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Jian-Pi-Yi-Shen recipe inhibits chronic kidney disease progression by ameliorating lysosomal injury and modulating autophagy through stress granules

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Abstract: To investigate the mechanisms by which Jian-Pi-Yi-Shen Recipe (JPYSR) affects chronic kidney disease (CKD) progression, focusing on stress granules (SGs), lysosomal integrity and autophagy regulation. A CKD model was induced in mice using a 0.2% adenine diet and treated with JPYSR (15.60 g/kg/day) via gavage. Renal function was assessed using serum creatinine (Scr) and blood urea nitrogen (BUN) levels. Pathological changes were evaluated using PAS and Masson's trichrome staining. Protein and gene expressions were analyzed using Western blot, qPCR, immunohistochemistry and immunofluorescence. In vitro studies were conducted on human renal tubular epithelial cells (HK2). Statistical analyses were performed using GraphPad Prism (version: 9) software. CKD progression was associated with lysosomal impairment and reduced autophagy. JPYSR treatment significantly improved renal function, reduced pathological changes, decreased renal fibrosis, promoted SG formation, alleviated lysosomal damage and maintained baseline autophagy. Inflammation was also diminished, as confirmed by *in vitro* experiments. JPYSR may slow CKD progression and reduce renal fibrosis by modulating SG formation, lysosomal function and autophagy levels.

Keywords: Jian-Pi-Yi-Shen recipe, chronic kidney disease, stress granules, lysosomal injury, autophagy, fibrosis.

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INTRODUCTION

Chronic Kidney Disease (CKD) is considered a global health concern of the current world. It is estimated that around 843.6 million people worldwide in 2017 were struggling with CKD but currently, the ratio exceeds 850 million across the globe and global age-standardised death rates per 100000 from 2006 to 2016 is 18.2% (Jager *et al*., 2019). Global burden disease (GBD) has projected that CKD will be the fifth on the list of mortalities by 2040 (Foreman *et al*., 2018). Renal fibrosis is a primary pathological driver of CKD progression and therapies targeting renal fibrosis can effectively slow its progression (Zhang *et al*., 2021). However, these treatments are often expensive and can place a significant financial burden on families. As a result, there is a growing interest in cost-effective alternatives, such as traditional Chinese medicine, which has been widely accepted in long-term clinical studies (Li and Wang, 2005). One such traditional Chinese formula, the Jian-Pi-Yi-Shen Recipe (JPYSR), has been used for decades in China to treat CKD. Preclinical studies on JPYSR have demonstrated its potential to improve CKD therapeutics (Lu *et al*., 2018; Liu *et al*., 2018).

Lysosomes are essential for cellular degradation and autophagy and their damage can significantly threaten cellular health (Papadopoulos *et al.*, 2020; Gomez-Sintes *et al.*, 2016). Damage to lysosomes, characterized by membrane disruption, leads to increased Galectin-3 expression, impaired autophagy and accumulation of pathologic products, contributing to disease progression (Jia *et al*., 2020). Lysosome-dependent sphingolipid metabolism plays a crucial role in both hereditary and non-hereditary glomerular diseases, including Alport syndrome and diabetic nephropathy (Prentzell *et al.*, 2021; Tee *et al.*, 2003). High glucose levels in diabetes-induced CKD cause autophagic insufficiency and lysosomal dysfunction, leading to podocyte apoptosis. Reducing mammalian-Target of Rapamycin (mTOR) levels by targeting the Raptor protein in mTOR complex 1 can prevent podocyte injury and glomerular dysfunction in diabetic patients (Yang and Wang, 2021).

Stress granules (SGs), formed in response to stress, can reverse lysosomal damage and improve autophagy (Wang *et al*., 2022). Ras GTPase-activating-binding proteins 1/2 (G3BP1 / G3BP2) are the responsible components of SGs (Mazroui *et al.*, 2006). It has been shown that G3BP is present on the cytoplasmic surface of lysosomes and binds to the lysosomal membrane under steady-state (Prentzell *et al*., 2021; Wang *et al*., 2023), stress granules are selectively formed near damaged lysosomal membranes and act as plugs to promote vesicle stabilization and survival, enabling endosomal stabilization and repair (Bussi *et al*., 2023). In addition,

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lysosomal damage induces stress granule formation to a range of stimuli (Panas *et al*., 2016; Jia *et al*., 2022). SGs attach the tuberous sclerosis complex (TSC) to the lysosome and inhibit primarily the activation of the metabolic master regulatory mechanism target of rapamycin complex-1 (mTORC1) by amino acid and insulin and affect autophagy (Prentzell *et al*., 2021).

Recent studies highlight that improving autophagy through factors such as AMPK activation and mTORC1 inhibition can effectively treat CKD and renal fibrosis (Ruby *et al*., 2023). Recent studies highlight that improving autophagy through factors such as AMPK activation and mTORC1 inhibition can effectively treat CKD and renal fibrosis

MATERIALS AND METHODS

Preparation of Jian-Pi-Yi-Shen recipe

The raw herbs in different dosage as shown in Table 1 were thoroughly mixed and subsequently boiled in ddH_2O, then filtered at a very concentrated level and frozen for the experiment.

Animals

A total of (n=18) SPF-grade C57 male mice of age 6 weeks, weighing 20-28 grams, were purchased from the animal resource facility of the Guangdong Medical Laboratory Animal Centre. They were acclimatized and then randomized into three groups (n=6/group): control, CKD, and CKD-JPYSR. The CKD was induced by 0.2% adenine in the chow diet for 3 weeks and then 15.60 g/kg/d of JPYSR was administered by gavage. The treatment started in the third week of CKD and ended in the seventh week. At the end of the study, sodium pentobarbital (50mg/kg body weight, intraperitoneally) was injected into all mice to be anesthetized, the eyes were removed for blood, and the mice were cervically dislocated and executed before achieving consciousness. Kidneys from each mouse were rapidly removed for further analysis.

Cell

Human renal tubular epithelial cells-2 (HK2) were selected, and the cultured cells were treated with DMEM/F12 media containing 10/100 parts of fetal bovine serum (FBS) + 1/100 parts of penicillin-streptomycin and placed in a CO2 (5%) incubator at 37ºC. Then the cultured cells were divided into three groups; i: control, ii: TGF-β1, and iii: TGF-β1+JPYSR group. The Fibrosis in mice was induced by 10 ng TGF-β1 and 1mg/ml JPYSR intervention. After the screening, the viable cells were counted by CCK8. The cells were collected after 48 hours for further analysis.

Serum biochemistry

At first, the collected blood from mice was centrifuged at 2,000 rpm /10 min at 4°C to obtain serum. The collected serum was used to evaluate the Serum creatinine (Scr) and blood urea nitrogen (BUN) levels, which were assayed by following the manufacturer's instructions in using the Serum Creatinine Assay Kit and Urea Nitrogen Assay Kit.

Histological analysis

Paraffin-enclosed kidney sections were subjected to two different staining: Periodic-acid-Schiff (PAS) staining and Masson's trichrome staining. It is used to identify the pathological injury of renal and tubulointerstitial fibrosis. After the experiment, the images were captured by Zeiss microscope and ZEISS ZEN software 3.10 (Zeiss, Germany). All images were captured at the same magnification, × 200, scale bar = 200 μm.

Western blotting

Renal cortex or HK2 cells were homogenized in media RIPA used as lysis and extraction buffer containing a protease inhibitor. Firstly, equal amounts of renal cortex lysates were loaded for 6% and/or 8% SDS-PAGE gel electrophoresis, which is transferred to nitrocellulose membranes (NM). In 5% skimmed milk, the lysates were incubated in fibronectin, α-smooth muscle actin, type-IV collagen, eukaryotic initiation factor-2e, p-eif2α, G3BP1, Galectin3, lysosomal associated membrane protein-2, TSC2, p-mTOR, mTOR, Raptor, Autophagy Related-5, Beclin, p-p65, p65, p-IκBα, IκBα, and Vinculin proteins with primary antibodies overnight at 4°C on next day, the concoct was further incubated with horseradish peroxidase (HRP)-coupled secondary antibody, the blots/gels were visualized by using a ChemiDoc MP imaging. Densitometry was used to quantify the bands using Image Lab software version 5.

Immunohistochemistry (IHC)

Paraffin-enclosed kidney tissue cut into 4 μm/piece. After deparaffinization, every slide was dipped into 3% hydrogen peroxide in order to block endogenous peroxidase activity and then the slides were placed in 10% goat serum (to avoid unnecessary antibody binding) for 60 minutes at 37°C. Subsequently, the slides with primary antibodies were incubated overnight at 4°C. After treatment with Signal Stain Boost Detection Reagent, slides were washed carefully with Signal Stain Diaminobenzidine (DAB) Substrate to produce a brown-colored product. Integrated optical density (IOD) values of positively stained areas were calculated using ImagePro Plus (6.0 version) software. All images were captured at the same magnification, × 200, scale bar =200 μm.

Immunofluorescence

HK2 cells were thoroughly adhered with 4% para formaldehyde in 35 mm Confocal Dishes and kept for 15 min and after that permeabilized with a known media; Triton X-100 (0.2%, 15 min). The cells were carefully washed twice with PBS media and then incubated overnight with primary antibody and Alexa Fluor coupled secondary antibody and DAPI media (0.1 g/ml, 5 min) for staining. A confocal microscope was used to capture

fluorescent images. All images were captured at the same magnification, × 200, scale bar = 200 μm.

Real-time fluorescence quantitative PCR

Mouse kidney RNA or HK2 cell RNA was collected by following the instructions on the RNA extraction kit. RNA was initially reversely transcribed and then amplified by real-time quantitative polymerase chain reaction (qRT-PCR). The following primers were used: mouse-G3BP1: forward 5'-CTCAGCCGCGCAGATTTT ATG-3', reverse 5'-CCACCCCGTCAGAGTT-3'; mouse-TSC2: forward 5'-AATCCCAGGTGTGCAGAAGG-3', reverse 5'- CCTCCACTGCATGCTCCTC-3; mouse-Raptor: forward 5'-ATGTGCACAGCCCATTCTT-3', reverse 5'-CGACAGGGCCAAGCTCA-3'; mouse-Atg5: forward 5'-AAAGATGTGCTTCGAGATGTGT-3', reverse 5'-CACTTTGTCAGTTACCAACGTCA-3'; mouse-Beclin: forward 5'- GTGCGCTACGCCCAGATC-3'. Reverse 5'- GATGTGGAAGGTGGCATTGAA-3', mouse-β-actin: forward 5'-GGCTGTATTCCCCTCCATC G-3', reverse 5'- CCAGTTGGTAACAATGCCATGT-3'. Homo-G3BP1: forward 5'- CTATCCTCGGTGCTGTGG TG-3', reverse 5'-AGTCAAATATGTCCAAACCTACGC-3'; homo-TSC2: forward 5'- AAAACCAAACAGCGCGA GAT-3', reverse 5'- AAAACCAAACAGCGCGAGAT-3; homo-Raptor: forward 5'- TTGTCTGTCGGCATCTTC CC -3', reverse 5'- CACCACCAGCTCGCTGTC -3'; homo-Atg5: forward 5'-CTGGAGTCCTGCTACCGC-3', reverse 5'- TCAACCAAAGCCAAACCTACT-3'; homo-Beclin: forward 5'- CAGTGTTCCCGTGGAATGGA-3'; reverse 5'- GTATAACGGCAGCTCCTTAGATT-3', homo -β-actin: forward 5'- CATGTACGTTGCTATCCA GGC -3', reverse 5'-CTCCTTAATGTCACGCACGAT-3'. β-actin protein was used as an internal reference for normalization and all experiments were performed in replicates.

Ethical approval

This study was approved by the Animal Ethics Committee of Shenzhen Traditional Chinese Medicine Hospital Animal Center (Approval No. ZXJZ20220428001).

STATISTICAL ANALYSIS

Data from the experiment was collected and analysed by using GraphPad Prism (version: 9) software. The data expressed as mean (±) standard error of the mean (SEM). Data were compared using Student's t-test; one-way analysis of variance (ANOVA) and Tukey's post hoc test; $p<0.05$ ($*p<0.05$; $**p<0.01$) was considered as statistically significant whereas $p>0.05$ was statistically insignificant.

RESULTS

Effects of JPYSR on kidney function indicators and structure in CKD Mice

Scr and BUN are two known indicators, used to determine kidney function. The experimental results showed that both Scr and BUN levels were significantly (P<0.01) increased in the CKD group than the control group (fig. 1a, 1b). The JPYSR significantly alleviate the elevated Scr and BUN in CKD mice (P<0.01). PAS staining showed progressive necrosis of renal tubular epithelial cells in CKD mice. The fibrosis area (%) significantly elevated in CKD but significantly decreased CKD with the treatment of JYPSR (fig. 1c). The tubular injury score significantly increased in CKD but significantly decreased CKD with the treatment of JYPSR (fig. 1d). The PAS (blue) and Masson (red) staining improved necrosis of renal tubular epithelial cells in CKD but recovered with JYPSR treatment (fig. 1e).

Effect of JPYSR on the expression of fibrotic proteins in CKD mice

Renal fibrosis was characterized in ECM, such as FN, ColIV and α-SMA aggregated in large quantities in the renal interstitium. Western blot showed that FN, ColIV, and α-SMA were significantly expressed as up-regulated in the CKD group but significantly down-regulated in the CKD group after treatment with JPYSR (fig. 2a), and IHC and q-PCR also corroborated that expression of ColIV and FN in CKD was markedly increased where the expression of ColIV, and FN in CKD was decreased with the treatment of JYPSR (fig. 2b, 2c).

Effects of JPYSR on SGs in the kidneys of CKD mice

Eif2/ (p) phosphorylation is a classical pathway activator of SGs formation, and G3BP1 is a core protein of SGs formation. The results showed that G3BP1and eif2α phosphorylation expressions were significantly reduced in CKD mice (fig. 3a) and eif2α phosphorylation and G3BP1 expression were significantly elevated in the JPYSR group (fig. 3a), which was further verified by IHC (fig. 3b) and q-PCR (fig. 3c) results.

Effects of JPYSR on Autophagy in the kidney of CKD mice

Autophagy, an evolutionarily highly conserved intracellular degradation system, is closely associated with the development of renal fibrosis. Western blot results showed that the autophagy-related proteins such as Beclin and Atg5 expression were significantly reduced and mTOR phosphorylation level increased significantly accompanied by elevated expression of Raptor where after the treatment of JYPSR the expression level of Beclin and Atg5 significantly increased, and the reduced the phosphorylation of mTOR and accompanied by an expression of Raptor (fig. 4a). The IHC and qRT-PCR results showed that the mRNA expression of the Raptor gene was significantly increased, and the mRNA expression of Beclin and Atg5 was significantly decreased before the treatment, whereas the treatment of the JPYSR significantly reduced the mRNA expression of the Raptor gene and where significantly increased the mRNA expression of Beclin, Atg5 (fig. 4b, 4c).

Table 1: The herbal composition of JPYSR

Plant name	Chinese name	Dosage
Astragalus mongholicus Bunge	Huang-Qi	30 g
Atractylodesmacrocephala Koidz.	Bai Zhu	10 g
Dioscorea oppositifolia L	Shan Yao	30 g
Cistanche deserticola Ma	Rou Cong Rong	10 g
Wurfbainia compacta (Sol. ex Maton) Skornick. and A.D. Poulsen	BaiDou Kou	10 g
Salvia miltiorrhiza Bunge	Dan Shen	15 g
Rheum palmatum L.	Da Huang	10 g
Glycyrrhiza glabra L.	ZhiGan Cao	6 g

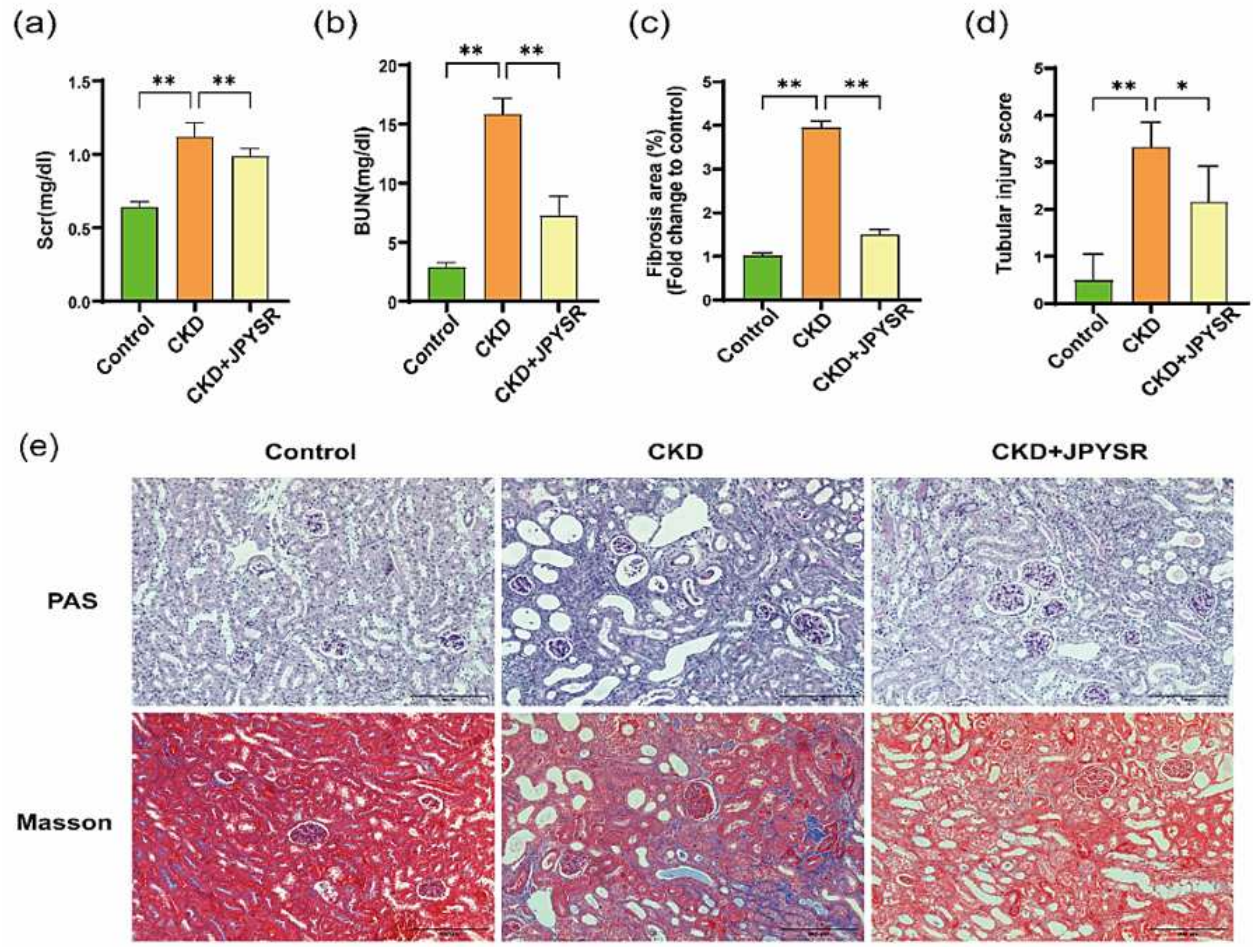

Fig. 1: (a-e): Changes in Renal Function and Renal Structure in Mice. (a) The peripheral blood Scr level in mice (n=6); (b) The peripheral blood BUN level in mice (n=6); (c) The fibrosis area (%) in mice (n=6); (d) The level of renal tubular injury in mice and (e) PAS staining and Masson staining of mouse kidneys (n=3);*p<0.05, **p<0.01.

Protective effect of JPYSR on cytosolic lysosomes in the kidney of CKD mice

Lysosomal damage is a common feature of many diseases; LAMP2 and Galectin3 are lysosomal markers and lysosomal damage markers, respectively, Western blot results showed that Galectin3 expression elevated significantly in the CKD group, and LAMP2 decreased significantly, whereas Galectin3 protein expression decreased after JPYSR treatment, and LAMP2 expression level rise (fig. 5a, P<0.01). Lysosome also widely recognized as the primary signaling platform for the inhibition of mTORC1 by the TSC complex; Western blot showed that TSC2 expression was significantly decreased in the CKD group and the inhibition of TSC2 expression was improved after JPYSR treatment (fig. 5a, P<0.01). The IHC results corroborated that the expression of TSC2 in the renal tubular region was elevated after JPYSR treatment (figs. 5b, 5c, 5d, P<0.05, P<0.01).

Effects of JPYSR on inflammatory pathways in the kidneys of CKD mice

The NF-κb pathway is vital in immune regulation and inflammation and the phosphorylation of p65 and IκBα is a significant indicator of the activation of this pathway.

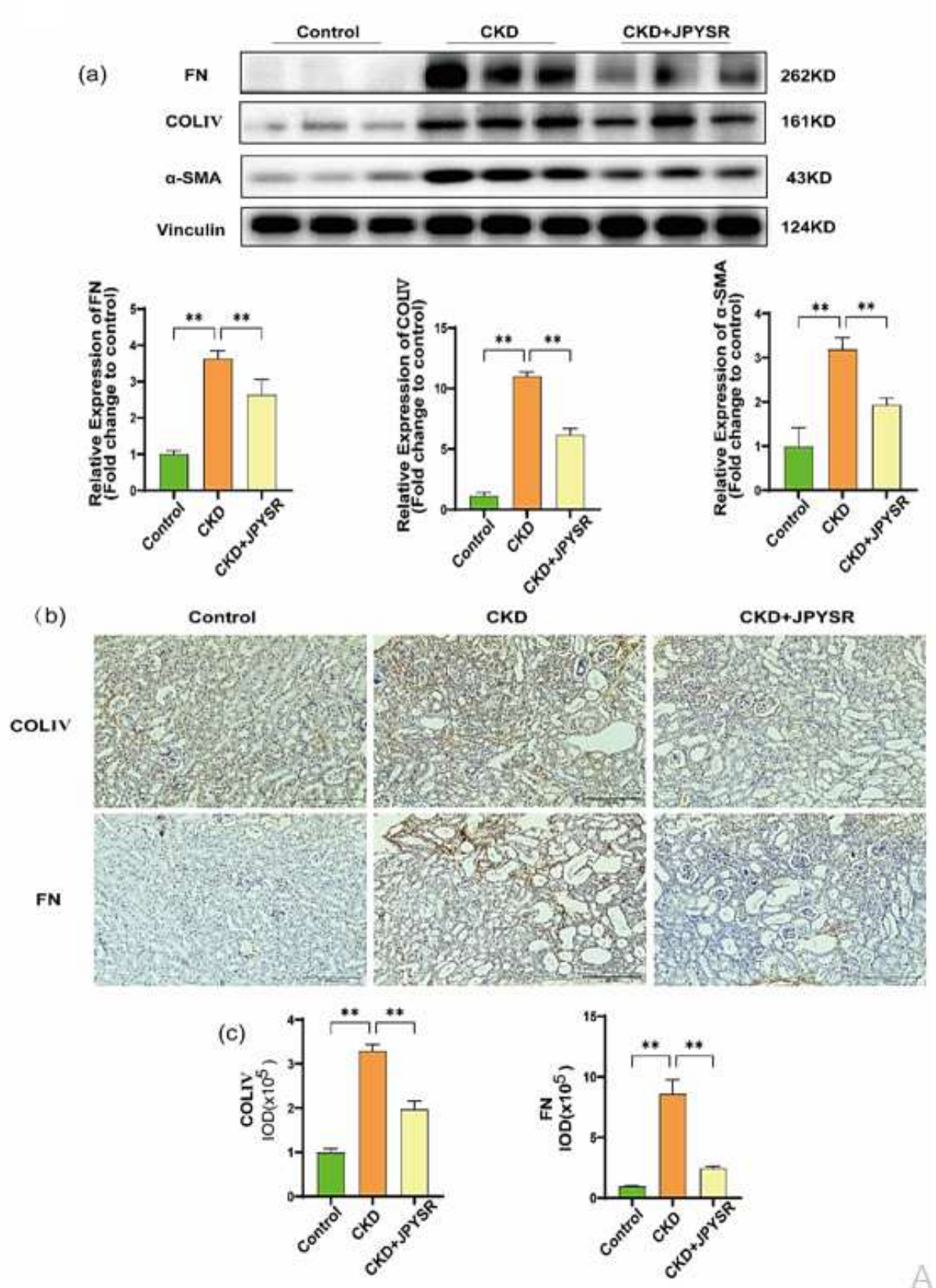

Fig. 2: Levels of Renal Fibrosis in Mice. (a) Western blot analysis of mouse kidney Fn, ColIV and α-SMA; (b) Immunochemical staining of mouse kidney Fn and ColIV; (c) Relative quantitative intensity of mouse kidney Fn and ColIV; (n=3) *p<0.05, **p<0.01.

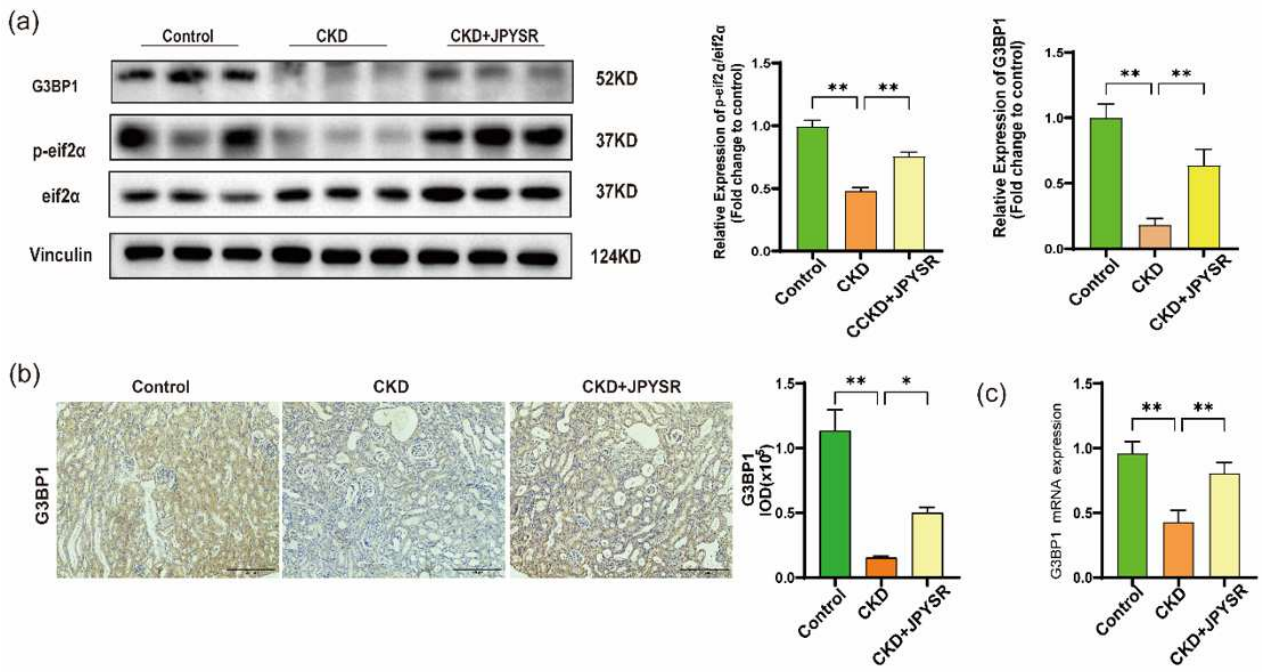

Fig. 3: Aggregation Levels of SGs In Mouse Kidney. (a) Western blot analysis of G3BP1 and eif2α phosphorylation in mouse kidney; (b) Immunochemical staining and relative quantitative intensity of mouse kidney G3BP1; (c) mRNA levels of mouse kidney G3BP1; (n=3), *p<0.05, **p<0.01.

Western blot analysis showed that the phosphorylation of p65 and IκBα was increased significantly in the CKD group and the treatment of JPYSR explicitly inhibited the phosphorylation of p65 and IκBα (fig. 6, **p<0.01).

Effects of JPYSR on lysosomal injury and nf-κb pathway in fibrosis induced by TGF-β1 Stimulation of HK-2 Cells in vitro

TGF-β1 is widely used as a classical inducer to study the transdifferentiation of renal tubular epithelial cells to fibroblasts. We found that TGF-β1 stimulated groups of ColIV, α-SMA, and Vimentin expression were significantly elevated (fig. 7c, 7d, *p<0.05, **p<0.01) accompanied by significant increases in Galectin3 (fig. 9b, fig. 10a, *p<0.05, **p<0.01) an indicator of lysosomal damage and p65 and IκBα phosphorylation in the NF-κb pathway. In contrast, the expression of the above indicators was suppressed in the JPYSR intervention group (fig. 8a, 8b, *p<0.05, **p<0.01).

Effects of JPYSR On the formation of SGs and Autophagy In vitro TGF-B1 Stimulated HK-2 cell induced fibrosis model

We also similarly examined stress granule formation and autophagy levels in each group under the TGF-β1-induced renal tubular epithelial cell fibrosis model and found that the expression level of G3BP1 and the phosphorylation level of eif2α were significantly decreased in the induction group and that stress granule aggregation was significantly ameliorated in the JPYSR treatment group (fig. 8a, 8c, *p<0.05, **p<0.01) and the immunofluorescence results showed a significant localization with LAMP2 and G3BP1 in HK2 cells (fig. 9a). In addition, the expression of autophagy pathway-related proteins Atg5 and Beclin decreased in the induced group, the expression of its upstream TSC2 was suppressed, mTOR phosphorylation was activated, and the expression of Raptor, a vital protein of the mTORC1 complex, increased at the same time, which was consistent with the phenomenon of autophagy inhibition; whereas, the expression of Atg5, Beclin, and TSC2 in the JPYSR treatment group was significantly elevated compared with that of the stimulated group. The mTOR phosphorylation situation was suppressed, and the expression of the vital protein Raptor of the mTORC1 complex decreased flush with that of the blank control group (fig. 10a, *p<0.05, **p<0.01). Intracellular RNA expression levels remained consistent with protein (fig. 10b, *p<0.05, **p<0.01. JPYSR has an excellent protective effect on renal tubules in *in-vitro* cellular experiments that corroborate altered autophagy levels (fig. 10c).

DISCUSSION

Autophagy is a catabolic process in which cytoplasmic components are delivered to lysosomes (suicide bags) for degradation. Autophagic cell death is a protective mechanism of the body. Diabetic patients can regulate renal dysfunction through autophagy (Zheng *et al*., 2021). However, in a long-term hyperglycemic state, the increase of autophagy within cells can also cause renal cell toxicity (Ma *et al*., 2020).

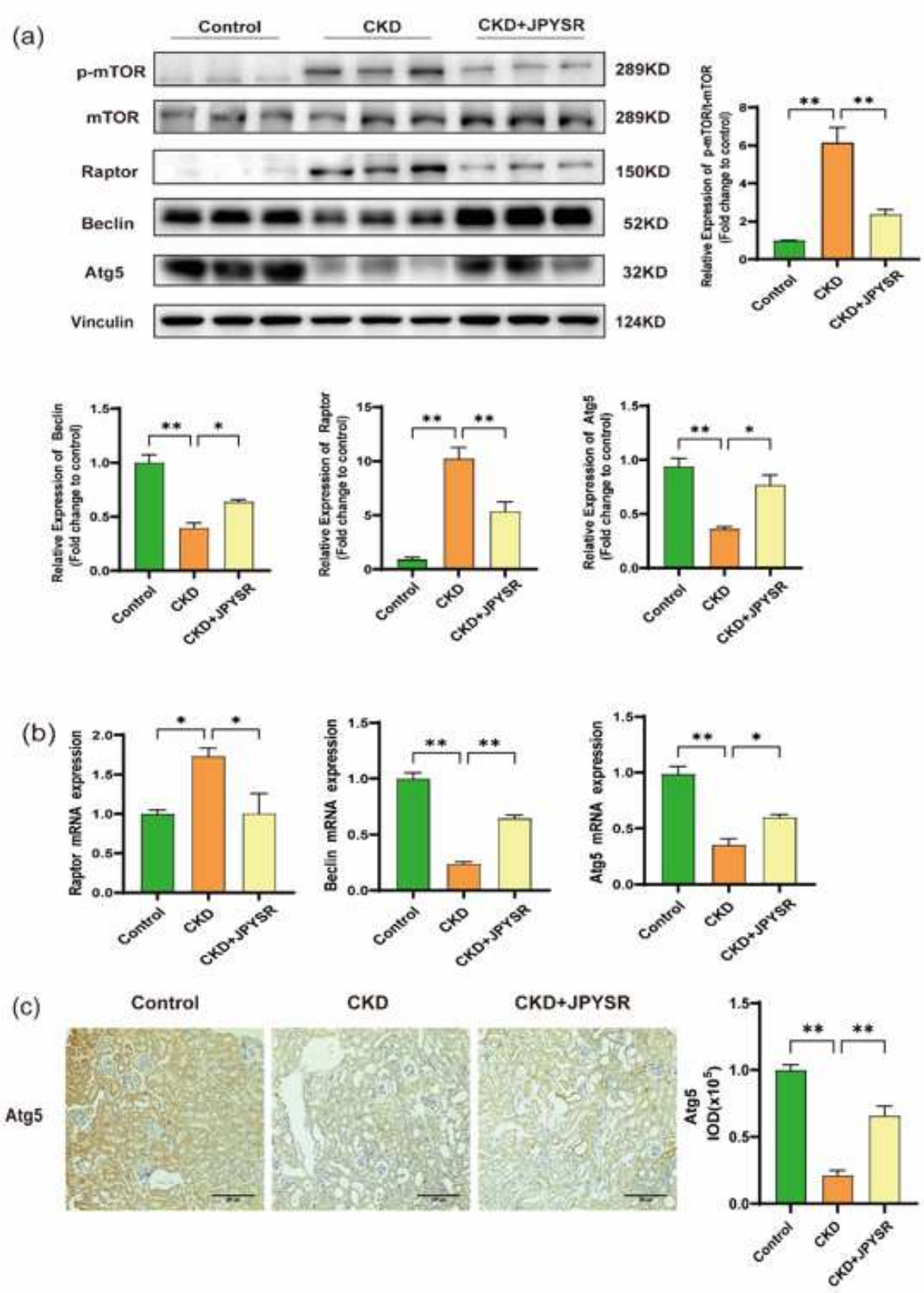

Fig. 4: Mouse Kidney Autophagy Levels. (a) Western blot analysis of mouse kidney mTOR phosphorylation levels, Raptor, Atg5 and Beclin; (b) Relative mRNA levels of mouse kidney Raptor, Atg5 and Beclin; (c) Immunochemical staining and relative quantitative intensity of mouse kidney Atg5; (n=3), *p<0.05, **p<0.01.

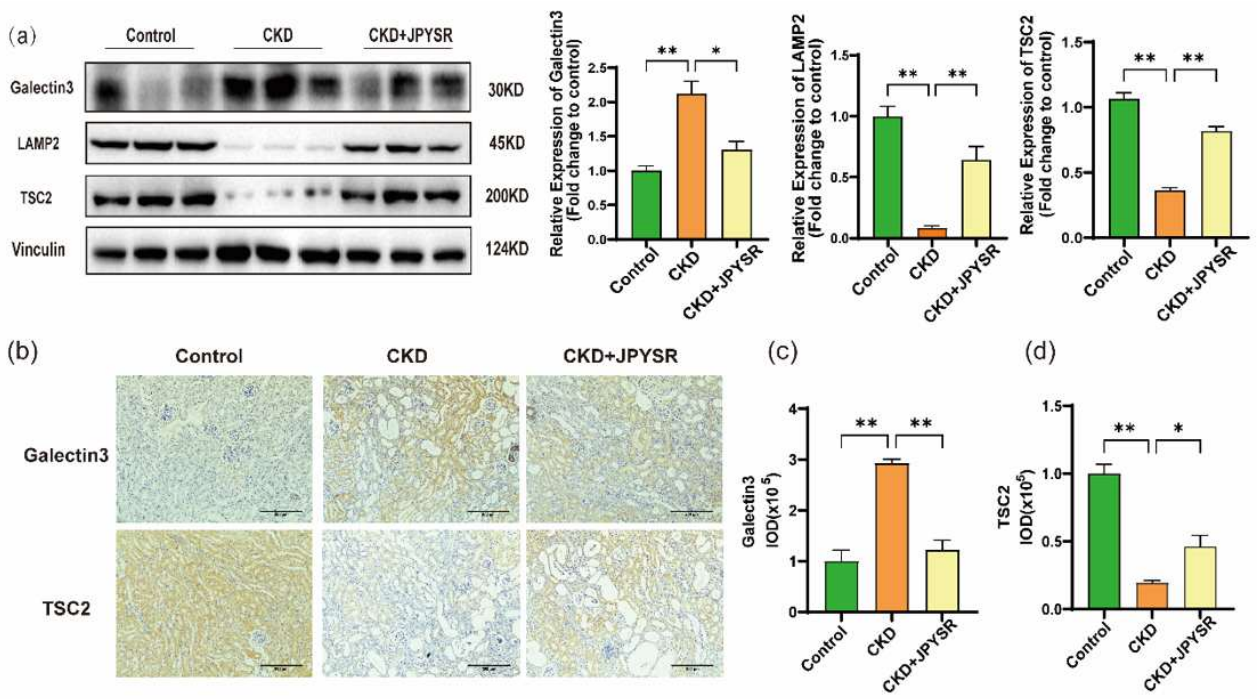

Fig. 5: Lysosomal Damage in Mouse Kidney. (a) Western blot analysis of mouse kidney Galectin3, LAMP2 and TSC2; (b) immunochemical staining of mouse kidney Galectin3 and TSC2; (c) relative quantitative intensity of mouse kidney Galectin3; (d) relative quantitative intensity of mouse kidney TSC2; (n = 3), *p<0.05, ** p<0.01. All images are displayed at the same magnification, ×200, scale bar = 200 μm.

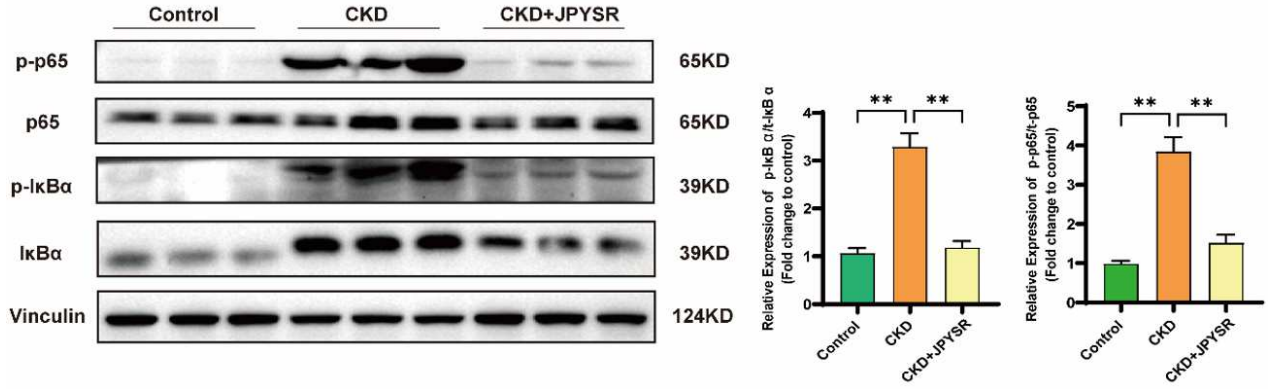

Fig. 6: Mouse Kidney NF-κb Pathway Expression Levels. Western blot analysis of mouse kidney p65 and IKBα phosphorylation levels, (n=3).

Studies have shown that autophagy affects the process of renal fibrosis through different pathways (Liang *et al*., 2022). Autophagy is a homeostatic mechanism of cellular metabolism (Li *et al*., 2021). The protein expressions of autophagy-related proteins Atg5 and Beclin in mice with chronic kidney disease were inhibited (fig. 5), while the NF-κb pathway was activated (fig. 6), accompanied by obvious renal injury and fibrosis (fig. 1). Moreover, the activation/regulation of autophagy has a protective effect on renal cells under stress, but autophagy dysfunction and external or internal damage to the kidney will eventually activate the inflammatory pathway, leading to renal injury and more severe renal fibrosis, which is consistent with previous studies (Li *et al*., 2016; Peng *et al*., 2019).

The treatment of JPYSR significantly improved the autophagy level, renal function and pathological damage in mice and significantly reduced renal interstitial fibrosis, indicating that JPYSR has an excellent protective effect on renal tubules. In vitro cell experiments also confirmed the change in autophagy level (fig. 10c).

As the primary site of cellular degradation, the normal lysosomal function is complementary to resume cellular activity and maintain the homeostatic integrity of the cell (Yang and Wang, 2021). Improving lysosomal function and restoring autophagic flux are crucial for the normal operation of the organism (Zheng *et al*., 2020).

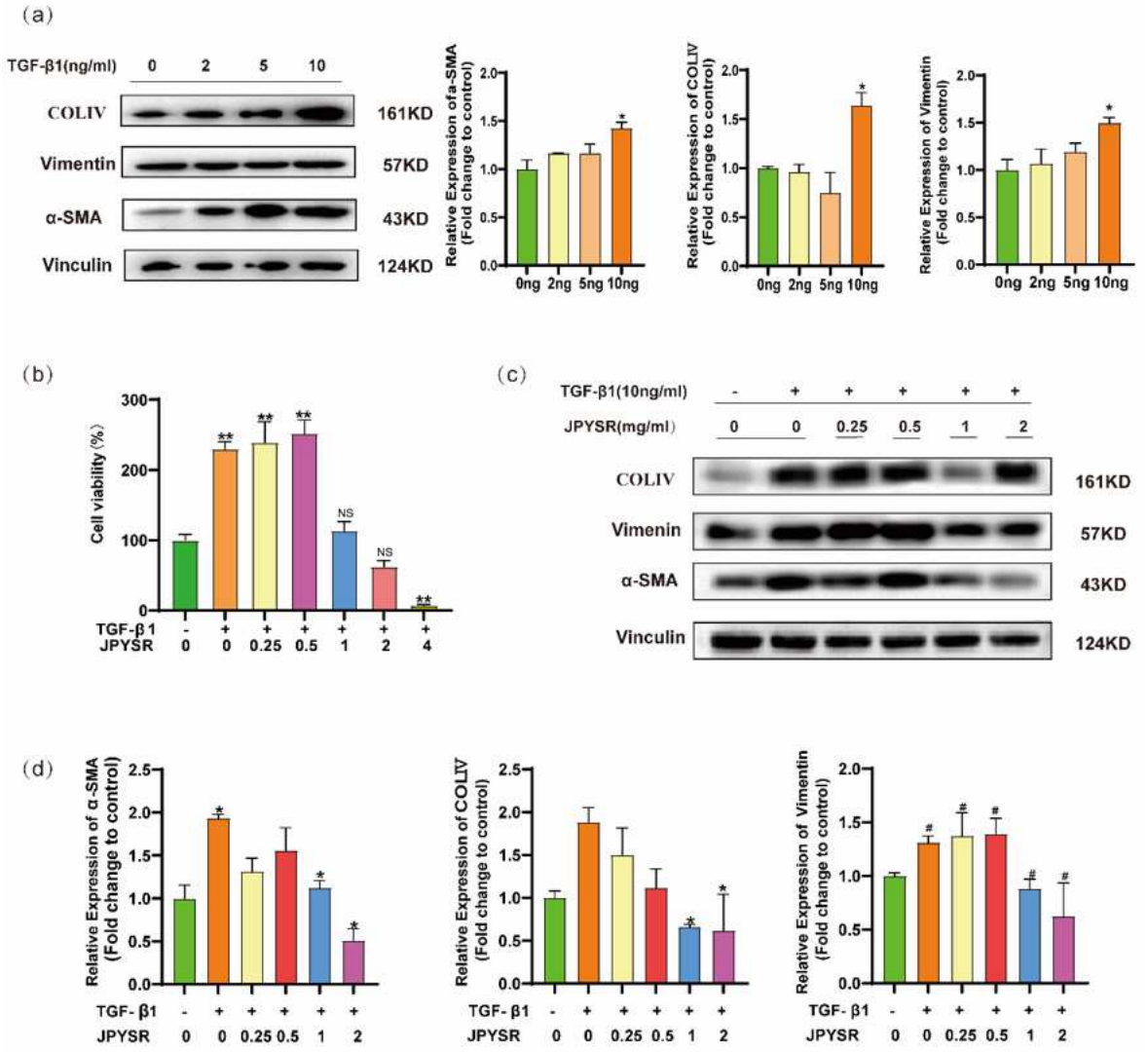

Fig. 7: Screening of Drug Concentration in Cellular Assay. (a) Western blot analysis of Fn, ColIV, and Vimentin of HK2 cells at 0ng, 2ng, 5ng and 10ng TGF-β1 concentrations; (b) CCK8 assay for HK2 cell viability at 0mg/ml, 0.25mg/ml, 1mg/ml, 2mg/ml and 4mg/ml concentrations of JPYSR at 10ng TGF-β1 induction; (c,d) Western blot analysis of Fn, Col-IV and Vimentin in HK2 cells treated with 10ng TGF-β1, 1mg/ml JPYSR; *p<0.05, **p<0.0.

Research indicates that lysosomal dysfunction plays a crucial role in the development of various kidney diseases. Impaired lysosomal function leads to autophagy inhibition, which in turn causes histological lesions in the kidney and accelerates glomerular sclerosis (Tagawa *et al*., 2016; Osicka *et al.*, 2000). The present study's results demonstrated a significant increase in Galectin3 expression and a decrease in LAMP2 in the kidneys of CKD mice. A significantly high expression of Galectin3 (fig. 4) was also observed in HK2 cells in the in vitro experiments (fig. 9, 10), suggesting that there is severe lysosomal impairment in the CKD model. This may result in a decrease in baseline autophagy, which may lead to the aggravation of renal tubulointerstitial extra cellular matrix (ECM) deposition and increased fibrosis.

At the same time, the current showed that the formation of SGs, regulates various stress responses, modulates signaling pathways, and maintains intracellular homeostasis, which is inhibited in the fibrotic environment. The expression of its core protein, G3BP1, significantly reduced the expression of protein and RNA levels. The current study showed that the phosphorylation level of eif2α was significantly reduced in the CKD but the level increased in the kidneys after the treatment of JPYSR. It indicate that JPYSR can promote the formation and aggregation of SGs (fig. 3, 8)

It has been demonstrated that SGs selectively form near damaged lysosomal membranes and act as plugs that promote vesicle stabilization and survival, allowing endosomal stabilization and repair. Li *et al*. (2023) identified that G3BP1 regulates autophagy in NP cells with the TSC2 complex inactivation of the mTOR signaling pathway and similarly, the current study showed that G3BP1 co-localization with LAMP2 in HK2 cells

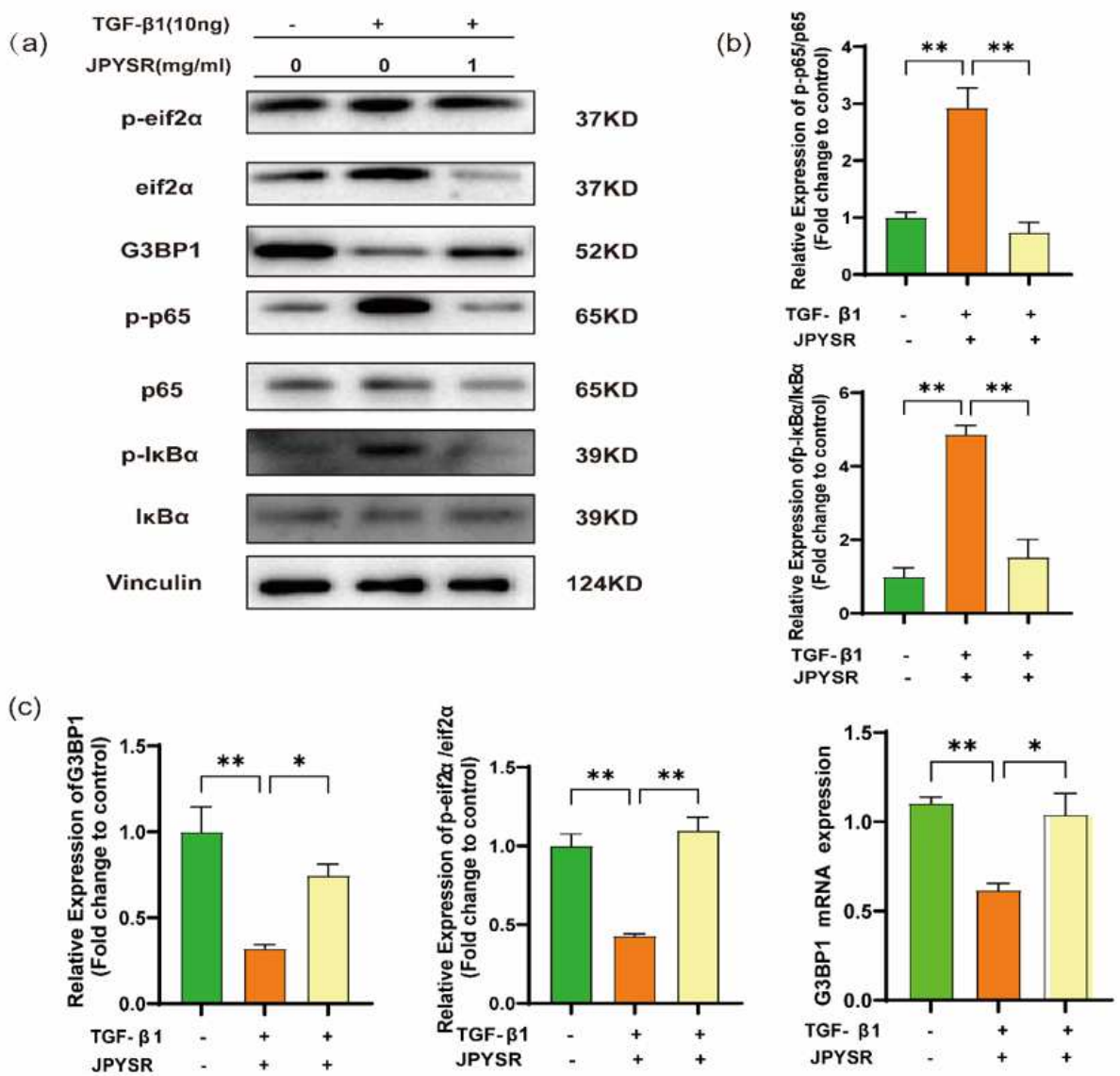

Fig. 8: Reduced Formation of SGs and Activation of Inflammatory Pathways in Fibrosis-Treated HK2 Cells, Improved by JPYSR Treatment. (a) Western blot images of fibrosis-treated HK2 cells, p-eif2α, eif2α, G3BP1, p-p65, p-IκBα, IκBα; (b) Western blot quantification of p65 phosphorylation and IKBα phosphorylation; (c) Western blot quantification of eif2α phosphorylation and G3BP1 blot quantitative analysis and G3BP1 mRNA expression levels; **p*<0.05, ***p*<0.01.

(fig. 9a), suggesting that SGs lysosomal localization. Further, the current study showed that TSC2 expression was suppressed in the model group of mice, including both protein and RNA (fig. 4); however, its downstream phosphorylation level of mTOR was subsequently increased, and the expression level of the signature protein of the mTORC1 complex, Raptor, was increased in parallel (fig. 5). That result suggests that the decrease in SG formation under the renal fibrosis model leads to a decrease in TSC complex formation, which promotes the activation of rapamycin complex 1, inhibiting autophagy expression. The inhibition of TSC2 expression was reversed by JPYSR treatment, and the downstream mTOR phosphorylation level also showed a decreasing trend, with a concomitant decrease in the expression level of Raptor, and the activation of autophagy in the treatment of fibrosis. The in vitro study results are consistent with the *in vivo* experiment (fig. 10). Thus, it is reasonable to assume that reduced SG formation and increased lysosomal damage are concomitant and that SGs can inhibit the activation of the mTORC1 via amino acids of the TSC complex and insulin. Thus, G3BP1 is known as a core component of SGs and a key component of lysosomal signaling. The treatment of JPYSR promotes the formation of SGs, thereby attenuating lysosomal damage in renal intrinsic cells and maintaining baseline autophagy in cells. In addition, Cui *et al*. (2020) found that LAMP2 is an essential component of lysosomes and

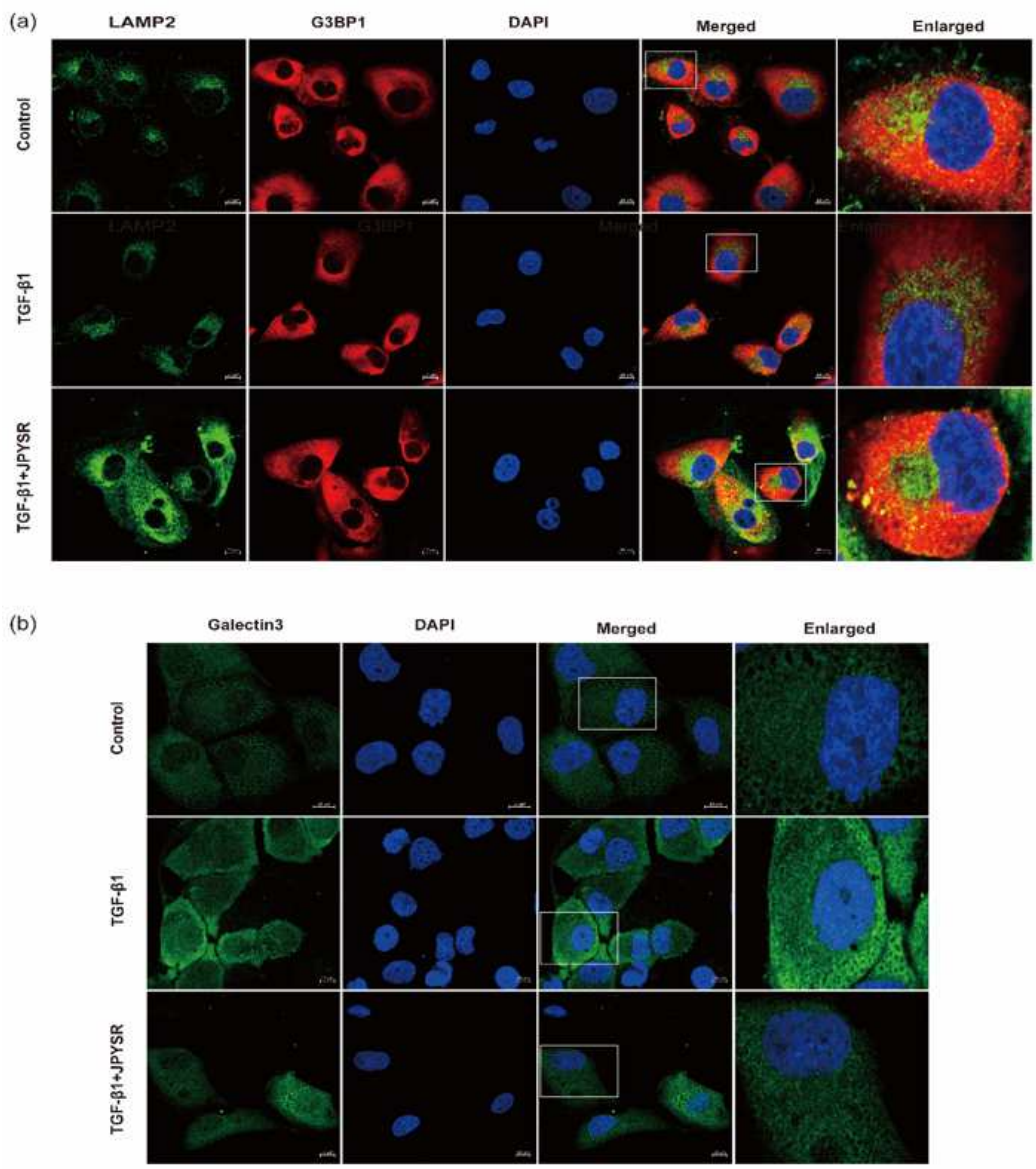

Fig. 9: JPYSR Ameliorates Lysosomal Damage and SGs Formation in HK2 Cells After Fibrosis Treatment. (a) Representative immunofluorescence images of G3BP1 (red) and LAMP2 (green); (b) Representative immunofluorescence images of Galectin3.

an important protein to promotes the progression of autophagy. It has been recognized as an essential protective agent against lysosomal permeabilization (LMP, a manifestation of lysosomal damage) and over expression of LAMP2 can improve lysosomal damage and reverse autophagic flux blockade. We found LAMP2 over expression in HK2 cells after JPYSR treatment and whether JPYSR also improves lysosomal injury and maintains autophagy by promoting LAMP2 over expression needs to be further investigated.

The current study showed the role of SGs in CKD for the first time. Further, it showed that the presence of lysosomal damage and autophagy inhibition in CKD. However, our current study still has some limitations. This study was limited to the autophagy occurrence levels by detecting the expression of related proteins without further understanding the specific autophagy type. Likewise, the potential mechanism by which G3BP1 regulates the TSC-mTOR signaling pathway remains unclear. In addition, the specific roles of SGs and autophagy in chronic kidney disease and the changes in therapeutic targets of JPYSR require further investigation.

CONCLUSION

In conclusion, JPYSR can promote the aggregation of autophagosomes under stress conditions, improve lysosomal damage and thereby inhibit the activation of inflammatory pathways, thus improving fibrosis and slowing the progression of chronic kidney disease.

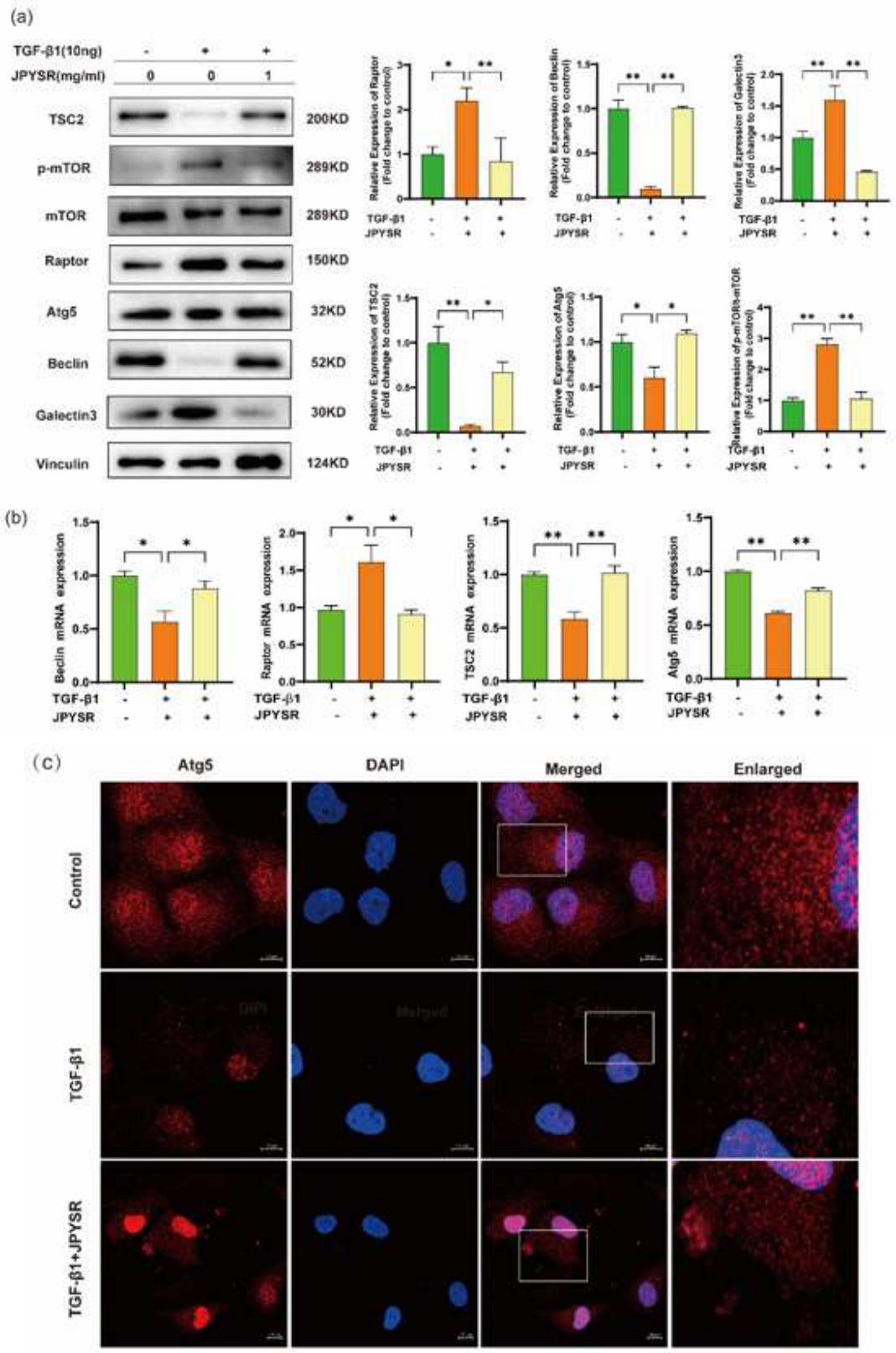

Fig. 10: JPYSR Ameliorates Lysosomal Damage and Autophagy in HK2 Cells After Fibrosis Treatment. (a) Quantitative levels of Galectin3, TSC2, p-mTOR/mTOR, Raptor, Atg5, Beclin proteins in HK2 cells by Western blot; (b) Expression levels of TSC2, Raptor, Atg5, Beclin mRNA in HK2 cells; (c) Representative immunofluorescence of Atg5 images; **p*<0.05, ***p*<0.01.

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