

## Original Article

# Matrine attenuates high glucose-induced podocyte damage by inhibiting HMGB1-associated TLR4-NF- $\kappa$ B signaling

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**Abstract:** Podocyte damage plays a pivotal role in the development of diabetic nephropathy (DN). Matrine (MAT), a natural compound extracted from *Sophora flavescens* Ait, has been reported to provide anti-inflammatory and reno-protective effects in adriamycin-induced nephropathy and cyclosporin A-induced chronic nephrotoxicity in rats. However, the effects of MAT on high glucose (HG)-induced renal damage have not been elaborated. The main objectives of the current study were to investigate the effects of MAT on HG-induced podocyte damage, examining whether the effects of MAT are associated with inhibition of high mobile group box 1 (HMGB1)-associated toll like receptor (TLR) 4-nuclear factor (NF)- $\kappa$ B signaling. HG-cultured mouse podocytes were treated with MAT. Cell viability was measured using MTT assays. Levels of TLR4, phosphorylated NF- $\kappa$ B p65 (p-p65), and phosphorylated inhibitors of NF- $\kappa$ B alpha (p-I $\kappa$ B $\alpha$ ) were detected using Western blotting. NF- $\kappa$ B p65 (p65) DNA-binding activity was detected using an NF- $\kappa$ B family EZ-TFA transcription factor assay kit. Levels of HMGB1, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured via ELISA assays. In addition, recombinant HMGB1 (rHMGB1) and a HMGB1 inhibitor ethyl pyruvate (EP) were used to detect the roles of HMGB1 in HG-induced podocyte damage. Results showed that HG-incubation decreased the viability of podocytes and increased levels of HMGB1, TLR4, p-I $\kappa$ B $\alpha$ , p-p65, p65 DNA-binding activity, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . However, MAT treatment significantly attenuated the above alterations induced by HG-incubation. MAT showed no effects on levels of HMGB1 in normal glucose (NG)-cultured podocytes. In addition, results showed that rHMGB1 incubation decreased podocyte viability and activated TLR4-NF- $\kappa$ B signaling in NG-cultured podocytes. Moreover, the effects of MAT on podocyte viability and TLR4-NF- $\kappa$ B signaling were markedly reversed by co-incubation with rHMGB1. Present data indicates that activation of HMGB1-TLR4-NF- $\kappa$ B signaling plays a crucial role in HG-induced podocyte damage. Cytoprotective effects of MAT on HG-cultured podocytes were shown to be associated with inhibition of HMGB1-associated TLR4-NF- $\kappa$ B signaling.

**Keywords:** Matrine, cytoprotective effects, high glucose, podocyte, HMGB1, TLR4, NF- $\kappa$ B, cytokine

## Introduction

Diabetic nephropathy (DN), an important complication of diabetes mellitus, is the leading cause of end-stage renal disease, worldwide. It affects 30-40% of type 2 diabetic patients and 15-25% of type 1 diabetic patients [1]. The mechanisms by which diabetes mellitus induces DN are complex. They have not been fully understood. Specific medicines for DN treatment have not been developed. Many recent studies have shown that inflammation plays a crucial role in DN [2, 3]. Anti-inflammatory treat-

ment has been regarded as a novel therapeutic strategy against DN [4].

Toll-like receptors (TLRs) are important regulators of inflammatory response, abundantly expressed in immune and non-immune cells. TLRs are found in the kidneys, playing a great role in inflammation-associated renal damage, including DN [5]. Regarding TLRs, overexpression of TLR4 has been observed in diabetic patients [6], DN animal models [7] and renal cell lines exposed to high glucose [8]. TLR4 expression has been positively correlated with renal dam-

age in DN [9, 10]. Therefore, TLR4 signaling is involved in renal inflammation and tissue injury in diabetic nephropathy [11]. Moreover, TLR4 can trigger activation of NF- $\kappa$ B, which plays a central role in regulating expression of the genes of multiple inflammatory mediators, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . These cytokines are involved in the pathogenesis of DN [2, 3]. In addition, deficiencies of TLR4 [12], TLR4 antagonist [13], and some agents [10] that inhibit overexpression of TLR4 have exhibited protective effects against diabetic nephropathy, according to recent studies. Thus, TLR4-NF- $\kappa$ B signaling is a novel therapeutic target for anti-inflammatory strategies against DN.

HMGB1, originally described as a DNA-binding protein, has recently been recognized as a key mediator in inflammatory response. Multiple receptors, such as TLR2, TLR4, and receptors for advanced glycation end products, may be involved in HMGB1-mediated cellular activation [14]. HMGB1 signaling through these receptors can promote the activation of NF- $\kappa$ B signaling, leading to inflammatory damage. Increased production of HMGB1 has been reported in renal damage, including kidney ischemia reperfusion injuries [15] and sepsis-induced kidney injuries [16]. Kim et al. first reported the association between HMGB1 and DN [17]. In a recent study, Zhang H et al. found that HMGB1 inhibitor glycyrrhizic acid attenuates kidney injuries in streptozotocin-induced diabetic rats [18]. Many other recent studies have proven that upregulation of HMGB1 exacerbates renal damage in DN, as well as other renal damage, by stimulating inflammatory and immune response through TLR4 pathways [18-20]. Moreover, downregulating HMGB1-mediated TLR4-NF- $\kappa$ B pathways has exhibited protective effects against renal damage in diabetic kidney disease [18, 21], as well as other renal disorders [22]. Thus, converging evidence has indicated that HMGB1 is an important therapeutic target for regulation of TLR4-NF- $\kappa$ B signaling and for management of renal damage.

Matrine (MAT), a natural compound extracted from *Sophora flavescens* Ait, has been tested in various diseases, examining its multiple pharmacological activities. It has exhibited anti-inflammatory activity in many inflammation-associated disorders, such as endotoxin-induced acute liver injuries in mice [23], ovalbumin-induced allergic airway inflammation in asth-

matic mice [24], and type II collagen-induced arthritis in rats [25]. Moreover, a study by Sun D et al. suggested that MAT could inhibit NF- $\kappa$ B signaling in airway epithelial cells and asthmatic mice [26]. Zhang B et al. found that MAT reduced the production of HMGB1, TNF- $\alpha$ , and IL-6 in LPS-induced acute lung injury in mice [27]. In addition, the reno-protective activities of MAT have been recently reported. Xu Y reported that MAT ameliorated adriamycin-induced nephropathy in rats by modulating Th17/Treg balance [28]. Jing Y et al. demonstrated that MAT attenuated cyclosporin A-induced chronic nephrotoxicity in rats [29]. However, whether MAT can attenuate HG-induced renal damage has not been elucidated.

The present study aimed to investigate the effects of MAT on HG-induced damage to podocytes. Podocytes play a pivotal role in maintaining glomerular filtration function, contributing to the development of DN if damage is present. The present study also investigated whether the effects of MAT on HG-stimulated podocytes are associated with inhibition of HMGB1-associated TLR4-NF- $\kappa$ B signaling.

### Materials and methods

#### *Culturing of mouse podocytes*

Conditional immortalized mouse podocytes were obtained from Jiniou Bio Company (Guangzhou, Guangdong, China). The podocytes were cultured, as described previously [30]. Before differentiation, the podocytes were grown on collagen-coated dishes at 33°C with 5% CO<sub>2</sub> in RPMI1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), containing 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 10 U/mL interferon- $\gamma$  (Sangon Biotech, Shanghai, China). To induce proliferation, they were cultured at 37°C without  $\gamma$ -interferon in collagen-coated plates for 12-14 days (medium was replaced every 2 days). Reaching 75% confluence, the podocytes were cultured in serum-free RPMI 1640 medium for another 24 hours for synchronization. The following experiments were performed with differentiated podocytes.

#### *Experimental protocol*

(Experiment 1: Evaluation of toxic effects of MAT on podocytes): Excluding the cytotoxicity of MAT on podocytes, differentiated podocytes

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were incubated with different concentrations of MAT (0, 0.1, 0.5, 1.0, or 2.0 mg/mL) (Nanjing TCM Institute of Chinese Materia Medica, Nanjing, China) for 12, 24, 48, or 72 hours, respectively. Cell viability was measured using MTT assays.

(Experiment 2: Effects of MAT on HG-induced cell injuries and TLR4-NF- $\kappa$ B activation): Differentiated podocytes were divided into 4 groups, including the normal glucose group (NG: 5 mM D-glucose), high glucose group (HG: 30 mM D-glucose), HG + MAT 0.5 mg/mL group (30 mM D-glucose + 0.5 mg/mL MAT), and HG + MAT 1.0 mg/mL group (30 mM D-glucose + 1.0 mg/mL MAT). The podocytes were cultured in RPMI 1640 with glucose and MAT, as described above, for 48 hours. Next, 25 mM mannitol was added into the medium of the NG group to compensate for osmolarity. Cell viability was measured using MTT assays. Levels of TLR4, p-I $\kappa$ B $\alpha$ , and p-p65 were measured using Western blotting. Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were measured using ELISA. Finally, p65 DNA-binding activity was detected using an NF- $\kappa$ B family EZ-TFA transcription factor assay colorimetric kit.

(Experiment 3: Effects of MAT on HMGB1 levels in podocytes with or without HG-stimulation): To observe the effects of MAT on levels of HMGB1 in NG and HG-cultured podocytes, differentiated podocytes were incubated in RPMI 1640, with 5 or 30 mM D-glucose and treated with 0.5 or 1.0 mg/mL MAT for 48 hours, respectively. The differentiated podocytes were divided into the following 6 groups: ① NG group: 5 mM D-glucose; ② NG + MAT 0.5 mg/mL group: 5 mM D-glucose + 0.5 mg/mL MAT; ③ NG + MAT 1.0 mg/mL group: 5 mM D-glucose + 1.0 mg/mL MAT; ④ HG group: 30 mM D-glucose; ⑤ HG + MAT 0.5 mg/mL group: 30 mM D-glucose + 0.5 mg/mL MAT; and ⑥ HG + MAT 1.0 mg/mL group: 30 mM D-glucose + 1.0 mg/mL MAT. Moreover, 25 mM mannitol was used to compensate for osmolarity. Levels of HMGB1 in cell lysates were measured using ELISA.

(Experiment 4: HMGB1 involvement in mediating HG-induced cell damage and examination of the effects of MAT on podocytes): To detect the roles of HMGB1 in mediating HG-induced cytotoxicity and the effects of MAT on podocytes, differentiated podocytes were grouped

as follows: ① NG group: 5 mM D-glucose; ② HG group: 30 mM D-glucose; ③ rHMGB1 group: 5 mM D-glucose + rHMGB1 (1  $\mu$ g/ml; R&D system, Inc., USA); ④ HG + EP group: 30 mM D-glucose + EP (5 mM, Sigma Aldrich, St. Louis, MO); ⑤ HG + MAT group: 30 mM D-glucose + 1.0 mg/mL MAT; and ⑥ HG + MAT + rHMGB1 group: 30 mM D-glucose + 1.0 mg/mL MAT + 1  $\mu$ g/ml rHMGB1. Differentiated podocytes were incubated in RPMI 1640 with the above agents for 48 hours. Levels of cell viability, TLR4, p-I $\kappa$ B $\alpha$ , p-p65, p65 DNA-binding activity, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were then assessed.

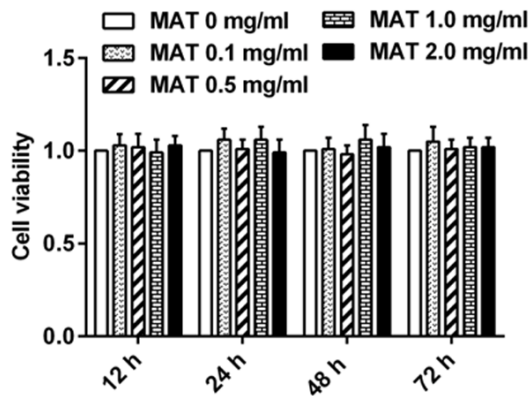
### MTT assays

In the current study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to assess the viability of podocytes. Briefly, at the end of each treatment period, the cells were incubated with MTT solution (0.5 mg/mL in PBS) (Sigma-Aldrich, MO, USA) for 4 hours at 37°C. After removing the MTT solution, 100  $\mu$ l DMSO was added to the wells, solubilizing the formazan crystals. Absorbance values were read using a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm, monitoring cell viability.

### Western blotting

After each specific treatment, the cells were harvested in lysis buffer containing protease and phosphatase inhibitors (Cell Signaling Technology, Inc., Danvers, MA, USA). The supernatant was collected, centrifuged at 15,000 g for 10 minutes at 4°C, and stored at minus 80°C. Concentrations of proteins were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For Western blot analysis, 40  $\mu$ g total proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with blocking buffer for 1 hour at room temperature, then incubated with 1:800 diluted anti-TLR4 antibody, 1:1000 diluted anti-p-I $\kappa$ B $\alpha$  antibody, 1:1000 diluted anti-p-p65 antibody, and 1:1000 diluted  $\beta$ -actin antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C. The membranes were washed 4 times for 10 minutes in the washing buffer with constant shaking. Subsequently, the membranes were incubated with a 1:5,000 dilution of secondary antibodies (Bioss Company, Beijing, China) for 1 hour with constant shaking.

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**Figure 1.** MAT did not exhibit cytotoxic effects on podocytes. Viability levels of podocytes were measured using MTT assays. Data are presented as mean  $\pm$  SD of 4 independent experiments performed in triplicate.

This was followed by washing. Bound antibodies were detected using enhanced chemiluminescence detection reagents (Thermo Scientific Pierce, Rockford, IL, USA). Protein band intensity levels were quantified using Image Lab software v.3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were normalized to the corresponding  $\beta$ -actin intensity.

### Measurement of p65 DNA-binding activity

The p65 DNA-binding activity was detected using an NF- $\kappa$ B Family EZ-TFA Transcription Factor Assay Colorimetric Kit (Millipore corp., Billerica, MA, USA), as described previously [31]. Briefly, nuclear extracts from the podocytes were added to plates, which contained biotinylated oligonucleotides with the wild-type consensus sequence (5'-GGGACTTCC-3') for NF- $\kappa$ B. The plates were incubated for 1 hour, then washed. Subsequently, the plates were probed with rabbit p65 primary antibody (1:1000 dilution) for 1 hour, followed by washing. They were then incubated with a 1:500 dilution of anti-rabbit horseradish peroxidase-conjugated antibody for 0.5 hours. After washing, the plates were further incubated with chemiluminescence substrate solution for 7 minutes before the reaction was stopped by the stop solution containing 0.5 M HCl. Samples were read with a microplate reader (Bio-Rad, Hercules, CA, USA).

### ELISA

Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the culture medium, as well as HMGB1 in cell lysates, were

measured using enzyme-linked immunosorbent assays (ELISA) with commercially-available ELISA kits (Xitang Company, Shanghai, China). All experiments were performed according to manufacturer instructions.

### Statistical analysis

Values are presented as mean  $\pm$  standard deviation (SD). Present data was analyzed with SPSS software 18.0 statistical software. Statistical differences among groups were evaluated with one-way ANOVA, followed by Tukey's HSD or Dunnett's T3 post-hoc tests.  $P < 0.05$  indicates statistical significance.

## Results

### MAT did not exhibit cytotoxic effects on podocytes

To observe the effects of MAT on the viability of podocytes, differentiated podocytes were incubated with 0, 0.1, 0.5, 1.0, or 2.0 mg/mL of MAT for 12, 24, 48, or 72 hours, respectively. MTT assay results suggest that MAT exhibited no cytotoxic effects at concentrations of 0-2.0 mg/mL when incubated for up to 72 hours (**Figure 1**).

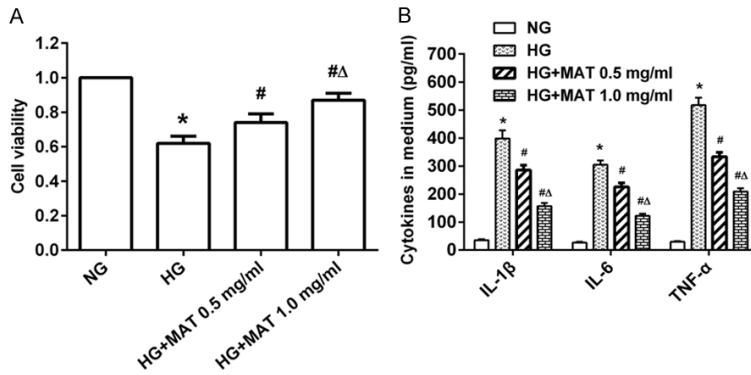
### MAT inhibits HG-induced cell injuries and cytokine production

Since high glucose can induce injuries to renal cells, the current study explored whether MAT had any effects on HG-induced injuries in podocytes. Differentiated podocytes with HG-incubation were treated with MAT for 48 hours. Results showed that 30 mM D-glucose decreased the viability of podocytes ( $P < 0.05$  vs. NG group). However, HG-induced reduction in cell viability was attenuated by MAT treatment ( $P < 0.05$  vs. HG group). The attenuation was MAT dose-dependent ( $P < 0.05$  vs. HG + MAT 0.5 mg/mL group) (**Figure 2A**).

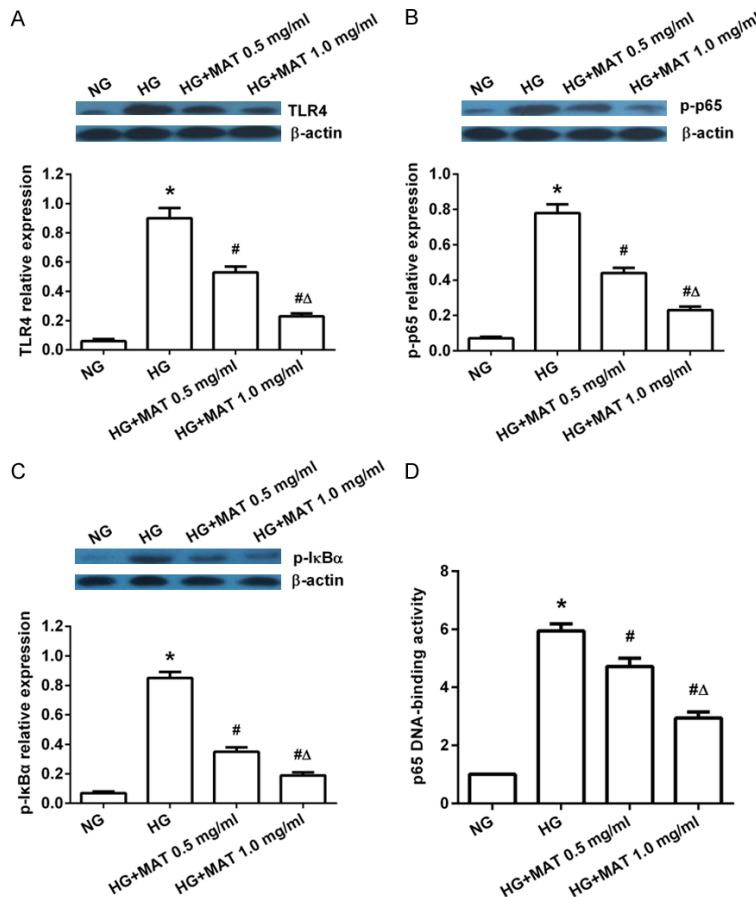
Inflammatory cytokines play a crucial role in the development and progression of DN. Thus, the current study also explored whether MAT showed any effects on cytokine production in podocytes with HG-incubation. Results indicate that HG-incubation increased the release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  to the culture medium from podocytes ( $P < 0.05$  vs. NG group). However, these increases in cytokine production were attenuated by MAT treatment ( $P < 0.05$  vs. HG



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**Figure 2.** MAT inhibited HG-induced cell injury and cytokine production. Viability levels of podocytes were measured using MTT assays (A); Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in culture medium were measured using ELISA assays (B); Data are presented as mean  $\pm$  SD of 4 independent experiments performed in triplicate. \* $P$ <0.05 versus NG group; # $P$ <0.05 versus HG group;  $\Delta P$ <0.05 versus HG+MAT 0.5 mg/mL group.



**Figure 3.** MAT inhibited HG-induced activation of TLR4-NF- $\kappa$ B signaling. Levels of TLR4 (A), p-p65 (B), and p-I $\kappa$ B $\alpha$  (C) were measured using Western blotting. Moreover, p65 DNA-binding activity was detected using an NF- $\kappa$ B family EZ-TFA transcription factor assay kit (D). Data are presented as mean  $\pm$  SD of 4 independent experiments performed in triplicate. \* $P$ <0.05 versus NG group; # $P$ <0.05 versus HG group;  $\Delta P$ <0.05 versus HG+MAT 0.5 mg/mL group.

group), in a dose-dependent manner ( $P$ <0.05 vs. HG + MAT 0.5 mg/mL group) (Figure 2B).

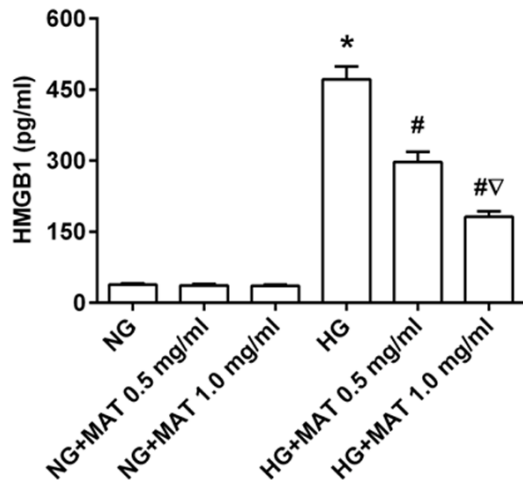
### *MAT inhibits HG-induced activation of TLR4-NF- $\kappa$ B signaling*

It has been reported that TLR4-NF- $\kappa$ B signaling is involved in HG-induced renal injuries and inflammatory response. Exploring the underlying mechanisms of MAT on viability and cytokine production in podocytes, the current study examined the effects of MAT on TLR4-NF- $\kappa$ B signaling. It was found that HG-incubation significantly increased levels of TLR4, p-I $\kappa$ B $\alpha$ , and p-p65 in podocytes (all  $P$ <0.05 vs. NG group) and elevated p65 DNA-binding activity ( $P$ <0.05 vs. NG group). Results indicate that HG induced activation of TLR4-NF- $\kappa$ B signaling in podocytes. However, HG-induced TLR4-NF- $\kappa$ B activation was significantly attenuated by MAT treatment (all  $P$ <0.05 vs. HG group), in a dose-dependent manner ( $P$ <0.05 vs. HG + MAT 0.5 mg/mL group) (Figure 3).

### *MAT inhibits HMGB1 release from podocytes with HG-stimulation, but not from podocytes with NG-stimulation*

HMGB1 is one of the ligands of TLR4. It can regulate the activity of TLR4-NF $\kappa$ B signaling. It was recently reported that HMGB1 may play a role in DN. Thus, the current study examined levels of HMGB1 in podocytes and the effects of MAT on HMGB1 levels. It was found that HG significantly elevated levels of HMGB1 in podocytes ( $P$ <0.05 vs. NG group). However, this elevation was greatly diminished by MAT

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**Figure 4.** MAT inhibited HMGB1 release from podocytes with HG-stimulation, but not from podocytes without NG-stimulation. Levels of HMGB1 were examined via ELISA assays. Data are presented as mean  $\pm$  SD of 4 independent experiments performed in triplicate. \* $P$ <0.05 versus NG group; # $P$ <0.05 versus HG group;  $\Delta P$ <0.05 versus HG+MAT 0.5 mg/mL group.

treatment, in a dose-dependent manner ( $P$ <0.05 vs. HG group). Moreover, MAT showed no marked effects on HMGB1 production in NG-incubated podocytes (**Figure 4**).

### *Inhibition of HMGB1 by MAT involved in mediating the effects of MAT on cell damage and TLR4-NF- $\kappa$ B activation in HG-cultured podocytes*

As shown above, MAT exhibited protective effects on HG-cultured podocytes. It also attenuated activation of TLR4-NF- $\kappa$ B signaling and inhibited the production of HMGB1. Thus, the current study next examined whether the effects of MAT on cell damage and TLR4-NF $\kappa$ B activation are associated with inhibition of HMGB1. As shown in **Figure 5**, rHMGB1 incubation decreased cell viability and activated TLR4-NF- $\kappa$ B signaling in NG-incubated podocytes ( $P$ <0.05 vs. NG group). In addition, EP, an HMGB1 inhibitor, significantly diminished HG-induced cell damage, TLR4-NF- $\kappa$ B activation, and cytokine release ( $P$ <0.05 vs. HG group). These findings suggest that HMGB1 plays a crucial role in HG-induced cell injury and TLR4-NF- $\kappa$ B signaling activation in podocytes. Additionally, it was found that the protective effects of MAT on podocytes were markedly abolished by co-incubation with rHMGB1 ( $P$ <

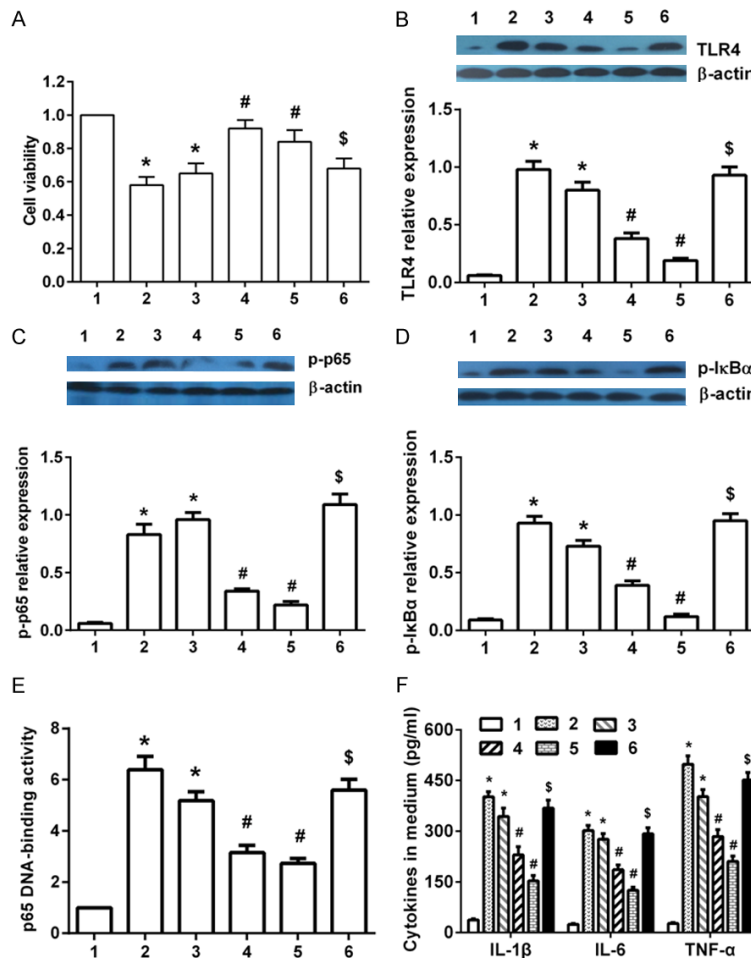
0.05 vs. HG + MAT group). Present results reveal that inhibition of HMGB1 by MAT should be involved in mediating the effects of MAT on cell damage and TLR4-NF- $\kappa$ B activation.

## Discussion

The present study provides new insight into the anti-inflammatory and reno-protective effects of MAT, offering evidence concerning the link between MAT and HMGB1 and downstream signaling pathways in HG-induced podocyte injuries. It was found that MAT attenuated reduction in cell viability, decreased the production of HMGB1, and inhibited activation of TLR4-NF- $\kappa$ B signaling. It also reduced the release of inflammatory cytokines in HG-incubated podocytes. Moreover, the effects of MAT were markedly abolished by co-incubation with HMGB1. In addition, HMGB1 inhibitor EP exhibited inhibitory effects on HG-induced injuries and TLR4-NF- $\kappa$ B activation. Taken together, these findings suggest that HMGB1 plays a crucial role in HG-induced podocyte injuries. Results also suggest that the protective effects of MAT on HG-incubated podocytes were, at least partially, associated with inhibition of HMGB1-mediated TLR4-NF- $\kappa$ B signaling pathways.

Podocytes are highly-specialized and terminally-differentiated epithelial cells. They form a major component of the glomerular filtration barrier [32]. It is believed that, at early onset of DN, there is podocyte drop-out. This provokes subsequent glomerular injuries. Therefore, podocyte damage has been regarded as a key pathogenic event leading to the development and progression of DN. Podocyte-targeted treatment is an important therapeutic strategy in the management of proteinuric kidney disease, including DN [33, 34]. MAT is a natural compound that has exhibited reno-protective effects, according to several studies [28, 29]. However, whether MAT provides any effects on diabetic nephropathy has not been investigated. The present study found that HG-incubation decreased the viability of podocytes, in accordance with previous studies [35]. However, MAT significantly attenuated HG-induced reduction in podocyte viability, in a dose-dependent manner. Previous reports have revealed that high glucose could arouse renal inflammation in diabetic patients [3] and animals [3, 36] *in vivo*, as well as in podocytes *in vitro* [37]. Pro-

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**Figure 5.** Inhibition of HMGB1 by MAT is involved in mediating the effects of MAT on cell damage and TLR4-NF- $\kappa$ B activation. 1: NG group; 2: HG group; 3: rHMGB1 group; 4: HG+EP group; 5: HG+MAT group; 6: HG+MAT+rHMGB1 group. The viability of podocytes was measured using MTT assay (A). Levels of TLR4 (B), p-p65 (C), and p-I $\kappa$ B $\alpha$  (D) were measured using Western blotting. Next, p65 DNA-binding activity was detected using an NF- $\kappa$ B family EZ-TFA transcription factor assay kit (E). Levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in the culture medium were measured using ELISA assays (F). Data are presented as mean  $\pm$  SD of 4 independent experiments performed in triplicate. \* $P$ <0.05 versus NG group; # $P$ <0.05 versus HG group; \$ $P$ <0.05 versus HG+MAT group.

inflammatory cytokines IL-1 $\beta$ , IL-6, and TNF- $\alpha$  have been well shown to exacerbate inflammatory cascades and promote renal damage in DN [2, 3, 36]. Consistently, the current study found that HG-incubation increased the release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  from podocytes. MAT has been reported to have anti-inflammatory activity. Zhang B et al. found that MAT alleviated LPS-induced acute lung injuries in mice by reducing production of TNF- $\alpha$  and IL-6 [27]. A recent report also indicated that MAT attenuated type II collagen-induced arthritis in rats by inhibiting the release of pro-inflammatory cyto-

kines, including IL-1 $\beta$ , IL-6, and TNF- $\alpha$  [25]. Similarly, the current study found that MAT markedly inhibited HG-induced release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  from podocytes. Many previous studies have confirmed that inhibition of pro-inflammatory cytokines by agents provides ameliorative effects on DN [3, 36]. Thus, it was assumed that inhibition of the release of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  by MAT would contribute to its protective effects on podocyte injuries.

Further exploring the mechanisms of MAT on pro-inflammatory cytokine release and podocyte injuries, the current study examined the effects of MAT on TLR4-NF- $\kappa$ B signaling. NF- $\kappa$ B is an inflammatory relevant transcription factor that plays a crucial role in the regulation of inflammatory response. In unstimulated cells, NF- $\kappa$ B is sequestered in the cytoplasm by its inhibitor I $\kappa$ B. Some specific signals can induce the degradation of I $\kappa$ B through phosphorylation. This is followed by the phosphorylation and translocation of p65 to the nucleus where it promotes expression of specific genes. Previous studies have revealed that NF- $\kappa$ B plays an important role in DN by promoting expression of pro-inflammatory

cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . Recent studies have shown that high glucose could induce the activation of NF- $\kappa$ B signaling in podocytes [8, 37, 38]. Similarly, the current study found that HG-incubation increased levels of p-I $\kappa$ B $\alpha$  and p-p65 and enhanced the p65 DNA binding activity in podocytes. However, activation of NF- $\kappa$ B signaling by HG-incubation was markedly diminished with MAT treatment. In addition to present findings of MAT on NF- $\kappa$ B signaling in podocytes, recent studies have also suggested that MAT attenuated inflammation by inhibiting NF- $\kappa$ B signaling in rats with

arthritis [25, 39], in rats with early brain injuries after experimental subarachnoid hemorrhages [40], in mice with airway inflammation [26], and in mice with LPS-induced acute lung injuries [27, 41]. TLR4 has been well demonstrated in DN and in activating NF- $\kappa$ B signaling [5, 6, 9, 42]. Studies have revealed that high glucose could promote expression of TLR4 in podocytes [8, 38]. Next, the current study investigated the effects of MAT on TLR4 expression in HG-incubated podocytes. It was found that HG-incubation led to a significant increase of TLR4 expression in podocytes, in accordance with previous studies [9]. However, overexpression of TLR4, in these HG-incubated podocytes, was significantly suppressed with MAT treatment. This was in accordance with the latest results, indicating that MAT inhibited TLR4 expression in porcine alveolar macrophages [43]. Collectively, present results suggest that MAT could inhibit activation of TLR4-NF- $\kappa$ B signaling in HG-incubated podocytes. Since many previous studies have demonstrated the beneficial effects on DN via inhibition of TLR4-NF- $\kappa$ B signaling, it was assumed that the attenuation of activation of TLR4-NF- $\kappa$ B signaling by MAT would contribute to the protective effects on podocyte injuries.

HMGB1 is one of the ligands of TLR4. HMGB1 has been reported to play a role in renal damage, at least partially, by stimulating inflammatory response through TLR4-NF- $\kappa$ B signaling pathways [15, 44]. HMGB1 has been regarded as an upcoming molecule for application in the management of DN [45, 46]. The present study found that HG-incubation promoted the production of HMGB1 in podocytes. MAT treatment significantly inhibited levels of HMGB1 in HG-incubated podocytes, but MAT showed no marked effects on levels of HMGB1 in NG-incubated podocytes.

Based on the above findings, MAT protected podocytes, attenuated TLR4-NF- $\kappa$ B activation, and inhibited HMGB1 expression in HG-incubated podocytes. The current study next examined whether the effects of MAT on podocytes are associated inhibition of HMGB1 expression. First, exogenous rHMGB1 induced podocyte injuries and TLR4-NF- $\kappa$ B activation in podocytes, which were incubated normal glucose. Second, inhibiting HMGB1 by EP diminished HG-induced podocyte injuries, TLR4-NF-

$\kappa$ B activation, and cytokine release. These findings confirm the crucial roles of HMGB1 in HG-induced podocyte injuries. Current results were in accordance with previous studies that demonstrated the involvement of HMGB1 in DN [44-46]. Additionally, the current study found that the effects of MAT on HG-incubated podocyte were markedly abolished by the addition of rHMGB1 to the medium. All converging results suggest that inhibition of HMGB1 is involved in mediating the effects of MAT on HG-incubated podocyte.

In conclusion, MAT can attenuate HG-induced podocyte damage, at least partially, by inhibiting HMGB1-associated TLR4-NF- $\kappa$ B signaling.

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### Disclosure of conflict of interest

None.

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