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# Role of Autophagy in Liver Fibrosis with *Qi* Deficiency and Blood Stasis Via the Nrf2-Keap1-Are Signaling Pathway

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**Abstract**

**Background:** To investigate the mechanism underlying the role of the Nrf2-Keap1-Are oxidative stress pathway in liver fibrosis related to qi deficiency and blood stasis.

**Methods:** A total of 30 Sprague–Dawley rats were randomly divided into a normal group, a *qi* deficiency and blood stasis group, and a Fuzheng Huayu treatment group. After death, body weights and liver wet weights were measured and liver sections were stained with Sirius red. RT-PCR was used to detect the mRNA expression levels of α-SMA, Nrf2, Keap1, and β-actin, and western blotting was used to detect the protein expression levels of α-SMA, LC3II, P62, and LC3II.

**Results:** The Ishak score for liver fibrosis in the *qi* deficiency and blood stasis group was higher than that in the liver fibrosis group ($P < 0.05$) and decreased significantly following Fuzheng Huayu therapy ($P < 0.05$). As determined by PCR, α-SMA mRNA levels were highest in the *qi* deficiency and blood stasis group and were significantly higher than those in the treatment group ($P < 0.05$). Nrf2 and Keap1 mRNA expression levels were lowest in the *qi* deficiency and blood stasis group but increased significantly after treatment ($P < 0.05$). Western blotting showed that α-SMA and LC3II levels were highest in the *qi* deficiency and blood stasis group ($P < 0.05$) and decreased significantly after treatment ($P < 0.05$). The expression levels of P62, Nrf2, and Nqo1 were lowest in the *qi* deficiency and blood stasis group and increased significantly after treatment ($P < 0.05$).

**Conclusion:** LC3II can down-regulate the expression of P62 in liver tissues of rats with *qi* deficiency and blood stasis, thereby inhibiting the activation of the Nrf2-Keap1-Are antioxidant stress pathway and aggravating liver fibrosis. However, this process can be reversed by strengthening *qi* and activating blood circulation to alleviate blood stasis.

**Keywords:** *Yiqi Huayu* therapy, *Qi* deficiency and blood stasis, Liver fibrosis, Autophagy, LC3II, Nrf2-Keap1-Are signaling pathway

## Background

Liver fibrosis is a common pathological basis of multiple chronic liver diseases, including schistosomiasis, alcoholic, viral, and nonalcoholic fatty liver diseases. The blocking and reversal of liver fibrosis at an early stage is the key to the prevention and treatment of liver cirrhosis [1]. It is generally believed that the pathological changes in liver fibrosis result from the activation of hepatic stellate cells (HSCs), resulting in an imbalance in extracellular matrix synthesis and degradation in the liver and excessive deposition [2]. Recent studies have shown that both oxidative stress and autophagy pathways contribute to the progression of liver fibrosis by inducing HSC activation [3, 4]. The Nrf2-Keap1-Are signaling pathway in humans is the primary cellular pathway that protects normal cells from reactive oxygen species, oxidative stress, and exogenous damage. By regulating target genes, the Nrf2-Keap1-Are pathway contributes to the protection against viral hepatitis, drug-induced liver injury, alcoholic liver diseases, nonalcoholic fatty liver diseases, and liver cancer [5]. Our preliminary experiments have indicated that *Yiqi Huayu* therapy can inhibit autophagy and therefore decrease the degree of liver fibrosis, although the specific mechanism and pathways underlying its effects require further verification. There is molecular cross-talk between the Nrf2-Keap1-Are signaling pathway and autophagy [6]. The initiation and progression of liver diseases are closely related to oxidative stress. Since the activation of the Nrf2-Keap1-Are

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pathway can antagonize the activation of HSCs, it can also inhibit the progression of liver fibrosis [7]. Therefore, the autophagy-regulating Nrf2-Keap1-Are oxidative stress pathway is likely an important therapeutic target for liver fibrosis.

Traditional Chinese medicine may be advantageous for the treatment of liver fibrosis. It is based on the concept of an overall, holistic approach as well as syndrome differentiation, with a focus on multi-target, multi-link, and multi-channel mechanisms and therapeutic characteristics. In Chinese medicine, liver fibrosis is classified as "hypochondriac pain," "swelling," and "stagnation," and the basic pathogenesis is a "*qi* deficiency, blood stasis, and phlegm accumulation." The most common clinical presentations of patients with liver fibrosis are *qi* deficiency and blood stasis. This is supported by our previous results showing that patients with these symptoms often exhibit a more severe degree of liver fibrosis. Although previous research has indicated that Yiqi Huayu therapy has an anti-fibrotic effect [8], few studies have evaluated the mechanism by which *Yiqi Huayu* therapy regulates autophagy and anti-oxidative stress. Therefore, in this study, the effect and mechanism of action of *Yiqi Huayu* therapy on liver fibrosis due to a *qi* deficiency and blood stasis were evaluated, with a focus on the Nrf2-Keap1-Are signaling pathway, to provide a basis for the development of new approaches for prevention and treatment.

## Materials and Methods

### Reagents

Carbon tetrachloride was obtained from Sinopharm Chemical Reagent Co., Ltd. (batch number: 20160621; Beijing, China). Olive oil was obtained from Sinopharm Chemical Reagent Co., Ltd. (batch number: 20161111;Beijing,China). Fuzheng Huayu Capsules were obtained from Shanghai Huanghai Pharmaceutical Co., Ltd. (batch number: 161103; Shanghai, China). The following primers were used: α-SMA, 5′-TAAGGCGGGCTTTGCT-3′, 3′-CGGATACTTCAGGGTCAGG-5′; Nrf2, 5′-CCATTTACGGAGACCC-3′, 3′-CACTGTGCCCTTGAGC-5′; Keap1, 5′-TCCTCCAGCCCAGTCTTT-3′, 3′-CCGTGTAGGCGAACTCAAT-5′. The following antibodies were used: Anti-α-SMA (ab5694; Abcam, Cambridge, UK), Anti-Nrf2 (SC-722, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Anti-Nqo1 (ab28947; Abcam), Anti-SQSTM1/p62 antibody (ab56416; Abcam), Anti-LC3B (L7543; Sigma, St. Louis, MO, USA), Anti-GAPDH (mab5465; Lianke Biotech, Hangzhou, China).

### Animals

A total of 30 male Sprague–Dawley (SD) rats, weighing 150 ± 10 g, were obtained from the Experimental Animal Center of Zhejiang Chinese Medical University. Prior to the experiment, rats were raised under normal feeding conditions.

### Generation of rat models of *qi* deficiency and blood stasis

For the generation of rat models of *qi* deficiency and blood stasis, a 40% oil emulsion was prepared by mixing CCl4 and olive oil at a ratio of 4:6, which was injected subcutaneously into rats twice daily at a dose of 0.3 ml/100 g. At the beginning of the 4th week, rats in the *qi* deficiency and blood stasis group were subjected to a swimming fatigue test. During the test, the rats were placed in a large bucket with smooth walls filled with water at 10°C; rats were forced to swim continuously until they sank. The test was conducted twice daily for 2 weeks to generate the model. After 6 weeks, 10 rats from the *qi* deficiency and blood stasis group were randomly selected and sacrificed. Liver tissues were extracted and evaluated by hematoxylin and eosin staining. Liver fibrosis in these tissues was observed under a microscope to determine whether the model generation was successful. The degree of liver fibrosis was evaluated according to the Ishak score, where grade VI indicates severe fibrosis. Rats in the group with liver fibrosis with *qi* deficiency and blood stasis were then randomly divided into a control group and a treatment group.

### Preparation of Fuzheng Huayu capsules

Rats with liver fibrosis in the *qi* deficiency and blood stasis group were intragastrically administered 2 ml of normal saline once daily. Rats in the treatment group were administered Fuzheng Huayu capsules at a scaled dose according to the "equivalent dose" method for experimental animals. Dissolved in normal saline, the Fuzheng Huayu capsule was intragastrically administered at a rate of 0.5 g/(kg·d) once per day for 4 weeks. During the drug intervention process, various properties, such as the mental state, activity, hair, appetite, and stool traits, were observed and recorded.

### Animal groups

A total of 30 SD rats were included in the study. Rats were provided free access to water and fed adaptively for 7 days before the experimental phase.

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These 30 SD rats were randomly divided into the following 3 groups using the random number table method: 10 rats in the normal control group, 10 rats in the liver fibrosis with qi deficiency and blood stasis group, and 10 rats in the Fuzheng Huayu treatment group.

### Serum biochemical marker assay

Serum biochemical markers were not evaluated in this study. Pathological analyses of liver tissue samples were used.

### Histology

At the end of 8 weeks, liver tissue samples were embedded in paraffin, serially sectioned, and stained with Sirius red. The remaining liver tissue was frozen in liquid nitrogen.

### RT-PCR detection

An RT-PCR assay was used to detect the mRNA expression of *α-SMA*, *Nrf2*, *Keap1*, and *β-actin*.

### Western blot analysis

A western blot analysis was used to detect the expression of α-SMA, LC3II, P62, Nrf2, Nqo1, and GAPDH.

### Statistical analyses

Statistical analyses were performed using SPSS22.0. Data are expressed as means ± standard deviation (x ± s). Groups were compared by one-way analysis of variance (LSD). $P < 0.05$ indicated statistical significance.

## Results

### Rat body weight and liver wet weight

Rats in the qi deficiency and blood stasis group had the lowest body weight and the highest liver wet weight ($P < 0.05$). Although there was no difference in body weight ($P > 0.05$), the liver wet weight was significantly lower in the treatment group than in the *qi* deficiency and blood stasis group ($P < 0.05$). The results are summarized in Table 1.

### Liver tissue pathology

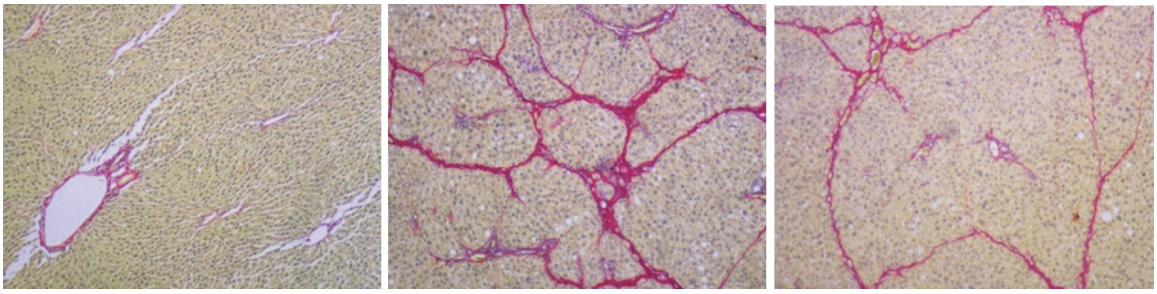
**Sirius red staining results.** In the normal group, the liver cells exhibited a regular arrangement, without fibrosis. The liver fibrosis groups exhibited obvious red collagen fibers. In the *qi* deficiency and blood stasis group, obvious red collagen fibers were visible. In the treatment group, there were fewer visible red collagen fibers. (Fugure 1, magnification: 200×).

### Liver fibrosis Ishak scores

Inflammation, liver fibrosis, and Ishak scores were consistently higher in the *qi* deficiency and blood stasis group than in the liver fibrosis group ($P < 0.05$). In addition, following Fuzheng Huayu therapy, these scores all decreased to a certain extent ($P < 0.05$).

**Table 1 Comparison of body weights and liver wet weights among groups (g, n = 10, x ± s).**

| Group | Body Weight | Liver Wet Weight |
|---|---|---|
| Normal group | 423.40 ± 30.18 | 10.95 ± 0.85 |
| *Qi* deficiency and blood stasis group | 375.80 ± 24.36*** | 11.31 ± 1.13 |
| Treatment group | 356.10 ± 20.45 | 10.34 ± 1.13# |



A B C

**Figure 1 Liver histopathology in each group**

A: Normal group, B: *Qi* deficiency blood stasis group, C: treatment group.

The results are listed in Table 2.

**mRNA expression**

The mRNA expression of *α-SMA* was highest in the qi deficiency and blood stasis group, and was significantly reduced after Yiqi Huayu therapy ($P < 0.05$). In contrast, the levels of *Nrf2* and *Keap1* were highest in the *qi* deficiency and blood stasis group, and increased significantly after Yiqi Huayu therapy ($P < 0.05$). The results are listed in Table 3.

**Western blotting**

**Relative protein expression levels.** The expression levels of α-SMA and LC3II were highest in the *qi* deficiency and blood stasis group and decreased substantially following Yiqi Huayu therapy ($P < 0.05$). In contrast, the expression levels of P62, Nrf2, and Nqo1 were highest in the *qi* deficiency and blood stasis group and increased significantly after Yiqi Huayu therapy ($P < 0.05$). The results are listed in Table 4.

**Western blotting results.** Bands for each protein in the western blotting analyses are shown below for the normal group, the *qi* deficiency and blood stasis group, and the treatment group (from left to right). GAPDH was used as the internal reference (Figure

**Table 2 Comparison of the degree of liver fibrosis among groups (score, n = 10, x ± s)**

| Group | Inflammation score | Liver fibrosis score | Total score |
|---|---|---|---|
| Normal group | 0 | 0 | 0 |
| *Qi* deficiency and blood stasis group | $14.2 \pm 1.7^{***}$ | $5.8 \pm 0.8^{***}$ | $20.0 \pm 1.6^{***}$ |
| Treatment group | $10.2 \pm 1.5^{***\#\#\#}$ | $2.7 \pm 0.9^{***\#\#\#}$ | $12.9 \pm 1.9^{***\#\#\#}$ |

$^{***}P < 0.001$ compared with the normal group; $^{\#}P < 0.001$ compared with the *qi* deficiency and blood stasis group.

**Table 3 Comparison of the mRNA expression of *α-SMA*, *Nrf2*, and *Keap1* among groups (n = 10, x ± s)**

| Group | *α-SMA* | *Nrf2* | *Keap1* |
|---|---|---|---|
| Normal group | 1.29 ± 0.04 | 1.14 ± 0.03 | 1.11 ± 0.03 |
| *Qi* deficiency and blood stasis group | $1.36 \pm 0.04^{*}$ | $1.09 \pm 0.02^{*}$ | $1.15 \pm 0.02^{*}$ |
| Treatment group | $1.25 \pm 0.01^{\#\#}$ | $1.16 \pm 0.02^{\#}$ | $0.17 \pm 0.01^{\#\#}$ |

Compared with the normal group, $^{*}P < 0.05$; compared with the qi deficiency and blood stasis group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

**Table 4 Comparison of the protein expression levels of α-SMA, LC3II, P62, Nrf2, and Nqo1 among groups (n = 10, x ± s).**

| Group | α-SMA | LC3II | P62 | Nrf2 | Nqo1 |
|---|---|---|---|---|---|
| **Normal Group** | 0.79 ± 0.10 | 1.84 ± 0.21 | 1.1 ± 0.23 | 1.81 ± 0.20 | 0.70 ± 0.12 |
| ***Qi* deficiency and blood stasis group** | $1.76 \pm 0.28^{***}$ | $3.01 \pm 0.57^{*}$ | $0.19 \pm 0.12^{**}$ | $0.72 \pm 0.42^{*}$ | $0.25 \pm 0.07^{*}$ |
| **Treatment group** | $1.03 \pm 0.18^{\#\#}$ | $2.04 \pm 0.30^{\#}$ | $0.67 \pm 0.32^{\#}$ | $1.67 \pm 0.53^{\#}$ | $0.72 \pm 0.35^{\#}$ |

Compared with the normal group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; compared with the qi deficiency and blood stasis group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

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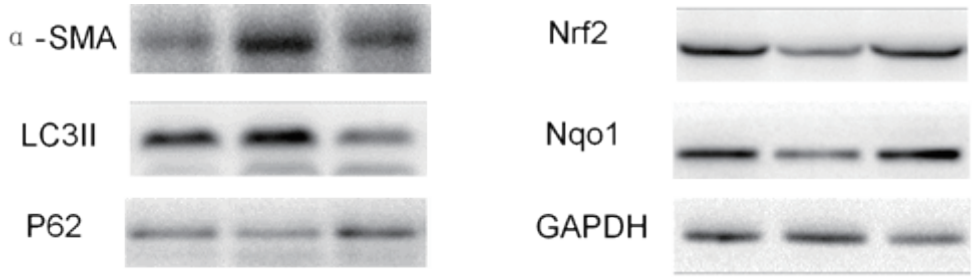


**Figure 2 Western blot bands**

2).

## Discussion

Liver fibrosis is caused by multiple factors, such as alcohol, fat, viruses, immune dysfunction, and genetic factors. The progression of liver inflammation is becoming increasingly common, and many patients can benefit from treatment. In the context of a lack of precise drugs resulting in disease reversal, traditional Chinese medicine can play a key role in the treatment of liver fibrosis. However, the mechanism by which traditional Chinese medicines protect against fibrosis remains unclear. Yiqi Huayu therapy is expected to regulate autophagy and anti-oxidative stress, providing insight into the anti-fibrotic mechanism.

In traditional Chinese medicine, liver fibrosis is classified as hypochondriac pain. Commonly caused by long-term illness, improper diet, emotional disorders, or external toxins, liver fibrosis can result in a variety of symptoms, including a lack of vitality, liver stagnation, and impaired qi dissipation. Without toxin removal, blood circulation is blocked, causing a qi deficiency and blood stasis. Yiqi Huayu therapy can improve liver function and alleviate the associated clinical symptoms [9]. We previously found that the level of autophagy is elevated in subjects with a *qi* deficiency and blood stasis. Strengthening *qi* to reinvigorate vital energy and activating blood circulation to dissipate blood stasis using Fuzheng Huayu capsules can decrease expression of the autophagy protein LC3II and increase the expression of p62, thereby inhibiting autophagy and preventing liver fibrosis. However, autophagy influences liver fibrosis by multiple mechanisms, and it can either promote or inhibit disease progression [10]. Autophagy has different effects on liver fibrosis via different pathways. In this study, we focused on the mechanism by which autophagy contributes to the treatment of liver fibrosis with qi deficiency and blood stasis via the Nrf2-Keap1-Are antioxidative stress pathway.

Oxidative stress refers to the deposition of reactive oxygen species and reactive nitrogen free radicals in the body after a noxious stimulus, which induces tissue injury and inflammation and thereby promotes liver fibrosis [11, 12]. Since Nrf2-Keap1-ARE is the primary antioxidant stress pathway, its activation plays a critical role in inhibiting inflammation and the progression of liver fibrosis [13]. As a downstream protein of ARE antioxidant elements, Nqo1 has antioxidant effects in various diseases, such as liver disease, coronary heart disease, and cancer [14-16]. Inflammation facilitates liver fibrosis, while the Nrf2-mediated signaling pathway plays an important role in the regulation of liver inflammation. After its activation, the Nrf2-mediated expression of antioxidant genes not only alleviates T cell-induced hepatocyte necrosis [17], but also inhibits dimethylnitrosamine-induced liver injury [18] and cadmium-induced liver injury [19]. Furthermore, a lack of Nrf2 can lead to the increased expression of CD133+ in hepatocyte-like cells, indicating that Nrf2 is a crucial regulator of liver precursor cells [20]. In conclusion, Nrf2 plays an important regulatory role in liver fibrosis with various underlying causes.

Based on Sirius red staining and Ishak scores in this study, the *qi* deficiency and blood stasis group was observed to have obvious liver fibrosis. However, the degree of fibrosis was alleviated by Fuzheng Huayu therapy. In addition, the mRNA and protein expression levels of α-SMA, an HSC activation marker, were higher in the *qi* deficiency and blood stasis group than in the control group, indicating that the HSC activation is enhanced in the group. Autophagy, as evaluated by LC3II, was also increased, thereby reducing the expression of the autophagy substrate P62. The effect of P62 is relatively weak due to competition with Keap1 for binding to Nrf2, which decreases the expression

of Nrf2 and Nqo1, downstream of the oxidative stress pathway. When the Fuzheng Huayu capsule was used to strengthen *qi* and attenuate stasis, the expression of the autophagy marker LC3II was inhibited, thereby increasing the expression of P62 and activating Nqo1 of the Nrf2/Keap1 antioxidative stress pathway. Since LC3-mediated autophagy can inhibit the Nrf2-Keap1-ARE anti-oxidative pathway, it is a strong candidate target for the treatment of liver fibrosis.

## Conclusion

In summary, our results identified the preliminary mechanism by which Yiqi Huayu regulates autophagy and the antioxidative stress pathway from a molecular biological perspective. Yiqi Huayu therapy in Chinese medicine reduces stasis and inhibits liver fibrosis, possibly by suppressing autophagy in sputum cells and activating the Nrf2-Keap1-Are signaling pathway. These results provide objective directions for syndrome differentiation in traditional Chinese medicine.

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