed in intrinsic kidney cells, such as podocytes, mesangial, tubular, and endothelial cells, after exposure to inflammatory stimuli, such as tumor necrosis factor (TNF) and angiotensin II. In the DN, NF-κB is localized in kidney tubular epithelial cells and correlated with proteinuria and infiltration of inflammatory cells in the interstitial [12]. NF-κB leads to the expression of monocyte chemoattractant protein-1 (MCP-1) [13], which is upregulated in kidney tissue. High MCP-1 levels in urine and macrophage influx describe the pathogenic role of MCP-1 in glomerular damage and progression of kidney disease [14].

The unavailability of biomarkers for elucidating the progression of DM complications is the main problem of DN. Tubular injury of the kidney can change the protein expression level, which has a possible role as a biomarker, such as kidney injury molecule-1 (KIM-1), E-cadherin, and vimentin. KIM-1 mRNA expression will increase in accordance with the severity of kidney dysfunction [15]. KIM-1 is a type-1 cell transmembrane glycoprotein comprising 6-cysteine, similar to immunoglobulin. KIM-1 can be used as a biomarker of tubulointerstitial damage because it is expressed in the apical membrane of epithelial cells of kidney proximal tubules in response to ischemia and toxicity [16]. Only a small amount of KIM-1 will be expressed continuously in normal kidneys [17]. Otherwise, kidney injury can be detected by alternated expression of E-cadherin and vimentin. During kidney damage, the cells are structurally altered, which separates the apical and basolateral compartments from the membrane [18]. Excretion of KIM-1 will increase in urine during kidney injury, and its titer is associated with the severity of kidney tubular injury, interstitial fibrosis, and inflammation [15]. This study elucidated the association between KIM-1 expression with inflammation and kidney injury in the kidney of the DM model.

2. Materials and Methods

2.1. Animal model of diabetes mellitus.

A total of 24 male Sprague Dawley rats (3 months old, 200 grams) were used in this study. Rats were placed in cages with a light-dark cycle of 12 hours. The diabetes mellitus (DM) model was induced by a single intraperitoneal injection of 60 mg/kg body weight (BW) Streptozotocin/STZ (Nacalai, 32238-91). Blood glucose level was quantified five days after injection to examine the success of the model. DM was defined by a blood glucose level higher than 200 mg/dL. Rats were kept in a light-and-dark cycle room with free access to water and chow. Ethical clearance was approved by the Ethical Clearance Committee of the Faculty of Medicine, Public Health and Nursing, known as FERCAP, with number KE/FK/0140/EC/2020.

Rats were divided into four groups based on the time of sacrifice: 1 month (DM1 group, n=6, 2 months (DM2 group, n=6), 4 months (DM4 group, n=6), and control group (n=6). The control group was given an intraperitoneal injection of NaCl 0.9%, which was then sacrificed after a specified time. For sacrifice, rats were deeply anesthetized using intramuscular injection of 100 mg/kg BW of ketamine. Then, the abdomen and thorax were opened, and the left ventricle was perfused with NaCl 0.9%. The right and left kidneys were harvested, the right kidney was kept in normal buffer formalin for paraffin making, and the left kidney was kept in RNA preservation solution (Favorgen, FATRR001) for RNA extraction.

2.2. Quantification of serum creatinine level.

Before sacrifice, blood was collected from the retro-orbital vein to quantify glucose and creatinine levels. Serum creatinine was measured with a kinetic test without deproteinization according to the Jaffe method (Creatinine FS; DiaSys®). Glucose level has been reported in our previous study [19].

2.3. Histological assessments for quantification of tubular injury and fibrosis area.

Paraffin slides were deparaffinized and then stained with Periodic acid Schiff (PAS) and Sirius Red to quantify tubular injury and fibrosis area, respectively. The tubular injury scores were determined through a semiquantitative scoring system. Ten fields were examined for each kidney, and the lesions were graded from 0 to 4 (0, no change; 1, changes affecting <25% of the section; 2, changes affecting 25-50%; 3, changes affecting 50-75%; and 4, changes affecting > 75%). The assessment was performed based on characteristics of tubular injury (tubular atrophy, tubular dilatation, loss of brush border, intraluminal casts, and interstitial inflammation). The score index of each rat was expressed as a mean value of all scores obtained.

Sirius Red staining was carried out to observe interstitial fibrosis and quantify interstitial fibrosis area fraction. Area fraction was measured in ten randomly selected fields with 400x magnification. Image J software was used for the quantification.

2.4. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR).

The total RNA from the kidney was extracted using Genezol solution (GENEzol™, GZR100) based on the manufacturer's protocol. RNA concentrations were quantified using a nanodrop. The synthesis of cDNA was done using a cDNA synthesis kit (SMOBio, RP1400).

RT-PCR was performed for these following genes with specific primers: E-cadherin (forward: 5′-CCAGCGACTGGTTCAGATCA-3′; reverse: 5′-GATGAAAACGCCAACAGGGG-3′), vimentin (forward: 5-ACCAGAGACGGACAGGTGAT-3′; reverse: 5′-CTTGCGCTCCTGAAAACTGC-3’), KIM-1 (forward: 5′-AATCCCTTGATCCATTGTTTTCTT-3′; reverse: 5′-GTGAGTGGACCAGGCACACA-3′), NF-κB (forward: 5-CACTCTCTTTTTGGAGGT-3′; reverse: 5’-TGGATATAAGGCTTTACG-3’), MCP-1 (forward: 5-GCTGTAGTATTTGTCACCAAGCTC-3′; reverse: 5′-ACAGAAGTGCTTGAGGTGGTT-3’), CD68 (forward: 5-TGTGTCCTTCCCACAAGCAG-3′; reverse: 5’-AAGAGAAGCATGGCCCGAAG-3’) and a housekeeping gene, ß-actin (forward 5′-GCAGATGTGGATCAGCAAGC-3′; reverse 5′-GGTGTAAAACGCAGCTCAGTAA-3′). For RT-PCR, we used Taq Master Mix (GoTaq®Green Master Mix, Cat No. M7122). PCR products were analyzed on 2% agarose gel with a DNA ladder (SMOBio, DM3200). Gene expressions were quantified with densitometric analysis using ImageJ software, with ß-actin being used to normalize the expression.

3. Results and Discussion

3.1. DM associated with decreased renal function and increased tubular injury.

Serum creatinine levels of DM2 and DM4 groups were significantly higher than control and DM1 groups.

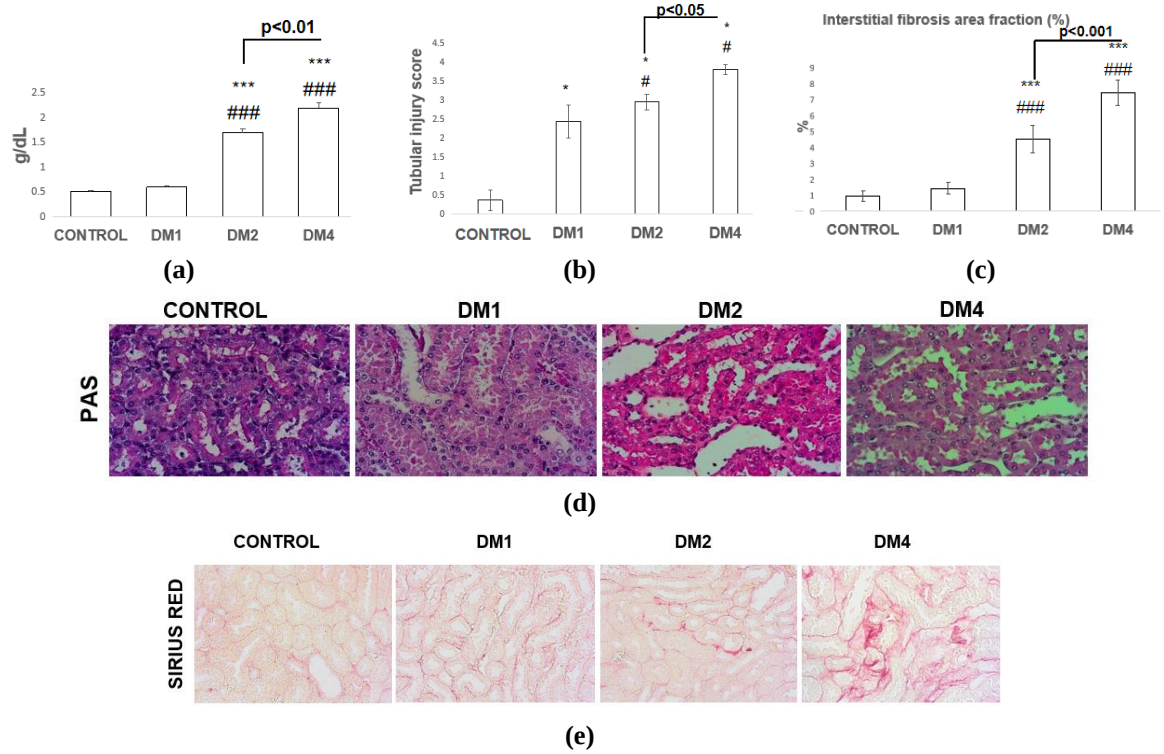

Figure 1. Serum creatinine level, tubular injury, and fibrosis area. (**A**) Serum creatinine level measurement showed DM progression is associated with deterioration of renal function with higher serum creatinine level found in DM groups; (**B**-**C**) Quantification of tubular injury score and interstitial fibrosis area fraction showed higher score and area fraction in DM groups; (**D**) PAS staining showed tubular injury with effacement of tubular epithelial cells and loss of brush border in DM groups; (**E**) Sirius Red staining showed positive red color as a sign of collagen accumulation in interstitial areas of DM groups. *p<0.05 vs control, ***p<0.001 vs control, #p<0.05 vs control, ###p<0.001 vs DM1 group.

Furthermore, the DM4 group demonstrated a higher escalation of serum creatinine levels compared to the DM2 group (Figure 1A). Furthermore, the deterioration of renal function was associated with higher tubular injury and interstitial fibrosis area fraction in DM2 and DM4 groups (Figure 1B and 1C). DM1 demonstrated a higher tubular injury score with no statistical difference in interstitial fibrosis area fraction compared to the control group. DM4 showed the highest tubular injury score and interstitial fibrosis area fraction among other groups. These results highlighted the progression of DM-induced damage to renal function and architecture (Figure 1D). Histological staining showed tubular injury in DM2 and DM4 groups, which was characterized by epithelial cell effacement with brush border loss (Figure 1D). Sirius red staining also revealed a positive red color, showing collagen accumulation in the DM2 and DM4 groups (Figure 1E).

3.2. DM is associated with the downregulation of E-Cadherin and upregulation of vimentin and KIM-1 mRNA expression.

RT-PCR analysis revealed downregulation of E-cadherin (a marker of epithelial cell integrity), as shown by significantly lower E-cadherin mRNA expression in the DM4 group compared to the control group (Figure 2B). The downregulation of E-cadherin was associated with the upregulation of vimentin mRNA expression in DM groups compared to control (Figure 2C). KIM-1 mRNA expression, as a marker of kidney injury, was also significantly higher in the DM4 group than in the control group (Figure 2D). There was no significant difference between DM1 and DM2 compared to control. Progression of DM might induce kidney injury, with the downregulation of epithelial cell markers and the upregulation of mesenchymal cell markers (Figure 2).

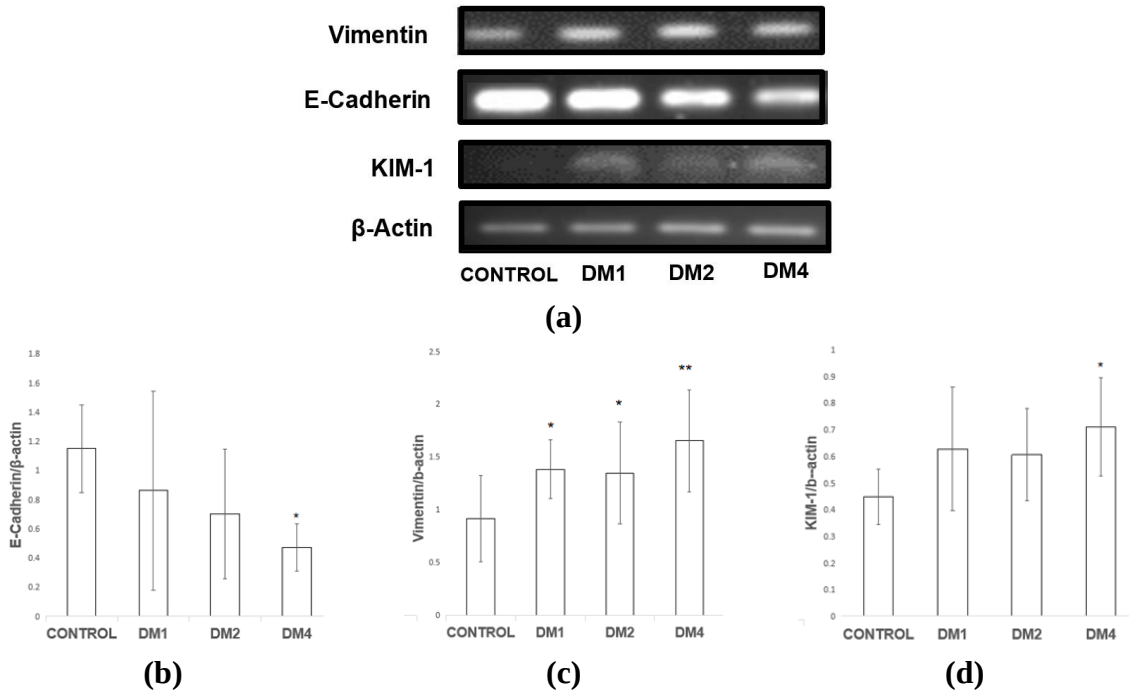

Figure 2. Upregulation of *E-cadherin*, *vimentin*, and *KIM-1* in DM groups. **(A)** Representative picture of electrophoresis gel of *E-cadherin*, *vimentin*, and *KIM-1* mRNA expression; **(B)** Densitometric analysis of *E-cadherin* mRNA expression. DM4 group demonstrated the lowest *E-cadherin* mRNA expression; **(C)** Densitometric analysis of *vimentin* mRNA expression. DM4 group demonstrated the highest *vimentin* mRNA expression; **(D)** Densitometric analysis of *KIM-1* mRNA expression. DM4 group demonstrated the highest *KIM-1* mRNA expression. *p<0.05 vs control, **p<0.01 vs control.

3.3. DM associated with upregulation of inflammatory mediator mRNA expression.

Next, we investigated the expression of inflammatory mediators, which showed increased NF-κB and MCP-1 mRNA expression in the DM4 group compared to the control group. Only the DM4 group had significantly higher NF-κB and MCP-1 mRNA expression than the control group. This result was associated with significantly higher CD68 mRNA expression as a marker of macrophages in the DM4 group than in the control group (Figure 3).

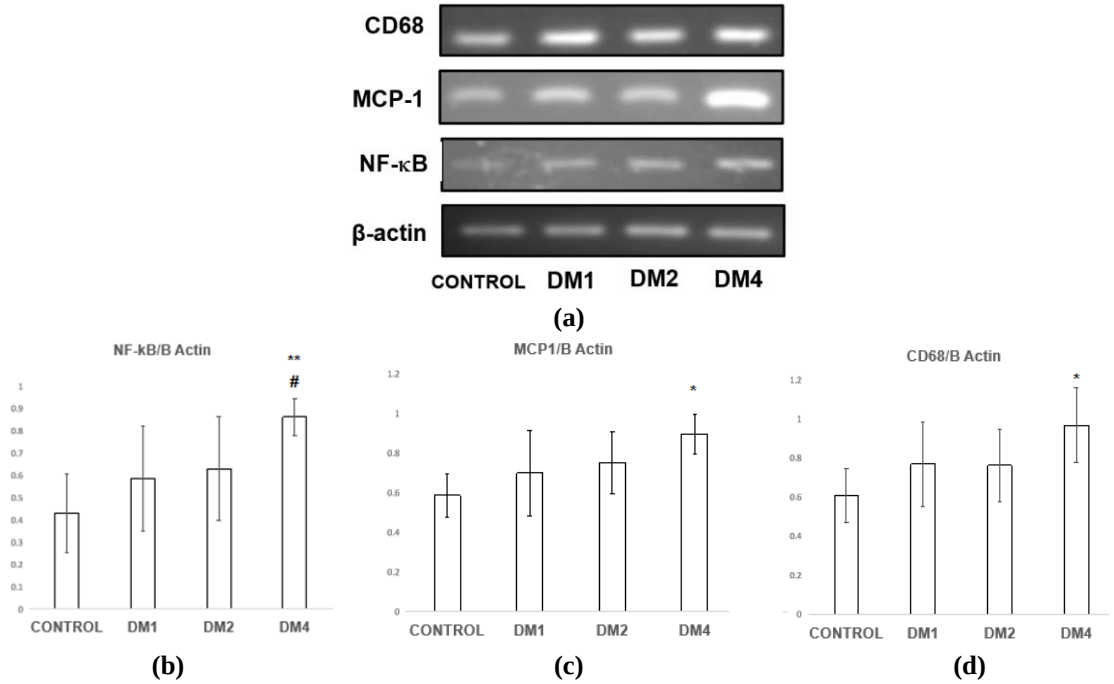

Figure 3. Upregulation of *CD68*, *NF-κB*, and *MCP-1* in DM groups. **(A)** Representative picture of electrophoresis gel of *CD68*, *NF-κB*, and *MCP-1* mRNA expression; **(B)** Densitometric analysis of *NF-κB* mRNA expression. DM4 group demonstrated the highest *NF-κB* mRNA expression; **(C)** Densitometric analysis of *MCP-1* mRNA expression. DM4 group demonstrated the highest *MCP-1* mRNA expression; **(D)** Densitometric analysis of *CD68* mRNA expression. DM4 group demonstrated the highest *CD68* mRNA expression. *p<0.05 vs control, **p<0.01 vs control, #p<0.05 vs control, p<0.001 vs DM1 group.

Our study revealed kidney function deterioration and tubular injury after DM associated with KIM-1 and inflammatory mediator upregulation. Downregulation of E-Cadherin after DM induction indicated a high tubular injury score in the kidney. E-cadherin expression can be used as an early biomarker of tubular injury. Reduction of E-cadherin expression in injury is caused by ischemia and, through cell apoptosis, increases activation of proteolytic enzymes in diabetic nephropathy [20]. Downregulation of E-Cadherin is also associated with the upregulation of vimentin as a mesenchymal marker [21], which might reveal an epithelial-to-mesenchymal transition (EMT) process. EMT is known as one of the mechanisms of kidney injury, which is characterized by: 1. loss of epithelial cells adhesion [22] (showed by reduction of E-cadherin expression); 2. actin reorganization and de novo α-SMA expression; 3. basal tubular membrane damage by matrix metalloproteinase (MMP); 4. increase of cell migration [23] and interstitial matrix invasion. In this research, we found a lower expression of E-cadherin and a higher expression of vimentin in our model [24,25]. Vimentin may appear due to damaged kidney tubules in diabetes. Chronic hypoxia, inflammation, and high glucose levels will cause tubular epithelial damage through EMT [26]. EMT is closely related to kidney fibrosis [27], in which tubular epithelial cells produce extracellular matrix (ECM), which is characteristic of mesenchymal phenotype [28].

Detection of kidney tubular injury as a complication of DN becomes the problem in evaluating DN. Our results demonstrated KIM-1 mRNA upregulation in the kidney of the DN model, which might represent kidney tubular injury and fibrosis as characterized by DN. In DN, KIM-1 upregulation occurred in the chronic period, especially in the DM4 group. Research showed that KIM-1 only indicates damage of kidney tubules during the advanced stage [29] and was not correlated with urine albumin-creatinine ratio (UACR) or estimated glomerular filtration rate (eGFR) [30]. It seemed that chronic induction of DM with higher tubular injury is associated with KIM-1 expression. KIM-1 is associated with the ischemic process, toxic renal injury, and fibrosis [31]. In this study, we found high KIM-1 expression, but it was not significant to prove the role of KIM-1 as an early screening marker of progressive kidney injury. However, it had a beneficial effect in elucidating tubular injury and fibrosis as a chronic complication of DN. KIM-1 expression can possibly be used as a kidney injury marker because its expression increased significantly from histopathological examination as well as blood tests [30]. KIM-1 is in a steady state to an undetected level in normal conditions [32].

On the other hand, KIM-1 expression increases in the proximal tubules of the surface of apical cells and can be detected in urine [33]. KIM-1 may also be detected in the blood and associated with epithelial cell polarity loss due to injury [34]. Exploration of KIM-1 expression in the blood and urine may provide more information about the role of KIM-1 in tubular injury and fibrosis in DN [35,36]. It may be performed in the next research. KIM-1 upregulation occurs and closely correlates with renal dysfunction severity and inflammation [15].

Our study also revealed inflammation with inflammatory mediators upregulation in the kidney after DN. Inflammation contributes to the occurrence of DM complications, such as diabetic nephropathy [37,38]. Hyperglycemia causes activation of NF-κB release of inflammatory cytokines by kidney tissue [39], such as IL-1, IL-6, TNF-α, and MCP-1, then inducing macrophage infiltration [40]. NF-κB might also be expressed by mesangial cells after high glucose treatment, which induces reduction of glucagon-like peptide-1 receptor (GLP-1R), which easily activates NF-κB and MCP-1 [41]. During hyperglycemia conditions, pyruvate kinase M2 (PKM2) phosphorylation can activate the NF-κB signaling pathway, which induces upregulation of intercellular adhesion molecule-1 (ICAM-1) and infiltration of inflammatory cells [42].

This study also demonstrated a reduction of E-Cadherin, which represented epithelial cell injury associated with upregulation of inflammatory signaling, especially NF-KB and MCP-1. DN in the *Psammomys obesus*, as an ideal animal model to study diabetes mellitus temporal evolution, demonstrated activation of NF-κB and downregulation of E-Cadherin after high-calorie diet treatment [43]. DM model with STZ injection is also consistent with our result, which showed significant NF-κB upregulation compared to a control group without STZ injection [44]. NF-κB induces inflammation signaling, leading to macrophage infiltration, especially through MCP-1 [40]. Furthermore, MCP-1 is expressed by mesangial cells due to many stimulations, such as inflammatory cytokine, tumor necrosis factor-alpha (TNFα) [45], immune complex, metabolite (glucose, glycation end products) [46] and danger-associated molecular patterns (DAMPs). Other cells also are known to express MCP-1, such as podocytes, endothelial cells, and kidney tubular epithelial cells. Kidney biopsy from DN patients showed upregulation of MCP-1 expression and macrophage infiltration in the glomerulus and tubulointerstitial areas [47]. Moreover, MCP-1 upregulation is associated with proteinuria, glomerular damage, and kidney disease progression in human [14, 48].

MCP-1 is considered a cytokine for initiating kidney inflammation and fibrosis [49], which finally leads to diabetic nephropathy due to its effect on monocyte recruitment into kidney tissue [44]. DM increased the expression of *CD68* [50] and *MCP-1* [45] in the kidney tubules and interstitial space, which was attenuated with Vitamin D treatment [51]. CD68 expression demonstrated macrophage infiltration. Our study revealed the upregulation of both MCP-1 and CD68 as macrophage markers in the DM model, which may be associated with renal function deterioration and KIM-1 expression. Furthermore, macrophage infiltration in the interstitial areas correlates with not only the glomerular filtration rate (GFR) stage and albuminuria [52] but also proteinuria and serum creatinine levels [53]. Systemic inhibition of MCP-1 can modify the characteristics and differentiation of the macrophages, reduce albuminuria, and restore glomerular endothelial glycocalyx [54].

4. Conclusions

This study highlighted that KIM-1 upregulation in the kidney may be associated with tubular injury and inflammation in the chronic diabetes condition. These findings may provide information to DN as a complication of diabetes.

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Conflicts of Interest

The authors declare no conflict of interest.

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