Sinkihwan-gamibang ameliorates puromycin aminonucleoside-induced nephrotic syndrome

Lee Hyeon Kyoung 1, 2, Jang Youn Jae 1, 2, Na Se Won 1, 2, Kim Hye Yoom 1, 2, Han Byung Hyuk 1, 2, Lee Yun Jung 1, 2, Lee Ho Sub 1, 2, Yoon Jung Joo 1, 2*, Kang Dae Gill 1, 2*

1 Hanbang Cardio-Renal Syndrome Research Center, Wonkwang University, Jeollabukdo 54538, Republic of Korea; 2 College of Oriental Medicine and Professional Graduate School of Oriental Medicine, Wonkwang University, Jeollabukdo 54538, Republic of Korea

Available online 20 Mar., 2022

[ABSTRACT] Nephrotic syndrome (NS) is a kidney disease characterized by hypertriglyceridemia, massive proteinuria, hypo-albuminemia and peripheral edema. Sinkihwan-gamibang (SKHGMB) was recorded in a traditional Chinese medical book named “Bangyakhappyeon (方藥合編)” and its three prescriptions Sinkihwan, Geumgwe-sinkihwan, and Jesaeng-sinkihwan belong to Gami-bang. This study confirmed the effect of SKHGMB on renal dysfunction in an NS model induced by puromycin aminonucleoside (PAN). The experimental NS model was induced in male Sprague Dawley (SD) rats through injection of PAN (50 mg·kg⁻¹ via the femoral vein. SKHGMB not only reduced the size of the kidneys increased due to PAN-induced NS, but also decreased proteinuria and ascites. In addition, SKHGMB significantly ameliorated creatinine clearance, creatinine, and blood urea nitrogen. SKHGMB relieved glomeruli dilation and tubules fibrosis in the glomeruli of the NS model. SKHGMB inhibited the protein and mRNA levels of the NLRP3 inflammasome including NLRP3, ASC, and pro-caspase-1 in NS rats. SKHGMB reduced the protein and mRNA levels of fibrosis regulators in NS rats. The results indicated that SKHGMB exerts protective effects against renal dysfunction by inhibiting of renal inflammation and fibrosis in NS rats.

[KEY WORDS] Sinkihwan-gamibang; Puromycin aminonucleoside; Nephrotic syndrome; Renal dysfunction; NLRP3 inflammasome; Fibrosis


Introduction

Proteinuria, hypoproteinemia, and ascites are main characteristics of nephrotic syndrome (NS) which is regarded as one of podocyte injury models [1]. Proteinuria is known to be an adverse event of primary podocytopathies due to an abnormality in the filtering barrier that contributes to renal inefficiency [2-3]. Proteinuria develops because of the integrity of the glomerular filtration barrier damages induced by podocyte injury [4]. Podocytes are known to terminally differentiate into visceral epithelial cells, a crucial component of the glomerular filtration membrane [5]. Podocyte injury can cause a reduced expression of cytoskeletal proteins, such as podocin and nephrin [6]. Podocyte injury also leads to cytoskeleton disorganization and foot process fusion, which results in the development of proteinuria and, subsequently, to renal damage [6-7].

Puromycin aminonucleoside (PAN) induces cellular oxidant injury and has been extensively used for modeling podocyte injury in rodents [8]. In the NS model induced by PAN, edema and ascites production resulted in a continuous decrease in tumor pressure, fluid leakage into the interstitial compartment, hyperaldosteronism, hypovolemia, and urinary sodium excretion [9-10]. The hydrolytic and transport activities of Na and K-ATPase reduced sodium excretion in the urine and were associated with a positive sodium balance and the generation of asctises [11]. Creatinine clearance (Ccr) is used as a marker of glomerular filtration rate (GFR) [12]. The case of reduced GFR, it increases the contribution of tubular secretion to creatinine excretion, while overestimation of GFR by endogenous creatinine clearance is prominent in patients with
renal dysfunction \(^{12-13}\).

In the PAN-induced NS model, inflammatory response is very important in pathogenesis, which regulates the NLRP3 inflammasome activation \(^{9}\). The NLRP3 inflammasome is a multi-protein complex and made up of apoptosis associated speck - like proteins including NLRP3, the CARD domain (ASC) and procaspase-1 \(^{14-16}\). The NLRP3 inflammasome is a main component in the immune response \(^{14}\) and acts as a member of the cytoplasmatic pattern recognition receptor family that plays a key role in inducing inflammatory factors, such as interleukin (IL)-1\(\beta\) and IL-18 \(^{15}\). Therefore, the NLRP3 inflammasome and proinflammatory cytokines, including IL-1\(\beta\) and IL-18, exert a direct effect on the renal tubular epithelium and lead to a reduction in renal function. IL-1\(\beta\) induces inflammation through activation of the transcription regulator nuclear factor-B (NF-\(\kappa\)B), which in turn engenders a variety of potentially fibrogenic actions including activation of protein kina (PKC), release of ROS, and production of pro-inflammatory cytokines, such as TGF-\(\beta\) \(^{17}\). TGF-\(\beta\) is a key profibrotic regulator which exerts various effects on all the cell types studied so far \(^{18}\). The Smad family is an essential component of the intracellular signaling pathway and acts as a transcription factor for TGF-\(\beta\)-mediated responses \(^{19}\). Extracellular matrix (ECM) including collagen IV and fibronectin is frequently used as the markers of fibrosis, and the synthesis of ECM is strongly induced by TGF-\(\beta\) activation \(^{20}\). Recent studies have shown that connective tissue growth factor (CTGF, a profibrotic factor) is known to promote abnormal deposition and upregulated in renal fibrosis \(^{21}\).

In Korean traditional medicine, renal deficiency is classified into three major deficiencies: deficiency of kidney yang, deficiency of kidney yin, and insufficiency of kidney qi. Sin-kihwan-gamibang (腎氣丸加味方, SKHGMB) is classified as Sin-kihwan (腎氣丸, SKH), Geumgwe-sinkihwan (金櫃腎氣丸, GSH), and Jesaeng-sinkihwan (濟生腎氣丸, JSH). SKHGMB is known to be effective in treating chronic nephritis, such as pollakiuria, generalized edema, and decreased renal function (方藥合編, 1884). Among the drugs constituting the SKHGMB, JSH was reported to improve proteinuria, hypoproteinemia, and hyperlipidemia, and relieved azotemia in NS patients with acute renal failure \(^{22}\).

However, the pharmacological mechanism of SKHGMB has not been clearly reported in NS. Thus, this study was conducted to investigate the possible effects of SKHGMB against NS.

**Material and Methods**

**Animal treatment**

All animal experiments followed the national institutes of health guide and was approved by Wonkwang University IACUC (WKU 20-22) on March 30, 2020. Seven-week-old male Sprague Dawley rats (180–190 g) were purchased from Koatech (Pyeongteak, Korea). The animals were randomly divided into the following groups: i) control group, ii) PAN group, iii) LOS (PAN + LOS 30 mg·kg\(^{-1}\)·d\(^{-1}\)) group, iv) SKH (PAN + SKH 100 mg·kg\(^{-1}\)·d\(^{-1}\)) group, v) GSH (PAN + GSH 100 mg·kg\(^{-1}\)·d\(^{-1}\)) group, and vi) JSH (PAN + JSH 100 mg·kg\(^{-1}\)·d\(^{-1}\)) group. The rats underwent a 12 h light/dark cycle. PAN was intravenously injected once at a dose of 50 mg·kg\(^{-1}\), while the control group was intravenously injected with normal saline alone. The rats were orally administered with distilled water, losartan (LOS), or SKHGMB once daily for seven days.

**Drugs and chemicals**

PAN was obtained from SIGMA-ALDRICH (Saint Louis, MO, USA), and losartan was purchased from TCI (TCI, Tokyo, Japan) as a positive control drug.

**SKHGMB preparation**

SKHGMB (Table S1) consists of SKH, GSH, and JSH. SKH is composed of 320 g of Rehmanniae Radix Preparata, 160 g of Dioscorea polyostachya, 160 g of Cornus offficinalis, 160 g of Schisandra chinensis, 120 g of Poria cocos, 120 g of Alisma cannaliculatum, and 120 g of Paeonia suffruticosa. GSH is composed of SKH, 120 g of Achyranthes aspera, and 120 g of Plantago ovata. JSH is composed of GSH, 120 g of Cinnamomum verum, and 120 g of Aconitum carmichaeli. The herbs were purchased from the Herbal Medicine Cooperative Association of Iksansi, Jeonbukdo. The prescription was boiled with 3 L of distilled water at 100 °C for 2 h. The supernatant was concentrated using a rotary evaporator (N-11, Tokyo Rikakikai, Tokyo, Japan) to obtain powder. The extract was completely dried using a freeze dryer and diluted to an appropriate volume before oral administration. Dried SKHGMB was kept at 4 °C until use.

**Monitoring of renal function**

The animals were sacrificed one week after the first dosing. Urine samples were collected over 24 h to measure renal function parameters, including creatinine, sodium, potassium, and osmotic pressure, etc. Blood samples were collected immediately after decapitation and osmolality, creatinine (CRE), blood urea nitrogen (BUN), triglyceride (TG), and albumin (ALB) were analyzed. The levels of plasma CRE, BUN, TG, and ALB were measured using an automatic clinical chemistry analyzer (FUJI DRI-CHEM NX700, Tokyo Fujifilm Co., Japan). The creatinine concentrations of plasma and urine were measured using a colorimetric method with a spectrophotometer. (Milton Roy, Rochester, NY).

**Western blot analysis**

Proteins (40 μg) extracted from kidney tissue were separated by 10% SDS-PAGE. The nitrocellulose membranes were blocked with a 5% BSA buffer and then incubated with primary antibodies for cryopyrin (NLRP3), ASC, procaspase-1, IL-1\(\beta\), TGF-\(\beta\), p-Smad2, collagen I, collagen IV, and \(\beta\)-actin (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were detected using a secondary goat anti-rabbit IgG or goat anti-mouse-IgG conjugated with horseradish peroxidase. Protein expression was analyzed by the IBright Imaging System (iBright FL100, Thermo Fisher Scientific, Waltham, MA).
RNA preparation and quantitative reverse transcription-PCR (RT-qPCR)

The cellular RNA was extracted using Trizol reagent (Ambion, Carlsbad, CA). The sequence of the primers is presented in supplementary data (Table S2). PCR amplification was performed by the SYBR Green method [23] using an Applied Biosystems real-time PCR system (Applied Biosystems, Foster city, CA). Each sample was measured in triplicates. The expression of each gene was determined using GAPDH (housekeeping gene).

Periodic acid Schiff staining

The kidney tissues were fixed in 10% formalin for one week, and then sectioned at 5 μmol·L⁻¹ after paraffin insertion. After fixing on the slide, it was stained using the Periodic Acid Schiff (PAS, Sigma). In the kidney tissues, histological changes to the inner medulla, outer medulla and cortex were analyzed by light microscopy (EVOS™ M5000, Thermo Fisher Scientific, Waltham, MA).

Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean ± standard error (SE). Statistically significant differences between the groups were determined using Student’s t-test. The value of P < 0.05 was considered significant.

Results

Effects of SKHGMB on BW, KW/BW, and HW/BW

PAN-induced rats are commonly used for experimental NS research. As shown in Table 1, the body weight (BW) (P < 0.001) was lower in the PAN group than that in the control group. However, there was no significant differences between the PAN groups and the SKHGMB-administered group groups (Table 1). Kidney weight/body weight (KW/BW) was significantly higher in the PAN group than that in the control group (Fig. 1A, P < 0.001). GSH significantly decreased KW/BW compared to PAN group (P < 0.05). In addition, Fig. 1B shows that compared to the control group, the heart weight/body weight (HW/BW) increased in PAN rats. However, this elevation was reduced by oral administration of SKH (P < 0.01).

Effect of SKHGMB on renal function parameters

To evaluate the effects of SKHGMB on renal function, Table 1 Effect of SKHGMB on BW (mean ± SE, n = 8)

<table>
<thead>
<tr>
<th></th>
<th>0 d</th>
<th>1 d</th>
<th>7 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont.</td>
<td>191.6±1.7</td>
<td>201.1±2.6</td>
<td>240.3±1.2</td>
</tr>
<tr>
<td>PAN</td>
<td>190.1±1.4</td>
<td>187.6±1.9</td>
<td>227.1±1.8***</td>
</tr>
<tr>
<td>LOS</td>
<td>185.4±1.3</td>
<td>183.6±0.6</td>
<td>221.1±1.4#</td>
</tr>
<tr>
<td>SKH</td>
<td>188.3±1.6</td>
<td>184.7±1.7</td>
<td>228.5±1.6</td>
</tr>
<tr>
<td>GSH</td>
<td>189.4±1.7</td>
<td>189.4±1.1</td>
<td>227.7±1.5</td>
</tr>
<tr>
<td>JSH</td>
<td>192.2±1.6</td>
<td>187.9±1.2</td>
<td>229.7±1.0</td>
</tr>
</tbody>
</table>

***P < 0.001 vs control; #P < 0.05 vs PAN

Fig. 1 Effects of SKHGMB on KW/BW and HW/BW. Data are expressed as mean ± SE (n = 8). *P < 0.05, ***P < 0.001 vs control; †P < 0.05, ‡P < 0.01 vs PAN
the levels of plasma creatinine, BUN, TG, and ALB were measured. As shown in Fig. 2, the PAN group showed increased plasma BUN and TG levels. However, the plasma BUN levels were significantly reduced by oral administration of GSH and JSH (Fig. 2A). Similarly, the plasma TG levels decreased after oral administration of GSH and JSH (Fig. 2B). In addition, the plasma ALB levels in PAN group were markedly lower than that in the control group, but increased by JSH (Fig. 2C). As a result of the measurement of plasma creatinine, an indicator of renal damage, SKHGMB (SKH, GSH, and JSH) effectively reduced the plasma creatinine level in PAN group (Fig. 2D). In addition, Ccr levels were up-regulated by SKHGMB (SKH, GSH, and JSH) compared to PAN group (Fig. 2E).

**Effects of SKHGMB on proteinuria and ascites**

To determine the effects of SKHGMB on PAN-induced NS, urinary protein excretion and ascites volume were measured. As shown in Fig. 3A, urinary protein excretion increased to the highest on day 5 in the PAN group, which was remarkably reduced by LOS (P < 0.05) and SKHGMB (SKH, GSH, and JSH). As shown in Fig. 3B, ascites volume remarkably increased in the PAN group (P < 0.001), but markedly decreased after administration with SKH, GSH, and JSH.

**Effect of SKHGMB on renal morphology**

A decrease in nephrin is known to be associated with the progression of renal diseases. The effect of SKHGMB on nephrin protein expression by NS was investigated through Western blot analysis. As a result, Figs. 4A and 4B shows that the level of nephrin protein significantly decreased in the glomeruli of PAN-induced NS rats, which then remarkably increased after administration with SKHGMB (SKH, GSH, JSH).
To determine the effects of SKHGMB on the structural histological changes and renal morphology, the tissue sections were examined by PAS staining. As a result, the PAN group showed renal structural damage such as matrix expansion of the renal glomerulus and formation of cortical vacuoles. However, administration of SKHGMB (SKH, GSH, and JSH) improved glomerular injury of the renal cortex compared to PAN group (Fig. 4C). Additionally, SKHGMB treatment ameliorated PAN-induced tubular dilation, tubular epithelial injury, cast formation, and debris accumulation in the outer and inner medulla.

### Effect of SKHGMB on regulating inflammation

Changes in the expression of the NLRP3 inflammasome by SKHGMB were detected by Western blot analysis. Among NLRP3 inflammasome signaling factors, the expression of NLRP3, ASC, caspase-1, and IL-1β protein was activated in response to PAN injury \((P < 0.01)\). However, NLRP3, ASC, and IL-1β protein expression was inhibited by SKHGMB (SKH, GSH, and JSH) \((P < 0.01, \text{Figs. 5A and B})\). In addition, administration with GSH and JSH markedly decreased caspase-1 protein expression compared to PAN group \((P < 0.01)\). Similarly, compared with the control group, the expression of NLRP3 mRNA increased ~4.95-fold in the PAN group (Fig. 5C). However, NLRP3 mRNA expression was markedly reduced by oral administration of SKHGMB (SKH, GSH, and JSH) \((P < 0.001)\). In particular, it was confirmed that GSH administration improved fibrosis in the kidneys to the control level.

### Discussion

SKHGMB was recorded in a traditional Chinese medical book named “Bangyakappyeon (方藥合編)” and three prescriptions, SKH, GSH, and JSH, belong to Gamibang. SKH, an herbal medicine formulation, was traditionally used to strengthen the ‘Yang’ and ‘Yin’ of the kidneys \([24]\). SKH has been reported to be used for diabetes complications.
and for the treatment of glomerulonephritis \cite{24-25}. However, there is no report about the effects of SKHGMB on renal dysfunction. Therefore, this study was performed to confirm whether SKHGMB improves the renal function in PAN-induced NS.

NS is usually caused by damage to the renal glomerulus \cite{26}. PAN-induced NS is characterized by increases in proteinuria, albumin (ALB), and Ccr levels in the kidneys \cite{27}. In this study, SKHGMB was administered in a PAN-induced NS rat and the results showed that SKHGMB ameliorated renal function biomarkers, such as Ccr, plasma CRE, TG, and urinary protein excretion. These results suggested that SKHGMB is effective at improving NS through regulating renal function biomarkers.

The kidney sections were stained with PAS to confirm the effects of SKHGMB on tubular injury. According to previous reports, the epithelial-mesenchymal transition differentiation (EMT) and renal damage of tubular epithelial cells (TECs) were observed in the NS model \cite{28}. In this study, glomerular injury was also found in the PAN group, and vacuole formation was observed in the cortex of the kidneys. However, SKHGMB improved glomerular injury by inhibiting vacuole formation and ameliorating EMT and renal injury of TECs in NS rats.

In another study, nephrin was observed to be an essential molecule that maintains the function of the glomerular capillary wall and is involved in proteinuria induction \cite{29}. Western blot analysis was performed to clarify whether nephrin is redistributed within podocytes or whether the expression of nephrin protein decreases. The reduced expression of nephrin revealed that SKHGMB treatment exhibited a reversing effect on the PAN-induced NS model. These results suggest that SKHGMB was effective since the expression of nephrin was largely negatively correlated with the degree of proteinuria, which may be an indirect consequence of proteinuria inhibition.

The NLRP3 inflammasome mainly causes the inflammation and dysfunction of the kidneys. Interrupting the activation of the NLRP3 inflammasome can control renal inflammatory injury \cite{30}. In the present study, Western blot and RT-qPCR analyses revealed that SKHGMB reduced PAN-induced NLRP3 inflammasome formation. This result showed that SKHGMB significantly inhibited the PAN-induced NLRP3 inflammasome, which then led to the maturation of proinflammatory cytokines, such as IL-1β. Therefore, these results suggested that SKHGMB decreased the inflammatory response by reducing NLRP3 inflammasome formation. Some studies have reported that NLRP3 is required for an optimal TGF-β/R-Smad signaling activation \cite{30}. TGF-β, one of the main regulators of fibrosis, is a well-known inducer of EMT in several organs \cite{31-32}. Overexpression of TGF-β in various tissues induced changes in fibrosis, and the corresponding TGF-β/Smad signal played a key role in fibrosis in other organs and tissues \cite{33}. This study revealed that SKHGMB reduced the protein and mRNA levels of the fibrosis regulators compared to PAN group. Therefore, these results suggested that SKHGMB decreases PAN-induced renal fibrosis.
Fig. 6 Effect of SKHGMB on the expression of fibrosis regulators in the kidneys. Protein expression of fibrosis including TGF-β, p-smad2, collagen I, and collagen IV (A) in the kidneys were analyzed by Western blot analysis and quantitative assessments of NLRP3 expression (B). Relative levels of TGF-β, fibronectin, CTGF, COL4, smad2, and smad3 mRNA (C) in the kidneys were analyzed by real-time RT-qPCR analysis. Data shown summarize three independent experiments. Data are expressed as mean ± SE. *P < 0.05, **P < 0.01, ***P < 0.001 vs control; †P < 0.05, ††P < 0.01, †††P < 0.001 vs PAN

Conclusion

SKHGMB has a protective effect against renal dysfunction by inhibiting renal inflammation and fibrosis. In particular, GSH extract is proved to be very effective in improving renal function in NS model. Thus, SKHGMB is considered to be a good treatment agent for NS.

Supplementary Materials

Supplementary information can be acquired by e-mail to corresponding author.

References


