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• BASIC RESEARCH •

Effects of Yigan Decoction on proliferation and apoptosis of hepatic stellate cells

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Abstract

AIM: **To investigate the effects of Chinese herb Yigan Decoction on proliferation and apoptosis of the hepatic stellate cells (HSC) *in vitro*.**

METHODS: **The study *in vitro* was carried out in the culture of HSC lines. Various concentrations of Yigan Decoction were added and incubated. Cell proliferation was detected with MTT colorimetric assay. Cell apoptosis was detected by electron microscopy, flow cytometry and TUNEL.**

RESULTS: **The proliferation of HSC was inhibited by Yigan Decoction, which depending on dose and time significantly. The HSC proliferation rates of groups at the end concentrations 144 and 72($g\cdot L^{-1}$) were 21.62% and 40.54% respectively, significantly lower than that of normal control group($P<0.01$). The HSC proliferation rates of groups at the end concentrations 36, 18 and 9($g\cdot L^{-1}$) were 54.05%, 45.95% and 51.35% respectively, lower than that of control group ($P<0.05$). When the end concentration was 4.5$g\cdot L^{-1}$, the proliferation rate was 83.78%, which appeared no significant differences compared with control group. At the same concentrations of 18$g\cdot L^{-1}$, the inhibitory effects of Yigan Decoction at 24h, 48h and 72h time point were observed, the effects were time-dependent, and reached a peak at 72h. Meanwhile, it was showed that the inducing effects of Yigan Decoction on HSC apoptosis were dose-dependent and time-dependent. The apoptosis index(AI) was detected by TUNEL. After Yigan Decoction had been incubated for 48h at the end concentration of 18$g\cdot L^{-1}$, the AI (14.5±3.1)% was significantly higher than that of control group (4.3±1.3)% ($P<0.01$). When visualized under transmission electron microscopy, some apoptotic stellate cells were found, i.e. dilated endoplasmic reticulum, irregular nuclei, chromatin condensation and heterochromatin ranked along inside of nuclear membrane. By flow cytometry detection, after HSC was treated with Yigan Decoction at different concentrations of 36, 18 and 9($g\cdot L^{-1}$) for 48 h, AI (%) were 13.3±3.2, 10.7±2.7 and 10.1±2.5 respectively, which were significantly higher than that of control group(4.1±1.9) ($P<0.01$). At the same concentration of 18$g\cdot L^{-1}$ for 24h, 48h and 72h, AI (%) were 9.3±1.8, 10.7±2.7 and 14.6±4.3 respectively, which were significantly higher than that of control group ($P<0.01$).**

CONCLUSION: **Yigan Decoction could significantly inhibit HSC proliferation and increase the apoptosis index of HSC dose-dependently and time-dependently, which may be related to its mechanism of antifibrosis.**

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INTRODUCTION

As the mechanisms of hepatic fibrosis have been gradually clarified and thus, many attempts to treat hepatic fibrosis have been made recently. But up to date there is still no effective way to treat hepatic fibrosis[1]. Recent insights[2-11] into the molecular pathogenesis of hepatic fibrosis and the efficacy of TCM have provided hope for the foreground of successful therapy, such as compound 861[12], Kangxianfang[13], Fuzhenghuayu decoction[14-16],Ganyanping[17] etc. Yigan Decoction, which was designed in our lab, was used in clinical setting for nearly twenty years. Our clinical practice has revealed that it has a marked curative effect on treating chronic liver diseases[18]. Hepatic sinusoidal cells such as the hepatic stellate cells (HSC),endothelial cells or Kupffer cells are deeply involved in hepatic fibrogenesis or fibrolysis. Recent studies have made their morphology and functions clear. A wealth of evidence now indicates that HSC is the key to produce fibrosis which served as the major source of fibrillar and nonfibrillar matrix proteins. Quiescent HSC synthesize low levels of matrix proteins, but as a result of injury, HSC could be proliferated and transformed to myofibroblast, a process termed activation[4]. So to inhibit HSC activation and proliferation and induce apoptosis of the activated HSC is one of the most important strategies for preventing and curing liver fibrosis. HSC cultured in uncoated plastic plates in vitro spontaneously undergo activation and share the similar features of cell activation *in vivo*[19]. This culture-induced activation has been extensively studied as a model of the activation secondary to liver fibrogenesis. In order to testify the action of Yigan Decoction on liver fibrosis and investigate its mechanism, the effects of Chinese herb Yigan Decoction on the proliferation and apoptosis of HSC in vitro were observed.

MATERIALS AND METHODS

Cell culture

HSC lines (CFSC) (established by Professor Greenwel) were provided by Southwest Hospital, Third Military Medical University. The phenotype was activated HSC[20, 21]. Cells were cultured in RPMI1640 (Gibco) medium plus 100mL·L^{-1} fetal calf serum, penicillin 1×10^5U·L^{-1} and streptomycin 100mg·L^{-1}, and kept in a controlled atmosphere (5% CO_2) incubator at 37℃.

Drug treatment

Yigan Decoction consists of Radix Salviae Miltiorrhizae, Radix Angelicae Sinensis, Radix Paeoniae Rubra and Hirudo, etc. The decoction was made by Hebei Institute of Gastroenterology, containing 0.72g of crude herbs in each milliliter. Exponentially growing cells were seeded in plates for 24h and treated with Yigan Decoction at

various concentrations (144, 72, 36, 18, 9 and 4.5g·L^{-1}) for 24h, 48h and 72h, respectively. The cells not treated with this drug served as control cells.

Dose-dependent effects of Yigan Decoction on cell proliferation

Colorimetric MTT assays[23] were used to observe cell proliferation. HSC were incubated in 96 well plates. The concentration of cells was modulated to 1×10^{8}·L^{-1}. After cultured for 24h, Yigan Decoction was added in different concentrations such as 144, 72, 36, 18, 9 and 4.5 (g·L^{-1}) for 48h. Each group was arranged three duplicate wells. After the supernatant was extracted and 0.05% MTT solution 10ìL was added to all wells for 4h, DMSO 100μL was added for coloration. After a few minutes at room temperature to ensure that all crystals were dissolved, the optic-metric density (OD) was read on ELISA reader at test wavelength of 570nm and referent wavelength of 630nm.

Time-dependent effects of Yigan Decoction on cell proliferation

The cultured cells were grown up to logarithmic growth phase, digested with 2.5g·L^{-1} trypsin. The concentration of cells was modulated to 1×10^{8}·L^{-1}. HSC were incubated in 24 well plates. Each well was added with 1mL. After being cultured for 24h, Yigan Decoction (50μL) was added into wells at the same end concentration of 18g·L^{-1}. Cell proliferation was observed at different times, i.e., 24h, 48h and 72h after the herb was treated, each was arranged three duplicate wells. The number of HSC was counted at the end of each time.

Apoptosis examined by TUNEL

In situ cell death detection kits were purchased from Boehringer Mannheim Company, Germany. The cells were adjusted to a density of 2×10^{3} cells/cm^{2}, added to 24-well plates with cover glass-slides in 0.5mL each well. After being incubated with Yigan Decoction at the end concentration of 18g·L^{-1} for 48h, the glass slides were taken out, rinsed, fixed and stained. The negative control with omission of TUNEL enzyme was designed according to the manufacturer's manual. The cells stained with dark brown nucleus were considered as positive cells. Ten optical fields, about 500-1000 cells were selected randomly and counted in each glass-slide under the high magnification (×400) microscope. Apoptosis Index (AI) = (apoptotic cells/ total cells)×100%.

Apoptosis examined by transmission electron microscopy

HSC were treated with Yigan Decoction at the end concentration of 18g·L^{-1} for 48h. Then the cells were centrifugated and fixed in glutaraldehyde for observation of transmission electron microscopy.

Apoptosis examined by flow cytometry

HSC were treated with Yigan Decoction at the end concentration of 36, 18 and 9(g·L^{-1}) for 24h, 48h and 72h. Cells were digested by 2.5g·L^{-1} trypsin, washed by PBS, fixed by cold ethanol at 4℃ and dyed with PI (propidium iodide), and then were analyzed by flow cytometry.

Statistics

Results were expressed as mean ± SD ($\bar{x}\pm s$). Differences between groups and differences over times were analyzed using analysis of variance and Newman-Keuls methods where appropriate. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Dose-dependent inhibitory effects of Yigan Decoction on cell proliferation

Yigan Decoction could significantly inhibit HSC proliferation dose-dependently compared with the control group (Table 1). The inhibitory effects of groups at concentrations of 144 and 72(g·L^{-1}) were stronger than those of groups at concentrations of 36, 18 and 9(g·L^{-1}), and no obvious inhibitory effect was found at the herb concentration of 4.5g·L^{-1}.

Time-dependent inhibitory effects of Yigan Decoction on cell proliferation

At the end concentration of 18g·L^{-1}, HSC were significantly inhibited compared with control group. The number of cells was manually counted. The effect was time-dependent, and reached a peak at 72h (P<0.01) (Table 2).

Apoptosis of HSC induced by Yigan Decoction examined by TUNEL

Based on the above results that Yigan Decoction could inhibit HSC proliferation at concentrations of 9-144g·L^{-1} from 24h to 72h, we selected the 18g·L^{-1} of Yigan Decoction and 48h affecting period as experimental conditions so as to better compare with the results of the herb on HSC lines. After Yigan Decoction had been incubated for 48h, the AI 22.5±7.1% was significantly higher than that of control group 4.3±1.3% (P<0.01). Apoptotic cell is characterized by compaction of nuclear chromatin and condensation of cytoplasm. By TUNEL stain the most condensed cells demonstrated evidence of DNA fragmentation and were strongly stained (Figure 1, arrowed).

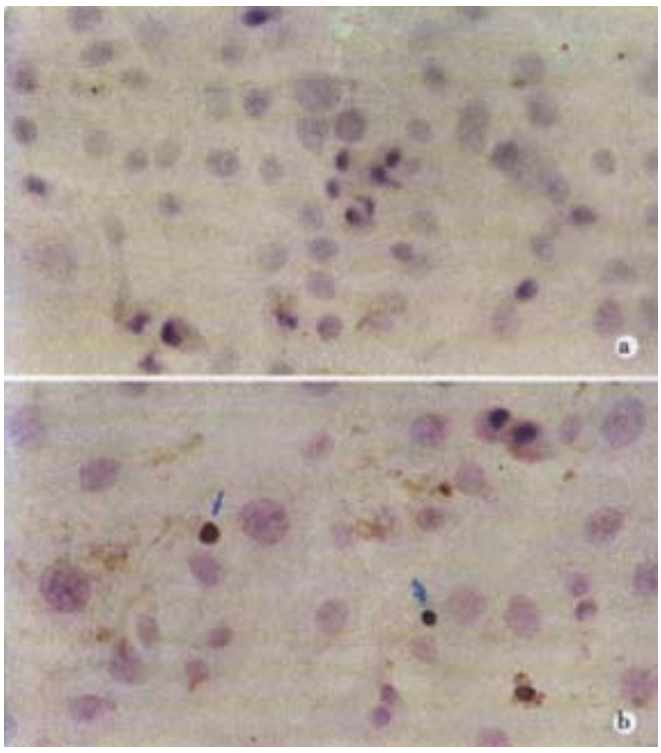

Figure 1 (A) Control HSC (TUNEL×400). (B) Apoptotic cell characterized by compaction of nuclear chromatin and condensation of cytoplasm (TUNEL×400). By TUNEL stain the most condensed cells demonstrated evidence of DNA fragmentation and were strongly staining (iü).

Apoptosis of HSC induced by Yigan Decoction examined by transmission electron microscopy

After 24~48h treatment of Yigan Decoction, we visualized under inverted microscopy. Small rounded cells were observed on the surface of the monolayer. These cells could be displaced by agitation of the tissue culture plate, demonstrating that they were very loosely adherent to the monolayer and that some were detached and floating in the culture supernatant (Figure 2). These features are compatible with HSC that have undergone programmed cell death or apoptosis[24].

When visualized under transmission electron microscopy, the apoptotic HSC were found, i.e. dilated endoplasmic reticulum, irregular nuclei, chromatin condensation and heterochromatin ranked along inside of nuclear membrane. All of these features are characteristic of apoptosis and distinguish programmed cell death from necrosis (Figure 3).

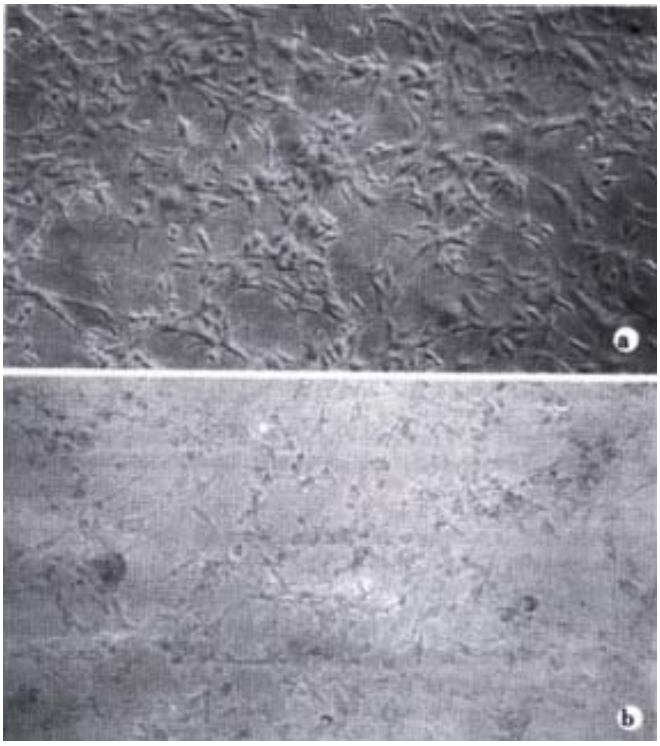

Figure 2 Morphological changes of HSC observed by light microscopy treated by Yigan Decoction. (A) Untreated cells (×200); (B) some HSC got round, detached and floating in the culture supernatant after exposure to 18g·L^{-1} Yigan Decoction for 48h (×100).

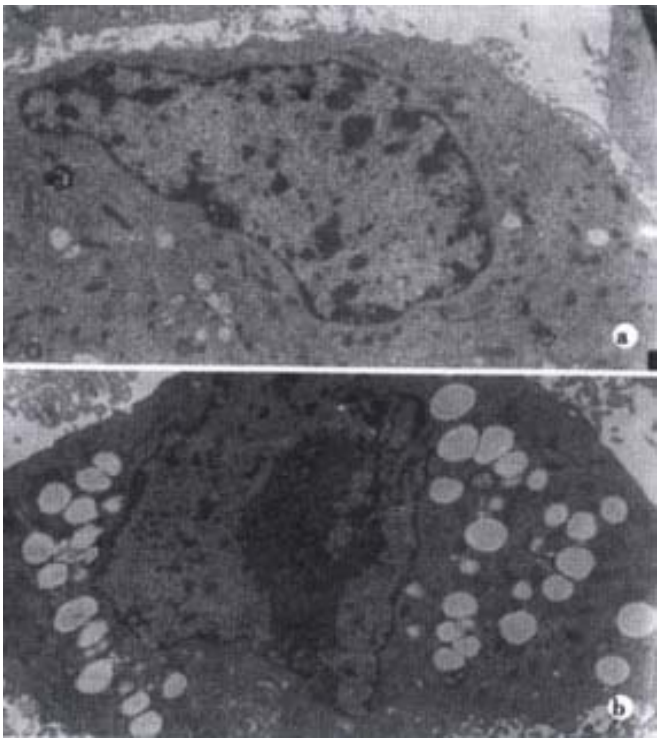

Figure 3 Ultrastructures of HSC with or without treatment with Yigan Decoction. Following 48h treatment of Yigan Decoction, dilated endoplasmic reticulum, irregular nuclei, chromatin condensation and heterochromatin ranked along inside of nuclear membrane could be found (A 5000×). Untreated cells (B 5000×).

Apoptosis examined by flow cytometry

After HSC were treated with Yigan Decoction at different concentrations of 36, 18 and 9(g·L^{-1}) for 48h, the apoptosis rate was significantly higher than that of control group (P<0.01) (Table 3). Our experiments showed that Yigan Decoction could increase the apoptosis rate dose-dependently and time-dependently compared with the control group. (Table 3 and 4, Figure 4).

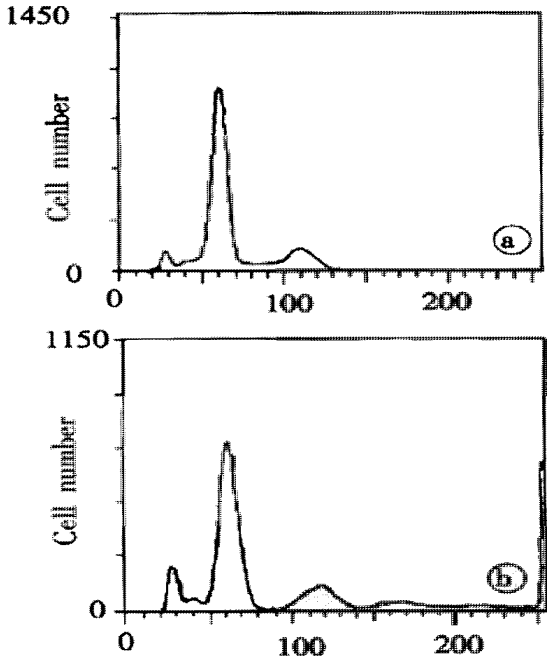

Figure 4 Flow cytometry changes.(A)Control group.(B)Yigan Decoction group.

Table 1 Effects of Yigan Decoction on HSC proliferation ($\bar{x}$±s)

Groups	Herb concentrations (g·L^{-1})	ODs	proliferation rates (%)
Yigan Decoction	144	0.08±0.02^b	21.62
	72	0.15±0.04^b	40.54
	36	0.20±0.03^a	54.05
	18	0.17±0.02^a	45.95
	9	0.19±0.05^a	51.35
	4.5	0.31±0.04	83.78
Control		0.37±0.03	100

$^a P$<0.05, $^b P$<0.01 *vs* control group.

Table 2 Effects of Yigan Decoction on HSC proliferation (×10^8·L^{-1}) ($\bar{x}$±s)

Groups	24h	48h	72h
Control	1.440±0.124	2.288±0.178	2.335±0.146
Yigan Decoction	1.203±0.112	1.258±0.098^b	1.359±0.079^b

$^b P$<0.01 *vs* control group.

Table 3 Apoptosis indexes of Yigan Decoction on HSC ($\bar{x}$±s)

Groups	Herb concentrations(g·L^{-1})	apoptosis indexes (%)
Yigan Decoction	36	13.3±3.2^b
	18	10.7±2.7^b
	9	10.1±2.5^b
Control		4.1±1.9

$^b P$<0.01 *vs* control group.

Table 4 Time-dependent effects of Yigan Decoction on HSC apoptosis ($\bar{x}$±s)

Groups	24h	48h	72h
Control	4.5±1.3	7.1±1.9	8.0±1.8
Yigan Decoction	9.3±1.8^b	10.7±2.7^b	14.6±4.3^b

$^b P$<0.01 *vs* control group.

DISCUSSION

Recent studies have found clearly the key role of HSC in developing liver fibrosis. HSC could activate, proliferate and largely synthesize various components of extracellular matrix in chronic liver disease, that would lead to liver fibrosis[22]. Studies suggest that HSC numbers are controlled by apoptosis in addition to proliferation during progressive fibrosis and particularly during recovery from fibrosis. The key event in the process of injury-fibrosis-recovery sequence is the loss of activated HSC mediated by apoptosis[23]. Therefore to induce apoptosis of activated HSC is one of the most important therapeutic strategies for liver fibrosis. Recent evidence has demonstrated that a mechanism to eliminate activated HSC in culture and in animal models is inducing apoptosis. Interestingly, activated HSC are more sensitive to some mechanisms of apoptosis than quiescent HSC. Such a mechanism may serve in the future to eliminate the undesirable activated HSC without affecting their normal quiescent counterpart.

The CFSC used in present study is activated HSC. So it shared some features with activated HSC *in vivo* and could be used as a desirable cell model for the test of antifibrotic drugs. Although the desirable antifibrotic drugs have not been found up to now, some herbal decoctions have been reported to prevent fibrogenesis effectively, showing a good prospect of using Chinese herbs in treating chronic liver diseases[24]. It was reported that Radix Salviae Miltiorrhizae (RSM) can inhibit fibroblast cells[25]. RSM can prevent liver fibrosis if it is used for a long time[26]. Large doses of RSM can activate collagenase and help reduce extracellular matrix. The level of P Ⅲ P and laminin were decreased in patients with liver disease after treatment of RSM. Studies demonstrated that long-term treatment of RSM for 10-12wk can reduce portal vein, spleen diameters and blood flow, but the velocity of blood flow did not change[27]. These results demonstrated the advantage of Chinese herbs in treating chronic hepatic diseases. Yigan Decoction, one of formally produced herbs, could prevent extracellular matrix production and deposit in CCl_4 induced rat liver fibrosis[28], and improve fibrotic liver structure and liver function in patients with chronic hepatitis or cirrhosis[18]. It is mainly composed of RSM, Radix Angelicae Sinensis (RAS), Radix Paeoniae Rubra and Hirudo, which have the actions of promoting blood circulation, removing blood stasis, shrinking the liver and spleen.

The present results suggest that Yigan Decoction could significantly inhibit HSC proliferation and increase the apoptosis rate of HSC dose-dependently and time-dependently compared with the control group ($P<0.01$). After Yigan Decoction had been incubation for 48h, the apoptosis rate of HSC was 27.5% compared with 7.1% in the control group ($P<0.01$). Our findings strongly suggest that inhibition of HSC proliferation and induction of HSC apoptosis may play an important role in the antifibrotic actions of Yigan Decoction. Our data may provide a new idea for the future development of therapeutic antifibrotic strategies by means of traditional Chinese medicine.

The antifibrotic effect of Yigan Decoction has been well proved by the results of experimental and clinical studies. However, Chinese herbs have very complicated components and their metabolisms are not well clarified yet. There's still a long way to go. With the ever-increasing in-depth studies about the pharmacodynamics, pharmacokinetics, reasonable combinations of drugs, the best preparation form and dosages of each herb and courses, Yigan Decoction is expected to be an effective antihepatofibrotic drug.

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