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Seropharmalogical effects of Fuzheng Huayu decoction on rat Ito cell morphology and function in culture

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Abstract

AIM: To investigate the mechanisms of anti-liver fibrosis actions of Fuzheng Huayu (FZHY) decoction, which acts to strengthen the body' s resistance and promote blood circulation.

METHODS: Ito cells were isolated from rats and cultured. Serum samples were collected from healthy (normal) rats after administration of FZHY decoction and added to the subcultured cells. The effects of FZHY decoction on the Ito cells were investigated by contrast microscopy (to observe cell morphology), [^{3}H]Pro incorporation assay (cell viability), [^{3}H]TdR incorporation and MTT colorimetric assay (cell proliferation), and [^{3}H]Pro incorporation and collagenase digestion (collagen synthesis rate).

RESULTS: The rat sera samples from rats treated with FZHY decoction had no influence on Ito cell morphology, but improved cell viability and markedly inhibited cell proliferation and collagen synthesis. The magnitude of these effects showed dependence on treatment dosage and drug concentration in serum.

CONCLUSION: The seropharmalogical method can be efficiently used to investigate the pharmacological mechanism of anti-fibrotic traditional Chinese herbs and formulae. Inhibition of Ito cell proliferation and collagen synthesis may be two of the major mechanisms underlying the anti-fibrosis actions of the FZHY decoction.

Key words: Fuzheng Huayu; Liver fibrosis; Ito cell; Collagen synthesis

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INTRODUCTION

Ito cells (also known as hepatic stellate cells, fat-storing cells or hepatic lipocytes) play a key role in liver fibrosis, the main hallmark of chronic liver diseases. In the chronic liver disease condition, Ito cells are activated, proliferative and synthesize large amounts of various components of the extracellular matrix, which may lead to liver fibrosis[1]. When cultured on uncoated plastic plates, Ito cells undergo activation and share the similar features of cell activation that are observed in the *in vivo* condition[2]. Our previous study[3] demonstrated that Fuzheng Huayu (FZHY) decoction, which acts to strengthen the body's resistance and promote blood circulation, exerts a protective effect on the liver in CCl_4-induced fibrotic rats, suggesting its potential for improving liver status and function in patients with cirrhosis. In order to investigate the actions of FZHY decoction on liver cells, the seropharmalogical method was applied to an *in vitro* (culture) system with rat Ito cells to determine the effects on cell morphology and function (*i.e.* cell viability, proliferation and collagen synthesis).

MATERIALS AND METHODS

Animals

Wistar male rats, weighing 350-500 g, were purchased from the Shanghai Science Academy.

Reagents

Minimum essential medium (MEM; developed by Eagle), 199 medium (M199) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco, USA. Pronase E, type IV collagenase, metrizimide and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co., USA. L- [5-^{3}H] proline ([^{3}H]Pro) was purchased from Amersham Co., England.

Drug

The primary components of FZHY decoction are Cerdeceps, semen Persiciae and Radix Salviae Miltiorr hizae. The decoction fluid extract, containing 2.703 g of each of the above raw herbs per g of fluid, was supplied by the Shanghai Zhonghua Pharmaceutical Factory.

Table 1 Effect of drug sera on Ito cell viability ($\bar{x} \pm s$)

Group	*n*	[^{3}H]Pro (cpm/well)
Controls	4	3610.18 ± 99.47
G	4	6578.13 ± 1690.95[a]
G	4	9606.33 ± 950.85[a]
13.8 g	4	4560.26 ± 2026.83

[a]$P < 0.05$ *vs* controls.

Table 2 Effect of drug sera on Ito cell proliferation ($\bar{x} \pm s$)

Group	*n*	[^{3}H]TdR (cpm/well)	MTT (OD_{570})
Controls	4	2864.50 ± 239.15	0.4206 ± 0.016
G	4	1979.65 ± 76.76[a]	0.4625 ± 0.06
G	4	1882.05 ± 112.08[a]	0.3475 ± 0.040[a]
13.8g	4	1781.93 ± 49.69[a]	0.3268 ± 0.081[a]

[a]$P < 0.05$, *vs* controls. MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.

Table 3 Effect of sera in different proportions to medium on Ito cell proliferation ($\bar{x} \pm s$)

Concentration	*n*	Controls	Drug sera	Inhibitive rate (%)
5%	4	1481.90 ± 168.03	703.03 ± 138.37[b]	52.56 ± 9.34
10%	4	2013.88 ± 628.87	637.33 ± 214.53[b]	68.35 ± 10.68
20%	4	2709.88 ± 788.19	480.20 ± 264.31[b]	82.27 ± 9.75

[b]$P < 0.01$ *vs* controls.

Table 4 Influence of drug sera on Ito cell collagen synthesis (%, $\bar{x} \pm s$)

Concentration	*n*	Intracellular		Extracellular	
		Controls	Drug sera	Controls	Drug sera
5%	6	0.42 ± 0.18	0.26 ± 0.10	4.02 ± 1.63	3.12 ± 30.33
10%	6	0.48 ± 0.16	0.25 ± 0.06[a]	4.49 ± 1.28	2.71 ± 1.04[a]
20%	6	0.58 ± 0.26	0.24 ± 0.08[a]	4.80 ± 1.47	2.59 ± 1.06[a]

[a]$P < 0.05$ *vs* controls.

Preparation of drug serum

The FZHY fluid extract was diluted to concentrations of 2.3%, 4.6% and 13.8% (w/v) with distilled water. Healthy (normal) rats were administered one of the dilutions orally at 10 mL/kg wt, twice, at an interval of 2 h. Control rats were given normal saline. One hour after the last administration, blood was collected from the inferior vena cava of the rats under sterile conditions. The samples were centrifuged at 1700 × *g* for 30 min at 4 ℃. The separated sera were then combined from rats that had received the same dosage of drug. The combined samples were mixed thoroughly, inactivated by incubating in a 56 ℃ water bath for 30 min, and stored in -70 ℃ freezer.

Cell isolation and culture

Ito cells were isolated from livers of Wistar rats and cultured according to the modified Friedman method as previously described[4]. Briefly, the cells were identified by immunofluorescent staining with desmin antibody and according to typical appearance under light microscopy. The recovery rate was 2.5 × 10^7 per liver, purity > 95%, and viability > 98%, as determined by the trypan blue exclusion assay. The primary Ito cells were expanded in culture, with passaging using 0.25% trypsin 0.02% EDTA; upon reaching confluence, the cells were subcultured with M199 containing 10% fetal calf serum in a humid CO_2 incubator with 5% CO_2 and 95% air.

Cell proliferation assay

The [^{3}H]TdR incorporation method[5] was used to assess cell proliferation. Confluent subcultured Ito cells in 24-well plates were incubated with M199 containing 5%, 10% or 20% (v/v) drug sera respectively. After 48 h, [^{3}H]TdR was pulsed in at 2.5 μCi/well and the cells were incubated for an additional 24 h, after which the cells were harvested for measurement of cpm by a Wallac 1410 Scintillator (Beckman, USA).

Colorimetric MTT[6]

Confluently subcultured Ito cells in 96-well plates were incubated with 100 μL M199 containing drug serum. After 68 h, MTT solution (5 g/L in PBS) was added to each well at 10 μL per 100 μL medium and the cells were incubated for an additional 4 h. Next, acid-isopropanol (100 μL of 0.04 *n* HCI in isopropanol) was added, mixed thoroughly and allowed to incubate for a few minutes at room temperature to dissolve the dark blue crystals. The plates were read on an ELISA reader at wavelength of 570 nm.

Cell viability assay

Cell viability was indicated by intracellular protein synthesis[7]. Specifically, the cells were processed as described above for the [^{3}H]TdR incorporation method, but with use of the [^{3}H]Pro (2.5 μCi/well) instead of [^{3}H]TdR.

Cell collagen synthesis rate assay

Greets' method[8] was used to assess the collagen synthesis rate. Specifically, confluently subcultured Ito cells in 6-well plates were incubated with M199 containing the drug sera. After 48 h, the culture medium was replaced with DMEM containing 5 μCi/mL [^{3}H]Pro, 100 mg/L β-aminopropionitrile, and 50 mg/mL ascorbic acid. After 24 h, the new culture medium and cells were collected respectively. The cells were processed for whole cell extract. The samples were dialyzed thoroughly and reacted with collagenase. Measurement of the total radioactivity in the samples (cpm-t), and the radioactivity in the samples treated with collagenase (cpm-c) and without collagenase (cpm-b), was made by liquid scintillation spectrometry. The amount of collagen produced (*i.e.* the fraction of collagenous protein) was expressed as percentage of total radiolabeled protein and calculated using the formula:

$$\% \text{ collagen} = 100/[5.4 \times (cpm_t\text{-}cpm_c)/(cpm_c - cpm_b) + 1]$$

RESULTS

Effects of drug serum on Ito cell morphology

Both normal rat and drug serum could sustain cell survival and growth, and there was no significant difference in cell shape. Compared with the fetal calf serum control treatment, normal rat serum and drug serum also showed no different influence on cell shape.

Effects of drug serum on Ito cell viability

The drug serum from the rats treated with 2.3 g and 4.6 g doses improved cell [^{3}H]Pro incorporation, while the latter producing a greater improvement (Table 1).

Effects of drug serum on Ito cell proliferation

Effects of sera from rats treated with different drug doses All sera from the different dose groups were added to Ito cells in 10% (v/v) proportion of culture medium. The drug sera inhibited cell [^{3}H]TdR incorporation and transformation of MTT to formazan, and this inhibitive effect was dose-dependent (Table 2).

Effects of sera concentration on Ito cell proliferation The drug sera derived from the 4.6% (w/v) group were prepared in 5%, 10% and 20% (v/v) proportion to M199 and added to the Ito cells, respectively. The results showed that the inhibitive effect on cell proliferation was enhanced as the drug concentration in M199 increased (Table 3).

Effect of drug serum on Ito cell collagen synthesis

The drug sera derived from the 4.6% (w/v) group were added to cells in 5%, 10% and 20% (v/v) proportion to M199. The 10% and 20% proportions inhibited both intracellular and extracellular collagen synthesis, and showed a greater tendency of dose dependence (Table 4).

DISCUSSION

It is well known that Ito cells play a central role in liver fibrogenesis, and inhibition of Ito cell activation is one of the main targets for

anti-fibrosis therapy[9]. Although the optimal anti-fibrosis drug has not been found yet, some herbal decoctions have been reported to prevent fibrogenesis effectively. Traditional Chinese Medicine (TCM) has shown a brilliant future in its application, in particular. FZHY decoction has been shown to prevent extracellular matrix production and deposits in CCl_4- or DMN-induced fibrotic rats, with potential to improve fibrotic liver structure and function in patients with chronic hepatitis or cirrhosis[3].

Chinese herbs have very complicated components, and their pharmacokinetics remain unclear. Therefore, it is difficult to investigate their pharmacological actions *in vitro* by direct addition of herbs or their rough extracts to cultured cells, because the direct addition method could change the *in vitro* environment (such as medium osmotic pressure and pH value, *etc*.) or cause loss of effective metabolites of the herbs or exert an action of unabsorbed substances. In clinical practice, most herbs are administered orally and absorbed into the blood circulation where they exert their functions. Therefore, the serum pharmacological method of indirect addition of a drug serum to culture systems *in vitro* could overcome the above shortcomings; in this way, when the drug is applied the herbal actions can be studied and their real pharmacological effects determined[5].

We found that drug serum collected 1 h after two administrations of FZHY decoction in rats could be applied to perform convenient and efficient investigations[10]. In this study, the drug serum showed no obvious influence on Ito cell morphology, but could improve cell viability. Thus, the drug serum had no cytotoxicity, but produced an effect of "strengthening sufficiency". This also indicated that the seropharmalogical method could reflect the practical effect of Chinese herbs.

Ito cell activation was characterized in this study by increased total cell number (proliferation) and enhanced per cell fibrogenesis. [^{3}H]TdR incorporation represents the cellular DNA synthesis rate and the cleavage of MTT represents the activity of dehydrogenase enzymes in active mitochondria[6], both of which reflect cell proliferation. Cell collagen synthesis consists of two processes: Intracellular translation and hydroxization, followed by extracellular excretion and processing. The [^{3}H]Pro incorporation and collagenase digestion methods reflect both the intracellular and extracellular collagen production processes for individual cells. In this study, the drug serum decreased the Ito cells' [^{3}H]TdR incorporation and MTT cleavage, and inhibited both intracellular and extracellular collagen synthesis per cell. These effects were related to the drug dose and drug serum concentration. Thus, FZHY decoction could inhibit Ito cell proliferation and collagen synthesis, and prevent cell activation. This may be one of the major mechanisms of the anti-fibrotic actions of FZHY decoction.

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