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Research Article

Keywords: TEG, hepatic stellate cells, liver fibrosis rats, autophagy, AMPK-mTOR pathway

Posted Date: April 4th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1504132/v1

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Taurine, EGCG and Genistein regulate AMPK-mTOR pathway to inhibit hepatic stellate cells autophagy and alleviate liver fibrosis in rats

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Abstract Activation of hepatic stellate cells (HSCs) is the most important factor in the hepatic fibrosis progression. Autophagy is closely related to the activation of HSCs. Based on our previous works, this research tried to explore the anti-fibrotic mechanism of combination therapy with Taurine, Epigallocatechin gallate（EGCG）and Genistein, referred to as TEG, by monitored autophagy in HSCs and CCl_4-induced liver fibrosis rats. Transmission electron microscopy showed that there were many autophagosomes and autophagy lysosomes in HSCs and fibrotic rats. After TEG intervention, autophagosomes are decreased, HSCs proliferation is inhibited and liver tissue damage is repaired. Western blot and IHC results found that TEG reduced the expression of autophagy marker proteins LC3-II and Beclin1 in HSCs or fibrotic rats. mRFP-GFP-LC3 showed that green fluorescence increased after TEG treat HSCs. AMPK-mTOR pathway analysis revealed that TEG inhibited LC3, Beclin1, AMPK(Adenosine 5'-monophosphate (AMP)-activated protein kinase) and mTOR

genes and phosphorylated protein expression in HSCs. Our results indicate that TEG anti-fibrotic may be associated with reduce autophagosomes by inhibit AMPK-mTOR pathway.

Keywords: TEG; hepatic stellate cells; liver fibrosis rats; autophagy; AMPK-mTOR pathway

Introduction

Liver fibrosis is a common pathological occurrence and increases the risk of cirrhosis, hepatic carcinoma and liver failure[1]. HSCs are the most important cells in the process of liver fibrosis[2]. At resting state, HSCs have a lot of lipid droplets which can store vitamin A and regulate the lipid metabolism balance in liver[3]. Once the liver is damaged, often lipid metabolism homeostasis is broken, which stimulate the release of inflammatory factors and cause HSCs activation[4]. When HSCs is activated, intracellular lipids are lost and cells have the ability to secrete ECM(extracellular matrix), thereby promoting liver fibrosis[5]. At present, an important means of anti-fibrosis is to inhibit HSCs proliferation or to convert them from activation to resting state[6].

Autophagy is an evolutionarily conserved cellular process and an important pathway regulating the cells homeostasis[7,8]. Studies have shown that HSCs activation is associated with autophagy and inhibition autophagy can reduce liver fibrosis[9, 10]. When autophagy is inhibited, triglyceride content in lipid droplets increases and HSCs activation is inhibited[11]. Adopted autophagy inhibitor to treat HSCs or eliminated autophagy-related gene Atg5, the number and volume of lipid droplets significantly increased. It is indicates that autophagy may provide energy for HSCs[12].

AMPK-mTOR is a classical autophagy upstream signal regulatory pathway. Inhibiting AMPK pathway can decrease autophagy[13, 14]. Recent studies have reported a close relationship between AMPK-mTOR pathway and HSCs activation [15, 16]. Jin[17] found that activating AMPK signaling induces autophagy to activate HSCs under hypoxic conditions. Resveratrol could improve dietary methionine-choline

deficiency-induced nonalcoholic steatohepatitis which mechanism may be related to the autophagy AMPK pathway[18].

Taurine, EGCG and Genistein are natural products. Sang[19]found that Genistein enhanced TRAIL-induced A549 adenocarcinoma cell death by inhibiting autophagy flux. Zhang[20] reported that Taurine has a protective effect on ARPE-19 cell autophagy. Li[21] found that EGCG pretreatment down-regulated hepatocyte apoptosis and autophagy in ConA-induced hepatitis. Our previous studies have indicated that combination of Taurine, EGCG and Genistein could significantly alleviate the progression of liver fibrosis in rats, and also inhibited HSCs proliferation more than a single drug[22-24]. However, whether the combination therapy inhibits autophagy to achieve anti-fibrosis is unclear.

In this research, in *vivo* and *vitro,* we applied carbon tetrachloride-induced liver fibrosis rat model and HSCs for research subjects. After TEG intervention, we observed the intracellular autophagosomes and biological changes, and explored the AMPK-mTOR pathway affect autophagy and anti-hepatic fibrosis. However, all of which may be reveal the mechanism of combination therapy anti-fibrotic and provide new ideas for liver fibrosis treatment.

Materials and methods

Chemicals and reagents. Taurine was obtained from Chengdu kelong Chemical Reagent Factory (Sichuan, China) with a purity of 99%. EGCG was obtained from Sichuan Yuga Tea Development Co. Ltd. (Sihuan, China) with a purity of 98%. Genistein was obtained from sigma (USA) with a purity of over 98%.

Cell culture and treatment. As described previously[25], HSC-T6 cells were cultured in high glucose DMEM medium (containing 10% fetal bovine serum) and incubated at 37°C and 5% CO2. Cells were passaged via trypsinization every 3 days. The experiment was divided into 4 groups: ①Control group (complete medium), ② Combination drugs group (TEG: Taurine 30μg/ml + EGCG 35μg/ml + Genistein 7μg/ml), ③ Autophagy inhibitor group (Bafilomycin A1, 10.5μmol/l, haoran,

Shanghai, China),④Combination drug + autophagy inhibitor group (TEG+Baf-A1, concentration as above), ⑤ TEG+ Compound C (AMPK inhibitor, 0.5μmol/L, MCE, China)

Animals and animal care. A total of 50 adult male Sprague-Dawley (S-D) rats weighing 250 g were purchased from the Experimental Animal Center of Guangxi Medical University. The study was approved by the Ethics Committee of Guangxi Medical University. Animals feeding conditions, modeling and medication are kept our previous research[26].

Experimental design. The experiment was divided into: ①Control group (n = 10, peanut oil only twice a week for 8 weeks and saline once a day for 6 weeks), ②CCl_4 model group (n = 10, 2 ml/kg CCl_4, twice a week for 8 weeks and saline once a day for 6 weeks), ③Combination drugs group (n = 10, Taurine 100 mg/kg + EGCG 15 mg/kg + Genistein 10 mg/kg, once daily for 6 weeks), ④Autophagy inhibitor (Baf-A1) group (n = 10, 1.5 mg/100 g, once daily for 6 weeks), ⑤TEG + inhibitor group (n = 10, Taurine 100 mg / kg + EGCG 15 mg/kg + Genistein 10mg/ kg+ Baf-A1 1.5 mg/100g, once daily for 6 weeks). All rats were sacrificed with anesthetized and liver tissues were collected after experiments completed.

Rat liver histopathology. As described previously[27], briefly, fresh tissue from the right lobe of rat liver was fixed in 5% paraformaldehyde solution for 24 h, then embedded in paraffin and routinely sectioned. HE staining was used to observe the pathological changes of liver tissue, and Masson staining was used to observe the collagen fibrosis. The experiments were repeated in triplicate.

Observation of autophagosomes by transmission electron microscopy. HSC-T6 and liver tissues were fixed with 3% glutaraldehyde for 2 h, rinsed with PBS, then fixed with 2% osmium tetroxide and subjected to gradient dehydration with ethanol. Next，cells or tissues samples were infiltrated with epoxy resin, embedded, polymerized,

sectioned, stained and observed for changes of autophagosomes under a transmission electron microscope.

Measurement of cell viability. HSC-T6 was seeded in a 96-well culture plate. After inoculation for 24 h, each group was added with 100μl TEG, Baf-A1 and TEG+Baf-A1. After continuing to culture for 24 h, all the liquids in the 96-well culture plate were aspirated. The CCK-8 reagent and the complete medium were mixed into a reaction solution at a volume ratio of 1:9 and added to the wells. After incubation for 2 h, the living cells at 450 nm were detected using a Microplate Reader. The number was repeated three times independently.

Autophagic lysosomes by acridine orange staining. HSC-T6 were collected by trypsinization and adjusted to 1×10^5/ml cell concentration. Each well added 2 ml cell suspension. After 24 h, each group added to 2 ml TEG, Baf-A1, TEG+Baf-A1 and complete medium cultured for 24 h, the cells were removed and washed three times with PBS. After fixed with 95% alcohol for 15 min and with 1% acetic acid for 30 s, the cells immediately added acridine orange staining for 30 s, 0.1 M $CaCl_2$ for 2 min, and rinsed with PBS for 3 times. Finally, the cells sealed with glycerin and observed under inverted fluorescence microscope. The changes of autophagy lysosomes in HSCs were observed and the experiment was repeated 3 times.

Western blot analysis. Briefly, as described previously[27], western blot validations were performed on LC3, Beclin1, AMPK, p-AMPK, mTOR, p-mTOR. Total protein of liver tissues and HSC-T6 cells were extracted using RIPA buffer, and the protein content was determined. Total proteins were loaded onto the SDS-PAGE electrophoresis gels (5%-12%) and then transferred to PVDF membranes by electroblotting at 120Ma and 90min. Following blocked in 5% non-fat milk powder and shake for 30 min. Wash three times with TBST and then incubate overnight at 4°C in the primary antibody. Wash three times with TBST and incubate for 1h at room temperature in the secondary antibody. Finally, the PVDF membrane was placed in an

Odyssey Infrared Imaging System for scanning analysis to calculate the gray value. Each experiment was repeated 3 times with different samples.

mRFP-GFP-LC3 analysis. HSC-T6 were seeded into 96-well plates according to the manufacturer's instructions, experimental grouping as above. When the cell confluence rate reached 50%, mRFP-GFP-LC3 lentivirus was transfected into HSC-T6. After 12 h of culture, the old medium was removed. Then add the TEG and Baf-A1 intervene the HSC-T6 for 24 h. Red fluorescent protein (RFP) and green fluorescent protein (GFP) were observed using a fully automated fluorescence inverted microscope system. Each experiment was repeated 3 times with different samples.

Immunohistochemistry. After fixed in 5% paraformaldehyde solution for 24 h, the rat liver was embedded in 4-μm paraffin, routinely sectioned and dewaxed by gradient. Single staining was performed using rabbit anti-rat antibodies: LC3 and Beclin1. Positive staining areas were quantified using Image J software.

RNA extraction and quantitative real-time PCR. The total RNA was extracted from HSC-T6 using Trizol reagent. The cDNA was synthesized using a reverse transcription kit according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was used to detect the expression of the LC3-II, Beclin1, AMPK and mTOR genes. GAPDH as the reference gene. All samples were detected by real-time PCR using ABI 7500 and repeated three times independently.

Statistical analysis. Collected data were treated by 22.0 software using one-way analysis of variance (ANOVA) followed by Tukey's test contrasts and conveyed as mean ± standard deviation. Difference was significant at $P < 0.05$.

Results

TEG attenuate CCl_4-induced liver fibrosis and inhibit HSCs activation. The HE and

Masson staining images showed, compared with the normal group, the model group intertwined complex blue-green reticular streaks. In TEG group, whatever, the fibrous tissue proliferation in the tube area was reduced, the fiber spacing was narrowed, and the formation of inflammatory fat vacuoles was significantly reduced (Figure 1-A).

The proliferation of HSCs was detected by CCK-8 kit. Compared with the control, TEG inhibited cell proliferation. The proliferation rate of the TEG + Baf-A1 group was the lowest (Figure 2-A).*TEG reduce the production of autophagosomes and autophagy lysosome in vivo and vitro.* The HSCs and liver tissues were collected and observed under transmission electron microscopy. *In vitro*，we found not only autophagosomes wrapped necrotic organelles and proteins, but the autophagosomes have been necrotized in the combination drugs group. The red arrows indicate the autophagosomes and autophagososomes (Figure 2-B). Compared with normal liver tissue, many autophagosomes appeared in the model group. After TEG treatment, the number of autophagosomes was decreased (Figure 2-C).

In HSCs, acridine orange staining shows the red fluorescent region was exceptionally obvious. The cell morphology was intact in the control group. Nevertheless, the TEG + Baf-A1 group had only weak red fluorescence (Figure 3-A). All above indicated that autophagy has occurred in fibrosis rats or HSCs. However, after TEG treatment, autophagy is inhibited.

Effects of TEG on autophagy proteins LC3-II and Beclin1 in liver tissue and HSCs. The results of IHC (Figure 1-B) showed, compared with the normal group, the positive regions of LC3 and Beclin1 protein were increased in the liver fibrosis model group. After TEG treatment, the expression of LC3-II and Beclin1 were decreased in rats. The positive region of LC3 and Beclin1 protein was the least expressed in TEG+ Baf-A1 group. Western blot results (Figure 4-A) showed that the expression trends of LC3 and Beclin1 were similar to those of immunohistochemistry. *In vitro*, TEG also inhibited LC3-II and Beclin1 expression (Figure 4-B). We investigated the double drugs or single drug to interfere HSCs. The results also showed drugs have reduced

LC3-II and Beclin1. Anyway, TEG inhibited autophagy proteins were greater than double drugs or single drug in HSCs (Figure 5-A).*TEG inhibit autophagic flow by reducing autophagosome in HSCs.* After TEG or Baf-A1intervene HSCs, the LC3-II in the TEG + Baf-A1 group was lower than in the Baf-A1 group (Figure 5-B). The mRFP-GFP-LC3 double fluorescence results showed that, compared with the control, the other three groups appeared obvious green fluorescence, among of which the TEG + Baf-A1 group had the strongest green fluorescence. The Merge image also emerged the yellowish fluorescence in the combination drugs group(Figure 3-B).

TEG inhibit HSCs autophagy via AMPK-mTOR signaling pathway. The RT-PCR results showed that, compared with the control group, the TEG inhibited LC3, Beclin1, AMPK and mTOR mRNA levels in HSCs, GAPDH as an internal reference gene (Figure 6-A). Meanwhile, after intervention the HSCs, western blot results showed that the expression of p-AMPK and p-mTOR was decreased and increased, respectively (Figure 6-B). It demonstrated that TEG inhibit autophagy by the AMPK-mTOR pathway in HSCs.

Discussion

Inhibiting HSCs proliferation or converting HSCs from activation to quiescence that becomes an important strategy at against fibrosis[28]. Our previous works demonstrated that combination therapy with Taurine, EGCG and Genistein exhibits a protective effect against liver fibrosis *in vivo and vitro*[27, 29]. Similarly, in the present study, the histological and cytological data clearly showed combination therapy could alleviate liver damages, decrease collagen deposition, and inhibit cell proliferation. All which indicated that combination therapy showed a significant anti-fibrosis.

Studies have shown that HSCs activation is associated with autophagy which promotes catabolism of lipids in fibroblasts to maintain energy balance and renews certain organelles[30]. Thereby, blocking autophagy can inhibit HSCs proliferation and reduce liver fibrosis. Denardin[31]reported that the extract of purple cherries could

eliminate liver fibrosis by inhibiting autophagy and caused the death of activated HSCs. Seo[32]found that phospholipase D1 inhibited collagen I accumulation to achieve anti-fibrosis by inducing autophagic in HSCs. In this research, on transmission electron microscopy, there were many autophagosomes in liver fibrosis rats and activated HSCs, but the number of autophagosomes decreased after TEG treatment. The same results showed on acridine orange staining. We also observed the cell proliferation was hindered in HSCs and the damaged liver tissue was repaired in rats, respectively(results from HE and Masson). From above results, we can speculate that liver fibrosis is associated with autophagy and TEG may inhibit HSCs proliferation by blocking autophagy. In addition, our research found that combination drugs inhibited autophagy in HSCs more than double or alone drugs. So, it was further shown that the combination drugs anti-fibrosis is closely related to autophagy.

Autophagy is a cyclic process that involves various formation, includes phagocytic vacuoles, autophagosomes and autophagic lysosomes degradation and so on[33]. LC3 and Beclin1 are markers for monitoring autophagy[34], and involves in the autophagosomes formation[35]. After treated combination drugs, the expression of LC3-II and Beclin1 were significantly decreased in HSCs. It indicated that the combination drugs could inhibit autophagosomes synthesis. Normally, the expression level of intracellular LC3-II represents the total amount of synthetic autophagosomes and Autophagy lysosome[36]. At present, the autophagy assay of Bafilomycin A1(Baf-A1) is the most conventional and recognized method. Due to the effective inhibition of autophagosomal degradation by Bafilomycin A1, LC3B-II detected by Western blot only represented the sum of the synthesized autophagosomes and autophagosomes under the condition of using Bafilomycin A1. Bafilomycin A1 is short for Baf-A1. In HSCs, the LC3-II expression in the TEG + Baf-A1 group was smaller than in Baf-A1 group. It indicates that TEG reduced autophagosome accumulation. *In vivo*, comparing with control group, the results of IHC and Western blot showed LC3 and Beclin1 were significantly increased in liver fibrosis rats. After the rats were treated with TEG or autophagy inhibitor, the LC3 and Beclin1 decreased and the impaired liver began to repair in fibrosis rats. According to *in vivo and vitro*,

the TEG slow down liver fibrosis by inhibiting HSCs autophagy in rats.

In order to explore the TEG impact on HSCs autophagy, we used mRFP-GFP-LC3 (a mRFP/GFP fusion protein) double fluorescence to assay autophagy. mRFP-GFP-LC3 is used to detect autophagic flow which can monitor the dynamic changes of autophagy by labeling and tracking LC3[37, 38]. When autophagy is activated, the GFP fluorescence signal is quenched after entering lysosome, but mRFP is still fluoresce[39]. Observing the intensity changes of RFP and GFP fluorescence can accurately determine the autophagy activity of cells[40]. In our experiment, GFP fluorescence quenching was reduced after TEG incubated HSCs. This implied that the drugs interfered activation of HSCs autophagy.

During HSCs are activated from a quiescent state, HSCs need to degrade lipid droplets to provide energy[41]. AMPK plays a key role in the regulation of energy metabolism[42]. Activated AMPK is able to inhibit mTOR activity, reduce cells energy consumption and ensure the energy supply of HSCs[13]. The existing researchers have found that AMPK-mTOR signaling pathway as the autophagy regulatory core[43]. In this experiment, Compound C, AMPK inhibitor was used as a control to observe TEG regulate AMPK-mTOR pathway in HSCs. The results found that combination drugs reduced the expression of AMPK and mTOR phosphorylation protein which indicate that TEG inhibited AMPK-mTOR pathway in HSCs. Meanwhile, TEG also reduced LC3 and Beclin1 gene expression. All these indicate that TEG inhibits HSCs autophagy through the AMPK-mTOR signaling pathway.

Taken together, *in vivo and vitro*, after TEG intervention, HSCs proliferation and autophagy were inhibited, and the impaired liver tissue turned to repair. Our data strongly suggested that TEG could protect liver against autophagosomes through inhibiting the AMPK-mTOR pathway, which should be the therapeutic mechanisms of combination therapy against liver fibrosis. In conclusion, our studies highlight the importance of converting HSCs from activation to quiescence by inhibiting cell autophagy for treatment of liver fibrosis, and provide a more precise and comprehensive perspective for clarifying the roles of combination therapy as a

promising agent for treatment of liver fibrosis.

Acknowledgements

Not applicable.

Funding

The study was supported by the National Natural Science Fund (81860344, 81460128) and Foundation of High-Incidence-Tumor Prevention & Treatment (Guangxi Medical University), Ministry of Education (GK2018-03, GKE2017-ZZ09)

Availability of data and materials

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

Conflicts of interest

There are no conflicts of interests.

Acknowledgments

The study was supported by the National Natural Science Fund (81860344, 81460128) , Foundation of High-Incidence-Tumor Prevention & Treatment (Guangxi Medical University), Ministry of Education (GK2018-03, GKE2017-ZZ09) and Project to improve the basic scientific research ability of young and middle-aged teachers in Guangxi Universities (2019KY0127）.

Authors' contributions

ML and SPS designed the current study. SPS, SCH, ZZG, DLH, YLL ZH performed the experiments and acquired/analyzed the data. XYC and CFM analyzed experimental data. ML and SPS wrote the manuscript. The final version of the manuscript was read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure 1. A: The cells in the liver tissue of rats with liver fibrosis were turbid and necrotic. After combined drug intervention, the liver tissue slowly returned to normal (HE/Masson stains, ×100). **B**: The results showed that the expression of LC3 and Beclin1 in the model group was increased compared with the normal group, but LC3 and Beclin1 was significantly decreased after combination drugs treatment.

Figure 2. A: The HSCs were divided into 4 groups and cells proliferation were detected by CCK-8 kit. The results showed combination drugs or autophagy inhibitor could block HSCs proliferate. Values are expressed as the mean ± SD. $^{**}P < 0.01$ vs

control group; #$P < 0.05$ vs model group. **B:** After combination drugs treatment, the HSCs were observed under transmission electron microscopy. Two-layer membrane autophagosomes and monolayer membrane autophagy lysosome were observed in control group. But the autophagy lysosomes were necrotic in TEG group. **C:** Hepatic fibrosis rats appeared many autophagosomes in the liver, and the number of autophagosomes decreased after TEG treatment.

Figure 3. A: Acridine orange staining showed that the TEG + inhibitor group had weak red fluorescence in HSCs. **B:** After drugs intervene HSCs 24 h, autophagy of each group HSCs were analyzed by mRFP-GFP-LC3. The mRFP-GFP-LC3 double fluorescence results showed that, compared with the control, the other three groups appeared obvious green fluorescence, among of which the TEG + Baf-A1 group had the strongest green fluorescence.

Figure 4. A: The effects of combination drugs on the expression of autophagy proteins LC3 and Beclin1 in liver tissues of rats by western blot. **B**: Combination drugs significantly inhibited the expression of LC3-II and Beclin1 in HSCs. All the results were expressed as the mean ± SD, ** P <0.01 vs control, ## P <0.01 vs model group, & P <0.05 vs TEG group.

Figure 5. A: The HSCs were divided into 8 groups: Control, TEG, T, E, G, TE, TG, and EG group. Combination three drugs (TEG) inhibited autophagy marker proteins were greater than double drugs (TE, TG or EG) or single drug (T, E or G) in HSCs. **B:** Combination drugs inhibit autophagic flow by reduce autophagosome production in HSCs. After drugs intervene HSCs 24 h, autophagy marker protein of each group HSCs were analyzed by Western blot. The expression of LC3-II in the TEG + Baf-A1 group was smaller than that Baf-A1 group. All the results were expressed as the mean ± SD, ** P <0.01 vs control, ## P <0.01 vs model group, & P <0.05 vs TEG group.

Figure 6. A: Combination drugs inhibit HSCs autophagy via AMPK-mTOR signaling pathway. RT-PCR results showed that the combination drugs inhibited the expression of LC3, Beclin1, AMPK and mTOR mRNA levels in HSCs. The expression of the above four genes in the TEG + compound C group were lower than that compound C group. **B**:Western blot results showed that the combination drugs can affect p-AMPK

and p-mTOR expression in HSCs. p-AMPK and p-mTOR were lowest in the combination drug + compound C group. All the results were expressed as the mean ± SD, *** P <0.001 vs control, ## P <0.01 vs TEG group.

Figures

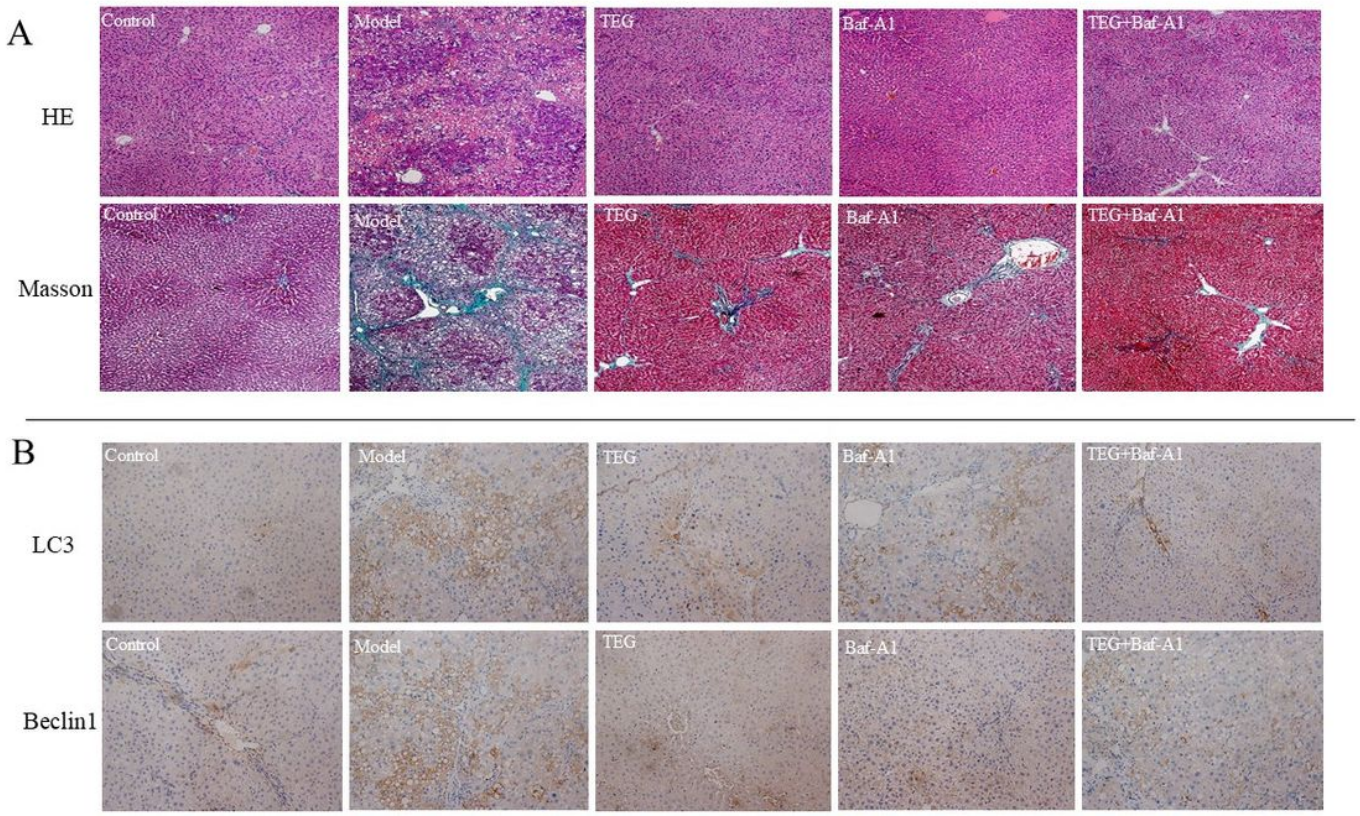

Figure 1

A: The cells in the liver tissue of rats with liver fibrosis were turbid and necrotic. After combined drug intervention, the liver tissue slowly returned to normal (HE/Masson stains, ×100). B: The results showed that the expression of LC3 and Beclin1 in the model group was increased compared with the normal group, but LC3 and Beclin1 was significantly decreased after combination drugs treatment.

Figure 2

A: The HSCs were divided into 4 groups and cells proliferation were detected by CCK-8 kit. The results showed combination drugs or autophagy inhibitor could block HSCs proliferate. Values are expressed as the mean ± SD. **P < 0.01 vs control group; #P < 0.05 vs model group. B: After combination drugs treatment, the HSCs were observed under transmission electron microscopy. Two-layer membrane autophagosomes and monolayer membrane autophagy lysosome were observed in control group. But the autophagy lysosomes were necrotic in TEG group. C: Hepatic fibrosis rats appeared many autophagosomes in the liver, and the number of autophagosomes decreased after TEG treatment.

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A: Acridine orange staining showed that the TEG + inhibitor group had weak red fluorescence in HSCs. B: After drugs intervene HSCs 24 h, autophagy of each group HSCs were analyzed by mRFP-GFP-LC3. The mRFP-GFP-LC3 double fluorescence results showed that, compared with the control, the other three groups appeared obvious green fluorescence, among of which the TEG + Baf-A1 group had the strongest green fluorescence.

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A: The HSCs were divided into 8 groups: Control, TEG, T, E, G, TE, TG, and EG group. Combination three drugs (TEG) inhibited autophagy marker proteins were greater than double drugs (TE, TG or EG) or single drug (T, E or G) in HSCs. B: Combination drugs inhibit autophagic flow by reduce autophagosome production in HSCs. After drugs intervene HSCs 24 h, autophagy marker protein of each group HSCs were analyzed by Western blot. The expression of LC3-II in the TEG + Baf-A1 group was smaller than that Baf-A1 group. All the results were expressed as the mean ± SD, ** P <0.01 vs control, ## P <0.01 vs model group, & P <0.05 vs TEG group.

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