Yu Nu compound regulates autophagy and apoptosis through mTOR in vivo and vitro

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Research

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Abstract

Background: Yu Nu compound (YNJ) is a traditional Chinese medicine widely utilized to treat type 2 diebetes, which has been reported to regulate autophagy. Abnormal podocyte autophagy and apoptosis could result in podocyte loss in diabetics nephropathy (DN). The mechanism of Yu Nu compound in DN is still unclear. Therefore, the study aims to investigate how Yu Nu compound exerts effects on DN rats.

Methods: GK rats were administered using YNJ with different dose once a day by gavage for 4 weeks. The renal cortex injury was observed by HE and electron microscope. Cell apoptosis of renal cortex was analyzed by tunnel staining. The mTOR, autophagy-related proteins and apoptosis-related proteins were detected by Western blot or Real-time PCR in vivo and vitro. MPC5 cells were exposed to high glucose (HG, 30mM) for 12h for simulating podocytes injury in DN. MPC5 cells were treated by serum containing YNJ with different dosage. Cell activities and apoptosis were respectively detected through CCK8 assay and Flow cytometry.

Results: The results showed that the medium dose of YNJ has better effects on decreasing blood glucose and improving renal injury in GK rats, followed by decreasing mTOR levels. The autophagy levels were enhanced, accompanied with the increase of cell apoptosis in vivo and vitro. The decreasing endogenous mTOR could reverse the effects of YNJ on cell apoptosis and autophagy.

Conclusion : the study suggested that YNJ reduced the excessive autophagy and suppressed apoptosis through regulating mTOR. The maintenance of normal basal autophagic activity possibly based on the effect of YNJ on multiple target was essential for maintaining podocytes function.

Background

Yu Nu compound is a traditional Chinese medicine that has significantly therapeutical effects for patients with type 2 diebetes[1]. Yu Nu compound could reduce autophagy apoptosis of islet cells possibly through decreasing the activities of autophagy. Autophagy of high level for a long time could lead to autophagy of islet cells[2]. Autophagy plays considerable roles in the growth and development of podocytes[3, 4]. Podocytes are highly differentiated cells that belong to outer part of the glomerular basement membrane and form the last defence in the glomerular filtration barrier. Podocytes injury could disrupt the integrity of the filter membrane and cause proteinuria. Autophagy is complex in the pathogenesis of diabetes[5]. Early exposure of high glucose(HG) could induce autophagy[6]. However, the autophagy levels were reduced with the prolonged glucose exposure time. Podocytes death would occur when cell autophagy and apoptosis continue to show upregulation. Therefore, autophagy showed different effects with the time of cell exposure to HG.

However, cell apoptosis was gradually increased when cells were exposed to HG[7]. A study has showed that autophagy could be the initiator that triggers the apoptosis[8]. There were studies down that HG triggers mitochondria-dependent apoptosis pathway in DN and Autophagy induces cell apoptosis independent of Bim mediating pathway[9]. When autophagy is activated, LC3-I participates in ubiquitin-

like reaction and forms lipidized form of LC3 (LC3II) as structural proteins of autophagosomes. Atg12 and Atg5 plays vital roles in the extension of autophagy[10]. mTOR exists in mTOR1 and mTOR2 form in cells. The studies have suggested that the pathogenesis of diabetes is related to autophagy inhibition caused by activation of the m TOR signaling pathway[11, 12]. mTOR pathway is involved in regulating autophagy and apoptosis pathway in DN[13, 14]. Besides, mTOR pathway is involved in autophagy and apoptosis of podocytes exposed to high glucose (HG)[15]. The study indicates that mTOR phosphorylates autophagy-related protein to suppress initiation of autophagy[16]. Therefore, the study aimed to investigate how Yu Nu compound exerts functions in DN.

Materials And Methods

Animals

GK rats of SPF (n=45, age: 10 weeks, weight: 320±22g) were purchased (CAVENS, Changzhou, China. number of animal license: SCXK 2016-0010. Certificate number: 20170005000503). Wistar rats of SPF were purchased (Shanghai slack laboratory animal co. LTD, Shanghai, China. number of animal license: SCXK 2017-0005. Certificate number: 201827392). The rats were raised in Fujian university of traditional Chinese medicine laboratory animal center barrier system. After fed adaptively for 4 week, the diabetic rats were determined through the random blood glucose beyond 11.1mmol /L. The GK rats were divided into Yu Nu compound group (YNJ) and model group (GK). Ten Wistar rats of the same strain and age were used as normal group (control). The model and normal group were administered by gavage with normal saline. Simultaneously, the YNJ group was the appropriate dose according to body weight once a day for 4 weeks. Yu Nu compound consisted of gypsum, rehmannia, Radix Ophiopogonis, anemarrhenae and bidentata root. The rats were administered in 6.25 times the dose of adults (gypsum: 15g, rehmannia: 30g, Radix Ophiopogonis: 6g, anemarrhenae: 4.5g, bidentata root: 4.5g). Thus, the dose of Yu Nu compound used by the rats was: gypsum1.56g·kg^{-1}·d^{-1}，rehmannia: 3.125g·kg^{-1}·d^{-1}，Radix Ophiopogonis: 0.625g·kg^{-1}·d^{-1}，anemarrhenae: 0.469g·kg^{-1}·d^{-1}and bidentata root 0.469g·kg^{-1}·d^{-1}. The compounds were oaked into 1000ml water for 2h and boiled. The fluid was kept after simmer for 15 minutes.The remaining was added into 500ml water and used decoction to get fluid. The two fluid was mixed and condensed mixture of 1.25mg/ml, which was conserved at 4 ℃. After final administration, the rats were anaesthetized using 1% pentobarbital (40mg/kg) for collecting renal cortex. The part of renal cortex was put into 2.5% glutaraldehyde and 1.5% paraformaldehyde for the preparation and observation of electron microscope specimens. Besides, the part of renal cortex was fixed using 4% paraformaldehyde for HE staining. The rest was stored at -80℃ for RT-PCR and WB.

HE staining

The renal cortex sections were deparaffined with xylene and dehydrated using gradient alcohol. The sections were stained using Hematoxylin for 15min. Then, after hydrochloric acid alcohol differentiation, the sections were washed with water for10min. Eosin was used to stain the sections for 1min,which then

was sealed using Neutral balsam. The sections were observed and photographed under Nikon 55i microscope.

Electron microscope

Renal cortex was fixed using 3% glutaraldehyde ,1.5% paraformaldehyde and 0.1M PBS (PH 7.2) at 4℃ overnight. Afterwards, the renal tissue was washed with 0.1M PBS for three times, 30min each, which then was fixed The 1% osmium and 1.5% potassium ferricyanide at 4℃ for 1.5h. The renal tissue was dehydrated through a series of process including 50% alcohol for 10 min, 70% alcohol saturated uranium acetate dye at 4℃ overnight, 90 % alcohol for 10min, 90% alcohol-acetone for 10min, 90 % acetone for 10min and anhydrous acetone for 10 min. Then, the renal tissue was soaked in anhydrous acetone and epoxy resin 618 embedding agent (1:1) for 1.5 h , followed by epoxy resin 618 embedding agent at 35℃ for 3h. Finally, the tissue was stained by uranyl acetate and lead citrate respectively for 10min after being embedded, polymerization and sliced (70nm-80nm).

Real-time PCR

The total RNA of renal cortex was extracted using Trizol, the concentration of which was detected using ultraviolet spectrophotometer. The RNA was reversed into cDNA using Reverse transcription kit. The primers were designed using Primer Premier 5.0 software (β-actin R 5' TAGAGGTCTTTACGGATGTCAACGT3' ATG12 F: 5' GCTGAAGGCTGTAGGAGACACT3', ATG12 R: 5' GAAGGGGCAAAGGACTGATT3' M-TOR F: 5' ACGAGTTTGTTTTCCTCCTGAA3' M-TOR R: 5' AGAGAAGTTGGGTCATTGGCTA3'). The mRNA were amplified in ABI StepOne plus PCR instrument and their levels calculated using $2^{-\triangle\triangle Ct}$.

Western blot

The renal tissue was lysed with RIPA Lysis Buffer, which then was centrifuged at 12000r/min for 15min at 4℃. The protein concentration was detected using BCA method. The proteins were separated through electrophoresis and transfered into nitrocellulose membrane. The primary antibodies (anti-ATG12: 1:1000. anti-mTOR: 1:1000; anti-β-actin: 1:1000. Abcam, England) (anti-Beclin-1, LC3II: 1:1000. Santa Cruz) were incubated with the membrane at 4 ℃ overnight blocked by 5% skim milk. The secondary antibody (1:5000) was then incubated with membrane for 2h. The gray value of protein was analyzed using Image Lab 3.0 software and its relative expression was calculated in the ratio of the target protein and reference.

Tunnel staining

The paraffine sections of renal tissue was waxed with xylene and dehydrated by gradient using ethanol. The protease K (20μg/ml) containing no DNase was dropped into the sections at 20 ℃ for 30min. Then, the sections were washed using PBS twice. The sections were incubated with tunnel solution (50μl, Abcam, America) at 37℃ for 1h and then observed under a fluorescent microscope.

Cell line

MPC5 were cultured in RPMI1640 medium containing 10% fetal bovine serum at 37℃ with 5%CO_2. INF-γ was used to induce cells proliferation. When cells grew to 80% fusion, cells were digested with tyrisin and used to passage. Then, cells were cultured in medium containing no INF-γ at 37℃ with 5%CO_2, which was differentiated and matured in 10-14 days for further experiment. The cells were exposed to high glucose for 24 h.

The preparation of serum containing drug

The rats were administered by gavage using aforementioned drug once a week. The blood was collected by abdominal aorta under anesthesia 2h after final administration. After clotting, the serum was collected at 3000r/min for 10min, which then was put in the 56 ℃water for 30min. The serum was filtered and sterilized with disposable sterile filter of 0.22 μm, which then was stored in -20℃ for further experiments.

CCK8 assay

The cells were seeded into 96-well plate (5*10^3/ml) and pre-cultured for 24 h at 37℃ with 5% CO_2. DN cell models were divided into five groups (control, model, YNJF-Low: 1mg/ml, YNJF-Mid: 2mg/ml, YNJF-High: 4mg/ml). Each group was set 5 double holes. 24h after intervention, CCK8 solution (10μL, DOJINDO, Shanghai, China) was added to incubate with the cells for 2h. The OD at 450nm was detected by microplate reader.

Flow cytometry

The MPC5 cells were washed with PBS and digested using 0.25% trypsin. The supernatent was discarded after centrifugation at 1000r/min and PBS was added into cells. The cell suspension (1* 10^6 cells) were collected and centrifuged at 1000r/min for 5 min. 500 μl binding buffer was added after the supernatant was discarded. 5μl Annexin V-FITC and 5 μl propidium iodide were respectively added into cell suspension, which was incubated and kept in dark place for 10min. The cell apoptotic levels were analyzed through Flow cytometry (Beckmancoulter, America).

Plasmid transfection

Cells in logarithmic growth were digested using Trypsin and seeded into 24 well-plate. When cells grew to 70%. The mTOR interference plasmids (shRNA-mTOR) were transfected into cells by Lipofectamine ^{TM}2000 according to manufacture's protocols (Invitrogen, America). 4-6 h after tansfection, the cells were cultured in the medium containing no γ-IFN at 37℃ with CO_2. The cells were performed further experiments after transfection of 24h. The cells were exposed to HG or JNF for 12h.

Statistical analysis

The data were shown in mean±SD. The comparison among different groups was performed by one-way ANOVA, followed by Turkey's test. P<0.05 was considered as statistically significant.

Results

Yu Nu compound significantly reduced the blood glucose (BG) in GK rats

The random blood glucose of GK rats presented progressive increase to more than 20 mmol/L with the age (Table1). BG in Metformin group was significantly decreased one week after administration. However, BG showed significant decrement two weeks after YNJM administration and BG presented similar effects after five weeks of YNJH administration. The effects of YNJM on decreasing BG were significantly superior to other doses. Thus, the medium dose of YNJ had better effects on decreasing HG.

Table1 the blood glucose in different time after YJNF administration. data were shown as mean±SD. *p 0.05, **p 0.01 or ***p 0.001 comparing to Control group. $^{\triangle}$ p 0.05, $^{\triangle\triangle}$ p 0.01, or $^{\triangle\triangle\triangle}p$ 0.001.

Yu Nu compound improved renal injury

The rats in GK group had significantly increasing corpuscle and enlarged renal lumen compared with control group. Furthermore, the lining cells of renal capsule transited from monolayer flat cells to monolayer cuboidal cells comparing to control. There were no significant differences in the morphology of renal corpuscles and tubules between metformin and normal. Renal capsule and renal tubular lumen were larger in YNJH. Vascular spherule atrophied, renal capsule lumen was larger, and the lining cells of renal capsule migrated to monolayer cubic occasionally in YNJL. HE staining indicated that the effects of YNJM on improving renal injury were better than other YNJ group (Figure 1).

Comparison of renal ultrastructure in each group

The endothelial structure of fenestrated capillary in renal corpuscle was clear in Control and the podocyte protuberances enclosed the basement membrane of the capillary endothelium. The basal membrane thickness was approximately 0.113μm and tubules microvilli long and well arranged (Figure 2). In the GK group, the fenestration of endothelial cells was relatively disordered with the basal membrane thickness of about 0.357m. There were more autophagosomes in the cytoplasm of the podocytes, and more vacuolated structures in the epithelial cells of the renal tubules, but the microvilli were more orderly arranged (Figure 2). In the YNJM group, The fenestration of endothelial cells with basal membrane thickness of 0.257 m. However, there were still many vacuoles in the cytoplasm of the renal tubular epithelial cells, suggesting that YNJM could improve the ultrastructure of the GK rats' kidneys (Figure2).

Yu Nu compound significantly decreased cell apoptosis and autophagy

Tunnel staining showed that cell apoptosis was significantly reduced by YNJM administration in GK rats comparing to model group (Figure3C). The results of WB analysis indicated that the expression of proteins mediating autophagy LC3, Atg5 and Beclin1 was significantly reduced comparing to

Model(Figure 3B). The LC3□ expression could reflect the activities of autophagy. The Beclin-1 was also a marker protein for autophagy, which was positively correlated with autophagy level. In addition, Bcl-2 could inhibit Beclin-1-induced autophagy through competitive combination with Beclin-2. Besides, the proteins (Bax, cleaved-Caspase3) promoting apoptosis was markedly increased comparing to Model. Anti-apoptosis protein Bcl-2 showed increasing expression (Figure 3B). In addition, mTOR levels were significantly increased by YNJF treatment (Figure 3B).

Yu Nu compound significantly promoted proliferation and reduced apoptosis in podocytes exposed to HG

CCK8 assay showed that the YNJF of medium dose had better effects on promoting proliferation than other doses (Figure 4A). Furthermore, the apoptosis levels were significantly decreased comparing to other doses (Figure 4B). The result indicated that the YNJF of medium dose has better protective effects than other doses.

Yu Nu compound markedly regulated cell autophagy and apoptosis pathway

The expression of autophay-related proteins and apoptotic-related proteins was detected by WB (Figure 5A-C). The results showed that YNJF significantly decreased the ratio of LC3-□/LC3-□, accompanied by the decrease of Atg5, Atg12 and Beclin1 compared with Model. However, the medium dose showed better inhibitory effects than other doses (Figure 5A-C). The results of apoptosis-related proteins analysis implied that YNJF inhibited podocytes apoptosis possiblely through suppressing mitochodrial-mediated apoptosis pathway. mTOR was significantly increased comparing to Model (Figure5C).

Yu Nu compound affected podocytes apoptosis and autophagy via regulating mTOR

The aformentioned results suggested that YNJF with medium dose had relatively better protection effect than other doses. Therefore, the YNJF with medium dose was utilized for further transfection experiment. qPCR was used to evaluate the transfection efficacy of plasmids interfering mTOR expression. The result showed that shRNA -mTOR-1 had better effects on suppressing the expression of mTOR (Figure6A). The decrease of endogenous mTOR expression markedly reversed the effects of YNJF on podocytes proliferation, apoptosis and the ratio of LC3-□/□, which implied that the effects of YNJF on podocytes injury caused by HG could be attributed to the complexity of YNJF compound (Figure6 B-E).

Discussion

Podocytes possess higher autophagy levels in normal physiological conditions[17]. However, whether the autophagy activation could induce cell injury depending on the intervention. Our study showed that YNJF significantly decreased autophagy, which indicated that YNJF could maintain a moderate autophagy level in DN through mTOR pathway.

Although HG-induced podocytes autophagy has protective effects, the effects couldn't reverse podocyte damage. Persistent presence of pathological factors contribute to the continuous increase of autophagy level, which may break the balance of autophagy in podocytes and lead to excessive autophagy, which

eventually cause programmed death of podocytes autophagy[18]. A review shows that Basal autophagy is necesssary for maintenance of β cell function. Beyond that, due to the complexities of compound in traditional Chinese medicine, the pathologic process of diabetes can be interfered through the interaction of various mechanisms, which could avoid further damage caused by overuse or underuse of a single target.

The LC3, belin-1 and autophagy-related proteins(Atg) are involved in forming autophagosomes[19]. The ratio of LC3-II and LC3-I reflects levels of the autophagy flux or the autolysosomal degradation that was blocked[20]. Our study showed that the ratio of LC3-II/LC3-I was significantly decreased in podocytes exposed to HG through YNJF treatment.Therefore, the autophagy activities of podocytes induced by HG might be notably increased by YNJF. The study has demonstrated that STZ promotes LC3 expression not due to a blockade in autophagy influx in diabetes, which indicates increased autophagy activities in early diabetes[21]. Podocytes autophagy has been reported to exert protective effects under HG stimuli[22]. However, autophagy beyond a certain degree possibly accelerated cell apoptosis. Moreover. The time of HG-induced autophagy shows differences in different podocytes lines[21, 23].

Glycation end metabolites increase protein kinase activity and activate polyol pathway, which have been identified as the pathogenesis of classic diabetic nephropathy. Increased advanced glycation end products, protein kinase activities and activation of the polyol pathway are considered as classical pathogenesis of DN[24]. In addition, cell stress response including oxidative stress and endoplasmic reticulum stress, and mTOR, AMPK pathway are involved in the development of DN as well, which is thought as important causes of autophagy formation[23, 25, 26]. DN could be ascribed to abnormal hemodynamics and metabolism caused by HG stimulation.

Conclusions

All in all, our study indicated that the complexity of YNJF compound could reduce podocytes injury through acting on diverse targets and maintain moderate autophagy activities to avoid excess cell apoptosis through mTOR pathway.

Abbreviations

YNJ: Yu Nu compound. DN: diabetics nephropathy. HG: high glucose. Atg: autophagy-related proteins. AMPK: Adenosine 5'-monophosphate (AMP)-activated protein kinase.

Declarations

Ethics approval and consent to participate

All the experimental operations were approved by Fujian University of Traditional Chinese Medicine.

Consent for publication

No applicable.

Availability of data and materials

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

Conflict of interest

The authors declared no conflict of interest.

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Authors' contributions

Cg-H, GL, SZ, JZ: Collection, analysis and interpretation of data, manuscript writing. YC, Zp-H, Yf-Z : collection and interpretation of data. Cg-H, GL,:conception and design, interpretation of data, manuscript revision.

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Not applicable.

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Tables

Table1 the comparison of random blood glucose levels in each group （*mean ±SD*）

group	n	random blood glucose（mmol/L）					
		Before administration	After 1w of administration	After 2w of administration	After 3w of administration	After 4w of administration	After 5w of administration
Control	7	6.26±0.40	6.56±0.74	6.77±0.76	6.91±0.66	6.77±0.77	6.25±0.40
GK	7	15.396.26±4.6**	15.53±4.12**	19.37±5.90**	23.23±8.24***	20.03±6.38**	21.20±7.68**
metformin	7	15.01±3.43**	10.50±4.97$^{*\triangle}$	11.72±4.67$^{*\triangle}$	11.90±2.61$^{*\triangle\triangle}$	9.93±5.25$^{*\triangle\triangle}$	10.44±3.84$^{*\triangle\triangle}$
YNJH	7	15,27±3.21**	16.6±6.79**	16.93±4.95**	17.52±8.77**	16.37±7.04$^{**\triangle}$	14.44±6.48$^{**\triangle}$
YNJM	7	15.07±3.27**	17.24±9.00**	13.40±4.13$^{**\triangle}$	13.90±2.91$^{**\triangle\triangle}$	14.17±8.92$^{**\triangle}$	11.13±2.50$^{*\triangle\triangle}$
YNJL	7	15.09±3.24**	23.33±10.04$^{***\triangle}$	17.87±3.79**	23.36±7.49***	21.50±8.29***	19.57±8.59***

* $p<0.05$ or $^{**}p<0.01$ comparing to Control.$^{\triangle}p<0.05$ or $^{\triangle}p<0.01$ comparing to GK group at the same time point.

Figures

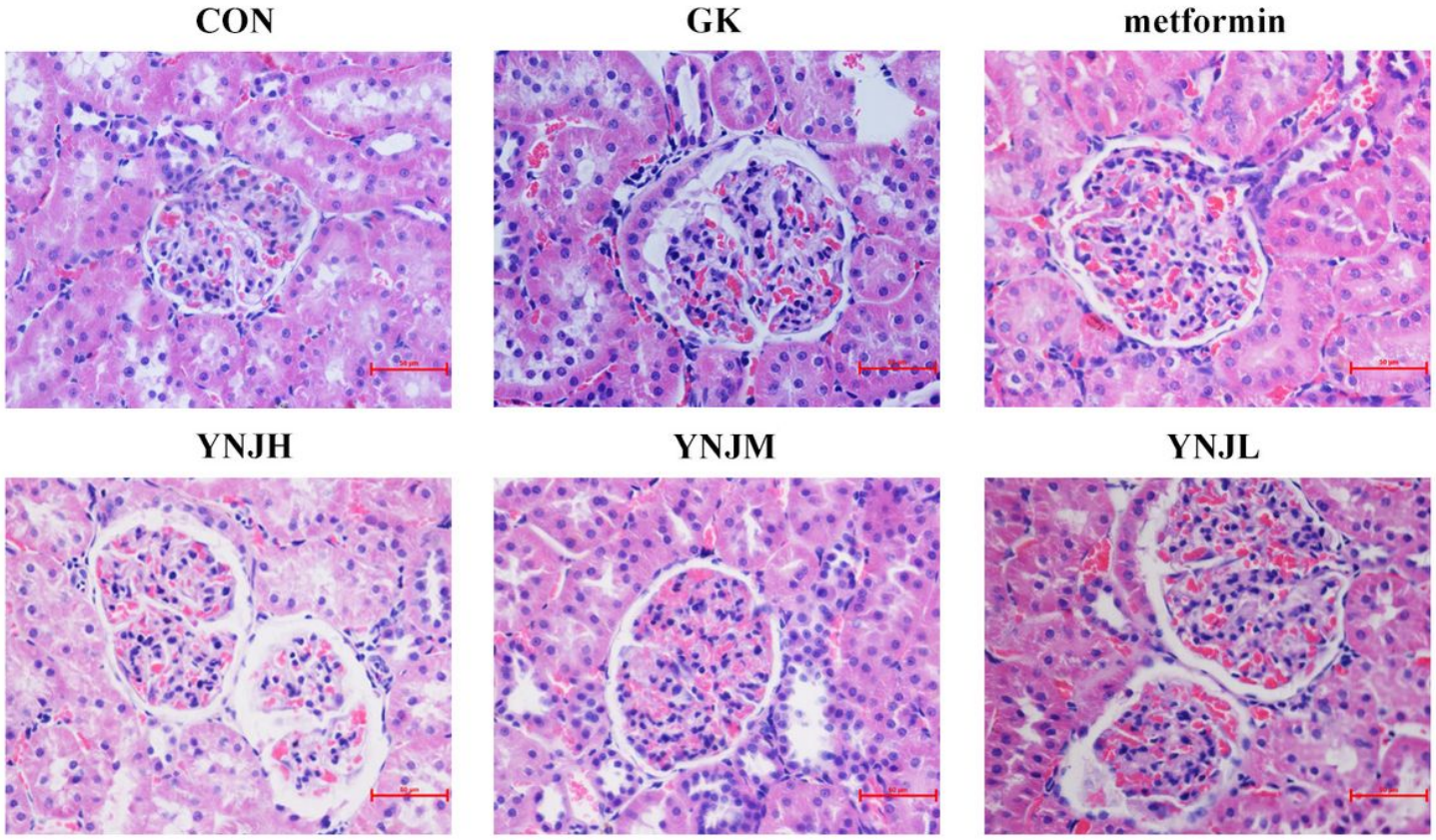

Figure 1

HE staining showed the renal injury. YNJH (high dose): 12.5 g.kg-1.d-1. YNJM(medium dose): 6.25g. kg-1.d-1. YNJL(low dose):3.125g. kg-1.d-1.

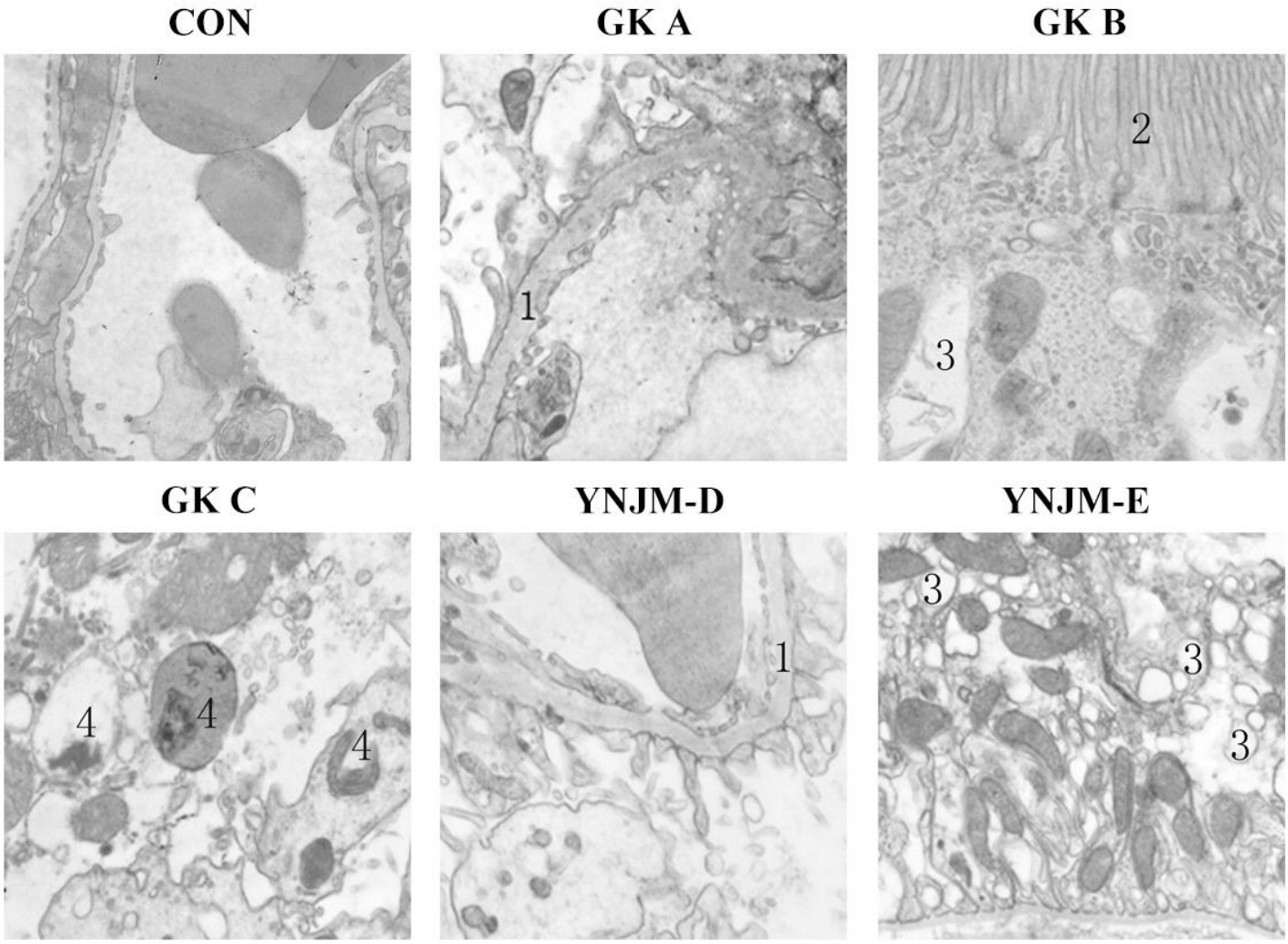

Figure 2

Electron microscope presented renal ultrastructure in different groups.

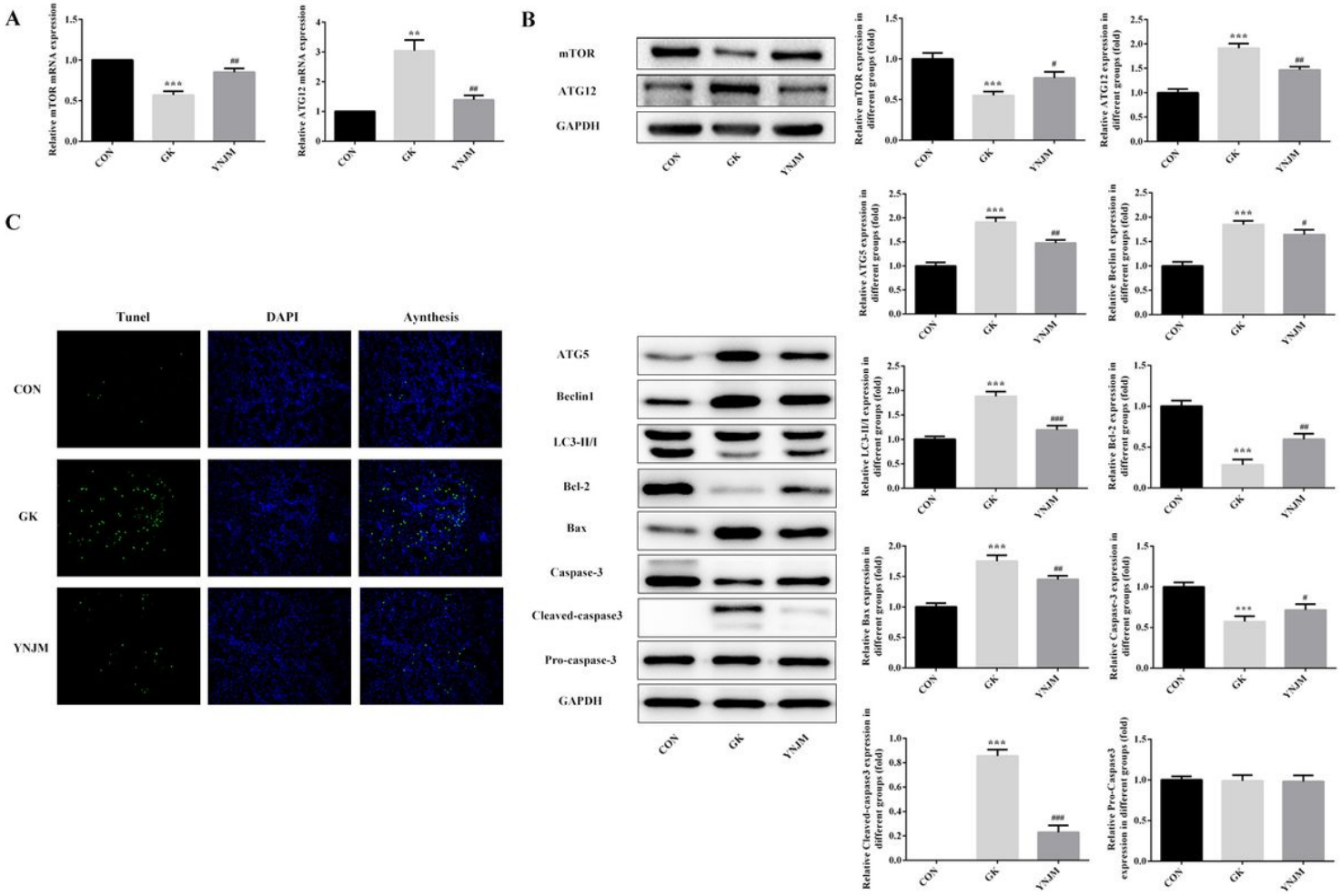

Figure 3

the autophagy and apoptosis-related proteins were detected by WB or RT-qPCR. Data were shown as mean±SD. *p<0.05, **p<0.01 or ***p<0.001 comparing to Control group. △ p<0.05, △△ p<0.01, or △△△p< 0.001.

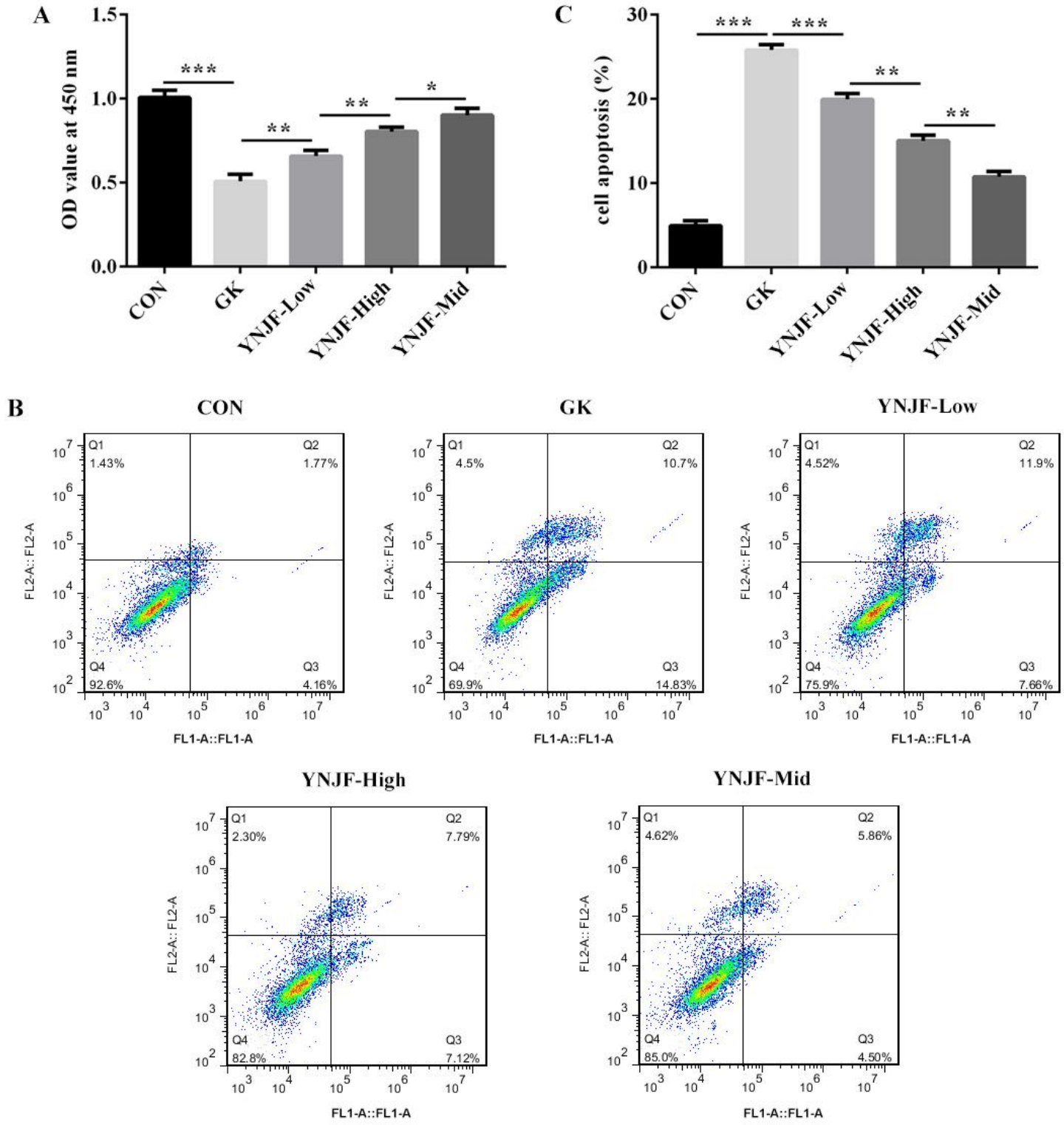

Figure 4

Yu Nu compound significantly promoted proliferation and suppressed apoptosis which respectively were analyzed by CCK8 assay and Flowe cytometry. data were shown as mean±SD. *p<0.05, **p<0.01 or ***p<0.001 comparing to Control group. △ p<0.05, △△ p<0.01, or △△△p<0.001.

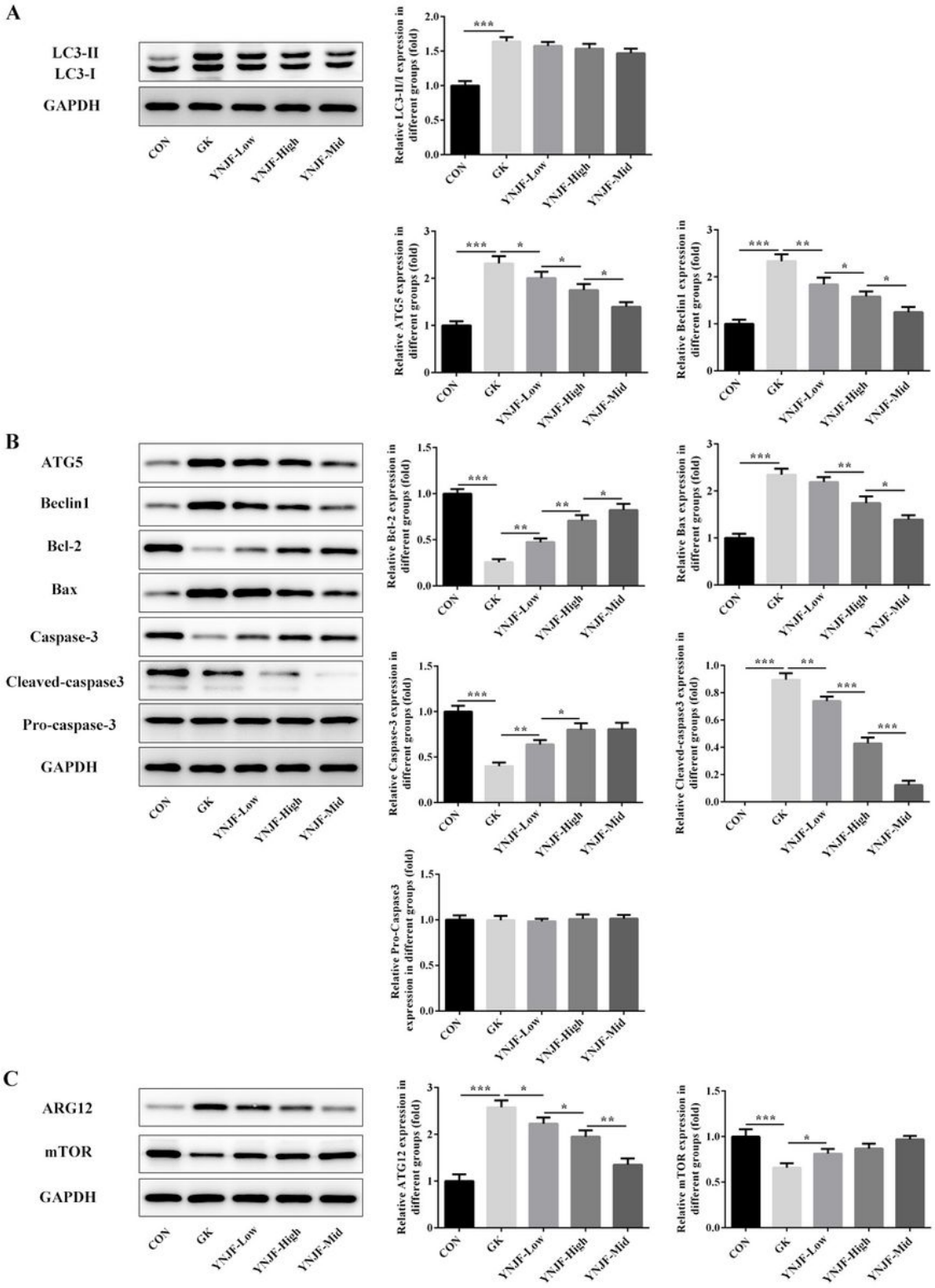

Figure 5

Yu Nu compound affected the expression of autophagy-related and apoptosis-related proteins. Data were shown as mean±SD. *p<0.05, **p<0.01 or ***p<0.001 comparing to Control group. △ p<0.05, △△ p<0.01, or △△△p<0.001.

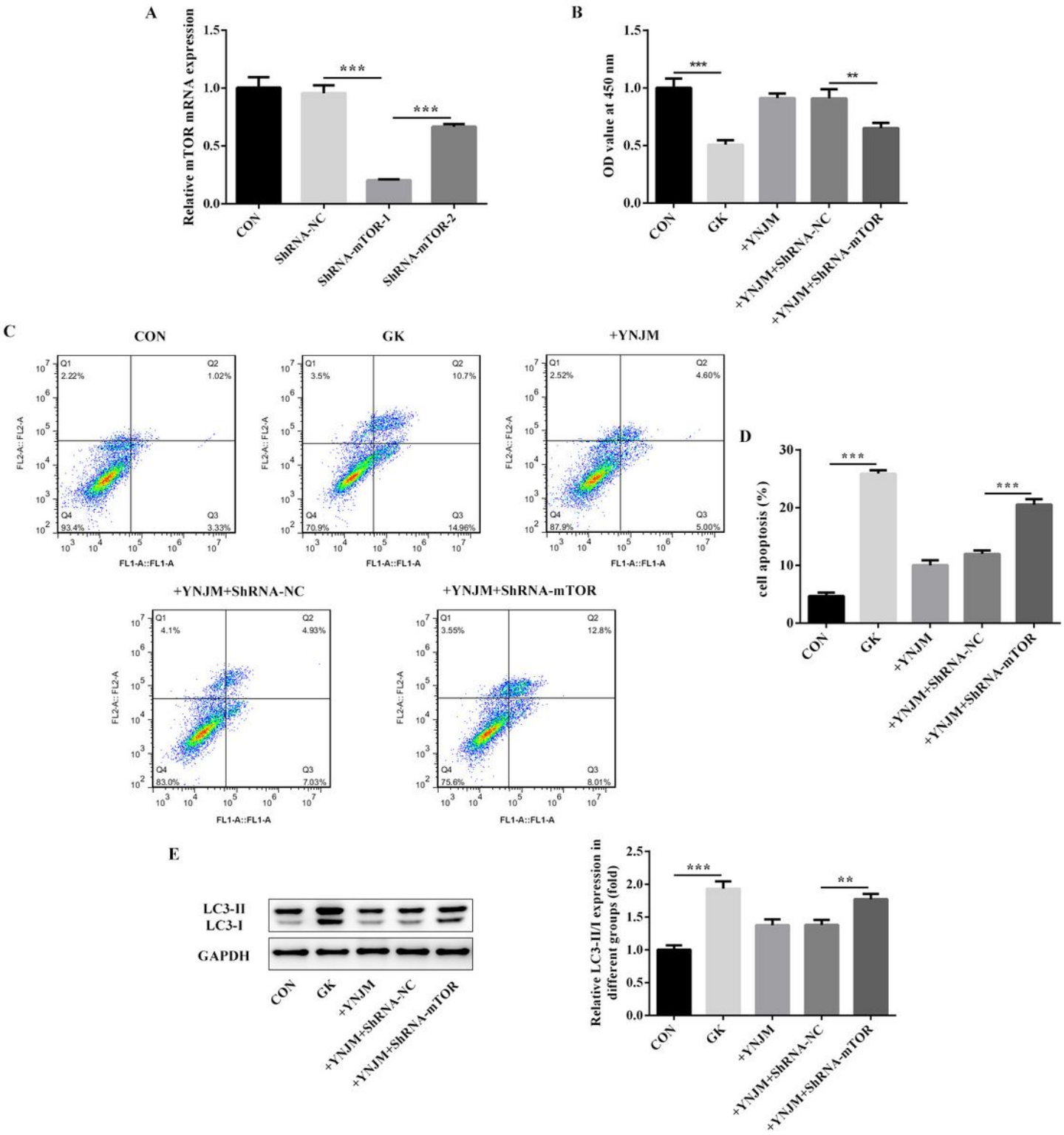

Figure 6

A shRNA-mTOR-1 or shRNA-mTOR-2 was respectively transfected into MPC5 cells. B-D mTOR knockdown reversed the effects of YNJF on proliferation and apoptosis. E mTOR knockdown reversed the effects of YNJF on autophagy. Data were shown as mean±SD. *p<0.05, **p<0.01 or ***p<0.001 comparing to Control group. △ p<0.05, △△ p<0.01, or △△△p<0.001.