

Preliminary study of the mechanism underlying how Jianshen Granules ameliorate renal failure in 5/6 nephrectomy model rats

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Research article

Keywords: antioxidant, 5/6 nephrectomy, chronic renal failure, transforming growth factor β 1, Jianshen Granule

Posted Date: April 21st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18615/v1>

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Abstract

Background

Chronic renal failure (CRF) is a worldwide public health concern, and at present, there are limited treatment options available. Jianshen Granules, a traditional Chinese herbal medicine, have been used clinically in the treatment of renal diseases for a large number of years in the Air Force Medical Center of the Chinese People's Liberation Army (PLA), and have shown great efficacy. In the present study, both the effectiveness and the mechanism of action of Jianshen Granules to treat CRF were investigated.

Methods

A rat model of CRF was established by 5/6 nephrectomy. Rats were administered distilled water, Uremic Clearance Granules (UCGs), or Jianshen Granules. During the administration period, the physiological state of the rats was observed, and the biochemical parameters of interest were measured. The pathology of the kidney tissues were assessed using hematoxylin and eosin, and Masson's staining. In addition, the expression level of transforming growth factor β 1 (TGF β 1) was detected. Human kidney cells (HKC cells) were used to investigate the effects of Jianshen Granules on apoptosis induced by hydrogen peroxide (H₂O₂), whereas cell viability was assessed by Cell Counting Kit8 (CCK8) assay. The level of apoptosis, the mitochondrial membrane potential (MMP), and reactive oxygen species (ROS) and Ca²⁺ levels were measured using flow cytometry.

Results

The results revealed that Jianshen Granules could reduce the levels of serum creatinine, blood urea nitrogen, alanine aminotransferase and aspartate aminotransferase, and the volume of urine in CRF rats. Renal histopathological examinations revealed that Jianshen Granules had an ameliorative effect on renal injury. In addition, Jianshen Granules led to a marked decrease in TGF β 1 levels in CRF rats. Following treatment with Jianshen Granules, cell viability and the level of the MMP increased, whereas the levels of ROS and Ca²⁺ were reduced significantly. The increased level of TGF β 1 was detected in the H₂O₂ treated group, although this increase was attenuated by treatment with Jianshen Granules.

Conclusion

Taken together, the results of the present study have shown that Jianshen Granules are able to ameliorate renal failure in CRF rats, and to inhibit apoptosis of HKC cells induced by H₂O₂ via downregulation of TGF β 1.

Background

Chronic renal failure (CRF) is a wellrecognized public health problem worldwide, characterized by evidence of renal damage or dysfunction[1]. CRF is characterized by the progressive loss of renal function, chronic inflammation, oxidative stress, vascular remodeling, and glomerular and tubulointerstitial scarring [2]. CRF has also been demonstrated to increase the risk of cardiovascular disease, diabetic nephropathy, and renal osteodystrophy[3, 4]. At the moment, kidney transplantation surgery and hemodialysis therapy are increasingly applied in clinical treatment; however, these therapies are expensive and difficult to be applied widely[5, 6]. Furthermore, these therapies may cause inflammatory reactions and oxidative stress during the course of treatment, thereby increasing the risk of complications. Therefore, it is important for clinical practice that effective and safe alternative therapies be sought after[7].

Traditional Chinese medicine (TCM) has abundant clinical applications and has demonstrable curative effects. Renal failure ensues largely as a consequence of endocrine disorders that lead to disturbance of the microcirculation. Based on TCM theory, renal failure is due to "deficient functioning of the spleen and kidney", "interior accumulation of dampness and turbidity", and "accumulated poison gathered in the kidney". It is characterized as one of the "consumptive diseases"[8].

Jianshen Granules, a traditional Chinese herbal medicine designed by the renowned TCM physician Dr Zhanmin Liu, working in Air Force Medical Center of PLA, has been used clinically in the treatment of renal diseases for a large number of years in the Air Force Medical Center of the Chinese People's Liberation Army (PLA). Jianshen Granules are composed of *Radix Rehmanniae*, *Cornus*, *Poria cocos*, *Astragali radix*, *Angelica sinensis*, *Salvia miltiorrhiza radix*, *Ligusticum chuanxiong* Hort, *lumbricus*, *Honeysuckle*, *Fried atractylodes* and *Rhubarb*. A clinical study conducted by the Air Force Medical Center on 61 patients with CRF revealed that the overall response rate of Jianshen Granules was 85.24%. Jianshen Granules not only enhanced recovery of the spleen and kidney, but were also demonstrated to clear stasis, promote the excretion of toxins and metabolic wastes from the body, and restore renal functions[9, 10].

Rodent models are commonly used in preclinical CRF studies[11]. The partially nephrectomized rat model has been used extensively to investigate archetypal pathological changes in CRF[12]. Residual kidneys of nephrectomized rats often show adaptive and compensatory growth following injury, which is similar to the development of human diseases.

The present study aimed to confirm the therapeutic effects, and delineate the underlying mechanism of action of, Jianshen Granules on kidney injury induced by 5/6 nephrectomy in rats, as well as effects on the damage caused to HKC cells.

Methods

Animals.

Sprague-Dawley (SD) male rats, aged 8 weeks with a body weight of 180-240 g were held at the National Beijing Center for Drug Safety Evaluation and Research. In accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), the rats were maintained under conditions of controlling temperature (<30 °C), humidity (<70%), and circadian circulation for 12 h. Each mouse was individually housed in a plastic cage with free access to high pressure-treated tap-water and standard rat food. The certificate number for the animals in these experiments was SCXK-2012-0004. Following the operation (see below), the activity levels of the rats and the healing of their surgical wounds were monitored daily.

All experimental procedures involving the animals were performed according to the protocols approved by National Beijing Center for Drug Safety Evaluation and Research, the approval number is IACUC-2018-035.

Animals groups and drug administration

Animals were randomized according to their body weight and divided into 2 groups prior to surgery: Animals with sham surgery, and animals with 5/6 nephrectomy[13]. The levels of serum creatinine (Scr) and blood urea nitrogen (BUN) were determined 2 weeks after the operation to establish whether or not the model had been successful. After confirming the model had been established successfully, the experimental rats were divided into the respective model groups: The uremic clearance granules group (UCG), Jianshen granule (Yucare, Beijing, China) low-dose group (JS-L), Jianshen granule middle-dose group (JS-M), Jianshen granule high-dose group (JS-H), and sham operation group, with 8 rats in each group. The UCG group was given a gavage of 3.6 mg/g/day premixing solution. (The dose of UGG was obtained by converting the dose of UCG in the instructions according to the dose-ratio table of human and animal body surface area ratio in "Pharmacological Experimental Methodology"[14]. The JS-L, JS-M, and JS-H groups were administered a gavage of 0.96, 1.92, and 3.84 mg/g/day premixing solution, respectively. (The dose of Jianshen Granules was calculated according to clinical dose). The sham and model groups

were provided with an equal volume of distilled water. Medicine was administered twice daily, and all groups received the medicine continuously over a period of 8 weeks.

Induction of renal failure: subtotal nephrectomy

Subtotal nephrectomy (5/6 nephrectomy) was performed in order to induce CRF. Rats were anesthetized with pentobarbital injection (i.p.), the dose of pentobarbital was 40mg/kg, and a dorsoventral incision parallel to spinal cord was made to expose the left kidney, which was then freed of connective tissue. The renal artery was ligated, and the upper and lower poles of the left kidney were cut out (2/3 nephrectomy). The cavity was closed by double sutures of muscle and skin using a non-absorbable surgical suture once the bleeding had ceased[12]. One week after the 2/3 nephrectomy, the right kidney was exposed and removed (i.e., 5/6 nephrectomy). In each case, benzyl penicillin was applied on sutures to prevent infections immediately following the surgery, Animals in the sham group underwent the same surgical procedure as above, except that the kidneys were not removed or cut: The kidneys were merely touched with forceps and threads. Similar post-operative care was also administered. After the operation, rats were placed individually in cages and were granted free access to food and water.

Biological detection

During the administration period, the physiological state of the rats was observed, and their body weight was recorded every week. In addition, the behavior, mental state, hair, and other physiological parameters of the rats were also monitored. Serum was collected from the fundus vein every 2 weeks, heparin was used as anticoagulant, and the plasma was subsequently separated by centrifugation at 13,000 *g* for 10 min at 4°C. Plasma was collected and used for estimation of the sought-after biochemical parameters, including Scr, BUN, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The plasma biochemical index was measured using a kinetic color test (i.e., the Jaffe method using an Olympus AU400[®] clinical chemistry analyzer). Immediately before and after the 4th, the 6th and the 8th week post-surgery, 2 ml urine sample was collected from all the

groups for analysis of the level of urine protein (UPr). Subsequently, the urine volume was measured during the last week of medicine administration.

A serum TGF- β 1 ELISA kit for rats (Shanghai Westang Bio-Tech Co., Ltd; Shanghai, China) was used to determine the TGF- β 1 level in the rat serum. In a glass tissue grinder, the rat kidneys were homogenized for 30 sec in prechilled methanol. The homogenate was centrifuged at 4°C for 20 min at 13,000 *g*, and the supernatant was retained.

All the rats were euthanized with pentobarbital injection (i.p.), the dose of pentobarbital was 130mg/kg. Then, the kidney remnants were taken out, weighed, teared off of the envelope, flushed with PBS, and fixed in 10% buffered formalin. Kidneys were then processed in paraffin. The pathologist, who was blinded to the treatment, duration, and genotype of the samples, examined the representative kidney sections of 3 rats from every group. Sections (5 μ m-thick) of tissue, following Masson and hematoxylin and eosin (H&E) staining, were evaluated according to a standard staining protocol. At high magnification (x 200), 6 complete glomeruli were randomly selected, histology scores were assessed according to morphological criteria, and the ratio of the proportion of the kidney affected by individual changes to the total area of the kidney sectioned was determined. To calculate the glomerular sclerosis index (GSI), histological scores were presented on an ordinal scale of 1-4. According to the lesion degree of renal tubules and glomerulus, the higher the score, the more serious the lesion.

Cell culture and treatment

HKC cells (a proximal tubule epithelial cell line) were grown in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in an incubator under a saturated humid atmosphere containing 95% air and 5% CO₂. Cells were passaged once every 3 days. The cells were purchased from National Infrastructure of Cell Resource (Beijing, China), and all experiments were performed on cells between passages 10-20. The HKC cells were pretreated with H₂O₂ (0.6

mM) for 4 h, and then co-treated with Jianshen Granule solution (at concentrations of 0.5, 1, 2 mg/ml) or UCG solution (1 mg/ml) for 24 h.

Cell viability analysis

Cytotoxicity was assayed in HKC cells grown in 96-well plates. Cells (1.5×10^5 cells/ml; 0.1 ml per well) were seeded into plates and allowed to grow overnight before replacement of the medium with serum-free medium supplemented with H_2O_2 for 4 h, and then co-treated with Jianshen Granule solution (0.5, 1, 2 mg/ml) or UCG solution (1 mg/ml) for 24 h. Subsequently, 10 μ l Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, Darmstadt, Germany) was added into each well and incubated for 1-4 h. The absorbance was read at 450 nm with a PerkinElmer Victor X Microplate Reader (PerkinElmer, Inc., Waltham, MA, USA). Reductions in optical density (OD) due to drug treatment were used to assess cell viability and normalized against control incubated in medium (100% viability).

Measurement of the mitochondrial membrane potential (MMP)

MMP was measured using flow cytometry, and the mitochondrial-specific cationic dye, JC-1. HKC cells (1.5×10^5 cells/ml) were plated in 12-well plates with H_2O_2 for 4 h, and then co-treated with Jianshen Granule solution (0.5, 1, 2 mg/ml) or UCG solution (1 mg/ml) for 24 h. Cells were harvested, washed twice with PBS, and incubated with 0.5 mL JC-1 (25 μ M) for 20 min at 37°C. MMP was assayed, and green (JC-1 monomer) and red (JC-1 aggregate) fluorescence were monitored at emission wavelengths of 525 and 595 nm, respectively. Changes in the ratio between measurements were indicative of changes in the MMP.

Measurements of intracellular ROS and Ca^{2+}

HKC cells (1.5×10^5 cells/ml) were plated in 12-well plates with the indicated concentrations of H_2O_2 for 4 h, and subsequently co-treated with Jianshen Granule solution (0.5, 1, 2 mg/ml) or UCG solution (1 mg/ml) for 24 h. Intracellular ROS and cytosolic Ca^{2+} were measured using the fluorescent probes Dichlorofluorescein diacetate (DCFH DA)

(Sigma-Aldrich, Darmstadt, Germany) and fluo-3-acetoxymethyl ester (Fluo-3-AM) (Biosea Biotechnology, Beijing, China), respectively, and a fluorescence-activated cell sorter. DCFH-DA is converted into a fluorescent compound in the presence of ROS. Fluo-3-AM was added to treated cells to measure Ca^{2+} . After treatment with the indicated drugs, cells were incubated with DCFH-DA (10 μM) for 20 min at 37°C in the dark (for the ROS assay) or Fluo-3/AM (5 $\mu\text{mol/l}$) for 30 min at 37°C (the Ca^{2+} assay), and the cells were then harvested and suspended in 500 μl HBSS. Intracellular ROS and Ca^{2+} were measured using a flow cytometer (excitation wavelength, 488 nm; emission wavelength, 535 nm).

Cell apoptosis

Apoptosis was also measured using Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) double-stained apoptosis detection kit (Biosea Biotechnology, Beijing, China). HKC cells were plated (1.5×10^5 cells/ml; 0.1 ml) in 12-well plates with the indicated concentrations of H_2O_2 for 4 h, and then co-treated with Jianshen Granule solution (0.5, 1, 2 mg/ml) or UCG solution (1 mg/ml) for 24 h. Cells were harvested, washed twice with ice-cold PBS, and then suspended in 200 μl ice-cold binding buffer. Subsequently, 10 μl HRP FITC-labeled annexin V and 5 μL PI were added to the cells. The cell suspension was gently mixed, and incubated for 15 min at room temperature in the dark. Apoptosis was monitored using flow cytometry (488 nm excitation wavelength), and the fluorescence intensity was measured at 530 nm (emission wavelength). Annexin V⁺/PI⁻ was used to document early apoptosis, whereas AnnexinV⁺/PI⁺ was used to assess the late apoptotic stages or necrotic cells.

RT-qPCR assay

HKC cells were treated with different concentrations of Jianshen Granule solution for the indicated times. Total RNA was extracted from kidney tissue and HKC cells using Invitrogen[®] TRIzol reagent (Thermo Fisher Scientific, Inc.), and cDNA was made by random hexamers. Quantitative PCR was monitored in a Real-Time PCR detection system

(Gene Amp 2400[®]; PerkinElmer, Inc.) with SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and relevant primers. The primer sequences are shown in Tables 1 and 2. The percentages of the above molecules were quantified using StepOne Plus[™] Real Time PCR (Applied Biosystems; StepOne Plus[™] 272006169).

Table 1 Primers of renal tissue

Renal tissue	Primers
TGF- β 1 F	GAAGGACCTGGGTTGGAAG
TGF- β 1 R	CGGGTTGTGTTGGTTGTAG
β -actin F	GGGAAATCGTGCGTGACATT
β -actin R	GCGGCAGTGGCCATCTC

Table 2 Primers of HKC cells

HKC cells	Primers
TGF- β 1 F	GGAAATTGAGGGCTTTCGCC
TGF- β 1 R	CCGGTAGTGAACCCGTTGAT
β -actin F	CGGCGCCCTATAAAACCCA
β -actin R	TCATCATCCATGGTGAGCTGG

Western blot assay

Proteins from kidney tissues and HKC cells were acquired and sonicated in RIPA lysis buffer. Aliquots (40 μ g) of proteins were subjected to SDS-PAGE (10% gels), and electro-transferred to PVDF membranes. The protein-containing PVDF membrane was blocked in blocking buffer (5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20; TBST) for 2 h, incubated with the primary antibody (see above) for 3 h, and subsequently incubated at 4°C overnight. The membranes were incubated with secondary

antibody (HRP-conjugated IgG for 1 h) and the protein bands were quantified using a luminescent image analyzer (GE Healthcare Bio-Sciences AB; Image Quant LAS 500; Sweden). The protein expression levels were normalized against GAPDH.

Statistical analysis

The data are expressed as the mean \pm SEM. $P < 0.05$ was considered to indicate a statistically significant value. All the statistical calculations were performed using Prism software package (GraphPad Prism, version 5) with unpaired or paired t-test, depending on the type of comparison being made.

Results

Animal model of nephrectomy

The rat CRF model was established by 5/6 nephrectomy. The levels of Scr and BUN in rat serum of the model group were found to be significantly higher than those of the sham group ($P < 0.05$). This suggested that the model had been built successfully (Fig. 1).

Figure 1

Pharmacodynamic evaluation

During the drug administration, the rats of the sham group were noted to be livelier and more energetic, with bright hair color and normal gains of weight. By contrast, the 5/6 nephrectomy model rats were mentally depressed, with slightly protruding eyeballs, dark hair color and a lower consumption of food. However, upon treatment of Jianshen granule and UCG, the mental state of the rats was gradually restored, which became similar to that of the sham operation group.

No significant differences in the weight of the rats before or after the nephrectomy were observed. However, the body weight of the rats in the nephrectomy group were found to be lower than those of the sham group 2 weeks after surgery ($P < 0.05$). By the end of the treatment cycle, the body weights of the rats in the JSL and JSM groups were significantly higher compared with that of the model group ($P < 0.05$). The body weights in the JSH group were lower compared with that of the model group, until after 7 or 8 weeks. Taken together, these results suggested that low or medium doses of Jianshen Granules enabled the CRF rats to retain their weights, whereas a high dose of Jianshen Granules had no effect (Fig. 2).

Figure 2

Changes in renal function can be assessed according to the levels of Scr and BUN. In the present study, the Scr and BUN levels were significantly increased in the model group compared with the sham group ($P < 0.05$). However, treatment with Jianshen Granules did lead to a significant reduction in the increases of Scr and BUN levels that were observed in CRF rats ($P < 0.05$), signifying that residual renal functions were protected, and renal failure symptoms were relieved (Fig. 3A and B). Furthermore, no differences were observed between the Jianshen group and the UCG group.

Changes in the levels of ALT and AST can be used to characterize the degree of damage of liver function. In these experiments, no significant differences were observed between the sham group and the other groups attributable to fluctuations in the ALT and AST values in each group, indicating that Jianshen Granules caused no damage to the liver function of CRF rats (Fig. 3C and D).

In terms of the analysis of the level of UPr, the UPr level of the model group was shown to increase rapidly, and the UPr level in the JS group was significantly lower compared with that in the model group ($P < 0.05$). The UPr content was lowest in the JSM group. Furthermore, the UPr level in the JS groups were lower compared with that in the UCG group (Fig. 3E). The volume of urine in the JS groups were also lower compared with that in the model group, while the JSM group had the lowest levels of all JS groups, also lower than the UCG group. These results suggested that Jianshen Granules may lead to an improvement in reabsorption of the renal tubules, and the effect was observed to be the best in the JSM group (Fig. 3F).

Figure 3

Histological analysis

The sham group appeared to have no obvious renal lesions, whereas the other groups exhibited different degrees of lesions, including renal membrane fibrosis, renal interstitium (i.e. interstitial fibrosis and chronic inflammation), and renal parenchyma lesions (i.e. renal tubule dilatation, protein tube type, peripheral glomerular fibrosis, glomerular mesangial hyperplasia and glomerular cysts deposition). Although the Jianshen Granule group also had similar pathological changes, these were significantly less severe compared with the other groups (Fig. 4A and B). According to the GSI, histological scores were determined (Fig. 4C). The model group rats were observed to have significantly higher scores compared with sham group, which indicated that severe renal lesions existed in the model group. The scores of the Jianshen Granule groups and the UCG group were lower compared with that of model group; taken together, these results demonstrated that Jianshen Granule and UCG treatment was able to relieve the lesions, and the JSM group experienced the best effect.

Figure 4

Previous research has shown that TGF β 1 is a key profibrotic growth factor involved in renal fibrogenesis, and an important factor among cytokines due to its upregulation in patients with chronic renal failure. In the present study, the TGF β 1 level was shown to be increased in the kidney tissues of 5/6 nephrectomy rats, whereas Jianshen Granule treatment led to a marked decrease in the level of TGF β 1. These results

were confirmed by western blot analysis of TGFβ1 (Fig. 5A). Similar results were also obtained by ELISA (Fig. 5C). In addition, the mRNA expression level of TGFβ1 was markedly reduced upon treatment with Jianshen Granules (Fig. 5B).

Figure 5

Cell function is influenced by Jianshen Granules

Hydrogen peroxide (H₂O₂) is able to cause oxidative damage to cells, leading to apoptosis. In order to evaluate the effects of Jianshen Granules on H₂O₂induced cell death, the viability of the HKC cells by CCK8 assay was first investigated, and it was identified that Jianshen Granules led to a significant improvement in the HKC cells' viability compared with H₂O₂ group ($P < 0.01$) (Fig. 6).

Figure 6

In addition, annexinV and PI staining were performed, and flow cytometry was used to distinguish living cells from apoptotic and necrotic cells. These experiments demonstrated that the number of apoptotic cells in the H₂O₂ group was significantly higher compared with those in the control group ($P < 0.01$), whereas the extent of apoptosis of HKC cells in the JSM, JSH and UCG groups was significantly lower compared with that in the H₂O₂ group ($P < 0.01$ or $P < 0.05$). However, no significant differences were observed among the JSM, JSH and UCG groups. These results revealed that treatment with Jianshen Granules was able to protect the HKC cells from H₂O₂induced apoptosis (Fig. 7A).

As a marker of oxidative stress, ROS participate in renal injury through oxidative stress and inflammatory reactions, and the levels of ROS are increased markedly in both acute and chronic renal failure [15]. In the present study, the levels of intracellular ROS in the H₂O₂ group were found to be significantly higher compared with those of the control group ($P < 0.01$) (Fig. 7B). In addition, the ROS level in the Jianshen Granule treatment group was also significantly lower compared with that in the H₂O₂ group ($P < 0.05$). Therefore, ROS production was shown to be effectively inhibited by Jianshen Granules.

When cells are stimulated by external stress, the mitochondrial function is compromised, with a decrease in the MMP, and an increase in the levels of ROS and the intracellular Ca²⁺ concentration [16]. In the present study, after HKC cells were stimulated by H₂O₂, the intracellular Ca²⁺ level increased significantly, whereas the MMP level decreased significantly ($P < 0.01$ and $P < 0.05$, respectively). After administration of Jianshen Granules or UCG, the Ca²⁺ concentration was reduced markedly, and the MMP level was elevated to an appreciable extent compared with that in the H₂O₂ group ($P < 0.01$). The decreases in Ca²⁺ and MMP caused by cell damage were also found to be alleviated following treatment with Jianshen Granules (Fig. 7C and D).

Figure 7

To further investigate the correlation between TGFβ1 and renal failure, the protein expression level of TGFβ1 was analyzed by western blot analysis, and the mRNA expression level of TGFβ1 was also assessed by RTqPCR in the HKC cells. In these experiments, the TGFβ1 level was increased in the HKC cells induced by H₂O₂, whereas Jianshen Granules were able to significantly decrease the protein and mRNA expression of TGFβ1 (Fig. 8).

Figure 8

Discussion

Although numerous studies have been published on CRF, effective drugs available for clinical treatment are very limited in supply [17, 18]. Treatment of CRF by TCM has elicited positive results, however, and this approach has led to a diversification of treatment methods, broadening developmental prospects for therapy[19]. To name but a few of the effects, TCM has had impressive achievements in relieving symptoms of CRF, protecting residual renal function, delaying the progress of the disease, and postponing the time of dialysis and the requirement for kidney transplantation, thereby greatly improving the quality of life for patients with CRF[20].

Jianshen Granules are a clinically effective prescription medicine, composed of 11 separate TCMs, and has been shown to be suitable for the treatment of early CRF. It has been used for 9 years as a hospital formulation in the Air Force Medical Center of PLA. The prescription helps to maintain kidney function, and the kidneys are stimulated with *Radix Rehmanniae* and *Cornus*, thereby and maintaining the remaining metabolic function of the kidneys. It can also nourish the blood, promote blood circulation, and improve the state of systemic deficiency via the presence of the ingredients *Poria cocos*, *Astragali radix*, and *Angelica sinensis*. Detoxifying turbidity and removing toxins in the body are accomplished by *Lumbricus*, *Honeysuckle*, and *Rhubarb*. Moreover, Jianshen Granules also have the function of being able to activate blood circulation, removing blood stasis and reducing glomerular fibrosis, courtesy of the presence of *Salvia miltiorrhiza* radix and *Ligusticum chuanxiong* Hort. The combination of the various traditional medicines not only improves the functioning of the spleen and kidney, but also removes blood stasis, reduces turbidity and detoxifies the blood, promotes the excretion of toxins in the body, and promotes the decomposition, transformation and recovery of the renal function of metabolic waste.

Scr and BUN are mainly excreted in the urine following glomerular filtration[21]. When the renal parenchyma is damaged, the glomerular filtration rate decreases, resulting in increases in the levels of Scr and BUN in the blood[22]. Therefore, Scr and BUN are the main indicators of clinical diagnosis of renal failure. In the present study, the Scr and BUN levels were significantly elevated in 5/6 nephrectomized rats, but their levels were reduced upon treatment with Jianshen Granules, suggesting that Jianshen Granules help to alleviate the symptoms of renal failure. Clinical trials have shown that reducing UPr may protect the kidneys; these results were corroborated by the present study, in which Jianshen Granules lowered the UPr in the 5/6 nephrectomized rats. It has also been demonstrated that Jianshen Granules have the function of protecting kidney function. ALT and AST may be used to evaluate whether liver

function has been compromised. In our experiments, the levels of ALT and AST in 5/6 nephrectomized rats of the Jianshen Granule group were not significantly different from those of the sham group, demonstrating that Jianshen Granules have no liver toxicity.

Tubulointerstitial fibrosis is a common pathological pathway for the progression of chronic kidney diseases to endstage renal failure caused by various etiologies[23]. TGFβ1 is a driving force of renal fibrosis[24]. Clinical data published previously have shown that TGFβ1 levels in the kidney and urine of patients with kidney diseases were markedly increased, and this increase was positively correlated with the degree of renal fibrosis [25]. In the present study, both the protein and mRNA expression levels of TGFβ1 in 5/6 nephrectomized rats were significantly increased, and these increases were inhibited upon treatment with Jianshen Granules. These findings indicate that Jianshen Granules may relieve the symptoms of renal failure by downregulating TGFβ1 expression.

It has been reported that oxidative stress is involved in damage to the glomerulus and tubulointerstitium [26], processes which lead to the chronic development of renal failure, ultimately leading to the end stage of renal failure[27]. When the body is under oxidative stress conditions, the balance between oxidation and antioxidation is disrupted. The increases in ROS levels in the cells exceed their scavenging capacity, which may lead to oxidative damage, abnormal protein expression, and cell damage[28, 29]. ROS induces apoptosis by altering the MMP. Furthermore, scholar[30] provided evidence that cell apoptosis, including experiments performed on renal tubular epithelial HKC cells, is a critical determinant of renal fibrosis, which eventually results in CRF. Constructing the cell model of oxidative stress injury by subjecting cells to H₂O₂ treatment is currently recognized and widely used[31]. The present study has shown that Jianshen Granules are able to inhibit H₂O₂induced apoptosis of the HKC cells. Although the intracellular ROS of HKC cells were markedly increased upon H₂O₂ treatment, these increases were reversed by treatment with Jianshen Granules. Furthermore, the Ca²⁺ level is in equilibrium when cells are in their normal state, but this balance is destroyed when the cells are damaged. It has been demonstrated that increases in Ca²⁺ concentration of the cells lead to cellular dysfunction, resulting in an imbalance of energy metabolism, which subsequently leads to the pathological state of the body[32]. It has been shown that increases in the Ca²⁺ concentration are negatively correlated with cell viability, indicating that cell death is associated with an increase in intracellular Ca²⁺ concentration. *In vitro* studies have confirmed that ROS lead to the change of intracellular Ca²⁺[33]. Numerous *in vitro* studies have demonstrated that ROS can induce extracellular Ca²⁺ influx, and they may therefore be convenient signal markers in regulating calcium signaling processes[34]. Mitochondria are the main site for the production of ROS, and also the main target of ROS. Numerous studies have shown that excessive ROS can cause oxidative damage to mitochondria, resulting in their abnormal function[35, 36]. The abnormalities of this function are mainly manifested in a reduction of MMP, mitochondrial DNA mutation, disruption of the mitochondrial respiratory chain, and so on [37]. In the present study, the increase in Ca²⁺ concentration, and concomitant decrease in MMP, caused by ROS were inhibited by Jianshen Granules, indicating that Jianshen Granules are able to inhibit cell injury.

During renal pathogenesis, apoptosis of HKC cells may lead to atrophy of renal tubular and interstitial fibrosis, which ultimately leads to the progression of chronic kidney disease to endstage renal failure[38, 39]. Previous studies have shown that preventing apoptosis of the HKC cells may effectively delay the progression of renal fibrosis, and reduce the damage caused to renal function[40, 41]. Apoptosis of HKC cells may be induced by TGF β 1. Previous studies have also shown that reducing the mRNA expression level of TGF β 1, and downregulating the expression of TGF β 1 protein, can reduce the apoptosis of HKC cells [42]. Therefore, TGF β 1 may be a therapeutic target for clinical prevention and treatment of renal tubular atrophy, alleviating the degree of renal fibrosis, and delaying renal dysfunction[43]. In the present study, upregulation of the levels of TGF β 1 mRNA and protein was inhibited upon treatment with Jianshen Granules. Therefore, downregulating TGF β 1 expression may have contributed to the apoptosis inhibition in HKC cells mediated by Jianshen Granules.

Our research also has certain limitations. Jianshen granules are composed of 11 kinds of traditional Chinese medicine, and the exact active ingredients cannot be determined at present. Next, we will use fingerprints to determine the active ingredients.

Conclusion

In conclusion, the present study has demonstrated that Jianshen Granules are able to reduce the levels of Scr, BUN and UPr in CRF rats, and thereby may protect the residual renal function, alleviating the symptoms of renal failure. Moreover, this medicine had no obvious side effects on liver function. Jianshen Granules are also able to inhibit apoptosis induced by H₂O₂. Taken together, the results of the present study have shown that Jianshen Granules may alleviate renal failure by inhibiting the expression of TGF- β 1. This study has confirmed the efficacy of Jianshen Granules with respect to therapeutic treatment of CRF, thereby providing important information for the clinical treatment of CRF.

Abbreviations

CRF, chronic renal failure; UCG, uremic clearance granule; ROS, reactive oxygen species; PLA, Chinese People's Liberation Army; TGF β 1, transforming growth factor β 1; TCM, traditional Chinese medicine.

Declarations

Ethics approval and consent to participate

All experimental procedures involving the animals were performed according to the protocols approved by National Beijing Center for Drug Safety Evaluation and Research, the approval number is IACUC-2018-035.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article. All figures can be used as supplementary information to the data.

Competing interests

The authors have no conflicts of interest to disclosure.

Funding

This work was supported by grants from Beijing Municipal Science & Technology Commission (No.Z161100001816008), the expenditure of the the study was supported by the funder.

Authors' contributions

L-N Du and H-L Yuan were contributed to the conceptualization, study design, and manuscript revision. Y Wu, X-X Zhang, J Kong, and H-J Lu contributed to the data collection, analysis and interpretation. X-X Zhang, H-Y Qiu involved in drafting manuscript.

Acknowledgements

The authors want to thank Dr. Qingxin Mu with his help in modifying the manuscript .

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Figures

Scr and BUN level before administration

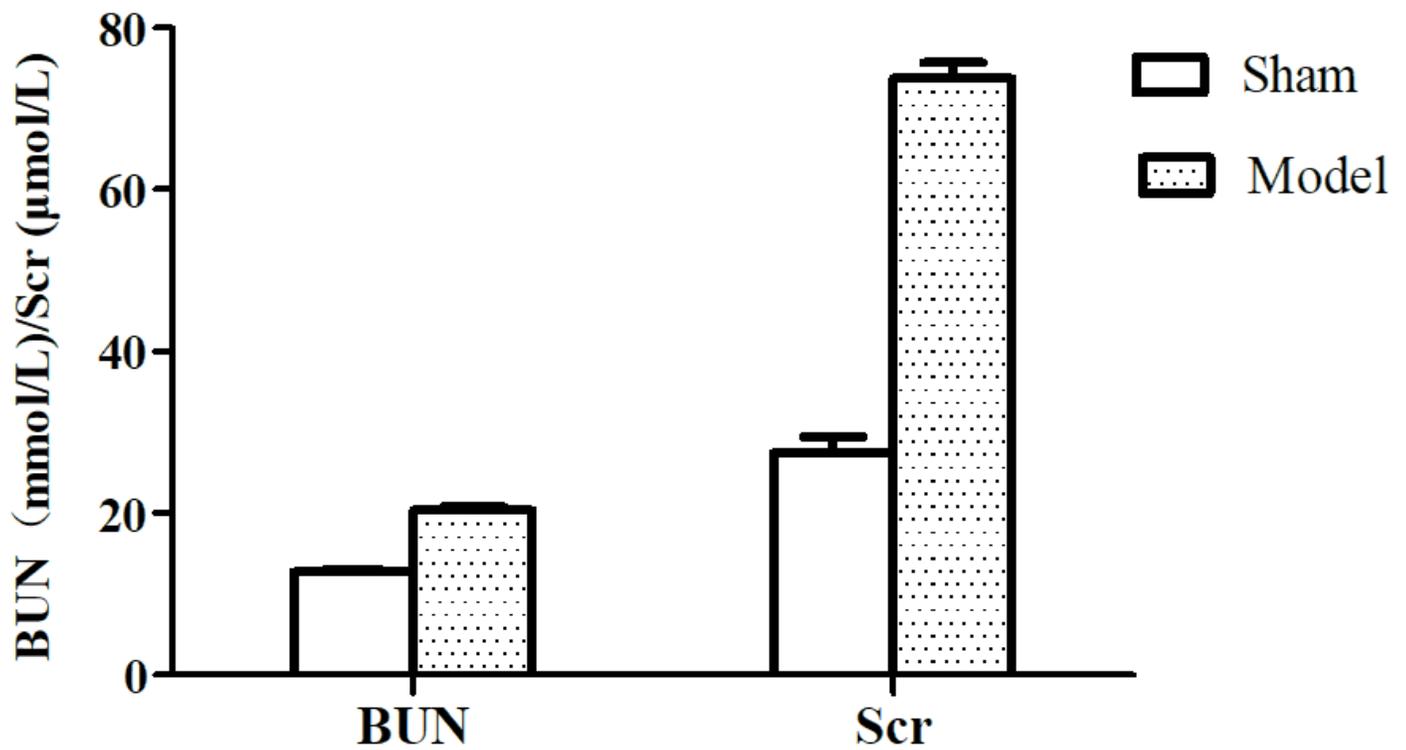


Figure 1

Scr and BUN levels of the rats in sham and model groups prior to administration of the medicine. Each value is expressed as the mean \pm S.E.M (n=8). *P<0.05 compared with the model group. Scr, serum creatinine; BUN, blood urea nitrogen.

Body weight of different groups (n=8)

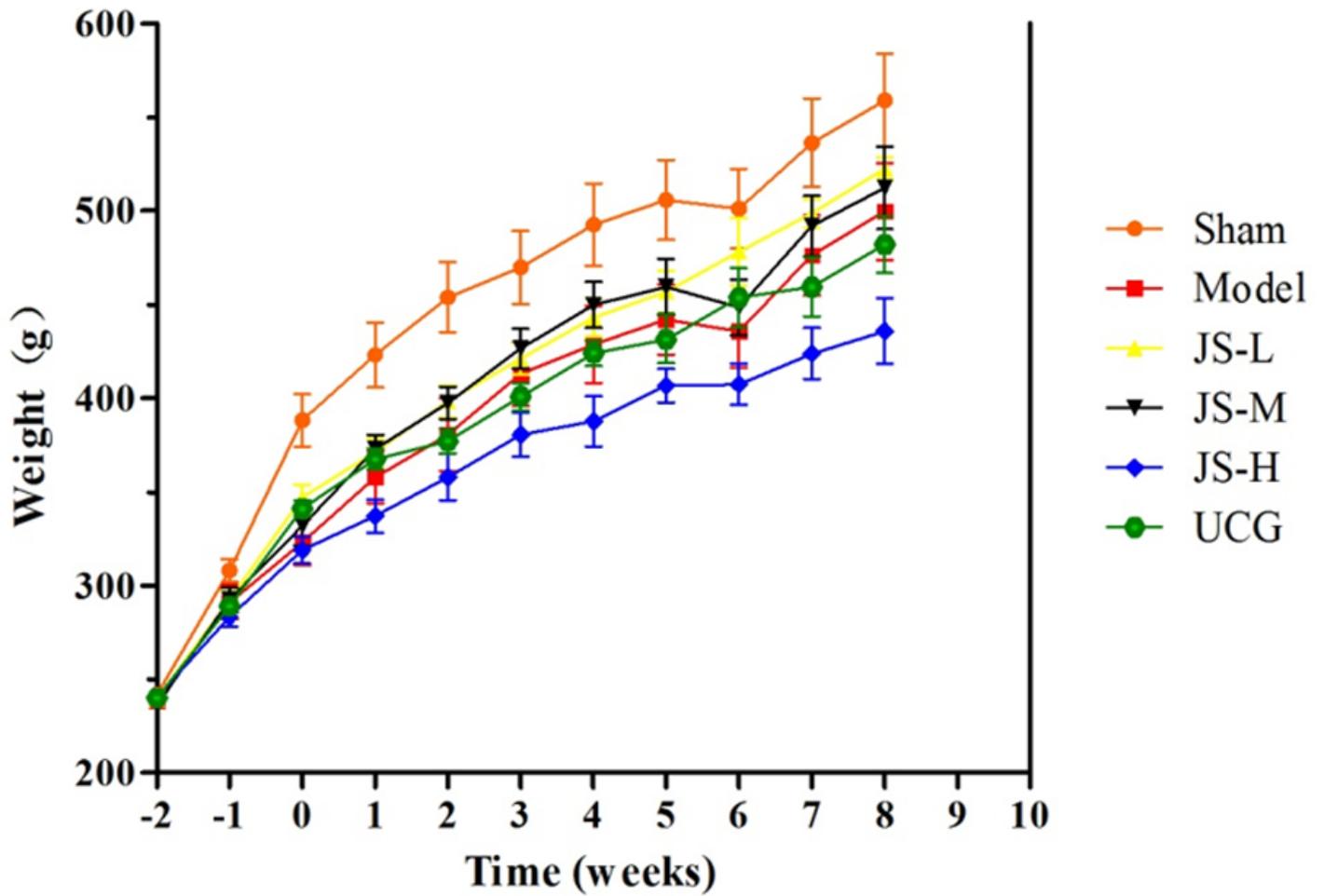


Figure 2

Effect of Jianshen Granules on body weight in the different experimental groups. Each value is expressed as the mean \pm S.E.M (n=8).

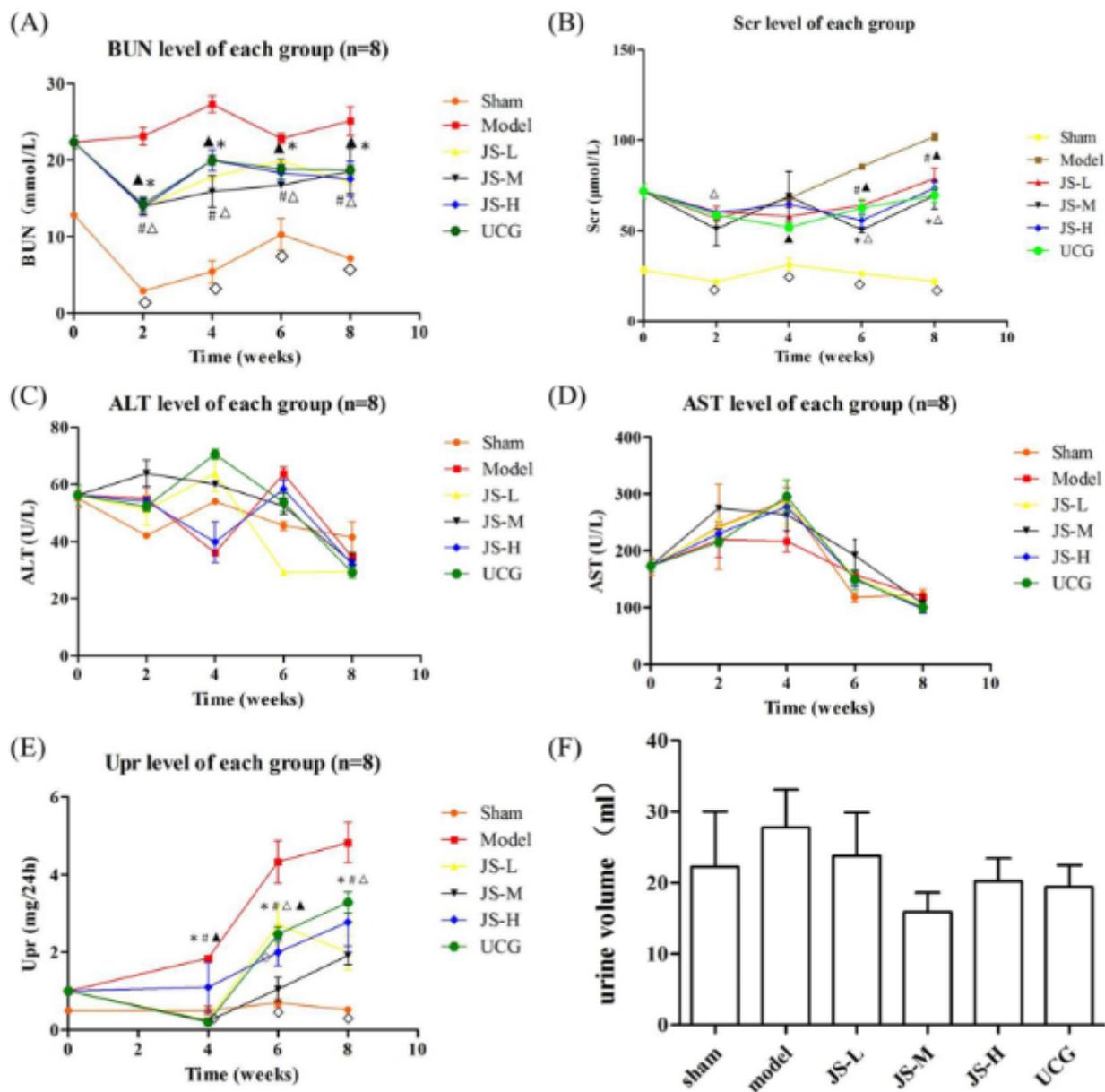


Figure 3

Effects of Jianshen Granules on rats subjected to 5/6 Nx. The levels of the following biochemical parameters were investigated: (A) BUN; (B) Scr; (C) ALT; (D) AST; (E) UPr; and (F) volume of urine. Each value is expressed as the mean \pm S.E.M (n=8). *P<0.05, the model group compared with the JS L group; #P<0.05, the model group compared with the JS M group; Δ P<0.05, the model group compared with the JS H group; \blacktriangle P<0.05, the model group compared with the UCG group; \square P<0.05, the model group compared with the sham group. For further details concerning the establishment of the model groups, see the Materials and methods section. 5/6 Nx, 5/6 nephrectomy; BUN, blood urea nitrogen; SCr, serum creatinine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; UPr, urine protein.

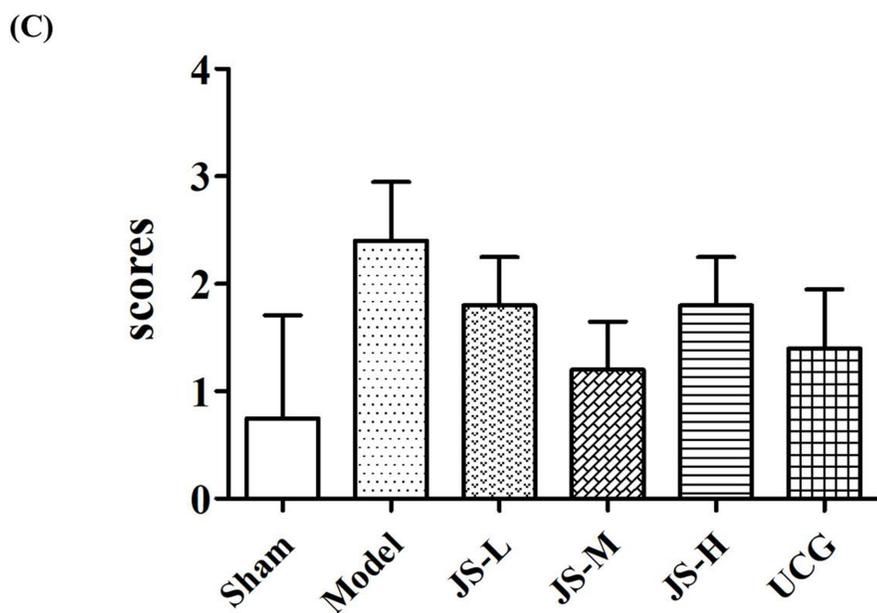
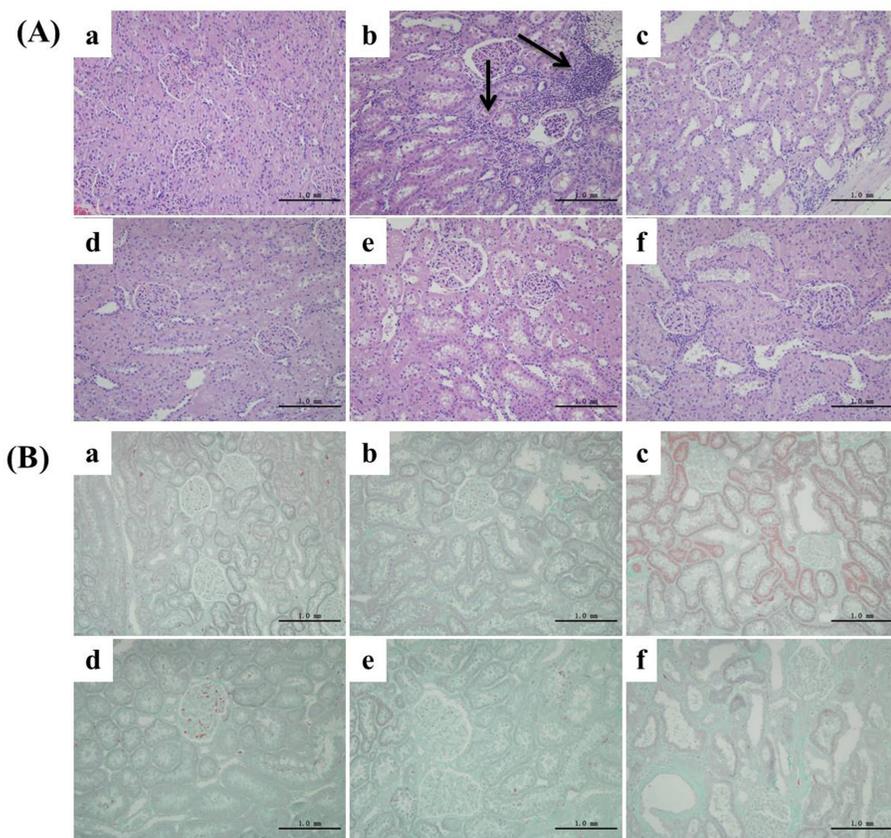


Figure 4

Histopathological changes in renal tissue obtained from 5/6 nephrectomized rats. Shown are the results from (A) H&E staining; (B) Masson's staining (magnification, x200). The black arrows represent tubular lesions, such as tubulointerstitial infiltration by inflammatory cells, tubular dilatation, tubular atrophy, and tubulointerstitial fibrosis. (C) Scores of Renal lesion tissue. (a) Sham group; (b) Model group; (c) J L

group; (d) J M group; (e) J H group; (f) UCG group. For further details concerning the establishment of the model groups, see the Materials and methods section.

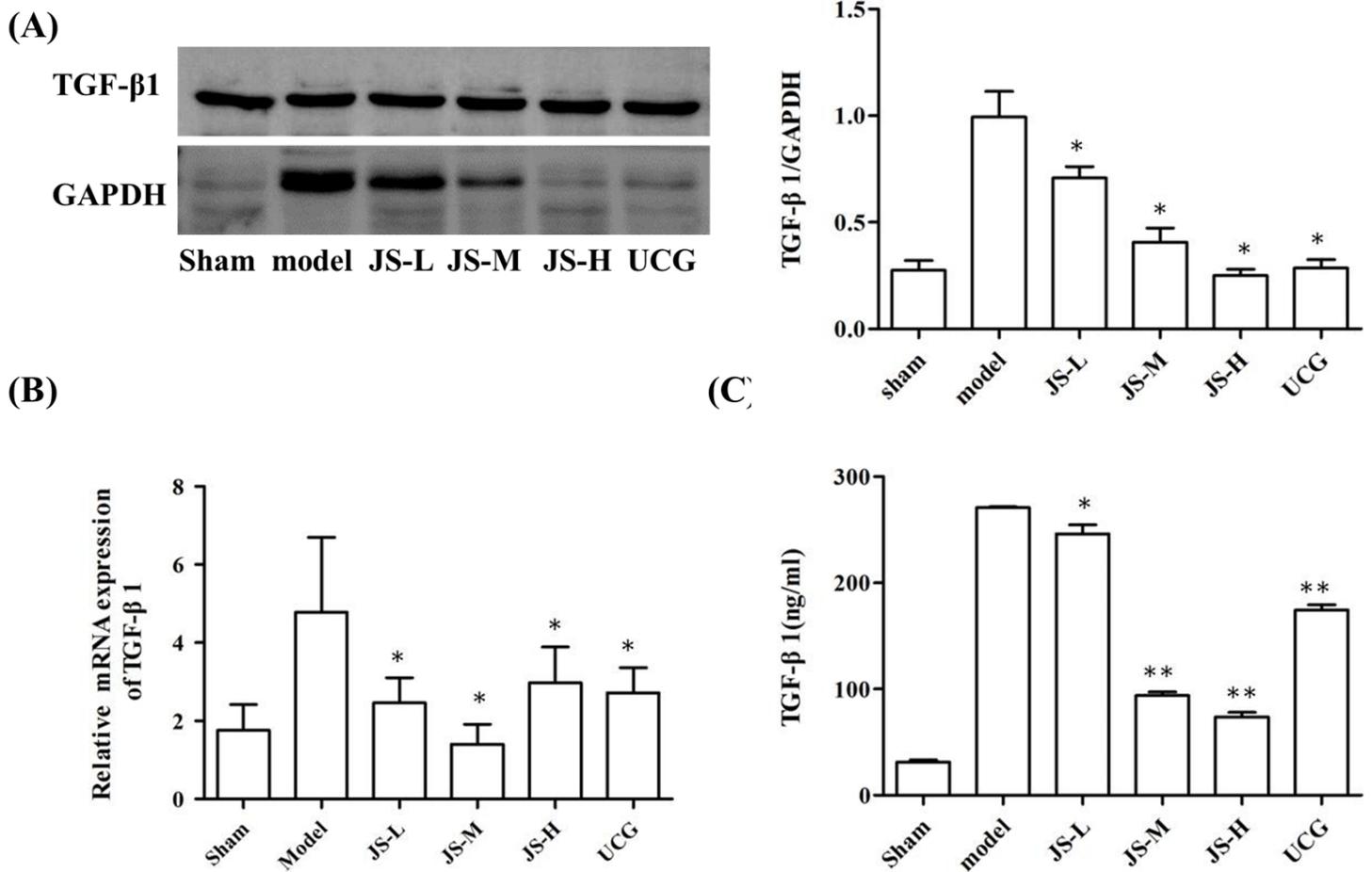


Figure 5

Effect of Jianshen Granules on the concentration of TGF β1 in the kidney tissues. Shown are (A) western blot analysis of TGF β1; (B) mRNA expression of TGF β1; and (C) TGF β1 expression analysis using an ELISA kit. Each value is expressed as the mean ± S.E.M (n=8). *P<0.05; **P<0.01 compared with the model group. TGF β1, transforming growth factor β1.

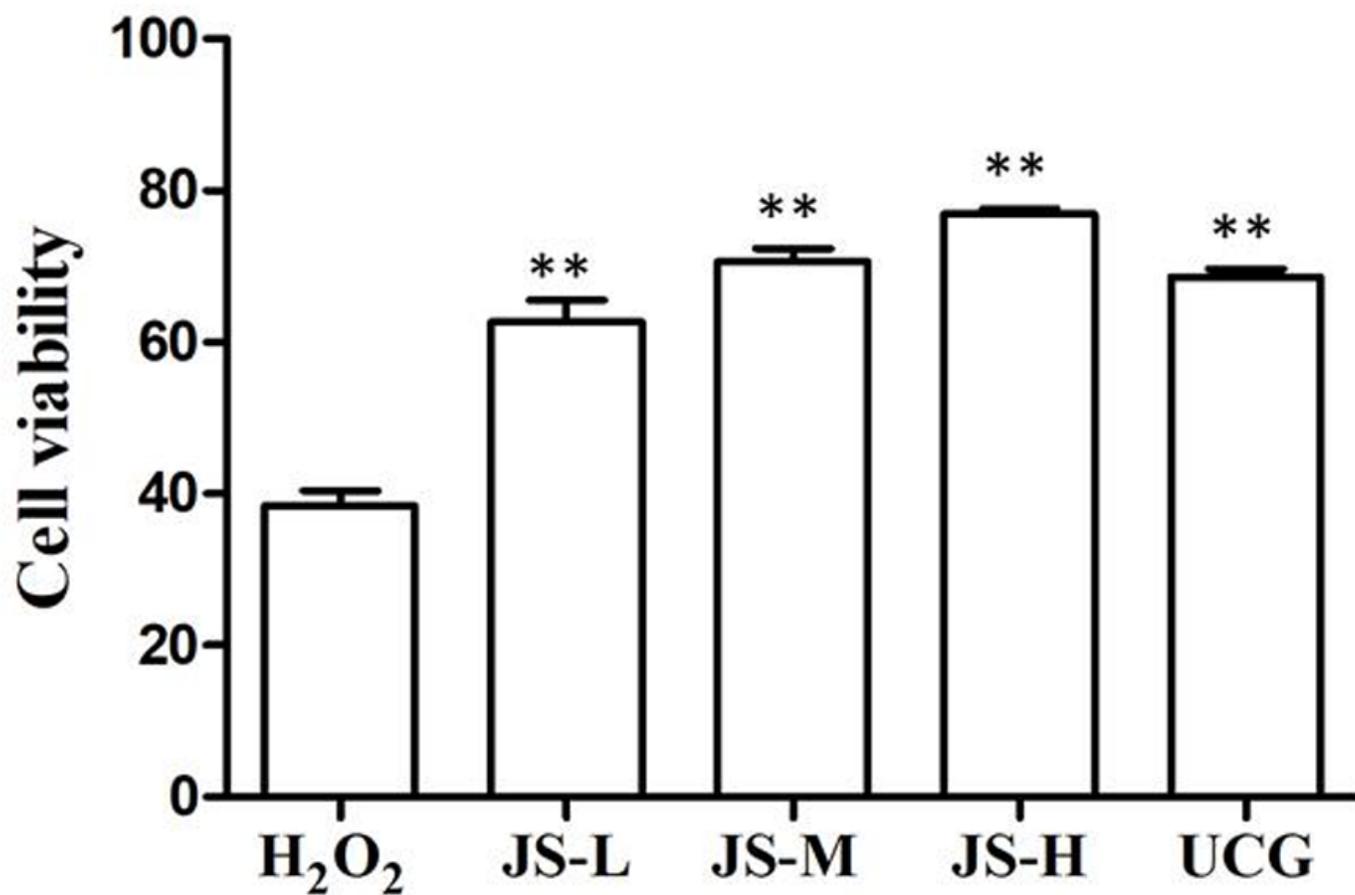


Figure 6

Effects of Jianshen Granules on the viability of HKC cells. Values are presented as the mean \pm S.E.M (n=8 per group). **P<0.01 compared with the H₂O₂ group.

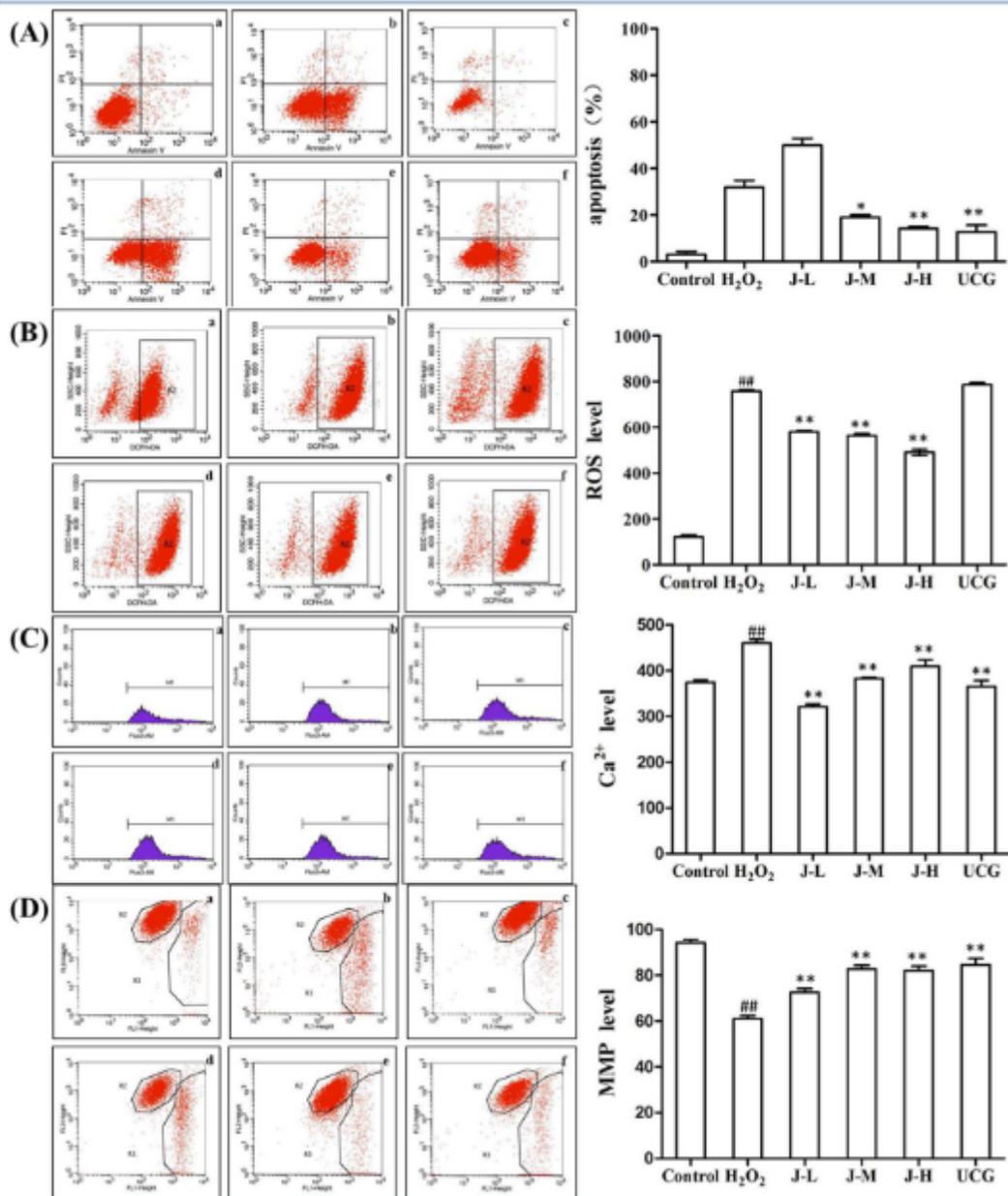


Figure 7

Effects of Jianshen Granules on H₂O₂ induced oxidative stress in HKC cells. Shown are the results of experiments that investigated (A) the extent of apoptosis; (B) level of ROS; (C) Level of Ca²⁺; (D) level of MMP [(a) Control group; (b) H₂O₂ group; (c) JS L group; (d) JS M group; (e) JS H group; (f) UCG group]. For further details concerning the establishment of the model groups, see the Materials and methods section. #P<0.05 compared with the control group; *P<0.05 compared with the H₂O₂ group. ROS, reactive oxygen species; MMP, mitochondrial membrane potential.

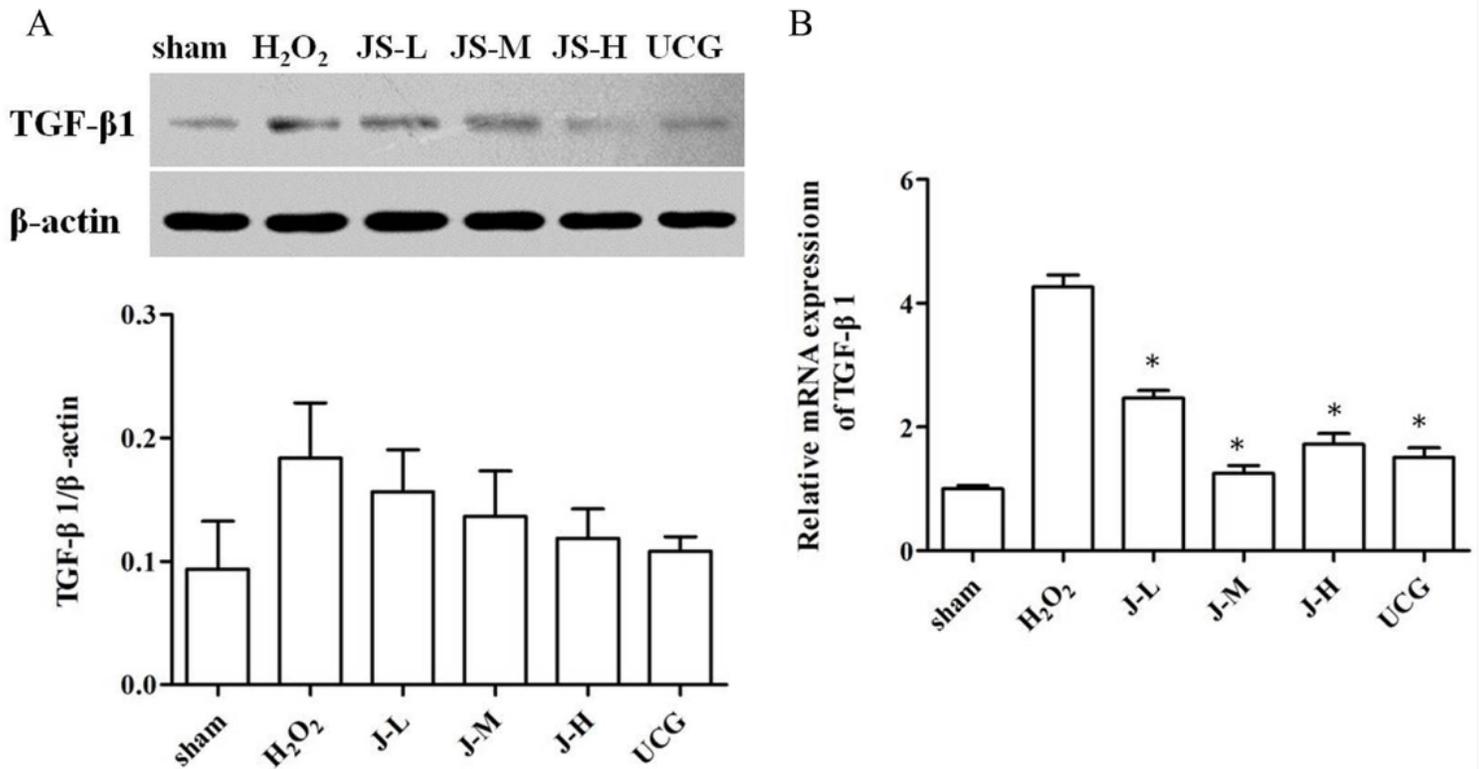


Figure 8

Effect of Jianshen Granules on the concentration of TGF β1 in the H₂O₂ induced apoptosis of HKC cells. Shown are the results of experiments that investigated (A) western blot analysis of TGF β1 and (B) mRNA expression of TGF β1. Each value is expressed as the mean ± S.E.M (n=8). *P<0.05 compared with the model group. TGF β1, transforming growth factor β1.

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