Original Article
Effect of Huazhuojiedu medicated serum on the proliferation and activation of hepatic stellate cells and the expression of PI3K and p-Akt in rats

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Abstract: To observe the effect of Huazhuojiedu medicated serum on the proliferation and activation of hepatic stellate cells, as well as the expression of PI3K and p-Akt in rats, and to explore the underlying mechanism of Huazhuojiedu prescription against hepatic fibrosis. Hepatic stellate cells harvested from rats were resuscitated and subcultured, followed by the intervention of Huazhuojiedu equivalent dose, Huazhuojiedu double dose, and positive drug (Compound Biejiaruangan Troche) medicated serum of rats. After *in vitro* culture, hepatic stellate cells were stimulated with 5 ng/mL transforming growth factor-β1. At 24, 48, 72 hours, the proliferation of hepatic stellate cells was detected with MTT assay; at 48 hours, α-SMA mRNA and protein expression in hepatic stellate cells were determined with RT-PCR assay and western blot analysis, respectively, to evaluate the activation of hepatic stellate cells; in addition, PI3K and p-Akt protein expression levels were also assayed with western blot analysis at 48 hours. The results showed that, 24-hour transforming growth factor-β1 stimulation significantly promoted the proliferation of hepatic stellate cells ($P < 0.01$). Each medicated serum inhibited the proliferation of hepatic stellate cells ($P < 0.01$). Huazhuojiedu equivalent dose had the similar inhibition effect with positive drug ($P > 0.05$), and Huazhuojiedu double dose achieved more apparent inhibition effect ($P < 0.01$). After 48 and 72 hours of transforming growth factor-β1 stimulation, hepatic stellate cells still proliferated significantly ($P < 0.01$), which was inhibited by each medicated serum ($P < 0.01$). Huazhuojiedu equivalent dose showed a weaker inhibition effect than positive drug ($P < 0.05$), and Huazhuojiedu double dose exerted a strong inhibition effect ($P < 0.05$). After hepatic stellate cells were stimulated with transforming growth factor-β1 for 48 hours, the expression of α-SMA mRNA and protein in hepatic stellate cells was significantly increased ($P < 0.01$); the medicated serums significantly down-regulated α-SMA mRNA and protein expression, and inhibited the activation of hepatic stellate cells ($P < 0.01$). Huazhuojiedu equivalent dose showed the similar inhibition effect with positive drug ($P > 0.05$), and Huazhuojiedu double dose exerted a significant inhibition effect ($P < 0.05$), which was stronger than Huazhuojiedu equivalent dose ($P < 0.05$). After hepatic stellate cells were stimulated with transforming growth factor-β1 for 48 hours, PI3K and p-Akt protein expression levels were increased ($P < 0.05$); each medicated serum down-regulated the elevated expression levels of PI3K and p-Akt ($P < 0.05$). Huazhuojiedu equivalent dose had the similar down-regulation effect with positive drug ($P > 0.05$), and Huazhuojiedu double dose achieved more apparent inhibition effect on PI3K expression ($P < 0.05$). Huazhuojiedu double dose significantly decreased the PI3K and p-Akt protein expression compared with Huazhuojiedu equivalent dose ($P < 0.05$). Huazhuojiedu medicated serum inhibits the proliferation and activation of hepatic stellate cells induced by transforming growth factor-β1 *in vitro*, reduces the expression of PI3K and p-Akt protein, and the mechanisms of preventing hepatic fibrosis is mediated by the intervention on PI3K/Akt pathway.

Keywords: Huazhuojiedu prescription, medicated serum, hepatic stellate cells, PI3K, p-Akt

Introduction

Phosphatidylinositol 3-hydroxy kinase (PI3K) is the second messenger involved in intracellular signal transduction, protein kinase B (Rac, Akt) is a serine/threonine protein kinase, the activated PI3K and Akt contribute to the regulation effect [1]. PI3K/Akt signaling pathway plays an important role in cell growth, proliferation, differentiation, and apoptosis [2], and is also criti-

cally related to biological behavior of hepatic stellate cells (HSCs) [3]. HSC proliferation and activation are the key link of hepatic fibrosis formation. Preliminary studies of our research group found that, Huazhuojiedu compound prescription could prevent hepatic fibrosis [4-6]. However, the underlying mechanisms remain unclear. This study aims to observe the effect of Huazhuojiedu medicated serum on the proliferation and activation of HSCs induced by transforming growth factor-β1 (TGF-β1) *in vitro*, as well as the expression of PI3K and p-Akt in rats, in a broader attempt to explore the underlying mechanism of Huazhuojiedu prescription against hepatic fibrosis.

Materials and methods

Cell line

Rat HSCs were kindly provided by Beijing Zhongyuan Company (Beijing, China) as a gift. The cell phenotype is stationary. HSCs were stored in liquid nitrogen.

Drugs

Huazhuojiedu prescription was consisted of Herba Artemisiae Scopariae, Rhizoma Coptidis, hypericum japonicum Thunb, Gold Theragran, Rhizoma Polygoni Cuspidati, Carapax Trionycis, Rhizoma Sparganii, integripetal rhodiola herb, Radix Salviae Miltiorrhiae, and Raidix Paeoniae Alba. Raw drugs were provided by Dispensary of Traditional Chinese Medicine, and concentrated in the Decoction Room, Hebei Provincial Hospital of Traditional Chinese Medicine (Hebei Province, China). After preparation, the solution was sterilized, packaged, and preserved at 4°C. Compound Biejiaruangan Tablet was purchased from Inner Mongolia Furui Medical Science Co., Ltd. (production batch number: 20120909).

Reagents

TGF-β1 (Peprotech Corporation, USA); high-glucose DMEM medium and trypsin (GIBCO, USA); fetal bovine serum (FBS) (Beijing Yansheng Technology Co., Ltd., Beijing, China); penicillin and streptomycin (North China Pharmaceutical Group Corporation); Thiazolyl blue tetrazolium bromide (MTT; Sino-American Biotechnology Company); RT-PCR reverse transcription kit, RNA extraction kit, and PCR primers (Tiangen Biotechnology (Beijing) Co., Ltd.); tetramethyle-thylenediamine (TEMED; Sigma, USA); dimethyl sulfoxide (DMSO; Beijing North Fine Chemicals Company); sodium dodecyl sulfate (SDS; Hebei Bohai Biological Engineering Co., Ltd.); rabbit anti-β-actin polyclonal antibody (Hangzhou Hua-An Biotechnology Co., Ltd.); rabbit anti-mouse α-smooth muscle actin (α-SMA), PI3Kp85α, p-Akt (Ser^{473}) phosphorylation antibody (Cell Signaling, USA); and HRP-conjugated goat anti-rabbit polyclonal antibody (Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd.) were used in this study.

Experimental instruments

Type HF-240 CO_2 incubator (Nature Company, USA); type XDS-1B inverted microscope (OLYMPUS, Japan); FOTODYNE gel imaging system (Image Corporation, USA); type VE-180 vertical electrophoresis tank and type VE-186 transfer electrophoresis tank (Shanghai Tanon Science and Technology Co., Ltd.); type DYY-III6B electrophoresis device (Beijing Liuyi Instrument Factory); type NDY-2000 detector (Thermo Corporation, USA); type GeneAmP9600 PCR instrument (TECHNE Company, British); type TS-2 shaker (Haimen Qilinbeier Instrument Manufacturing Company, China); type Sunrise enzyme-linked immunosorbent assay (TECAN Company, Switzerland) were used in this study.

Methods

Preparation of medicated serum

Eight clean, healthy, male Sprague-Dawley rats, weighing 200±20 g, were purchased from Experimental Animal Center of Hebei Medical University in China (license No. 1012133). Rats were given intragastrical administration of Huazhuojiedu equivalent dose 2.7 g/200 g • d (n = 2 rats), Huazhuojiedu double dose 5.4 g/200 g • d (n = 2 rats), Compound Biejiaruangan Tablet 1.25 g/200 g • d (n = 2 rats) and saline (n = 2 rats) in 8:00 am of every day according to body surface area (2 mL/200 g • d), for consecutive 10 days [7]. All rats were fed with normal diet, and abdominal aortic blood samples were harvested under sterile conditions 2 hours after the last administration [8]. The blood samples were sterilely packaged and the serum was fully precipitated, and then centrifuged at 12,000 rpm for 5 minutes. Subsequently the centrifuged serum was mixed and inactivated at 56°C water bath for 30 minutes,

sterilized using a 0.22 µm filter membrane, and stored at -70°C. Prior to the use, blood serum was rewarmed to 37°C, and each group was added with 20% serum for the culture of HSCs [9].

HSC cultured and grouping

Cell resuscitation and subculture: Stationary HSCs were removed from the liquid nitrogen, and rapidly thawed at 37°C water bath within 1 minute. Then the cells were placed in frozen tube and transferred to sterilized centrifuge tube containing 10 mL of 10% FBS-DMEM (high glucose) medium, followed by a centrifugation at 1,500 rpm for 5 minutes. After the supernatant was discarded, the cells were transferred to a culture bottle, which contained 3 mL of 10% FBS-DMEM, 1×10^5 U L penicillin, and 100 mg/L streptomycin, and cultured in 37°C, 5% CO_2 incubator. The cells were subcultured according to the growth state. When cultured cells reached an 80% confluence in a dense monolayer, the culture medium was discarded, the cells were rinsed twice with D-Hanks solution and digested with 2 mL of 0.25% trypsin for 30 seconds. The digestion was terminated upon the appearance of cell gap enlargement and cell retraction under an inverted microscope. 10% FBS-DMEM culture medium was added, to triturate cell suspension and subculture the cells at a ratio of 1:2 or 1:3. The subcultured cells were frozen for further use.

Cell grouping: HSCs were divided into five groups, normal control group (normal rat serum + normal saline), model group (5 ng/mL TGF-β1 + normal serum), positive control group (5 ng/mL TGF-β1 + Compound Biejiaruangan Tablet medicated serum), equivalent dose group (5 ng/mL TGF-β1 + Huazhuojiedu equivalent dose medicated serum), and double dose group (5 ng/mL TGF-β1 + Huazhuojiedu double dose medicated serum).

Effect of Huazhuojiedu medicated serum on the HSC proliferation detected by MTT assay

The subcultured HSCs at logarithmic growth phase were diluted to 2×10^4 cell/mL with DMEM culture medium, and seeded onto the 96-well culture plates with 200 µL in each well, in a 37°C, 5% CO_2 incubator for 24 hours. After the cells began to adhere, culture medium was replenished and the cells were divided into five groups, each group contained five holes. At 24, 48, 72 hours after cell culture, 5 mg/mL MTT was added, 20 µL/hole, to culture cells for additional 4 hours. Subsequently each well was added with DMSO 150 µL, shaking for 10 minutes. The absorbance was read at 490 nm with a microplate reader, and averaged to obtain the final result in each group.

Effect of Huazhuojiedu medicated serum on α-SMA mRNA expression as detected by RT-PCR assay

RNA extraction: HSCs at logarithmic growth phase were seeded onto the six-well culture plate at the density of 1×10^6 cells/hole and cultured with appropriate serum for 48 hours. Then cultured cells in each group were collected and total RNA was extracted with Trizol. (1) Original culture medium was discarded, cells were rinsed with PBS three times and cleavaged with 1 mL Trizol, then transferred to 1.5 mL RNase-free EP tubes, and placed on ice for 5 minutes. (2) Cells were mixed with 200 µL chloroform for 15 seconds, placed on ice for 5 minutes, and centrifuged at 4°C, 12,000 rpm for 15 minutes. (3) The supernatant was collected and transferred to a new EP tube, adding equal volume of isopropanol and gently mixing, then placed on ice for 10 minutes and centrifuged at 4°C, 12,000 rpm for 10 minutes. (4) After the supernatant was discarded, cells were precipitated with pre-cold 75% ethanol 1 mL, and centrifuged at 4°C 7,500 rpm for 5 minutes. (5) The supernatant was discarded, and the precipitations were dried naturally and dissolved in 20 µL DEPC water, finally stored at -80°C for further use.

Determination of RNA concentration and purity: 200 µL DEPC water served as a blank zero of nucleic acid quantification instrument. 2 µL RNA sample was added to 98 µL DEPC water, to measure sample concentration and absorbance value at 260 nm and 280 nm. The RNA at the purity ratio of 1.8-2.0 can be used for experiments.

Identification of RNA integrit: The EB-containing 1% agarose gel was prepared and electrophoresis tank was added with 1 × TAE buffer, immersing the gel. 2 µg RNA samples were mixed with 2 µL sample buffer, gel electrophoresis was run at 60 V voltages and transferred to 3/4 of the gels. 18S and 28S bands were

observed. When the width and brightness of 28S band were twice of that of 18S band, the boundary was clearly visible; we can perform RT-PCR.

cDNA synthesis: The DEPC-treated 0.2 mL EP tube was added with the following reagents: RNA 1 µg, oligo (dT) 18 primer 1 µL, DEPC-treated water 12 µL. The above reaction system (12 µL) was preheated to 65°C in PCR instrument and denatured for 5 minutes, then added with the following reagents: 5 × reaction buffer 4 µL, RiboLock™ RNase Inhibitor 1 µL, 10 mM dNTP mix 2 µL, RevertAid™ M-MuLV Reverse Transcriptase 1 µL. The above reaction system (20 µL) was placed in the PCR instrument, 42°C for 65 minutes and 70°C for 5 minutes, to synthesize cDNA. The cDNA solution was diluted 10 times and preserved at -20°C .

PCR amplification: The synthesized cDNA was taken as the template to amplify the target gene. The sequences were screened from the NCBI database and designed by NCBI BLAST, as follows: β-actin primer sequences: 459 bp, Upstream: 5'-CGC GAG TAC AAC CTT CTT GC-3', Downstream: 5'-GTA CAT GGC TGG GGT GTT GA-3', α-SMA mRNA primer sequence: 195 bp, Upstream: 5'-GCC ATC AGG AAC CTC GAG AA-3', Downstream: 5'-AGC TGT CCT TTT GGC CCA TT-3'.

PCR conditions: denaturation at 95°C for 5 minutes; 35 cycles of 95°C 30 s, 52°C 30 s, 72°C 1 min; followed by the extension at 72°C for 5 minutes. The PCR products were stored at -20°C.

Electrophoresis and gel analysis

10 µL RT-PCR products were mixed with the sample buffer 2 µL and DNA Marker 10 µL, which was then run electrophoresis in 1.5% agarose gel. The gray value of bands was scanned with Gel-Proanalysis3.1 software, and the ratio of α-SMA mRNA amplification band gray value to β-actin amplification band gray value reflects the expression levels of α-SMA mRNA.

α-SMA, PI3K and p-Akt protein expression detected by western blot assay

Extraction and quantification of HSC cell proteins: HSCs at logarithmic growth phase were seeded into the six-well plates at the density of 1×10^6 cells/hole, in 37°C, 5% CO_2 incubator for 24 hours. When the cells began to adhere and reached a 70% confluence, serum-free DMEM medium was added to culture cells at 37°C for additional 24 hours. Subsequently cells were rinsed three times with PBS, and the growth of the cells synchronized into resting phase. Accordingly the cells were cultured with the corresponding serum for 48 hours and rinsed with 1 × PBS twice. After the second washing, 0.5 mL PBS was left in each hole, the cells were scraped and placed into five 1.5 mL centrifuge tube for rapid centrifugation. After the supernatant was removed, cell lysates were mixed with protease inhibitors (PMSF) at the ratio of 100:1, and transferred to the EP tube on ice for 30 minutes. Then the cells were pipetting 30 times with 1 ml syringe, and centrifuged at 12,000 rpm for 20 minutes. After the supernatant was quickly drawn, the proteins were extracted and protein concentration was measured using BCA method according to the kit instructions. The quantitative protein was mixed with sample buffer at the ratio of 3:1 and boiling for 5 minutes, denatured and stored at -80°C.

SDS-PAGE electrophoresis: Irrigation and sampling: The denatured protein stored at -80°C were thaw on ice and polymerized with stacking gel, the comb was carefully removing, and each comb hole was added with 50 µg protein. At 90 V the electrophoresis lasted for 2.2 hours and was terminated when bromophenol blue reached the bottom of the separation gel.

Transferring the membrane: PVDF membrane was immersed in methanol for 4 minutes, washed with distilled water three times for 2 minutes each, and mixed with the gel and filter paper. The mixture was immersed in the transferring solution for 10 minutes. The filter paper, gel, and PVDF membrane were placed in electrotransfer clip, with the gel toward the anode and PVDF membrane towards the cathode, in the transferring tank. These mixtures were transferred to the membrane at 4°C for 2-3 hours under constant state. PVDF film was stained with 0.2‰ Ponceau, to observe protein transfer status.

Antibodies blocking: After TBST rinsing and blocked with 5% skim milk at room temperature for 2 hours, the incubation was performed with
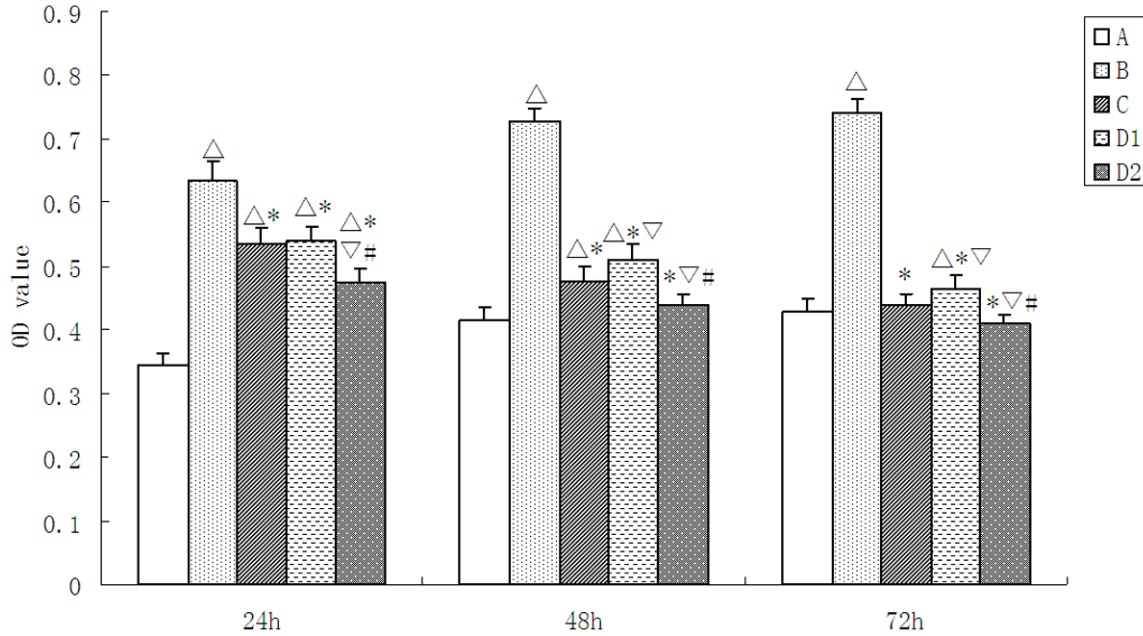
Figure 1. Effect of Huazhuojiedu serum on the proliferation of HSCs stimulated with TGF-β1. A: control group, B: model group, C: positive group, D1: Huazhuojiedu prescription equivalent dose group, D2: Huazhuojiedu prescription double dose group. ΔP < 0.05, vs. control group; *P < 0.05, vs. model group; ▽P < 0.05, vs. positive group; #P < 0.05, vs. Huazhuojiedu prescription equivalent dose group.

Table 1. Effect of Huazhuojiedu serum on the proliferation of HSC stimulated with TGF-β1 ($n = 5$, $\bar{x} \pm s$)

Group	24 h	48 h	72 h
A	0.346±0.017	0.416±0.019	0.429±0.021
B	0.633±0.030^Δ	0.728±0.021^Δ	0.740±0.022^Δ
C	0.534±0.027^Δ,*	0.477±0.022^Δ,*	0.438±0.018^*
D1	0.539±0.024^Δ,*	0.510±0.025^Δ,*,▽	0.464±0.022^Δ,*,▽
D2	0.473±0.024^Δ,*,▽,#	0.439±0.018^*,▽,#	0.410±0.013^*,▽,#

A: control group, B: model group, C: positive group, D1: Huazhuojiedu prescription equivalent dose group, D2: Huazhuojiedu prescription double dose group. ΔP < 0.05, vs. control group; *P < 0.05, vs. model group; ▽P < 0.05, vs. positive group; #P < 0.05, vs. Huazhuojiedu prescription equivalent dose group.

specific antibody (1:600 dilution in blocking solution) at 4°C overnight and with second antibody in the dark for 2 hours. Three TBST washing for 10 minutes each was given between the incubation.

Coloration: ECL luminescence detection systems were used for the analysis and scanning. The ratio of α-SMA, PI3K, p-Akt protein amplification band gray value to β-actin amplification band gray value represented the expression level of α-SMA, PI3K, p-Akt proteins. The results of three scans were averaged to obtain the final value.

Statistical analysis

Data were analyzed using SPSS 13.0 software and expressed as mean ± SD. Two-sample differences were compared using the *t*-test. Multi-sample differences were compared using one-way analysis of variance. The homogeneity of variance test was performed prior to analysis, and the least significant difference test was used if homogeneity of variance was met, while rank sum test was applied if homogeneity of variance was not met. A *P* < 0.05 was considered difference and *P* < 0.01 as significant differences.

Results

Effect of Huazhuojiedu medicated serum on the proliferation of HSCs

As shown in **Figure 1** and **Table 1**, after 24 hours of exogenous TGF-β1 stimulation and corresponding serum treatment, the proliferation of HSCs was significantly higher than that in normal control group (*P* < 0.01). Compared with model group, all medicated serums obvi-
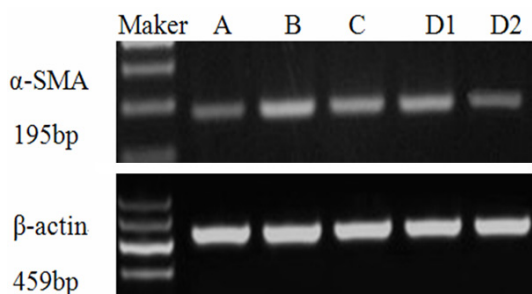
Figure 2. Effect of Huazhuojiedu serum on expression of α-SMA mRNA in HSCs stimulated with TGF-β1. A: control group, B: model group, C: positive group, D1: Huazhuojiedu equivalent dose group, D2: double dose group.

Table 2. Effect of Huazhuojiedu serum on expression of α-SMA mRNA and protein in HSC stimulated with TGF-β1 ($\overline{x}$±s)

Group	α-SMA mRNA (n = 5)	α-SMA protein (n = 3)
A	0.442±0.021	0.134±0.015
B	1.177±0.036$^\Delta$	0.241±0.017$^\Delta$
C	0.848±0.018$^{\Delta,*}$	0.180±0.010$^{\Delta,*}$
D1	0.836±0.022$^{\Delta,*}$	0.185±0.011$^{\Delta,*}$
D2	0.651±0.022$^{\Delta,*,\triangledown,\#}$	0.155±0.014$^{\Delta,*,\triangledown,\#}$

A: control group, B: model group, C: positive group, D1: Huazhuojiedu prescription equivalent dose group, D2: Huazhuojiedu prescription double dose group. $^\Delta P < 0.05$, vs. control group; $^*P < 0.05$, vs. model group; $^\triangledown P < 0.05$, vs. positive group; $^\#P < 0.05$, vs. Huazhuojiedu prescription equivalent dose group.

ously inhibited the HSC proliferation ($P < 0.01$). Huazhuojiedu equivalent dose had the similar inhibition effect with the positive drugs, without significant differences ($P > 0.05$). Huazhuojiedu double dose exerted more pronounced inhibition effect compared with positive control group ($P < 0.01$). Double dose was stronger than equivalent dose in the HSC proliferation ($P < 0.01$). At 48 and 72 hours, HSCs still proliferated in model group and Huazhuojiedu equivalent dose group, when compared to the normal control group ($P < 0.01$). The proliferation of HSCs was obviously inhibited by the treatment of corresponding medicated serum compared with the model group ($P < 0.01$). Huazhuojiedu equivalent dose showed a weaker inhibition effect than positive drug ($P < 0.05$), while Huazhuojiedu double dose exhibited a stronger effect than positive drug ($P < 0.05$). Huazhuojiedu double dose significantly inhibited the proliferation of HSCs when compared to equivalent dose ($P < 0.01$).

Effect of Huazhuojiedu medicated serum on the activation of HSCs

Expression of α-SMA mRNA in HSCs

48-hour TGF-β1 stimulation upregulated the α-SMA mRNA expression in HSCs compared with the normal control group ($P < 0.05$). The α-SMA mRNA expression levels were significantly inhibited by the treatment of corresponding medicated serum, when compared to model group ($P < 0.01$). There was no significant difference in the α-SMA mRNA expression between positive control group and Huazhuojiedu equivalent dose group ($P > 0.05$), while Huazhuojiedu double dose significantly reduced the expression than positive drug ($P < 0.01$). In addition, Huazhuojiedu double dose was stronger than equivalent dose in down-regulating α-SMA mRNA expression ($P < 0.01$) (**Figure 2**, **Table 2**).

Expression of α-SMA protein in HSCs

48-hour TGF-β1 stimulation upregulated the α-SMA protein expression in HSCs compared with the normal control group ($P < 0.05$). The α-SMA protein expression levels were significantly inhibited by the treatment of corresponding medicated serum, when compared to model group ($P < 0.01$). There was no significant difference in the α-SMA protein expression between positive control group and Huazhuojiedu equivalent dose group ($P > 0.05$), while Huazhuojiedu double dose significantly reduced the expression than positive drug ($P < 0.05$). In addition, Huazhuojiedu double dose was stronger than equivalent dose in down-regulating α-SMA protein expression ($P < 0.05$) (**Figure 3**, **Table 2**).

Expression of PI3K protein in HSCs

PI3K protein expression was elevated after treatment, compared with normal control group ($P < 0.05$). The PI3K protein expression levels were reduced by the treatment of corresponding medicated serum ($P < 0.05$), and significantly reduced by Huazhuojiedu double dose ($P < 0.01$), when compared to model group. There was no significant difference in the PI3K protein expression between positive control group and Huazhuojiedu equivalent dose group ($P > 0.05$), while Huazhuojiedu double dose reduced the expression than positive drug ($P < 0.05$). In addition, Huazhuojiedu double dose was stronger than equivalent dose in down-regulating PI3K protein expression ($P < 0.05$) (**Figure 4**, **Table 3**).
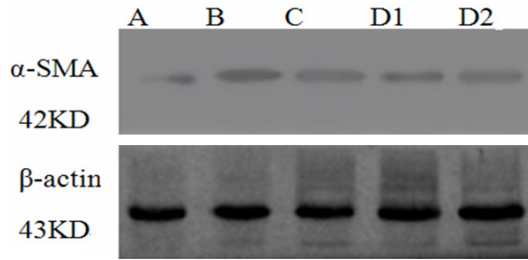
Figure 3. Effect of Huazhuojiedu serum on expression of α-SMA protein in HSCs stimulated with TGF-β1. A: control group, B: model group, C: positive group, D1: Huazhuojiedu equivalent dose group, D2: double dose group.
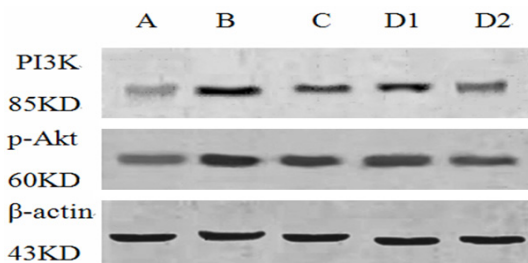

Figure 4. The expression of PI3K and p-Akt protein in HSCs stimulated with TGF-β1. A: control group, B: model group, C: positive group, D1: Huazhuojiedu prescription equivalent dose group; D2: Huazhuojiedu prescription double dose group.

Expression of p-Akt protein in HSCs

The p-Akt protein expression was elevated after treatment, compared with normal control group ($P < 0.05$). The p-Akt protein expression levels were reduced by the treatment of corresponding medicated serum ($P < 0.05$), and significantly reduced by Huazhuojiedu double dose ($P < 0.01$), when compared to model group. There was no significant difference in the p-Akt protein expression between positive control group, Huazhuojiedu equivalent dose and double dose groups ($P > 0.05$). In addition, Huazhuojiedu double dose was stronger than equivalent dose in down-regulating p-Akt protein expression ($P < 0.05$) (**Figure 4, Table 3**).

Discussion

Hepatic fibrosis is characterized by fibroblast proliferation and activation into myofibroblasts, leading to excessive ECM deposition in the liver [10]. Currently myofibroblasts are derived from the HSC activation and proliferation, portal fibroblasts, bone marrow mesenchymal stem

Table 3. The expression of PI3K and p-Akt protein in HSC stimulated with TGF-β1 ($n = 3$, $\overline{x} \pm s$)

Group	PI3K/β-actin	p-Akt/β-actin
A	0.717±0.053	0. 867±0.011
B	1.933±0.097$^{\Delta}$	2.022±0.087$^{\Delta}$
C	1.237±0.067$^{\Delta,*}$	1.439±0.028$^{\Delta,*}$
D1	1.404±0.028$^{\Delta,*}$	1.441±0.009$^{\Delta,*}$
D2	0.917±0.051$^{\Delta,*,\triangledown,\#}$	1.183±0.073$^{\Delta,*,\#}$

A: control group, B: model group, C: positive group, D1: Huazhuojiedu prescription equivalent dose group, D2: Huazhuojiedu prescription double dose group. $^{\Delta}P < 0.05$, vs. control group; $^{*}P < 0.05$, vs. model group; $^{\triangledown}P < 0.05$, vs. positive group; $^{\#}P < 0.05$, vs. Huazhuojiedu prescription equivalent dose group.

cells, epithelial-mesenchymal transition, and peripheral blood mononuclear cells [11]. Among them, HSC is the main source of hepatic fibroblasts/myofibroblasts, and it is regarded as the mediator of liver fibrosis formation. The HSC proliferation, activation and differentiation are regulated by a variety of factors. TGF-β1 is the most important pro-fibrotic cytokine during the process of liver fibrosis, it can activate HSCs, promote the myofibroblast differentiation, increase ECM synthesis, and inhibit ECM degradation, and TGF-β1 contributes to sustained activation during the liver fibrosis formation [12]. In this study, stationary HSCs were stimulated with TGF-β1 *in vitro* to simulate the formation of early liver fibrosis. The results showed that, HSC proliferation was more apparent at 24, 48, 72 hours after TGF-β1 intervention, Huazhuojiedu medicated serum inhibited HSC proliferation in a dose- and time-dependent manner. In addition, after 48 hours of TGF-β1 intervention, Huazhuojiedu medicated serum reduced the α-SMA mRNA and protein expression, thereby inhibiting the activation of HSCs.

The formation of liver fibrosis *in vivo* is a very complex process [13], involving various cells, intercellular matrix and neurotransmitters, which constitute a cumbersome network. In these networks, several signaling pathways attribute to mediate the development of liver fibrosis *in vivo* through different pathways and means. PI3K/Akt signaling pathway has attracted increasing attention in liver fibrosis studies, which is closely related with the fibrosis of a variety of tissues and organs [14] and plays a crucial role in cell survival, differentiation,

growth, and apoptosis [2]. PI3K is the second messenger associated with intracellular signaling conduction [15]. Akt is a serine/threonine protein kinase, the activated PI3K and Akt can interact to each other, but PI3K/Akt pathway is not the only way for the Akt activation [16]. TGF-β1 is the most powerful fibroblast growth factor in HSCs, it can promotes the synthesis of type I collagen through PI3K/Akt pathway with the assistance of p38MAPK and p70S6k [12]. The HSC proliferation is critically related to the PI3K activation and the Akt phosphorylation [17]. Therefore the expression of PI3K and p-Akt protein is often used as the study focus.

Some extracts and active ingredients of Chinese herbs can inhibit the expression of PI3K/Akt pathway-related proteins in the HSCs. Previous study [18] found that, Rhizoma Curcumae extract medicated serum could inhibit PI3K expression in the activated HSCs induced by PDGF; other scholars [19] have shown that, Danshensu inhibited the PDGF-BB-induced HSC proliferation and the expression of p-Akt protein, indicating that the mechanism of Danshensu associated with the Akt phosphorylation may be related to inhibition of PI3K/Akt signal transduction pathway. There is little evidence available on the role of compound Chinese herbs on liver fibrosis through the PI3K/Akt pathway. The results of this study found that, PI3K and p-Akt protein expression in Huazhuojiedu equivalent dose and double dose groups were significantly lower than that in model group. This evidence suggests that, Huazhuojiedu medicated serum inhibits PI3K and p-Akt protein expression, and accordingly interferes PI3K/Akt signal transduction pathway in a dose-dependent manner; PI3K protein expression in Huazhuojiedu double dose group was significantly lower than that in positive control group, suggesting the regulatory action on PI3K/Akt signaling pathway.

In summary, Huazhuojiedu medicated serum can inhibit HSC proliferation and activation induced by TGF-β1 *in vitro*, and downregulate the expression of PI3K and p-Akt protein. The mechanisms of anti-hepatic fibrosis depend on the intervention of PI3K/Akt pathway transduction.

Acknowledgements

Disclosure of conflict of interest

None.

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