Apamin inhibits renal interstitial inflammation and fibrosis via suppressing TGF-β1 and STAT3 signaling pathway *in vivo* and *in vitro*

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Research

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Abstract

Background Renal fibrosis is a progressive and chronic process that influences kidneys with chronic kidney disease (CKD), irrespective of cause, leading to irreversible failure of renal function and end-stage kidney disease. Among the signaling related to renal fibrosis, transforming growth factor-β1 (TGF-β1) signaling is a major pathway that induces the activation of myofibroblasts and the production of extracellular matrix (ECM) molecules. Apamin, a component of bee venom (BV), has been studied in relation to various diseases. However, the effect of apamin on renal interstitial fibrosis has not been investigated. The aim of this study was to estimate the beneficial effect of apamin in unilateral ureteral obstruction (UUO)-induced renal fibrosis and TGF-β1-induced renal fibroblast activation.

Results This study revealed that obstructive kidney injury induced an inflammatory response, tubular atrophy, and ECM accumulation. However, apamin treatment suppressed the increased expression of fibrotic-related genes, including α-SMA, vimentin, and fibronectin. Administration of apamin also attenuated the renal tubular cells injury and tubular atrophy. In addition, apamin attenuated fibroblast activation, ECM synthesis, and inflammatory cytokines such as TNF-α, IL-1β and IL-6 by suppressing the TGF-β1-canonical and non-canonical signaling pathways.

Conclusions This study shown that apamin inhibites UUO-induced renal fibrosis *in vivo* and TGF-β1-induced renal fibroblasts activation *in vitro*. Apamin inhibited the inflammatory response, tubular atrophy, ECM accumulation, fibroblast activation, and renal interstitial fibrosis through suppression of TGF-β1/Smad2/3 and STAT3 signaling pathways. These results suggest that apamin might be a potential therapeutic agent for renal fibrosis.

Introduction

Renal fibrosis is the ultimate common manifestation of progressive chronic kidney disease (CKD) leading to the irreversible destruction of kidney parenchyma and end-stage of renal failure [1, 2]. Renal fibrosis is characterized by the demolition of renal tubules, tubular atrophy, infiltration of immune cells, accumulation of myofibroblasts, and overproduction of extracellular matrix (ECM) resulting in renal tubular cell apoptosis and necrosis [3–5].

Among these characteristics, inflammatory response plays an important role in the progression of numerous acute and chronic renal injuries [6]. This reaction is induced as a protective response to a wide range of renal injuries, but ongoing inflammation promotes progressive renal fibrosis regardless of the underlying etiology [7]. To be more specific, inflammatory cell infiltration in the circulation is localized to the damaged tissue, inducing a renal inflammatory response. This in turn leads to the generation and secretion of inflammatory mediators and pro-fibrotic cytokines and growth factors. Inflammatory mediators mediate the cascade amplification and sustenance of inflammatory responses and cause apoptosis of the renal tubular cells, activation of myofibroblasts, renal tubular atrophy and renal

interstitial fibrosis. Therefore, inhibiting the inflammatory response may facilitate attenuating renal tubular epithelial cell apoptosis and interstitial fibrosis [8].

Representative inflammatory mediators include transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 [9]. Among these, TGF-β1 is a key effector leading to renal interstitial fibrosis and fibroblast activation [10, 11]. TGF-β1 stimulates fibroblast cell activation and induces ECM accumulation through its interaction with TGF-β receptors [12]. ECM accumulation is the end-result of increased matrix components secretion and deposition, and decreased degradation. Fibroblasts are the main sources of interstitial ECM components. Fibroblasts become activated during fibrosis, sometimes irreversibly. Long-term activated fibroblasts are called myofibroblasts and are characterized by the de novo expression of α-smooth muscle actin (α-SMA) [13]. Because of this reason, a massive increase in interstitial myofibroblast activation is believed to play a central role in the pathogenesis of tubulointerstitial fibrosis [14, 15].

The TGF-β1 signal is initiated when activated TGF-β1 binds to a TGF-β type Ⅱ receptor (TβRⅡ), a constitutively active kinase, leading to phosphorylation of the TGF-β type Ⅰ receptor (TβRⅠ). The activated TβRⅠ kinase then phosphorylates the downstream receptor-associated Smads, including Smad2 and Smad3. Then, the phosphorylated Smad2/3 forms a complex with Smad4, and translocate to the nucleus to control the transcription of target genes. [16, 17]. On the other hand, Smad7, an inhibitory Smad, exerts its negative effect on the TGF-β signaling through consequently conpeting with the Smad2/3 [11].

In addition, TGF-β-induced renal fibrosis is mediated by Smad-independent signaling pathways. Signal transducer and activator of transcription 3 (STAT3) is a representative mediator in the Smad-independent signal. STAT3 is a significant member of the STAT family (STAT14, STAT5a/5b, and STAT6) and mediates cell proliferation and survival. Various growth factors and cytokines can phosphorylate STAT3 tyrosine. The activated STAT3 form a dimer and translocate into the cell nucleus to regulate the transcription of target genes [18–20]. It has been reported that STAT3 phosphorylation mediates the activation of myofibroblast and the progression of renal interstitial fibrosis in unilateral ureteral obstruction (UUO) models [21, 22]. Therefore, it is thought that inhibition of TGF-β1 and STAT3 signaling can alleviate renal fibrosis via suppression of myofibroblast activation and ECM accumulation.

Bee venom (BV) therapy has been used to alleviate suffering and to treat a variety of inflammatory diseases since ancient times, including arthritis, bursitis, back pain, rheumatism, skin disease, and other chronic conditions [23, 24]. Apamin, a component of BV, is well known specifically a selective blocker of small conductance Ca^{2+}-activated K^+ channel (SK channel) as it binds to the pore of the channel [24, 25]. For several years, apamin has been studied as a specific SK channel blocker in the central nervous system [26]. Recently, other therapeutic effects of apamin have been announced. The anti-inflammatory effect of apamin accompanied by a reduction of seromucoid and haptoglobin levels has been reported [27]. In addition, apamin attenuates inflammatory responses in THP-1-derived macrophages [28], suppresses inflammatory cytokines in TNF-α- and IFN-γ-induced keratinocytes [29], and inhibits hepatic fibrosis in CCl_4-injected mice [30]. However, the effects and mechanisms of apamin in obstructive kidney

injury and TGF-β1-induced fibroblast activation has not been reported. Therefore, this study investigated the anti-inflammatory and anti-fibrotic effect of apamin using a UUO-induced mice model and a TGF-β1-induced NRK-49F cell model.

Results

Apamin attenuated renal interstitial fibrosis and improved kidney function in the UUO model

Among experimental animal models, the UUO research model is mainly used to investigate obstructed renal interstitial inflammation and fibrosis [31]. Untreated urinary obstruction can lead to tubular atrophy, interstitial inflammation and fibrosis, and, finally, irreversible kidney injury [32, 33]. To identify the beneficial effect of apamin on obstructive kidney injury, this study used a UUO-induced obstructive animal model. As shown in Fig. 1a, histological analyses showed that obstructive kidney exhibited interstitial immune cells infiltration, partial tubular expiation, and tubular atrophy. However, apamin treatment attenuated these morphology changes. Additionally, in representative trichrome images, apamin was able to suppress collagen accumulation caused by UUO-induced renal injury (Fig. 1a, d). In supplementary Fig. 2, we performed the western blotting analysis to investigate the beneficial effect of apamin on renal tubules injury. The expression of E-cadherin, as the epithelial cell marker, was decreased by obstructive kidney compared with normal kidney. In addition, the renal injury biomarker neutrophil gelatinase-associated lipocalin (NGAL) was increased in UUO mice. However, the expression of E-cadherin was increased and the expression of NGAL was decreased by apamin administration. Furthermore, to clarify the protective effect of apamin on renal function, blood urea nitrogen (BUN) and serum creatinine were measured using mice serum plasma (Fig. 1b and c). In UUO mice, the BUN and serum creatinine levels were increased, which means that UUO injury effectuated renal dysfunction. In contrast, apamin treatment indicated BUN and creatinine levels similar to normal kidney conditions.

Apamin inhibited renal interstitial inflammation response in UUO-injured mice

After obstructive injury, macrophage and T lymphocytes were infiltrated and the inflammatory response was intensified in kidney tissue. In addition, continuous inflammation plays an important role in the initiation and development of renal interstitial fibrosis [34, 35]. To examine the anti-inflammatory effect of apamin, the expression of TNF-α, IL-1β and IL-6 were analyzed by immune blot. The protein level of TNF-α, IL-1β and IL-6 were markedly increased in UUO mice (Fig. 2a-d). However, apamin treatment attenuated the UUO-mediated renal inflammatory cytokines.

Monocyte chemoattractant protein-1 (MCP-1) is widely known as a chemokine that has the ability to regulate infiltration and migration such as monocytes and macrophages. Monocytes collected by MCP-1 differentiate into macrophages and secrete inflammatory cytokines (TNF-a, IL-6, IFNs, etc.) to intensify the inflammatory response [36]. Based on this fact, to find out the efficacy of apamin on renal inflammation, F4/80 and MCP-1 were stained using immunohistochemistry staining. The expression of MCP-1 was increased in obstructive kidney (Fig. 2e, g). Furthermore, the $F4/80^{+}$ macrophages were

increased in UUO-injured kidney (Fig. 2f, h). Conversely, administration of apamin diminished MCP-1 expression, macrophage infiltration, and inflammatory cytokines.

Fibrotic gene expression and myofibroblasts activatin were reduced by apamin injection

A key step in renal fibrogenesis is the accumulation of myofibroblasts and ECM molecules. Excessive ECM deposition is the end-result of increased matrix elements secretion and accumulation, and decreased degradation. There are many components in the ECM matrix, including fibronectin, collagen I and others [13]. We next examined the expression of ECM in kidney tissues using western blot analysis. The results showed that the expression of α-SMA, vimentin and fibronectin significantly increased in the fibrotic kidney compared to normal mice. However, these changes were reversed by apamin administration (Fig. 3a-d).

Myofibroblasts show high proliferation and ECM secretion rates and play a key role in interstitial fibrosis in the UUO model [13]. To identify whether apamin suppresses myofibroblast accumulation in UUO kidneys, this study investigated the protein levels of fibroblast-specific marker-1 (FSP-1) using immunohistochemistry. The results showed that UUO-injured kidney increased the expression of FSP-1, as a myofibroblasts marker, whereas apamin administration suppressed this expression (Fig. 3e, f).

Apamin inhibited TGF-β1/Smad signaling pathway and STAT3 signaling pathway

TGF-β1 signaling is a key mechanism leading to fibroblast activation and renal interstitial fibrosis. This cytokine also induces Smad2/3 and STAT3 phosphorylation that regulate the transcription of target genes [12, 37]. To confirm the molecular mechanism of apamin in obstructive kidney, we investigated the protein expression of TGF-β1/Smad signal mediators and STAT3 transcription factor through western blotting analysis. As shown in Fig. 4, the UUO group exhibited significantly increased expression of TGF-β1, p-STAT3 and p-Smad2/3, whereas these expressions were inhibited in apamin treatment group.

Smad7 is an inhibitory regulator in the TGF-β1/Smad signaling pathway, which blocks the signal transduction of TGF-β1 via its negative feedback loop. Moreover, Smad7 efficiently prevents Smad2 and Smad3 interaction, and Smad-related protein expression [12]. To further investigate the effect of apamin on Smad7 signal, we analyzed the Smad7 expression using western blotting analysis. In the immunoblotting study, the protein expression of Smad7 was significantly decreased by UUO-injury. However, apamin treatment recovered the Smad7 expression (Fig. 4a, e).

Anti-fibrotic effect of apamin in TGF-β1-treated renal fibroblast cells

On the basis of the observation of apamin administration in UUO mice, we determined the anti-fibrotic effect of apamin on TGF-β1-induced kidney fibrosis in *in vitro* models. First, cell counting kit (CCK)-8 assay was executed to indicate the cytotoxicity of apamin at different doses. The normal rat kidney interstitial fibroblast cells (NRK-49F) were treated with 0.1, 0.5, 1, 2, 5, and 10 μg ml^{-1} of apamin for 6, 24 and 48 h. In the 6 or 24 h apamin treatment, all concentrations of apamin treatment did not alter cell viability (Fig. 5a, b). In the case of 48 h apamin treatment, NRK-49F cell viability was reduced at 10 μg ml^{-1} of apamin. However, 0.1, 0.5, 1, 2, and 5 μg ml^{-1} of apamin did not affect NRK-49F cell viability

(Fig. 5c). In accordance with this result and with a previous study [38], we selected the apamin concentrations (0.5, 1, and 2 μg ml^{-1}) and apamin treatment time (48 h) in the following experiments.

To determine the anti-fibrotic effect of apamin on TGF-β1-induced renal fibroblast activation, we investigated the protein expression of ECM products using immunoblotting analysis. After TGF-β1 treatment, the expressions of collagen Ⅰ and fibronectin were significantly increased. However, collagen Ⅰ expression was decreased in a dose-dependent manner in the apamin treatment groups. TGF-β1-induced expression of fibronectin was most decreased in the 0.5 μg/ml^{-1} concentration of apamin (Fig. 5d-f). Furthermore, to confirm the molecular mechanism of apamin in TGF-β1-treated cells, we examined the expression change of TGF-β1 signal mediators. TGF-β1 stimulation induced phosphorylation of STAT3 and Smad2/3, and expression of TGF-β1. Conversely, apamin significantly diminished TGF-β1-induced STAT3, Smad2/3, and TGF-β1 expressions in a dose-dependent manner. In the case of Smad7, this expression was decreased in TGF-β1 treatment renal cells. However, apamin treatment recovered the expression of Smad7.

Discussion

Development of kidney fibrosis is the hallmark of most progressive CKDs, irrespective of the cause, and is thought to be a common pathway leading to end-stage renal diseases [3, 4]. The development of end-stage renal disease requires renal transplantation due to irreversible loss of tissue and impaired kidney function. For this reason, CKD has increasingly become a major global public health concern and portends high rates of morbidity and mortality [39]. Therefore, it is important to prevent kidney interstitial fibrosis to prevent or slow the devastating CKD sequelae and progression to end-stage renal disease [40, 41].

While various drugs, mainly drugs targeting angiotensin II, slow the progression of CKD, the therapeutic armamentarium is still imperfect [41]. Hence, there is a critical need for new therapeutics to diminish kidney fibrosis and renal failure. Recently, drug discoveries for fighting kidney fibrosis have mainly focused on compounds that are specific for a protein kinase or particular receptor. Given that kidney fibrosis is associated with increased production of multiple growth factors/cytokines and the following activation of their receptors and signaling pathways, it is supposed that inhibitors with wide specificity might provide improved therapeutic benefits in renal fibrotic diseases [42].

Apamin is a specific component of BV that is known as a greatly selective blocker of calcium-dependent potassium channel [43]. This channel connects intracellular Ca^{2+} transients to changes of the membrane potential by inducing K + efflux following increases of intracellular Ca^{2+} during an action potential [44]. Kim et al. [29] have shown that apamin has anti-inflammatory effects against TNF-α- and IFN-γ-stimulated keratinocytes. In addition, previous studies have shown that apamin suppressed Smad-dependent and Smad-independent signaling pathways in liver fibrosis, and suppressed STAT signaling pathways in atopic dermatitis [29, 30]. Following these studies, we hypothesized that apamin was going

to have an anti-inflammatory and anti-fibrotic effect in renal interstitial fibrosis via suppressing the TGF-β1 and STAT3 signaling pathways.

To prove the hypothesis, this study investigated the therapeutic effect of apamin using UUO model and TGF-β1-treated fibroblast cells. Similar to other studies [32, 33], obstructive kidneys show interstitial inflammation, tubular injury and death, tubular atrophy, renal failure, and interstitial fibrosis by UUO injury. Increasing evidence shows that tubular epithelial cells play various roles in renal repair or progression to CKD. Continuous injury of tubular epithelial cells promotes production and release of bioactive mediators that induce renal interstitial inflammation and renal fibrosis [45]. Thus, injury of tubular epithelial cells is an important indicator in renal diseases. In this study, we evaluated the renal tubular cell injury via investigative the expression of epithelial cell marker and kidney injury marker. In UUO kidney, E-cadherin was decreased and NGAL was increased compared with normal kidney, indicating the induction of tubular cells injury. On the other hands, apamin treatment remarkably attenuated the renal epithelial cells damage. In addition, administration of apamin significantly improves kidney function and suppresses tubulointerstitial fibrosis, as evidenced by a diminution in plasma levels of creatinine and BUN and histopathological changes such as tubular atrophy, collagen deposition, and interstitial fibrosis induced by obstructive injury. Taken together, these findings suggest that, in mice, apamin protects from UUO-induced renal dysfunction, tubular cell injury and structural changes.

Renal interstitial inflammation is implicated as an important event in the initiation and progression of kidney fibrosis in CKD. The inflammatory response is characterized by infiltration of immune cells, activation of resident renal cells, excessive production of cytokines (including interstitial inflammatory, pro-fibrotic cytokines and growth factors), and renal tubular atrophy and interstitial fibrosis [46–48]. Several studies demonstrated that inhibiting the inflammatory response results in attenuation of renal tubular epithelial cell apoptosis and renal fibrosis [8, 46, 47]. In this study, we showed that the expression of TNF-α, IL-1β and IL-6 increased by obstructive injury, while administration of apamin reduced the inflammatory cytokines. Moreover, our previous research showed that apamin treatment suppressed inflammatory responses through inhibition of the NF-κB signal pathway in THP-1-derived macrophages [49]. Macrophages, a principal type of inflammatory cell, are recruited in all kidney disease. Recruited macrophages are associated with the induction of renal injury, repair, and fibrosis [40]. Furthermore, several studies have reported that macrophages induce the synthesize ECM molecules including fibronectin and collagen [50, 51]. In the current study, administration of apamin significantly suppressed the expression of F4/80, the macrophage marker, and MCP-1, the macrophage recruitment chemokine. Altogether, these results suggest that apamin has anti-inflammatory effect through inhibition of macrophage infiltration and cytokine production.

Based on these results, we thought that apamin would attenuate myofibroblast activation and ECM accumulation by inhibiting inflammatory responses. Some study remarked that interstitial deposition of macrophages and myofibroblasts is strongly associated with the progression of UUO injury [52]. In addition, proliferation of fibroblast with myofibroblast transformation induce excessive accumulation of the ECM component in kidney fibrosis [53, 54]. Similar to other studies, UUO mice observed the excess

deposition of myofibroblasts and ECM molecules. However, apamin administration showed that the accumulation of ECM, including α-SMA, vimentin and fibronectin, was decreased and proliferation of myofibrobalsts were diminished *in vivo* experiment.

TGF-β1, a key factor of the initiation and progression of renal fibrosis, induces tubular epithelial cell apoptosis, myofibroblasts activation, and excessive production of ECM molecules by binding to the TβR receptor [55, 56]. Furthermore, Liu et al. [57] showed that phosphorylation of Smad3 by TGF-β1 injury promotes STAT3 activation in the injured kidney. As previously reported, our results showed that TGF-β1, Smad2/3, and STAT3 were activated by obstructive injury, while apamin treatment reduced these signal mediators. In addition, TGF-β1 can induce phosphorylation of STAT3 which promotes the activation of renal fibroblasts and progression of renal fibrosis [37]. Based on these facts, we performed the *in vitro* experiments using TGF-β1 to investigate the molecular mechanism in more detail. Similar to other studies, TGF-β1 treatment increased the production of ECM molecules and the activation of TGF-β1 and STAT3 signaling pathways in renal fibroblasts cell. On the other hand, pre-treatment with apamin was shown to reduce the expression of TGF-β1, p-STAT3, and p-Smad2/3, and the production of collagen, and fibronectin by TGF-β1 treatment.

Furthermore, we also investigated the expression of Smad7 in *in vivo* and *in vitro* models. Smad7, as an inhibitory regulator in TGF-β1 signaling, prevents Smad-related protein expression by suppressing Smad2 and Smad3 interaction [58]. In addition, hyperactivation of TGF-β1 and Smad3 was concerned with progressive degradation of Smad7. More importantly, the disproportion of Smad3 and Smad7 was determined to be one of the important mechanisms in mediating the fibrotic response [12]. In the current study, the expression of Smad7 was decreased in UUO-injured mice and TGF-β1-treated renal fibroblast cells, while apamin administration restored Smad7 expression like as normal condition. As mentioned in Meng et al. [12], our results show that the rebalancing of the Smad3/Smad7 ratio by apamin may contribute to the suppression of renal fibrosis. Taken together, apamin administration was observed to reduce the activation of TGF-β1 and Smad2/3 and to increase the expression of Smad7 in renal fibrosis in the *in vivo* and *in vitro* models. It is thought that suppression of the TGF-β1 and STAT3 signaling pathways by apamin may contribute to the attenuation of myofibroblast activation and ECM production. Altogether, this study suggests that apamin inhibited kidney interstitial fibrosis by blocking various signaling pathways such as TGF-β1-canonical and TGF-β1-noncanonical signaling.

Conclusions

In conclusions, this study has demonstrated the anti-inflammatory and anti-fibrotic effect of apamin on UUO-induced renal fibrosis and TGF-β1-activated renal fibroblast models. This study shown that UUO-induced myofibroblasts activation and ECM accumulation were inhibited by apamin treatment. In addition, UUO-induced interstitial inflammatory response in renal tissue was reduced by apamin, and the expression of pro-inflammatory cytokines was also decreased. These results suggest that apamin may have a protective effect against renal fibrosis. Based on the results of *in vivo* study, we confirmed the effect of apamin on fibroblast activation by TGF-β1 *in vitro*. Apamin administration suppressed the

activation of fibroblasts by TGF-β1 and expression of fibrotic genes. In addition, this study showed that the expression of various fibrotic genes was significantly reduced through inhibition of Smad2/3 and STAT3 signaling. All the take together, the current study may be the first proof that apamin can be used for an anti-inflammatory and anti-fibrotic effect on renal fibrosis. Although further examination will be required to clarify a more detailed mechanism, these results suggest that apamin might be a potential therapeutic strategy to prevent renal fibrosis.

Materials And Methods

Experimental animals and drug treatment

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (EXP-IRB number: DCIAFCR-160705-6-Y). Six-week-old male C57BL/6 mice were purchased from Samtako (Osan, Korea) and housed in a controlled environment as humidity (55%) and temperature (22 ± 2˚C) under a 12 h: 12 h light-dark cycle. The mice were randomly subdivided into three groups (n = 5 per group) as follows: (1) group with surgical procedure similar to unilateral ureteral obstruction (UUO) but not subjected to ureteral ligation (sham); (2) performed UUO procedure group (UUO); and (3) performed UUO procedure and treated apamin group (UUO + apamin).

After seven days of acclimatization, the UUO operation was performed after anesthetizing the mice. The animal's abdominal cavity was exposed by a small incision and the left ureter was isolated and ligated with 5 – 0 silk sutures at two different sites: upper and lower. Apamin treatment at a concentration of 0.5 mg/kg was given via intraperitoneal injection twice a week. Eight days after the UUO operation, the kidneys were collected for various experiments.

Cell culture and drug treatment

Normal rat renal interstitial fibroblast cells (NRK-49F) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics at 37 °C in a 5% humidified CO_2 incubator. NRK-49F cells were seeded at 3×10^5 cells per 3 mL of complete medium in a 60 mm cell culture dish. After 24 h, the medium was changed to a serum-free medium containing the indicated concentrations of apamin (0, 0.5, 1, and 2 μg/ml). After 30 min, 5 ng/ml of TGF-β1 were added to the cells and co-cultured for 48 h. After 48 h, the cells were collected for western blotting.

Cell viability assay

The cell viability of the NRK-49F cells was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan). The cells were seeded in a 96-well plate at 5×10^3 cells per well and were pre-incubated for 24 h. Next day, the cells were treated with various concentrations of apamin for 6 h, 24 h and 48 h. After treatment, 10 μl of WST-8 solution [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to each well, and the cells were

incubated for an additional 4 h at 37 °C. The cell viability values were measured by absorbance at 450 nm using a microplate reader.

Creatinine and blood urea nitrogen

The blood samples were collected in tubes by cardiac puncture in all groups. All blood samples were coagulated for 1 h at room temperature (RT). Plasma was separated from whole blood using centrifugation (2,000 g, 20 min). The plasma samples were obtained from the supernatants after centrifugation method for blood urea nitrogen (BUN) and creatinine analysis. The plasma BUN was measured using a BUN-E kit (Asan Pharmaceutical, Seoul, Korea) and the serum creatinine was measured using a QuantiChrom™ creatinine assay kit (Bioassay Systems, Hayward, CA, USA). Analysis of the samples was carried out according to the manufacturer's recommended protocols.

Western blotting

Total protein samples were extracted from kidney tissues using protein extraction solution (Cell Lytic™ M, Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's recommendations. The protein concentration was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The protein samples were loaded onto gradient polyacrylamide gels (Bolt™ 4–12% Bis-Tris Plus Gels; Thermo Fisher Scientific, Waltham, Ma, USA) and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). After transfer, the membranes were blocked in 5% bovine serum albumin for 1 h at RT. The membranes were probed with a primary antibody overnight (4 ℃). Next day, the membranes were washed using a TBS-T buffer for 7 min on the shaker; the process was repeated three times. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h 30 min at RT. Repeat the wash step. The signals were detected using enhanced chemiluminescence detection reagents (Thermo Fisher Scientific). Signal intensity was analyzed using ChemiDoc™XRS + Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the Image Lab software (Bio-Rad Laboratories). The protein expression levels were normalized to GAPDH (Cell Signaling, Beverly, MA, USA) and β-actin. The primary antibodies used were as follows: anti-α-SMA, TNF-α, TGF-β1, fibronectin, collagen Ⅰ, IL-6 (Abcam, Cambridge, UK), anti-vimentin (BD Biosciences, San Jose, CA, USA), anti-IL-1β, Smad7, NGAL (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GAPDH, p-Stat3, t-Stat3, p-Smad2/3, t-Smad2/3, E-cadherin (Cell Signaling), and anti-β-actin (Sigma-Aldrich).

Histological and immunohistochemistry

After harvesting, the kidney tissues were immediately fixed in 10% formalin at RT and then embedded in paraffin. Thereafter, the paraffin-embedded tissues were cut into 4 μm sections. The thin sections were mounted on glass slides and deparaffinized. Kidney tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to standard protocol.

For the immunohistochemical stain, the paraffin-embedded sections on slides were deparaffinized with xylene and dehydrated in gradually decreasing concentrations of ethanol. The sections were incubated

with a primary antibody (1:100 dilution) for 1 h at 37 ℃. The signal was visualized using an EnVision System (DAKO, Carpinteria, CA, USA) for 30 min at 37 °C; 3,3'-diaminobenzidine tetrahydrochloride was used as the coloring reagent, and hematoxylin was used as the counter-stain. Primary antibodies were as follows: anti-F4/80, MCP-1 (Santa Cruz Biotechnology), anti-FSP-1 (Cell Signaling). All sections were processed by an indirect immunoperoxidase technique using a commercial EnVision System kit (DAKO) and counterstained with hematoxylin. All slides were scanned using Pannoramic® MIDI slide scanner (3DHISTECH, Budapest, Hungary).

Statistical analysis

All data are presented as means ± SE. A Student's t-test was used to assess the significance of the independent experiments. Differences with $p < 0.05$ were considered significant.

Abbreviations

α-SMA: α-smooth muscle actin; BUN: blood urea nitrogen; BV: bee venom; CKD: chronic kidney disease; ECM: extracellular matrix; FSP-1: fibroblast-specific marker-1; IL-1β: interleukin-1β; MCP-1: Monocyte chemoattractant protein-1; NGAL: neutrophil gelatinase-associated lipocalin; SK channel: small conductance Ca^{2+}-activated K^+ channel; STAT3: Signal transducer and activator of transcription 3; TβR□: TGF-β type □ receptor; TβR□: TGF-β type □ receptor; TGF-β1: transforming growth factor-β1; UUO: unilateral ureteral obstruction

Declarations

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Not applicable.

Authors' contributions

M.-G.G. and K.-K.P. participated in the design of the study. M.-G.G., H.-J.A. and H.G. performed the experiments. Y.-A.K. and S.M.H performed the data analysis. M.-G.G. and K.-K.P. drafted the manuscripts. All authors discussed, revised and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All protocols for animal study were approved by Institutional Animal Care and Use Committee of the Catholic University of Daegu (EXP-IRB number: DCIAFCR-160705-6-Y). All animal experiments have been performed in accordance with the ethical standards outlined in the Best Practice Guidelines on Publishing Ethics.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

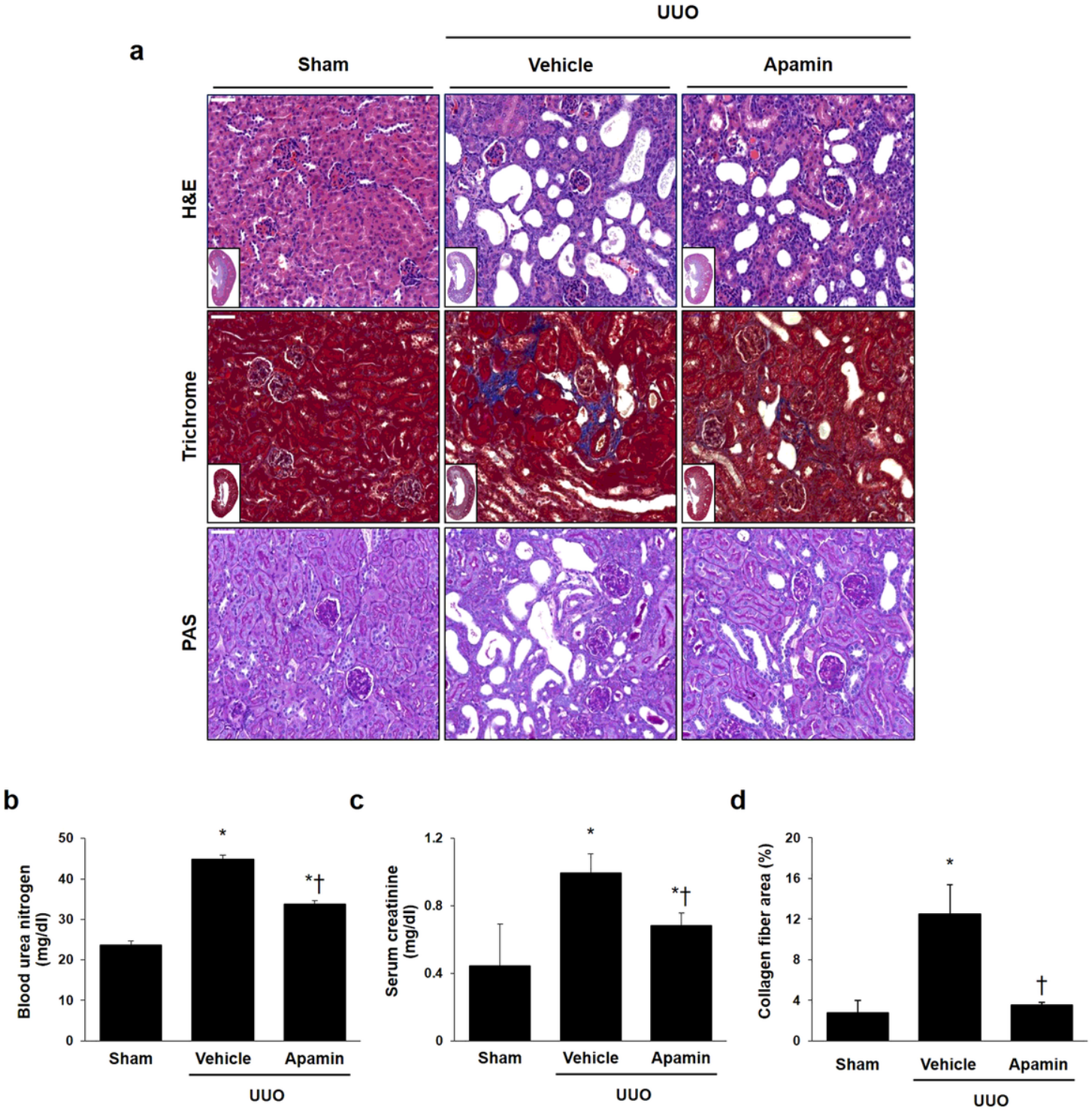

Figure 1

Therapeutic effect of apamin on histological alterations and renal function in unilateral ureteral obstruction (UUO) mice. a Histological sections of murine kidney stained with hematoxylin and eosin (H&E), Masson's trichrome, and periodic acid Schiff (PAS); scale bar 50 μm. b Blood urea nitrogen (BUN) and c creatinine were measured using mice plasma. d The semi-quantitative analysis of blue-stained collagen areas in trichrome staining for each group. *p < 0.05 compared to the normal control group; †p < 0.05 compared to the UUO group.

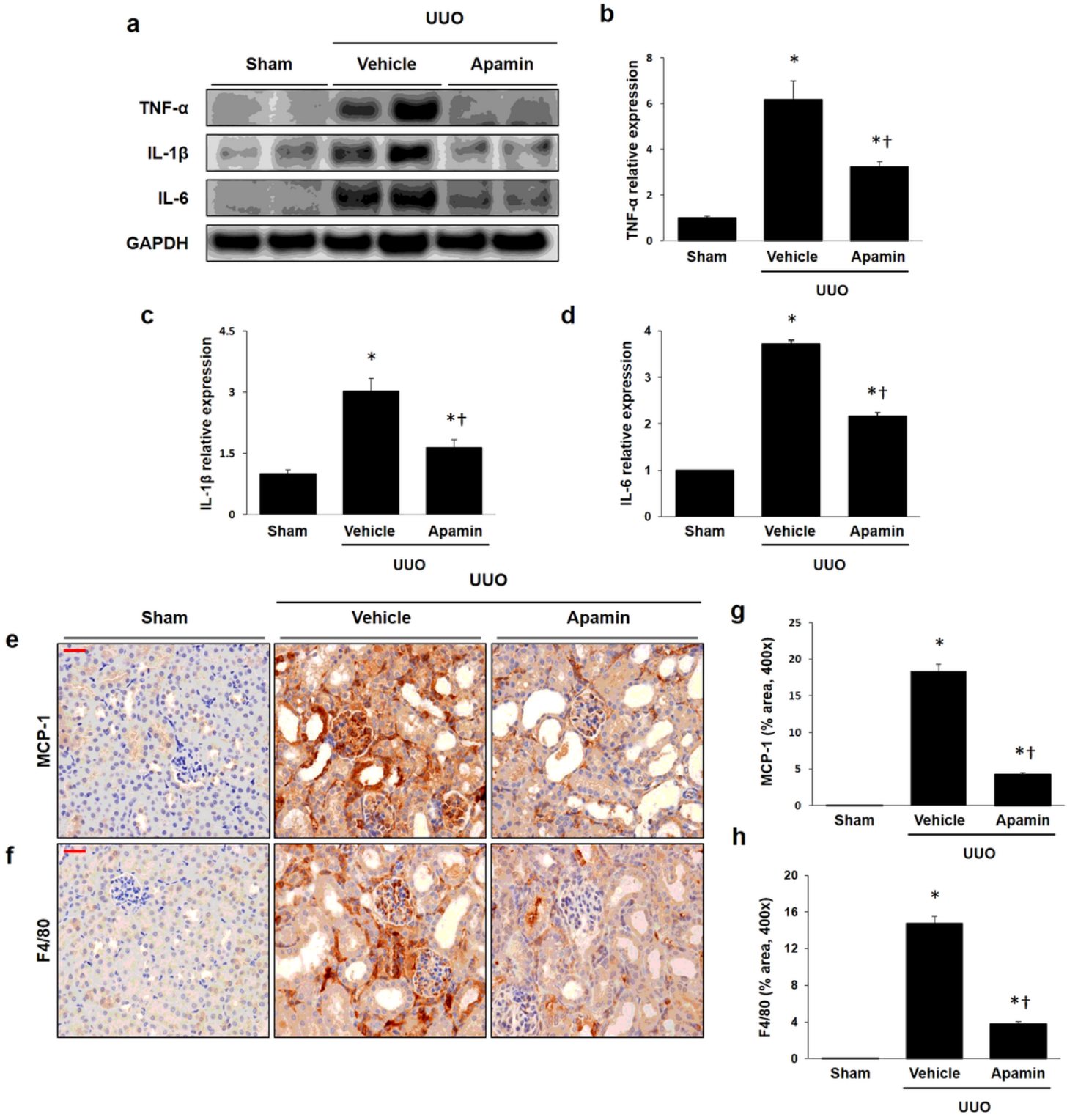

Figure 2

Effect of apamin on renal inflammation in obstructive kidney injury. a The representative image of western blot analysis shows TNF-α, IL-1β and IL-6 protein expressions in the mouse kidney. GAPDH was used to confirm equal loading of all protein samples. The graph shows the quantitative signal intensity of b TNF-α and c IL-1β d IL-6 normalized against GAPDH. Representative immunohistochemical staining images reveal the expression of e MCP-1 and f F4/80; original magnification ×400. The graphs g and h

show the percentage of MCP-1 and F4/80 positive area. *p < 0.05 compared to the normal control group; †p < 0.05 compared to the UUO group.

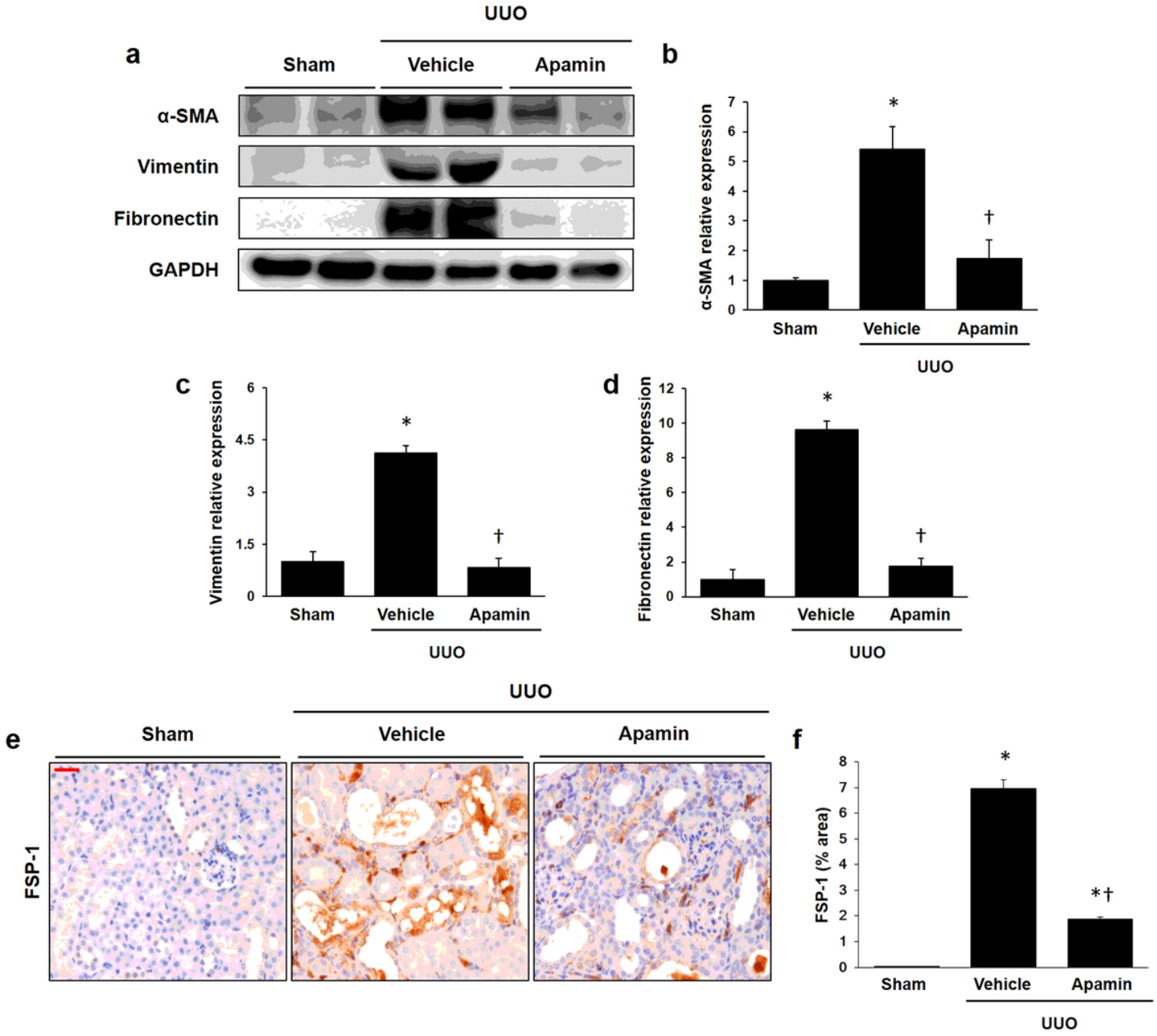

Figure 3

Apamin suppressed the renal interstitial fibrosis and myofibroblast activation in UUO-injured kidney fibrosis animal model. a The protein expressions of α-SMA, vimentin, and fibronectin were analyzed with western blotting analysis. The quantitative graphs show protein expression of b α-SMA, c vimentin, and d fibronectin normalized with GAPDH. e Representative image of immunohistochemical stain using anti-FSP-1 antibody. f The graph shows the FSP-1 positive area (percentage, %); original magnification ×400. *p < 0.05 compared to the normal control group; †p < 0.05 compared to the UUO group.

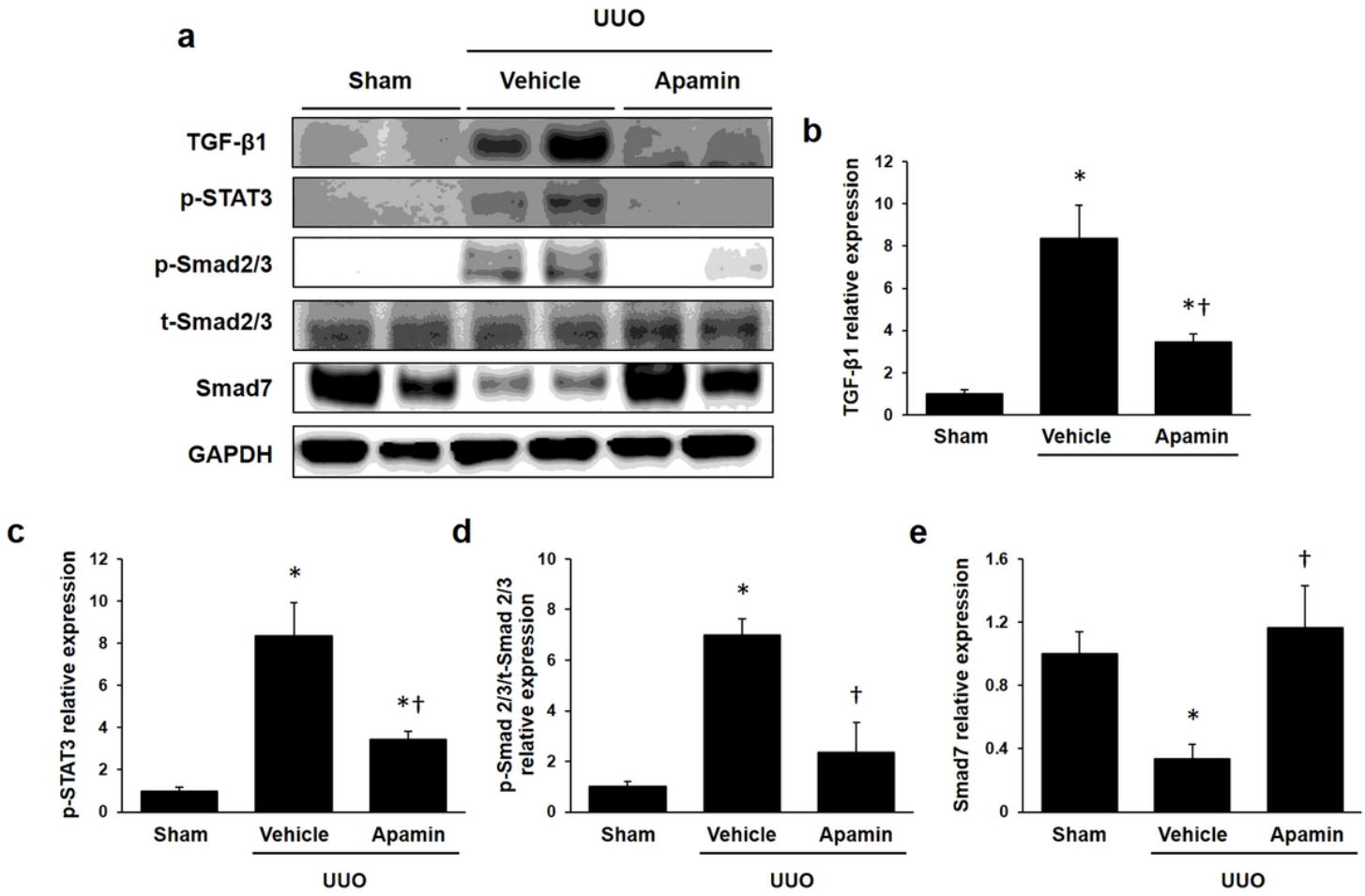

Figure 4

Inhibitory effect of apamin on STAT3 and TGF-β1/Smad signaling in fibrotic murine kidney. a The protein expressions of TGF-β1, p-STAT3, p-Smad2/3, t-Smad2/3 and Smad7 were analyzed by western blotting analysis. All western blot results were exhibited as representative of three independent experiments. The graphs summarize the quantification of b TGF-β1, c p-STAT3, d p-Smad2/3, and e Smad7, each normalized to GAPDH and t-Smad2/3. *p < 0.05 compared to the normal control group; †p < 0.05 compared to the UUO group.

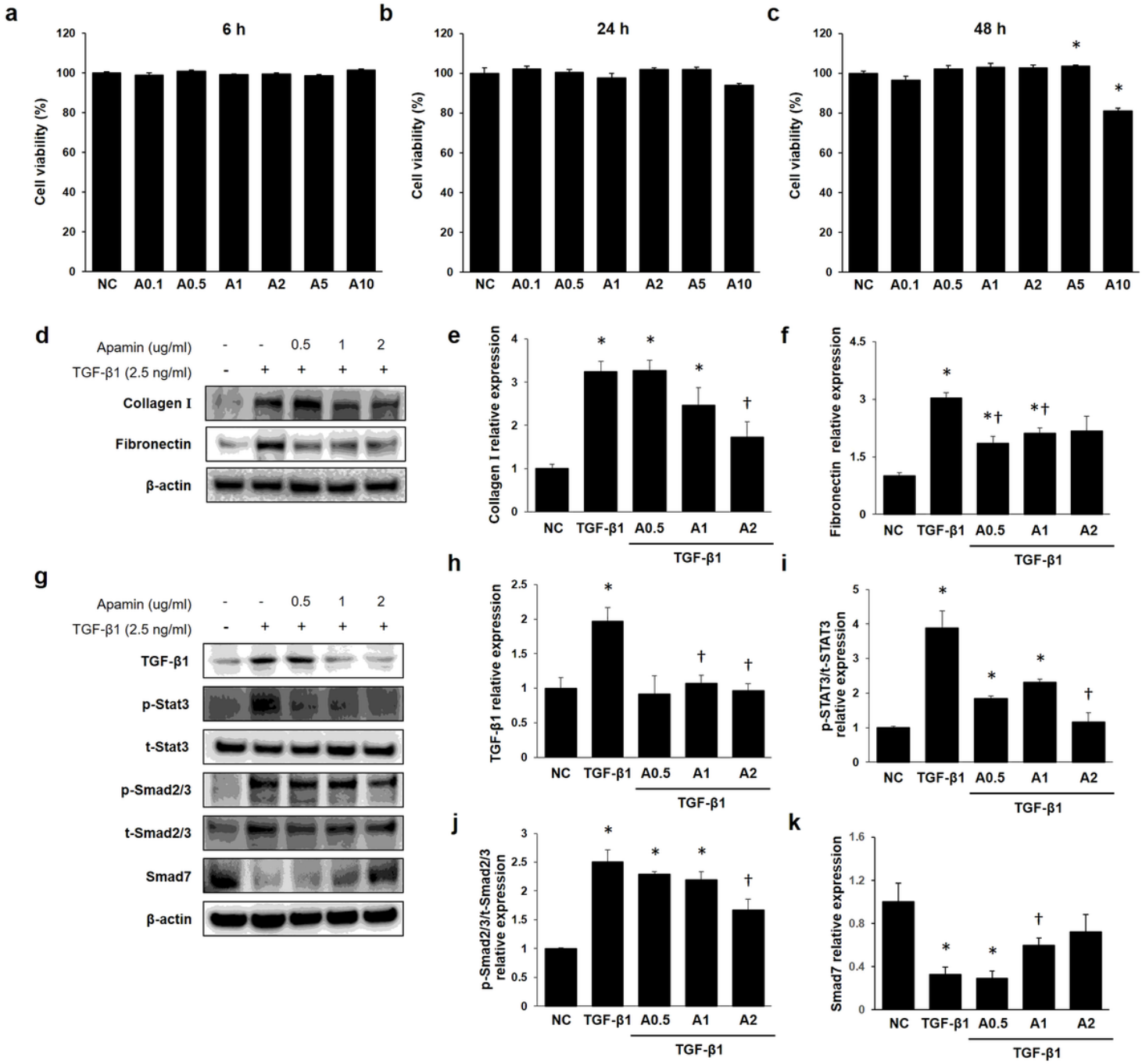

Figure 5

Anti-fibrotic effect of apamin on ECM accumulation and fibrotic signaling in NRK-49F cells. Cellular toxicity of apamin was analyzed using the CCK-8 method. These results show the cytotoxic effect of apamin on NRK-49F cells which were treated with 0.1, 0.5, 1, 2, 5, and 10 μg/ml of apamin for a 6 h, b 24 h, and c 48 h. d The protein expression of collagen Ⅰ and fibronectin were confirmed by western blotting analysis. β-actin was used to load the equal volume of all samples. e and f Quantification of western blotting results. g The representative image of western blotting analysis shows TGF-β1 signaling molecules and TGF-β1 non-canonical mediator expression. The graphs show the quantitative signal intensity of h TGF-β1, i p-STAT3, j p-Smad2/3, and k Smad7 normalized against β-actin, t-STAT3, and t-

Smad2/3. *p < 0.05 compared to normal control group; +: treated; −: un-treated; †p < 0.05 compared to the TGF-β1 treated group.

Supplementary Files

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