Original Article

Mechanism of Allicin for Improvement of Hepatic Fibrosis by Regulating Autophagy

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ABSTRACT

Objectives: To explore the effects of allicin on the expression of mitochondrial autophagy-related proteins in liver tissues of hepatic fibrosis (HF) rats. **Materials and Methods:** 45 SD rats were randomly divided into Normal, Model, Low, Middle and High groups. The HF rat model was replicated intraperitoneal injection of carbon tetrachloride and ethanol gavage for 4 weeks. Detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rat serum; HE and Masson staining to observe pathological changes; Evaluating LC 3B protein expression by Immunofluorescence; Western Blotting and qRT-PCR methods were used to detect PINK1, Parkin, Beclin 1, and LC3 gene and protein expression levels in rat liver tissues. **Results:** Compared with the Normal group, the degree of HF in the model group was obvious, the volume fraction of collagen increased significantly ($p<0.001$), the LC 3B protein expression significantly up-regulation ($p<0.001$), and the rat serum ALT and AST increased significantly ($p<0.001$, respectively). Up-regulation of PINK1, Parkin, Beclin 1, and LC 3B mRNA and protein expression and LC 3II/LC 3I ratio in liver tissues ($p<0.001$, respectively); Compared with the model group, Allicin treated groups had reduced HF degree, collagen volume fraction decreased significantly ($p<0.05$, respectively), LC 3B protein was significantly reduced ($p<0.05$, respectively), and liver tissue PINK1, Parkin, Beclin 1, and LC3B mRNA and protein expressions, and LC 3II/LC 3I ratio were down-regulated ($p<0.05$, respectively). **Conclusion:** Allicin could improve hepatic fibrosis. The mechanism might be related to down-regulating the expression of PINK1, Parkin, Beclin 1, and LC 3 gene and proteins and interfering with the level of mitochondrial autophagy

Keywords: Allicin, HF, PINK1, Parkin, Autophagy.

INTRODUCTION

Hepatic fibrosis (HF) is a process of scar formation and liver repair in response to several forms of chronic hepatic injury, which, as a clinically frequently-occurring disease, is characterized by abnormal extracellular matrix (ECM) deposition and represents an inevitable shift from chronic hepatic injury to cirrhosis or liver cancer.[1] Treatment of HF can reduce the incidence of hepatic cirrhosis and prevent liver cancer through a reversal of the disease process.[2] Despite the fact that HF can be reversed by causal and anti-HF treatment, no biological or chemical agent is currently available in clinical application. Garlic (Allium sativum) belongs to the amaryllis family (Amaryllidaceae), and its bulb has been traditionally used for disease control and prevention.[3] In recent years, the chemical pharmacology of garlic has received extensive attention and achieved real breakthroughs.[4] Allicin, also known as diallylthiosulfinate ($C_6H_{10}S_3$), has a relative molecular weight of 178.3, and as an active compound in garlic, it is derived from the amino acid alliin in a reaction catalyzed by the enzyme alliinase.[5] The bioactivities of allicin have been demonstrated by numerous studies, including antioxidant, anti-pathogenic microorganism, anti-inflammatory, free radical scavenging, anti-cancer cell proliferation, apoptosis regulating, hypocholesteremic and antithrombotic activities.[6] Mitochondrial autophagy (mitophagy) mediated by the

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PINK1/Parkin signaling pathway is a selective cellular activity involved in the development of HF and liver cancer as well as the selective clearance of damaged mitochondria to maintain cellular homeostasis; therefore, HF may be treated by regulating mitophagy via the PINK1/Parkin pathway.[7-8] Melatonin is reported to have antifibrotic properties as it plays a regulatory role in mitophagy in HF.[9] Fine particles less than 2.5 microns (PM2.5) regulate mitophagy via the PINK1/Parkin pathway, and mitophagy in turn supports the activation of hepatic stellate cells (HSCs) through degradation of lipid droplets to accelerate the development of HF.[10] Currently, further studies are needed to clarify the molecular mechanisms of mitophagy and HF.[11] The molecular mechanism responsible for the therapeutic effect of allicin on HF is not fully understood. Against this backdrop, an HF rat model was established and treated by allicin in this study to investigate the effect of allicin on HF from the perspective of mitophagy, which hopefully can provide a research basis for the clinical treatment of HF.

MATERIALS AND METHODS

Laboratory animals

Male SPF Sprague-Dawley rats (n =45) were provided by the Laboratory Animal Center of Nantong University. Laboratory Animal Certificate No.: SCXK (S) 2014-0001. Body Weight: (160±20) g.

Drugs and Reagents

Xindi Allitride Injection (Jinan Limin Pharmaceutical Co., Ltd., H20046064); the mother liquid allitride injection was diluted to concentrations of 10%, 5%, and 1% with sterile sesame oil; chloral hydrate (Leagene Biotech Co., Ltd., Batch No.: 1113A20); PFA (Leagene Biotech Co., Ltd., Batch No.: 1125A20); TRIpure Reagent (Beijing Aidlab Biotechnologies Co., Ltd., Batch No.: 272026AX); EvaGreen Express 2×qPCR MasterMix-ROX, oneScript™ cDNA Synthesis Kit (Abm, Canada, Batch No.: G234). Alanine Transaminase (ALT) Assay Kit (Nanjing Jiancheng Bioengineering Institute, Batch No.: 009-2); Aspartate Transaminase (AST) Assay Kit (Nanjing Jiancheng Bioengineering Institute, Batch No.: C010-2); PINK1 and Parkin primary antibodies (Thermo, USA, Batch No.: MA51153, PA532539); Beclin 1 and LC3B primary antibodies (Abcam, UK, Batch No.: ab231341, ab51520); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Nanjing EnoGene, Batch No.: E1C604-2); ECL Chemiluminescence Kit (Beyotime, Batch No.: P0018A); HRP-labeled anti-rabbit IgG secondary antibody (goat), RIPA lysis buffer, restained protein marker, and BCA Protein Concentration Assay Kit (Nanjing EnoGene, Batch No.: E8HA1001-100, E1WP106, E1WP402, E1WP2011); developer and fixing solutions were purchased from Nanjing EnoGene Biotech Co., Ltd. Primers were synthesized by Shanghai Ruijie Co., Ltd. Details of the primers are as shown in Table 1.

Table 1: Primer information.

Gene name	Primer sequence	Size (bp)
β-actin	F: 5'-TGCTATGTTGCCCTAGACTTCG-3'	240
	R: 5;-GTTGGCATAGAGGTCTTTACGG-3'	
Parkin	F: 5'-CAACAGGTACCAGCAGTATG-3'	162
	R:5'-CGGCAGAAAACGAACCCACA-3'	
PINK1	F:5'-CAGAAAAACAAGCAAGTGTC-3'	203
	R:5'-CCCTTTGAGACGACATCTGG-3'	
Beclin-1	F:5'-CTTCAATGCGACCTTCCATATC-3'	258
	R:5'-CCAGAACAGTACAACGGCAACT-3'	
LC 3B	F: 5'-AGCGATACAAGGTGAGAAGC-3'	140
	R:5'-CAGGAGGAAGAAGGCTTGGTTA-3'	

Instruments

TL998-IV Real-Time PCR Analyzer (Xi'an Tianlong Science and Technology Co., Ltd.); Nano-200 Ultra-Micro Nucleic Acid Protein Analyzer (Hangzhou Allsheng Instrument Co., Ltd.); TC1000-G PCR Gradient Thermal Cycler and D3024R High Speed Refrigerated Micro Centrifuge (SCILOGEX, USA); BSA224S Electronic Analytical Balance (Sartorius, Germany); EPS-300 Electrophoresis Apparatus, VE-186 Transfer Protein Electrophoresis Tank, and VE-180 Micro Vertical Electrophoresis Tank (Shanghai Tanon Science and Technology Co., Ltd.); TY-80B Transferable Decoloration Shaker (Jintan Ronghua Instrument Manufacture Co., Ltd.); WH 986 Silent Mixer (Qilinbeier Instrument Manufacturing Co., Ltd.); UV5100 UV Visible Spectrophotometer (Shanghai Metash Instruments Co., Ltd.); MK3 Enzyme Mark Instrument (Thermo, USA).

Methods

Grouping, modeling, route of administration and sample collection

This study adopted a modeling method by reference to a previous study.[12] The rats received 1.5 mL/kg 50% CCl4 in olive oil solution twice per week via intraperitoneal injection and 10 mL/kg 30% ethanol every other day via gastric lavage for 8 weeks. The normal group was given sesame oil and normal saline in equivalent

volumes during the same period. Following model replication, drug intervention was performed via gastric lavage at a dose of 10 mL/kg. The normal group and the model group were administered with normal saline via gastric lavage for 4 weeks. After intervention and fasting for 12 hr, each rat was intraperitoneally injected 0.3 g/kg 10% chloral hydrate for anesthesia to collect blood samples from the abdominal aorta for centrifugation at 4°C, 3000 r/min for 10 min to harvest blood serum, which was refrigerated at -80°C for future use. Part of the harvested liver tissue was fixed in 4% paraformaldehyde (PFA) and 2.5% glutaraldehyde (GA) for general pathology, Masson's trichrome staining, and LC3B immunofluorescence (IF) staining. The rest of liver tissue was stored at -80°C for western blotting (WB) and qRT-PCR.

Liver tissue pathology

The PFA-fixed liver tissue was embedded in paraffin to prepare tissue sections for HE and Masson's trichrome staining for liver tissue pathology using an optical microscope.

Serum liver function tests

An automatic biochemical analyzer was used for the determination of ALT and AST levels.

LC3B IF staining

After dewaxing and hydration, the paraffin-embedded liver tissue sections from each group were soaked in 0.01 mol/L citrate buffer solution for antigen retrieval for 15 min, which was followed by natural cooling. Then, 3% hydrogen peroxide (v/v) was added to block endogenous peroxidase activity for 15 min, and normal goat serum was used for blocking for 15 min. Subsequently, the primary antibody LC3B (diluted at 1:800) was added for overnight incubation at 4°C, the biotinylated anti-rabbit IgG secondary antibody (goat) was added for incubation for 30 min at 37°C, and the HRP-labeled streptavidin was added to incubate for another 30 min at 37°C before IHC staining with 3,3'-diaminobenzidine (DAB), after stain with hematoxylin, differentiation with glycolic acid, treatment with dilute ammonia solution, stepwise dehydration with ethanol, clearing with xylene, and mounting with neutral gum. A fluorescence microscope (×200) was used for observation of the staining procedures. The open-source software ImageJ was used for image analysis.

Determination of PINK1, Parkin, Beclin 1, and LC3 protein expression by the WB assay

Liver tissue was harvested for sample preparation and protein determination. Total protein extraction was performed for protein electrophoresis and trarsmembrane before overnight incubation at 4°C with corresponding target protein primary antibodies (PINK1, Parkin, Beclin 1, LC3, or GAPDH, 1:1000). After washing, the membrane was added to the corresponding secondary antibody (1:5000) to incubate at room temperature for 1 hr, followed by washing and treatment with the developer and fixing solution. The software Adobe Photoshop CS5 was employed to analyze the net optical density of each target band with GAPDH as the internal control.

Determination of PINK1, Parkin, Beclin 1 and LC3B mRNA expression by qRT-PCR

TRIpure reagent was applied to isolate total RNAs. The OneScript™ Reverse Transcriptase Kit and the OneScript™ cDNA Synthesis Kit were used for RNA reverse transcription, followed by PCR at 42°C for 50 min, at 85°C for 5 min. Then, cDNAs were stored at 4°C for PCR amplification after ice cooling. PCR amplification conditions were 95°C for 10 min, 95°C for 5 sec, and 60°C for 30 min, 41 cycles. The expression levels of all target genes were determined by qRT-PCR, and the relative content was calculated with the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The software SPSS 25.0 was used for statistical analysis. Results were expressed by "Mean±SD". Comparison of means among three or more groups was examined by one-way analysis of variance (ANOVA); comparison between two groups was examined by the LSD test. Comparison of ranked data was examined by the nonparametric test. $p<0.05$ was indicative of a level of statistical significance, and $p<0.01$ was considered statistically highly significant.

RESULTS

HE staining

Results of HE staining in the model group suggested the formation of denser, thicker fibrous tissues at the portal area, which extended outwards from the portal area or the central veins; in addition, fibrous septum formation was observed in a few rats. Compared with the model group, the allicin intervention groups (low-, middle-, and high-dose) exhibited regression of fibroplasia Figure 1.

Masson's trichrome staining

In the normal group, the hepatic lobules remained structurally intact, while few blue stained collagen fibers were found in the portal area and the central veins.

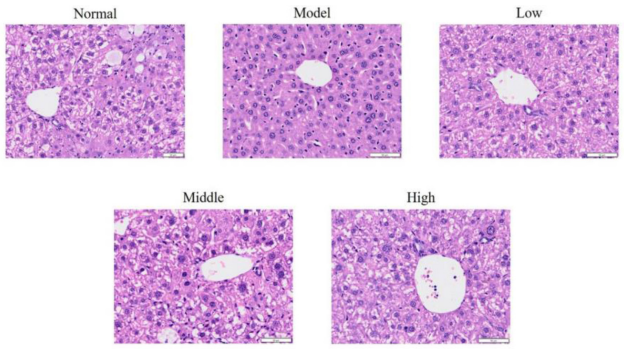

Figure 1: Effects of Allicin on the pathological changes of liver tissues by HE staining (200×).

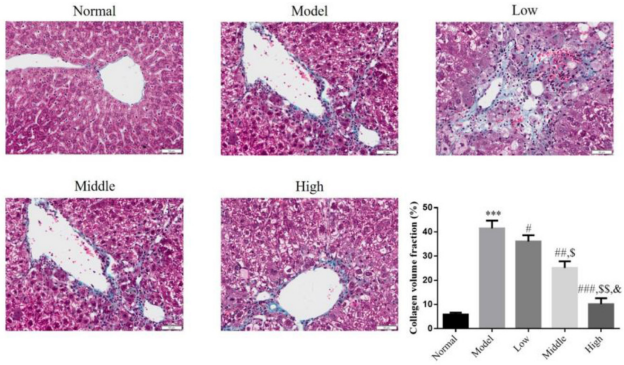

Figure 2: The Collagen volume fraction of difference groups in liver tissues by Masson staining (200×).

***: p<0.001, compared with Normal group; #: p<0.05, ##: p<0.01, ###: p<0.001, compared with Model group; $: p<0.05, $$: p<0.01, compared with Low; and: p<0.05, compared with Middle.

Compared with the normal group, the model group had a number of thicker, larger, blue stained collagen fibers and visibly wider fibrous septa in the portal area and the central veins. Compared with the model group, the intervention groups had thinner, smaller, blue stained collagen fibers, and the fibrous septa were substantially narrowed. See Figure 2. The collagen volume fraction (CVF) of the fibrous tissue in the liver (area of blue stained collagen fibers / total tissue area × 100%) was calculated using Image J. Compared with the normal group, the model group had a significantly greater CVF (p<0.001, Figure 2); compared with the model group, the low-, middle-, and high-dose intervention groups experienced significant reductions in their CVFs (p<0.05, respectively, Figure 2).

Effect of Allicin on the Liver Function of HF Rats

ALT and AST levels were significantly increased in the model group as compared with the normal group (p<0.001, Table 2) and markedly reduced in the low-, middle-, and high-dose intervention groups when compared with the model group (p<0.05, respectively, Table 2).

Table 2: Effects of Allicin on serum ALT and AST in HF rats (Mean±SD, *n*=9).

Group	Dose (g/kg)	ALT(U/L)	AST(U/L)
Normal	-	39.43±2.88	106.84±3.63
Model	-	87.87±3.37***	226.87±14.44***
Low	5.00	77.65±3.58#	219.48±15.09#
Middle	10.00	65.64±3.28##,$	184.63±12.39##,$
High	20.00	45.60±2.66###,$$,&	132.81±11.01###,$$,&

***: p<0.001, vs. Normal; #, p<0.05, ##: p<0.01, ###: p<0.001, vs. Model; $: p<0.05, $$: p<0.01, vs. Low; &: p<0.05, vs. Middle.

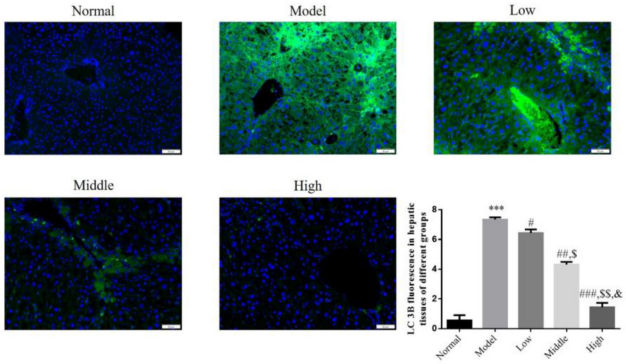

Figure 3: LC 3B fluorescence in hepatic tissues of different groups (200×).

***: p<0.001, compared with Normal group; #: p<0.05, ##: p<0.01, ###: p<0.001, compared with Model group; $: p<0.05, $$: p<0.01, compared with Low; and: p<0.05, compared with Middle.

Effect of allicin on LC3B protein expression in rat liver tissue

Compared with the normal group, the model group exhibited a significant increase in LC3B protein expression (p<0.001, Figure 3); following intervention with allicin, the LC3B protein expression levels were significantly reduced in the low-, middle-, and high-dose intervention groups as compared with the model group (p<0.05, respectively, Figure 3).

Effect of allicin on PINK1, Parkin, Beclin 1, and LC3B mRNA expression in liver tissue

Compared with the normal group, the model group experienced significant increases in PINK1, Parkin, Beclin 1, and LC3B mRNA expression in the liver tissue (p<0.001, respectively, Figure 4); compared with the model group, the low-, middle-, and high-dose intervention groups showed decreases in PINK1, Parkin, Beclin 1, and LC3B mRNA expression (p<0.05, respectively, Figure 4).

Effect of allicin on PINK1, Parkin, Beclin 1, and LC3B protein expression in liver tissue

Compared with the normal group, the model group had elevated PINK1, Parkin, and Beclin 1 protein

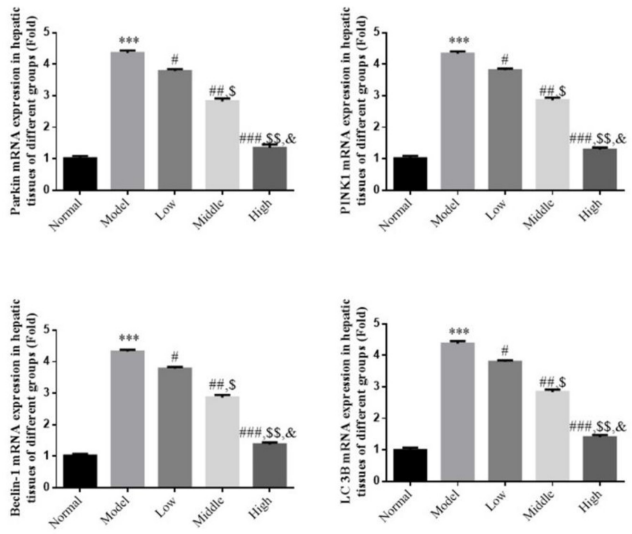

Figure 4: Relative gene expressions by qRT-PCR assay.

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with Low; and: $p<0.05$, compared with Middle.

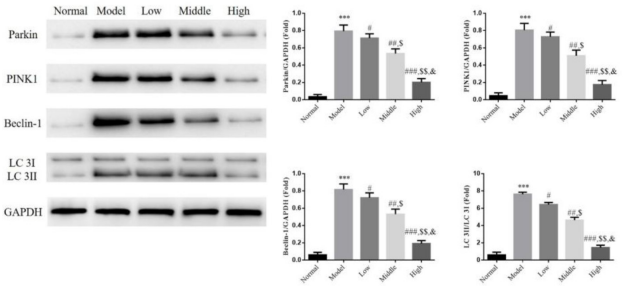

Figure 5: Relative protein expressions by WB assay.

***: $p<0.001$, compared with Normal group; #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$, compared with Model group; $: $p<0.05$, $$: $p<0.01$, compared with Low; and: $p<0.05$, compared with Middle.

expression and a greater LC3-I/LC3-II ratio ($p<0.001$, respectively, Figure 5); compared with the model group, the low, middle, and high-dose intervention groups experienced decreases in PINK1, Parkin, and Beclin 1 protein expression and the LC3-I/LC3-II ratio ($p<0.05$, respectively, Figure 5).

DISCUSSION

Autophagy is a self-survival mechanism. During the development of HF, HSCs are activated to produce massive collagen fibers with autophagy as the source of energy supply; liver regeneration largely depends on the autophagy of necrotic death of liver cells.[13] Mitophagy refers to the selective autophagy of mitochondria, which guarantees effective mitochondrial quality control and cell homeostasis through degradation of mitochondria of structural and functional abnormalities by autophagosome-lysosome fusion[14-15] to support cell function, survival, and regeneration. Mitophagy occurs in the process of early autophagy[16] to maintain mitochondrial structure and function,[17] while excessive autophagy may bring damage to mitochondrial function and induce cell death.[18] Mitochondrial fission and fusion play a critical role in the degradation of diseased mitochondria during mitophagy to protect the liver tissue.[19] Multiple genes and proteins are involved in the regulation of mitophagy.[20] Autophagy-related genes eventually come into play in the form of autophagosomes, which represents one of the morphologic characteristics of autophagy.[21] Beclin 1 plays a critical regulatory role in the process of autophagy.[22] LC3II targets autophagosomes to mitochondria to trigger mitophagy, which reveals the number of autophagosomes [23] and the close association with autophagosomal activity.[24] According to the determination of Beclin 1 and LC3B protein expression and the LC3-I/LC3-II ratio, it was found that in the liver tissue of HF rats, Beclin 1 and LC3B protein expression were increased, while Beclin 1 and LC3B mRNA expression were elevated to the levels higher than those in the normal group; in the intervention groups, particularly the high-dose intervention group (10%), allicin was shown to reduce Beclin 1, LC3B, and LC3-I/LC3-II when compared with the model group.

Mitophagy is regulated by the typical PINK1/Parkin signaling pathway[25] that has a close association with damaged mitochondria.[26] PINK1, as an outer mitochondrial membrane protein, is abundantly expressed in mitochondria and serves as a molecular receptor for mitochondrial damage, monitor and regulator for mitophagy and key player in the occurrence of mitophagy.[27] Structurally damaged mitochondria can lead to a decrease in the mitochondrial membrane potential and phosphorylation, activation and aggregation of PINK1 on the outer mitochondrial membrane to recruit Parkin molecules, activate Parkin protein and initiate mitophagy.[28] The activated Parkin protein enables the ubiquitination of diverse mitochondrial protein substrates.[25] The ubiquitinated substrates produce mitochondrial autophagosomes by binding to LC3-II.[23] In short, Parkin activation represents a mechanism of positive regulation for mitophagy. The expression levels of the mitophagy-related proteins PINK1 and Parkin in the liver tissue of HF rats were measured in this study, and the results showed that PINK1 and Parkin protein and mRNA expression were elevated in the model group, suggesting increased activation of mitophagy in HF rats; for the intervention groups, allicin was demonstrated to reduce PINK1 and

Parkin protein and mRNA expression, especially when a high concentration of 10% was administered.

CONCLUSION

In conclusion, the PINK1/Parkin pathway can be targeted for the treatment of HF considering the involvement of the PINK1/Parkin-mediated mitophagy in the development of disease. Allicin can suppress PINK1, Parkin, Beclin 1, and LC3B mRNA and protein expression in the liver tissue of HF rats and reduce the LC3-I/LC3-II ratio, which demonstrates the role of allicin to inhibit mitophagy, promote the biosynthesis of mitochondria and accelerate the recovery of liver tissue injury simultaneously and offers a new perspective and experimental basis for the treatment and research of HF.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

HF: Hepatic fibrosis; **ALF:** Alanine aminotransferase; **AST:** Aspartate aminotransferase; **GA:** Glutaraldehyde; **IF:** Immunofluorescence; **WB:** Western blotting; **ECM:** Extracellular matrix; **WB:** Western Blot.

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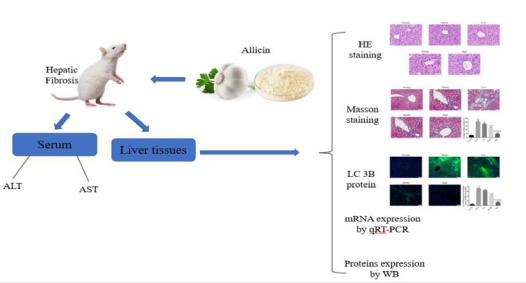
PICTORIAL ABSTRACT



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SUMMARY

The present research evaluated and revealed the treatment effect of Allicin in hepatic fibrosis in rats. Allicin, also known as diallylthiosulfinate ($C_6H_{10}S_3$), has a relative molecular weight of 178.3, and as an active compound in garlic, it is derived from the amino acid alliin in a reaction catalyzed by the enzyme alliinase. In our present study, 45 SD rats were randomly divided into Normal, Model, Low, Middle and High groups. The HF rat model was replicated intraperitoneal injection of carbon tetrachloride and ethanol gavage for 4 weeks. Detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in rat serum; HE and Masson staining to observe pathological changes; Evaluating LC 3B protein expression by Immunofluorescence; Western Blotting and qRT-PCR methods were used to detect PINK1, Parkin, Beclin 1, and LC3 gene and protein expression levels in rat liver tissues. Depending on these results, Allicin could improve hepatic fibrosis. The mechanism might be related to down-regulating the expression of PINK1, Parkin, Beclin 1, and LC 3 gene and proteins and interfering with the level of mitochondrial autophagy.

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