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Research Article
ShenSu III Decoction Ameliorates Angiotensin-induced Injury by Regulating Expression of Ang2, Tie2 and VEGFR2

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Abstract

Background and Objective: ShenSu III decoction (SSD), a Chinese herbal medicine formulation has demonstrated beneficial effects in decreasing the severity of proteinuria; however, the potential underlying mechanisms are still unclear. This study aimed to investigate the protective effects and mechanisms of SSD on glomerular endothelial cell (GEC) injury induced by angiotensin II (Ang II). **Materials and Methods:** Mouse GECs were divided into control group, Ang II group, Ang II+Hirudin group, Ang II+Benazepril group and Ang II+SSD group. Permeability of GECs in each group was measured by fluorescein isothiocyanate-labeled bovine serum albumin. The expression levels of angiopoietin 2 (Ang2), tyrosine protein kinase receptor 2 (Tie2) and vascular endothelial growth factor receptor 2 (VEGFR2) were determined by real-time PCR and western blot. Cell cycle was examined by flow cytometry. **Results:** The SSD significantly reduced Ang II-induced apoptosis and permeability of GECs ($p<0.05$). These protective effects were attributed to the down regulation of enhanced Ang2, Tie2 and up regulation of decreased VEGFR2 both induced by Ang II. **Conclusion:** The present results indicated that SSD attenuates apoptosis and permeability of GECs through the regulation of Ang2, Tie2 and VEGFR2 expression to protect against Ang II-induced injury.

Key words: ShenSu III decoction, angiotensin II, permeability, glomerular endothelial cells, proteinuria

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Proteinuria is considered as an independent risk factor for chronic kidney disease (CKD)[1]. Accumulating evidence indicated that impairment of glomerular filtration apparatus can result in proteinuria[2,3]. The glomerular filtration barrier (GFB) comprises of three major components: the glomerular basement membrane (GBM), podocytes and fenestrated glomerular endothelial cells (GECs)[4,5]. Among these, GECs controlling the major aspect of filtration are essential for glomerular capillary permeability. Injury to GECs leads to increased endothelial cell permeability, which subsequently contributes to the progression of proteinuria. The increased permeability also promotes the leakage of albumin from GFB[4].

Angiotensin II (Ang II) is a peptide hormone that causes vasoconstriction and increase in blood pressure. It is the sole biologically active downstream peptide of renin-angiotensin system (RAS) that regulated vascular injury through its actions on endothelial function. Ang II caused micro-vascular endothelial barrier injury, which is considered as a vital risk factor underlying renal diseases[6]. Recently an increasing number of animal studies and clinical trials have reported that traditional Chinese herbs could decrease the severity of proteinuria and decelerate CKD progression either by extending blood vessels or by contributing to anti-inflammation and anti-coagulation[7-10]. Moreover, effective ingredients in these herbs can activate the injured intrinsic renal cells and restore their function. In this way, proteinuria can be remitted and kidney function can be improved remarkably.

ShenSu III decoction (SSD) is a polyjuice potion composed of Chinese Thorowax (10 g), Scutellaria (10 g), Fourstamen Stephania (10 g), spreading hedyotis (10 g), astragalus (20 g), Chinese angelica (10 g), Eucommia ulmoides (10 g) and winged euonymus branchlet (10 g). Some clinical studies have reported the beneficial effects of SSD (such as; reduction in proteinuria severity and increase in plasma protein content) in the treatment of proteinuria in patients with focal segmental glomerulosclerosis[11]. However, the mechanism underlying SSD therapy for proteinuria remains unclear.

Therefore, in this study, the protective effects of SSD on angiotensin II (Ang II)-induced GEC injury were investigated. Results of this study would help in understanding the mechanism underlying SSD therapy for treating proteinuria.

MATERIALS AND METHODS

This study was conducted in First Teaching Hospital of Tianjin University of Traditional Chinese Medicine (Tianjin, China) from May, 2014-April, 2018.

Cell culture: Mouse GEC line was purchased from TianJin Biobay, Bio-tech Co., Ltd., (Tianjin, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS, Gibco, USA), 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin at 37< and 5% CO_2 in a humidified atmosphere.

Immunohistochemistry: Immunohistochemical analysis of Factor VIII and CD31 were performed according to Chen *et al.*[12]. Briefly, GECs were cultured on cover-slips in DMEM. The cells were washed thrice with PBS fixed with 4% paraformaldehyde followed by 3% H_2O_2 and incubated at room temperature for 10-15 min to quench endogenous peroxidase activity. After three washes with PBS, the cells were incubated with primary antibodies anti-CD31 and anti-VIII overnight at 4 . Next, they were incubated with secondary antibodies for 1 h at room temperature. Finally, images were captured with a fluorescence microscope (Leica, Germany).

Analysis of GEC permeability: The GEC monolayer was prepared by using Transwell insert chambers. The upper Transwell chamber was coated with 0.05% gelatin (BD Biosciences, San Jose, CA, USA) and added with 200 µL GEC (1.0×108/l), while the lower chamber was filled with 500 µL complete DMEM until the cells formed an adherent monolayer. Ten microliters of fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA) (stock of 1 mg mL^{-1}) was added to the upper chamber medium and cultured for 12 h at <37 and 5% CO_2. Media was harvested and absorbance was determined by using multifunctional flow cytometry in triplicate.

Treatment: The following concentrations of Ang II were added to the GECs and were incubated for 12 h to induce GEC injury: 0, 2, 5, 10, 20, 40 and 80 µg mL^{-1}. The concentration of Ang II that induced maximum GEC injury was selected for further experiments. The chemical compositions of SSD were examined by mass spectrometry analysis and the result was displayed in Supplementary Table 1. Ang II, hirudin and benazepril were purchased from Sigma-Aldrich (St-Louis, MO, USA). The GECs were divided into five groups: control group;

Table 1: Primers used in real-time PCR

Gene	Direction	Sequence (5'-3')	Size (bp)
Ang2	Upward	AGCACTATGATGCCAAGCCAAAAG	115
	Reverse	CTTGGTGTCATGGATAAAGGTGTTG	
Tie2	Upward	CGGCCAGGTACATAGGAGGAA	67
	Reverse	TCACATCTCCGAACAATCAGC	
VEGFR2	Upward	CGTTTTCAGAGTTGGTGGAGCATTT	151
	Reverse	ATACAGGAAACAGGTGAGGTAGGCA	
β-actin	Upward	GCCTTCCTTCTTGGGTAT	97
	Reverse	GGCATAGAGGTCTTTACGG	

Ang II group, stimulated by Ang II (5 μg mL^{-1}), Ang II+Hirudin group, stimulated by Ang II (5 μg mL^{-1})+Hirudin (10 μg mL^{-1}), Ang II+Benazepril group, stimulated by Ang II (5 μg mL^{-1})+ Benazepril (80 μg mL^{-1}) and Ang II+SSD group, stimulated by Ang II (5 μg mL^{-1})+SSD (80 μg mL^{-1}). In the treated groups, hirudin, benazepril and SSD were spread in Transwell chambers prior to incubation of cells for 48 h.

Western blot analysis: The GECs were cultured in serum-free medium for 24 h and then stimulated with or without Ang II. Total protein extracts were obtained by lysing cells and western blot analysis were performed according to previous studies[13] using following antibodies: polyclonal anti-Ang2 (1:500, Cat No. SAB1105001, Sigma-Aldrich), anti-Tie2 (1:1000, Cat No. 7403, Cell signaling technology) and anti-VEGFR2 (1:2000, Cat No. ab39256, Abcam). β-actin (1:5000, Cat No. CW0096A, Cwbio. Co., Ltd., Beijing, China) was used as internal control.

Real-time PCR: Real-time PCR was carried out according to descriptions elsewhere[13]. Briefly, total RNA was isolated using RNA isolation reagent (Invitrogen) and reverse transcribed with PrimeScript RT Reagent Kit (Cwbio. Co., Ltd., Beijing, China). Real time PCR was performed in 7500 RT-PCR system by (Applied Biosystems) using the SYBR Premix Ex *Taq* Kit with primers shown in Table 1. The GAPDH was used as internal control and relative expression was calculated by 2-Ct method[14].

Cell cycle analysis: Cells from different groups were harvested by EDTA-trypsin digestion and a single-cell suspension was prepared. Cells were then incubated in microtiter plates with fluorochrome labeled antibodies at 4°C for 40 min and analyzed by flow cytometry (BD, SanJose, CA).

Statistical analysis: All experiments were performed in triplicate; all statistical analysis were carried out by using SPSS software 15.0 (IBM, Armonk, NY, USA) for Windows. Data were expressed as mean±standard error (SEM). Comparison between two groups was conducted by Student's t-test and comparison among multiple groups was performed using one-way analysis of variance. A p-value less than 0.05 indicated statistical significance.

RESULTS

Immunohistochemical analysis displayed positive expression of VIII and CD31 in GECs: In order to characterize GECs, the expression of VIII and CD31 in GECs was detected by immunohistochemical staining. As shown in Fig. 1, after culture for 3 days, most cells acquired a spindle-shaped morphology. In addition, immunohistochemical staining of cells yielded positive results for the presence of both Factor VIII (Fig. 1a) and CD31 (Fig. 1b). These results suggested the endothelial character of GECs.

Ang II increased the permeability of GECs in a dose-dependent manner: To investigate the effect of Ang II on the permeability of GEC monolayer, the cells were treated with different concentrations of Ang II for 48 h. As shown in Fig. 2, the endothelial monolayer permeability gradually increased with the increase of Ang II concentration from 5-80 μg mL^{-1} and reached a peak at 80 μg mL^{-1}. The permeability at 20, 40 and 80 μg mL^{-1} Ang II was significantly higher than that in the control group ($p<0.05$). These results suggested that Ang II-induced vascular damage is dose-dependent.

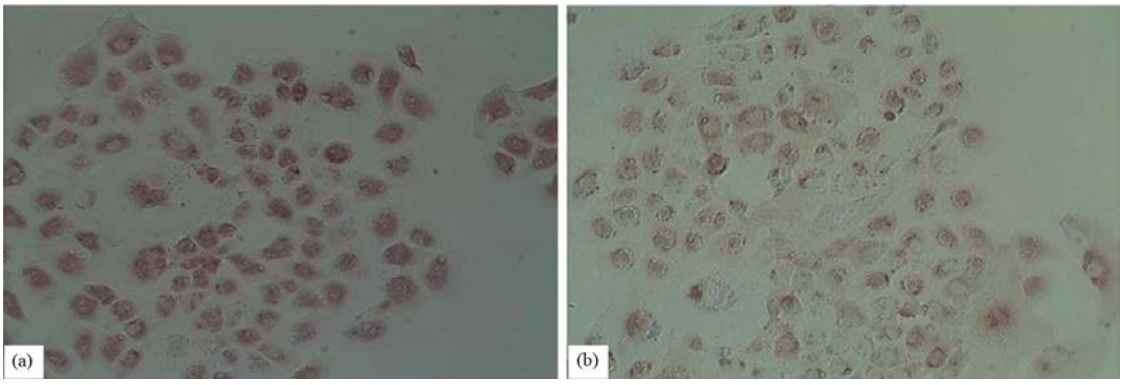

Fig. 1(a-b): Immunohistochemical staining of glomerular endothelial cells (GECs), (a) Immunohistochemical staining of factor VIII (Magnification 400×) and (b) Immunohistochemical staining of CD31 (Magnification 400×)

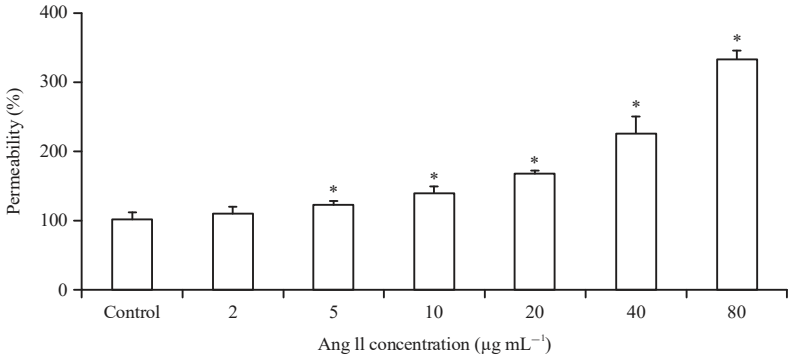

Fig. 2: Permeability of GECs after SSD treatment (at different concentrations) was measured by FITC-labeled BSA
Data were expressed as mean±SEM. *Indicated p<0.05 compared with control group as calculated by Student's t-test

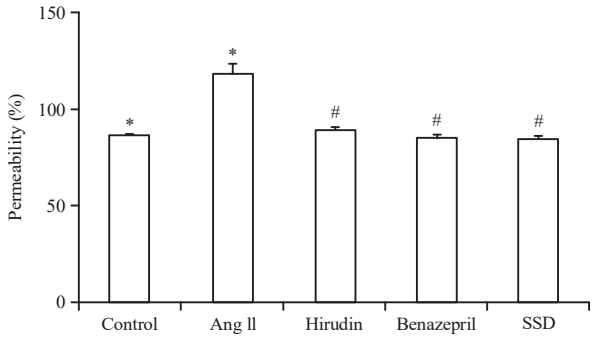

Fig. 3: Permeability of GECs after treatment with Ang II, hirudin, benazepril and SSD was measured by FITC-labeled BSA
Data were expressed as mean±SEM, *p<0.05 compared to control group as calculated by Student's t-test, #p<0.05 compared to Ang II group as calculated by Student's t-test, SSD: ShenSu III decoction, Ang II: Angiopoietin 2

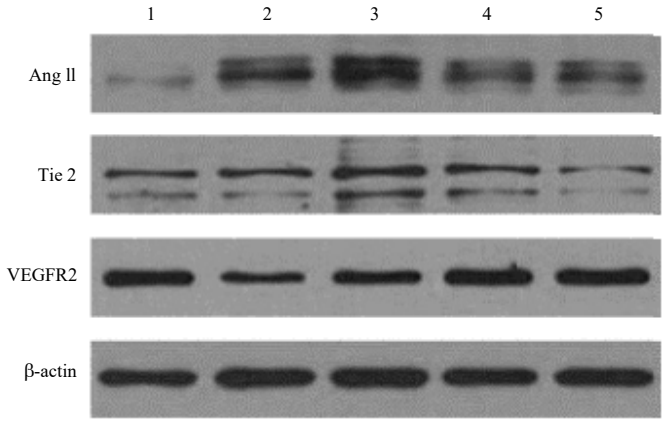

Fig. 4: Relative protein expression of Ang2, Tie2 and VEGFR2 in five groups. Protein expressions were detected by western blot
Lane 1: Control group, Lane 2: Ang II stimulation group, Lane 3: Hirudin treatment group, Lane 4: Benazepril treatment group and Lane 5: SSD treatment group

SSD ameliorates Ang II-induced permeability increase in GECs: The permeability of GECs in each group was evaluated. As shown in Fig. 3, Ang II stimulation significantly enhanced the permeability ($p<0.05$), whereas treatment with hirudin, benazepril and SSD significantly ameliorated the increased permeability ($p<0.05$).

SSD downregulates Ang2 and Tie2 and upregulates VEGFR2: The expression levels of Ang2, Tie2 and VEGFR2 were evaluated by western blot and real time PCR. As shown in Fig. 4, Ang2 was weakly expressed in control GECs while its expression was significantly enhanced by Ang II stimulation and markedly downregulated by SSD treatment. Ang II-induced Tie2 expression was also significantly reduced by SSD treatment. The VEGFR2 expression was inhibited by Ang II, but restored with SSD-treatment. These results showed that SSD ameliorates Ang II-induced permeability in mouse GECs via upregulation of VEGFR2 and downregulation of Ang2 and Tie2.

As shown in Fig. 5, compared to those of the Ang II group, the mRNA expression levels of Ang2 and Tie2 were upregulated in the Ang II-stimulated group and downregulated in the SSD-treated group (Fig. 5a and b, $p<0.05$), whereas VEGFR2 level was downregulated in the Ang II-stimulated group and upregulated in the SSD-treated group (Fig. 5c, $p<0.05$).

SSD enhances GEC proliferation and maturation: As shown in Fig. 6, Ang II stimulation could improve the proportion of cells in G1/G0 (69.33 vs. 89.52%, Fig. 6a and b) and reduced the proportion of cells in S phase compared to that in the control (23.71 vs. 5.86%, Fig. 6a and b). It is evident that hirudin, benazepril and SSD treatment led to decrease of the proportion of cells in G1/G0 phase induced by Ang II (81.74%, 76.04 and 79.31 vs. 89.52%; Fig. 6c-e) and increase of

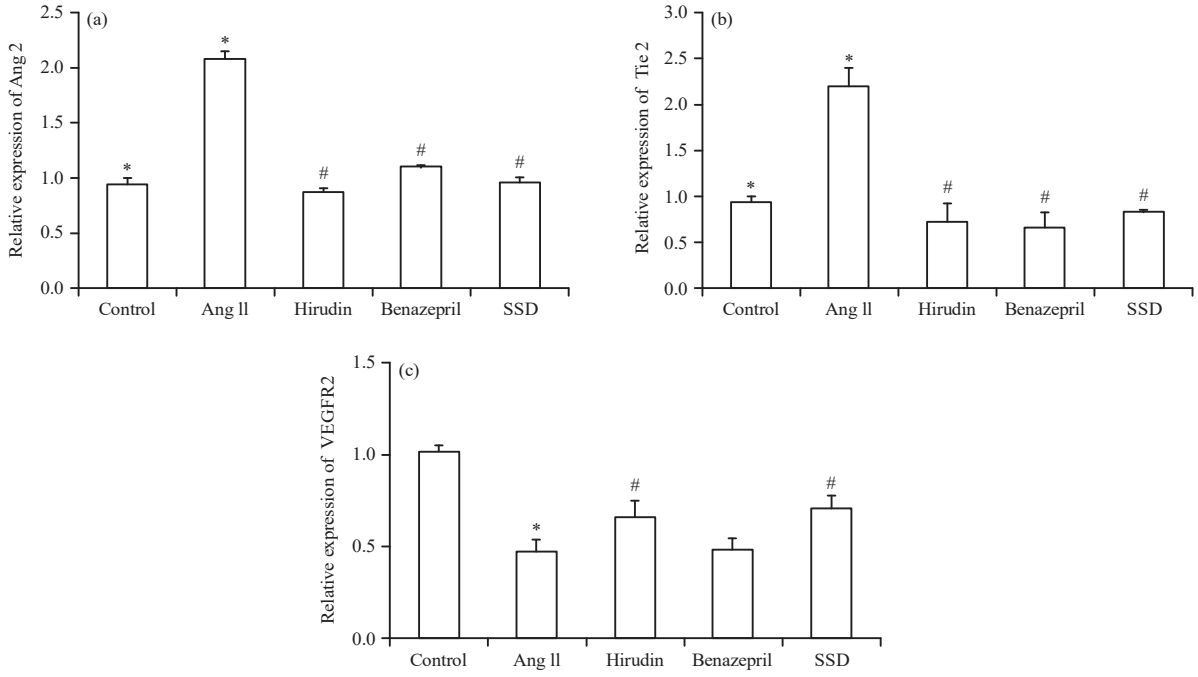

Fig. 5(a-c): Relative mRNA expression detected by real time PCR, (a) Ang 2, (b) Tir 2 and (c) VEGFR2

Data were expressed as mean±SEM. *p<0.05 compared to control group and #p<0.05 compared to Ang II group as calculated by Student's t-test, SSD: ShenSu III decoction, Ang II: Angiopoietin 2

the proportion of cells in S phase (11.60, 17.07 and 9.87% vs. 5.86%; Fig. 6c-e) indicating promotion of cell regeneration and maturation.

DISCUSSION

The present study investigated the protective effects of SSD on Ang II-induced GEC injury. Results showed that SSD ameliorated Ang II-induced GEC injury through reduction in permeability and recovery of cell cycle. Furthermore, SSD down regulated the AngII-enhanced Ang2 and Tie2 expression and unregulated the AngII-reduced VEGFR2 expression. The results suggested SSD might be effective in protecting AngII-induced injury. Ang II, as a vital component of RAS, increased capillary filtration pressure both directly and indirectly[15]. It also directly influenced the integrity of GFB by decreasing the synthesis of negatively charged proteoglycans and downregulation of nephrin[16]. In this study, the endothelial monolayer permeability gradually increased in an AngII dose-dependent manner (Fig. 2). Results suggested that increased Ang2 release from endothelial cells results in widening of vascular endothelial cellular slit and increase in vascular permeability. Treatment with benazepril, hirudin and SSD led to improvement of glomerular endothelial permeability to different extents. A possible mechanism of action of SSD could be via the regulation of Ang2, Tie2 and VEGFR2 by the endothelial cells.

Tie2-bound Ang2 triggers endothelial Tie2 phosphorylation, thereby enhancing neovascularization[17]. Ang2 supported endothelial cell adhesion, independent of Tie2 signaling[18] and is weakly expressed in glomeruli under normal physiological conditions. In the early stages of vascular injury, Tie2 was highly upregulated[19]. Ang2 blocked Ang1-activated endothelial surface Tie2 receptors, resulting in decreased vascular stability[20]. In addition, the injured endothelium also initiated platelet activation and coagulation cascade both having been implicated in the pathogenesis of glomerulosclerosis. Ang2 activity depends on the levels of vascular endothelial growth factor (VEGF). The VEGF acts by binding to the receptor tyrosine kinase VEGFR2. The VEGFR-2/flk-1/KDR pathway directly regulated Ang2 expression in presence of VEGF and enables Ang2 to begin early angiogenesis, improve blood vessel diameter, increase basal-layer endothelial cell mobility through vascular remodeling and stimulate vascular sprouting, growth, angiogenesis and proliferation[21,22]. In this study, Ang II significantly up regulated Ang2 and Tie2 downregulated VEGFR2 while SSD could ameliorate AngII-induced up regulation of Ang2 and Tie 2 and down regulation of VEGFR2. In particular, the SSD group performed relatively better than

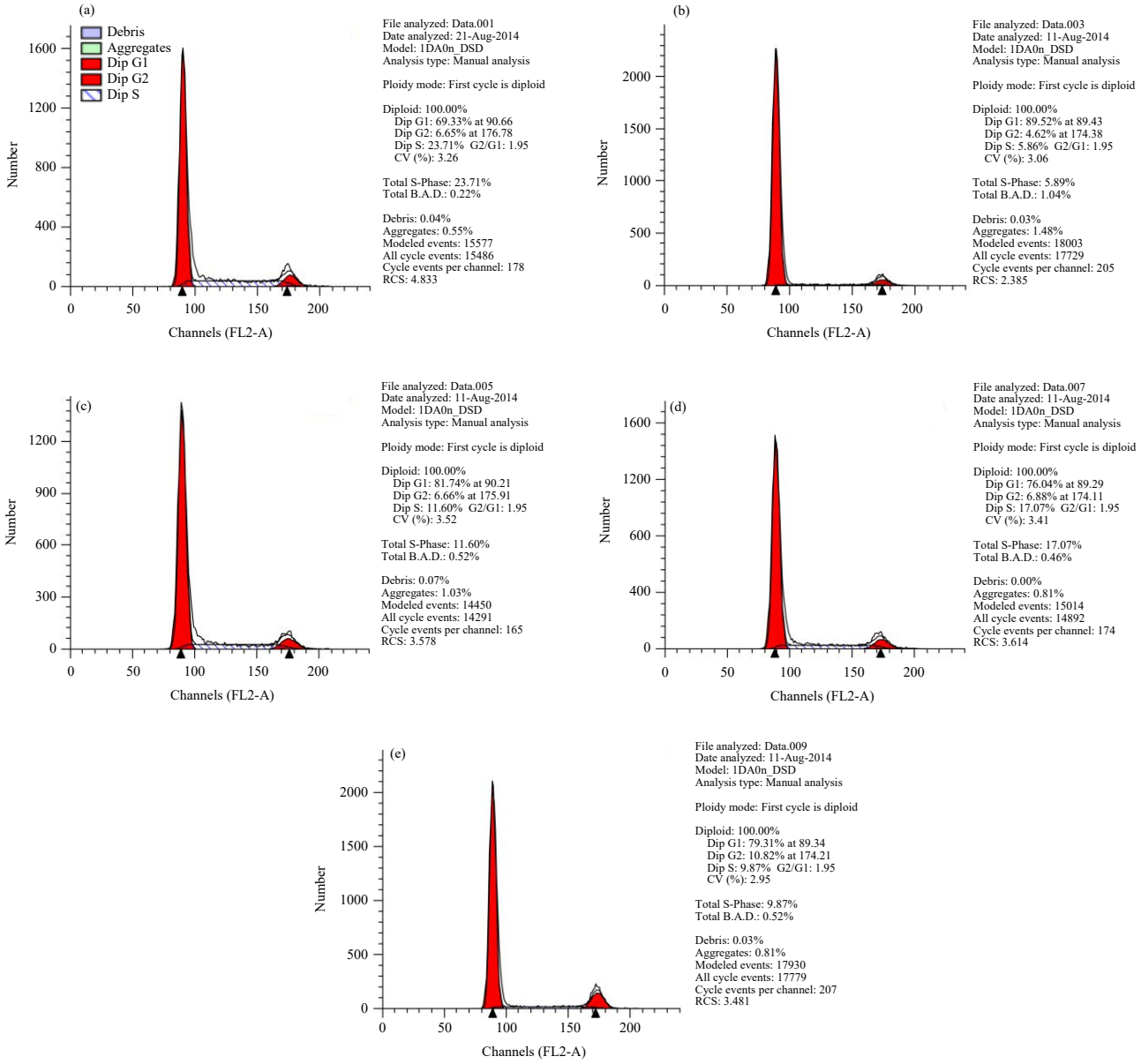

Fig. 6(a-e): Flow cytometry assay was performed to evaluate cell cycle, (a) Control group, (b) Ang II stimulation group, (c) Hirudin treatment group, (d) Benazepril treatment group and (e) SSD treatment group

the other groups. After being treated with SSD, vascular endothelial cellular slit was recovered and therefore, vascular stability was enhanced. Binding of Ang2 to Tie2 stimulated the neovascularization in the presence of VEGFR2, decreased vascular permeability and reduced the protein leakage.

CONCLUSION

This study indicated that SSD could attenuate GEC permeability through down regulation of Ang2 and Tie2 and up regulation of VEGFR2 to protect against Ang II-induced injury. SSD caused narrowing of the vascular endothelial cellular slit decreased vascular permeability and reduced protein leakage. Results of this study would help in understanding the mechanism underlying SSD therapy for treating proteinuria. However, further *in vivo* studies should be carried out to further validate the results of this study.

SIGNIFICANCE STATEMENT

This study discovered the Shensu III decoction could reduce apoptosis and AngII-induced permeability that can be

beneficial for glomerular endothelial cell injury and decrease the severity of proteinuria. This study will help the researchers to uncover the critical areas of treatment of proteinuria that many researchers were not able to explore. Thus a new theory on the mechanism underlying SSD therapy for treating proteinuria may be arrived at.

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