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Effect of Six flavonoids on proliferation of hepatic stellate cells *in vitro*[1]

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KEY WORDS fisetin; quercetin; apigenin; phloretin; hesperetin; chalcone; cell division; macrophages; platelet-derived growth factor

ABSTRACT

AIM: To study the effects of six flavonoids (fisetin, quercetin, apigenin, phloretin, hesperetin, and chalcone) on proliferation of hepatic stellate cell (HSC-T6 cells). **METHODS:** Cell proliferation was measured by crystal violet staining assay. **RESULTS:** Fisetin, quercetin, apigenin, phloretin, hesperetin, chalcone (6.25 – 50 $\mu mol \cdot L^{-1}$) inhibited the proliferation of HSC-T6 cells stimulated by serum, macrophage conditioned medium (MCM) and platelet-derived growth factor (PDGF) in a concentration-dependent manner. In the MCM-stimulated proliferation experiment, their IC_{50} were 21.48, 18.52, 19.75, 22.32, 30.32, and 30.85 $\mu mol \cdot L^{-1}$, respectively. In the PDGF-stimulated proliferation experiment, their IC_{50} were 9.47, 9.48, 9.25, 12.25, 25.22, and 30.40 $\mu mol \cdot L^{-1}$, repectively. **CONCLUSION:** The six flavonoids inhibited the proliferation of hepatic stellate cells.

INTRODUCTION

Hepatic stellate cells (HSC) play a central role in the liver fibrogenesis. During fibrosis, HSC are activated and undergo a phenotypic change to myofibroblasts, which are highly proliferative and synthesize most extracellular matrix components[1]. The proliferation of HSC is mainly stimulated by Kupffer/macrophage cell conditioned medium (MCM), especially platelet-derived growth factor (PDGF)[2,3]. Thus, development of specific inhibitors which can effectively block HSC proliferation are of particular therapeutic interest for hepatic fibrosis.

Fisetin, a flavonoid extracted from *Gleditsia sinensis* lam, exhibited inhibitory effects on myofibroblast proliferation using an *in vitro* crystal violet assay in our previous studies[4]. Here, we used HSC-T6 cells, a myofibroblast line, as target cells[5] and compared the effects of 6 flavonoids (fisetin, quercetin, apigenin, phloretin, hesperetin, and chalcone) on HSC-T6 proliferation in response to serum, MCM and PDGF, to provide the theoretical basis for further structural modification of flavonoids.

MATERIALS AND METHODS

Reagents Fisetin, quercetin, apigenin, phloretin, hesperetin, chalcone, and colchicine were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM) and other reagents were from Gibco.

Mice ICR Mice, ♀, weighing 28 g ± *s* 3 g, from the Animal Center of Second Military Medical University (Certificate № 28 – 48, grade Ⅱ) were used.

Cell culture HSC-T6, a myofibroblast line, which had the stable phenotype and biochemical characters, was kindly provided by Dr S L Friedman (Liver Center Laboratory, San Francisco General Hospital, USA). The cells were cultured in DMEM with 10 % calf serum at 37 ℃ in a humidified atmosphere of 5 % CO_2 + 95 % air.

Preparation of activated macrophage conditioned medium The macrophage conditioned medium (MCM) was prepared[6]. Briefly, the peritoneal exudate cells (PEC) from ICR mice were stimulated with calcimycin 1 $\mu mol \cdot L^{-1}$ and lipopolysaccharides 100 $\mu g/L$. Cells were washed with PBS three times and then incubated with DMEM for 24 h. The supernatants were stored at – 30 ℃ until use.

Cell proliferation HSC-T6 cells (1×10^4/well) were plated in 96-well microplate for 24 h. Cells were then incubated in DMEM with 10 % calf serum in the

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presence of serial concentration of the tested flavonoids. For the untreated controls, an equal amount of drug-solvent (DMEM containing 0.1 % Me_2SO) was added. After an 48-h incubation, cell density was measured by crystal violet assay and expressed as A_{595}[7].

In PDGF- and MCM-stimulated proliferation, the medium was removed after a 24-h incubation. Then DMEM with 0.4 % calf serum was added for another 48 h. Flavonoids and PDGF (10 $\mu g \cdot L^{-1}$) or MCM (1:4, v/v) were added into medium. After 24 h, cell density was measured.

The inhibitory rate was calculated:

Inhibition % (serum) =

$$(A_{Control} - A_{Drug}) / A_{Control} \times 100$$

Inhibition % (MCM or PDGF) =

$$(A_{Control} - A_{Drug}) / (A_{Control} - A_{Medium}) \times 100$$

Statistics Data were analyzed by ANOVA and t-test.

RESULTS

Effect of colchicine on growth of HSC-T6 cells The proliferation of HSC-T6 cells stimulated by serum was inhibited by colchicine, and the proliferation stimulated by MCM or PDGF was also inhibited by colchicine in a concentration-dependent manner (Tab 1).

Effect of flavonoids on growth of serum-stimulated HSC-T6 cells Six flavonoids (6.25 – 50 $\mu mol \cdot L^{-1}$) inhibited the proliferation of HSC-T6 cells in a concentration-dependent manner. ANOVA showed that 6 flavonoids could be divided into two groups on the basis of their anti-proliferation effects. One group containing fisetin, quercetin, apigenin, and phloretin was of stronger activities than another consisting of hesperetin and chalcone. (Tab 2)

Tab 1. Effect of colchicine on growth of HSC-T6 cells. $n=6$. $\bar{x} \pm s$. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* 0.

Colchicine nmol·L⁻¹	Absorbance at 595 nm		
	Serum (10 %)	MCM (1:4)	PDGF (10 μg·L⁻¹)
0	0.98±0.06	1.02±0.01	0.90±0.07
7.8125	0.91±0.10[a]	0.99±0.05[a]	0.88±0.02[a]
15.625	0.84±0.06[c]	0.90±0.02[c]	0.82±0.05[c]
31.25	0.67±0.06[c]	0.83±0.07[c]	0.75±0.04[c]
62.5	0.47±0.06[c]	0.78±0.05[c]	0.68±0.06[c]

Effect of flavonoids on proliferation of MCM-stimulated HSC-T6 cells The macrophage conditioned medium (1:4) markedly induced proliferation of HSC-T6 cells, the proliferation rate was 37.8 %. The six flavonoids, fisetin, quercetin, apigenin, phloretin, hesperetin, and chalcone inhibited the proliferation in a concentration-dependent manner. The IC_{50} were 21.48 (18.27 – 25.25), 18.52 (15.73 – 21.81), 19.75 (17.19 – 22.69), 22.32 (19.72 – 25.27), 30.32 (24.60 – 37.48), 30.85 (26.96 – 35.30), respectively (Tab 3).

Tab 2. Effect of flavonoids on growth of serum-stimulated HSC-T6 cells. $n=6$. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

Treatment/μmol·L^{-1}		A_{595}	Inhibition/%
Medium		0.96 ± 0.04	
Control		0.98 ± 0.06	
Fisetin	6.25	0.96 ± 0.07^a	2.04
	12.5	0.91 ± 0.03^b	7.14
	25	0.82 ± 0.04^c	16.33
	50	0.57 ± 0.05^c	41.84
Quercetin	6.25	0.95 ± 0.06^b	3.06
	12.5	0.87 ± 0.07^a	11.22
	25	0.66 ± 0.05^c	32.65
	50	0.48 ± 0.04^c	51.02
Apigenin	6.25	0.94 ± 0.04^a	4.08
	12.5	0.84 ± 0.02^c	14.28
	25	0.74 ± 0.06^c	26.53
	50	0.56 ± 0.05^c	42.86
Phloretin	6.25	0.96 ± 0.10^a	2.04
	12.5	0.85 ± 0.03^c	13.26
	25	0.72 ± 0.07^c	26.53
	50	0.50 ± 0.06^c	48.98
Hesperetin	6.25	0.94 ± 0.07^a	4.08
	12.5	0.91 ± 0.08^a	7.14
	25	0.87 ± 0.05^b	11.22
	50	0.72 ± 0.05^c	26.53
Chalcone	6.25	0.97 ± 0.06^a	1.02
	12.5	0.93 ± 0.04^a	5.10
	25	0.86 ± 0.03^c	12.24
	50	0.69 ± 0.06^c	29.59

Tab 3. Effect of flavonoids on proliferation of MCM-stimulated HSC-T6 cells. $n=6$. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

Treatment/μmol·L^{-1}		A_{595}	Inhibition/%
Medium		0.74 ± 0.01	
Control (MCM)		1.02 ± 0.01	
Fisetin	6.25	1.02 ± 0.02^a	0
	12.5	0.96 ± 0.02^c	21.43
	25	0.89 ± 0.02^c	46.28
	50	0.82 ± 0.02^c	71.43
Quercetin	6.25	0.98 ± 0.08^a	14.28
	12.5	0.94 ± 0.04^b	28.57
	25	0.84 ± 0.06^c	64.28
	50	0.74 ± 0.06^c	100.00
Apigenin	6.25	1.00 ± 0.08^a	7.14
	12.5	0.96 ± 0.08^a	21.43
	25	0.84 ± 0.02^c	64.28
	50	0.73 ± 0.03^c	103.57
Phloretin	6.25	0.99 ± 0.06^a	10.71
	12.5	0.96 ± 0.06^a	21.43
	25	0.89 ± 0.05^c	46.43
	50	0.77 ± 0.05^c	89.29
Hesperetin	6.25	0.99 ± 0.04^a	10.71
	12.5	0.94 ± 0.04^b	28.57
	25	0.90 ± 0.05^b	42.86
	50	0.84 ± 0.05^c	64.28
Chalcone	6.25	1.01 ± 0.05^a	3.57
	12.5	0.99 ± 0.06^a	10.74
	25	0.90 ± 0.07^b	42.85
	50	0.82 ± 0.07^c	71.43

Effect of flavonoids on proliferation of PDGF-stimulated HSC-T6 cells PDGF (10 μg/L) markedly stimulated the proliferation of HSC-T6 cells, the increase rate was 42.86 %. After adding fisetin, quercetin, apigenin, phloretin, hesperetin, and chalcone, the PDGF-driven proliferation was reduced concentration-dependently. The IC_{50} were 9.47 (8.20 – 10.95), 9.48 (8.24 – 10.91), 9.25 (8.24 – 10.38), 12.25 (10.45 – 14.39), 25.22 (20.95 – 30.36), 30.40 (25.73 – 35.93), respectively (Tab 4).

DISCUSSION

The proliferation of HSC was influenced by many factors, such as cytokines. Manipulation of these cytokines may constitute a significant new approach in the modulation of liver fibrosis by blocking the actions of these cytokines. We used an HSC cell line, HSC-T6 cells stimulated with serum, MCM, and PDGF as an experimental model for screening the agents that will effectively block HSC proliferation. In this model, the stimulation by serum, MCM, and PDGF may reflect the effects of the blood bioactive factors, the fibrogenic cytokines released by activated macrophages in local environment and the most mitogenic factor PDGF on HSC proliferation. Colchicine is a well-known anti-fibrotic drug in clinical treatment[8]. It was tested in this model as a positive control. The data demonstrated that colchicine could inhibit the proliferation of HSC-T6 cells stimulated by serum, MCM, and PDGF. It suggests that this model sounds feasible for screening anti-fibrotic agents *in vitro*.

Our results showed that six flavonoids, fisetin, quercetin, apigenin, phloretin, hesperetin, and chalcone reduced the increase of HSC-T6 proliferation derived by serum, MCM, and PDGF. Analysis of variance demonstrated that these six flavonoids were divided into two groups, one having high activities including fisetin, quercetin, apigenin, phloretin, and the other having low activities including hesperetin and chalcone. Having

Tab 4. Effect of flavonoids on proliferation of PDGF-stimulated HSC-T6 cells. $n=6$. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

Treament/μmol·L^{-1}		A_{595}	Inhibition/%
Medium		0.63 ± 0.03	
Control (PDGF)		0.90 ± 0.07	
Fisetin	6.25	0.82 ± 0.05^a	29.63
	12.5	0.72 ± 0.04^b	66.67
	25	0.67 ± 0.04^c	85.18
	50	0.62 ± 0.06^c	103.70
Quercetin	6.25	0.88 ± 0.03^a	33.41
	12.5	0.81 ± 0.04^b	55.33
	25	0.74 ± 0.04^c	89.25
	50	0.63 ± 0.06^c	100.00
Apigenin	6.25	0.82 ± 0.06^a	29.63
	12.5	0.73 ± 0.04^b	62.96
	25	0.64 ± 0.02^c	96.30
	50	0.58 ± 0.05^c	118.52
Phloretin	6.25	0.83 ± 0.06^a	25.93
	12.5	0.76 ± 0.07^b	51.85
	25	0.70 ± 0.06^c	74.07
	50	0.61 ± 0.07^c	107.41
Hesperetin	6.25	0.86 ± 0.07^a	14.81
	12.5	0.83 ± 0.08^a	25.93
	25	0.75 ± 0.07^b	55.56
	50	0.72 ± 0.08^c	66.67
Chalcone	6.25	0.89 ± 0.08^a	3.70
	12.5	0.83 ± 0.04^a	25.93
	25	0.79 ± 0.06^b	40.74
	50	0.72 ± 0.05^c	66.67

compared the structural features of these compounds, we found 4′-hydroxy group was closely related to high activity. When the 4′-hydroxy group was missing in chalcone or when it was changed to 4′-methoxy in hesperetin, their activities extensively decreased. Secondly, it did not malter if 1,4-benzopyranone ring moiety existed or not as similar activity was observed in both groups. These results indicate that the moiety 1,4-benzopyranone was not essential for their biological activities. PDGF is one of the important components in serum and accounts for 50 % – 70 % of the total macrophage-derived mitogenic activity[9,3]. The results in this report showed that the compounds with high activities inhibited proliferation of HSC-T6 cells at the highest concentration with inhibitory rates about 100 % – 120 % when stimulated by PDGF, about 70 % – 100 % by MCM, and about 40 % – 50 % by serum. All the above results suggest that inhibition of HSC proliferation by 6 flavonoids mainly arose from the blocking of the proliferative action induced by PDGF.

In summary, our results showed that these six flavonoids effectively blocked HSC proliferation and they may be beneficial in liver fibrosis. The relationship of structure-bioactivity may provide a basis for rational structure modification.

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6种黄酮类化合物对离体肝储脂细胞增殖的影响[1]

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关键词 黄颜木素；槲皮素；芹菜素；根皮素；橙皮素；查耳酮；细胞分裂；巨噬细胞；血小板源生生长因子

目的：研究黄颜木素、槲皮素、芹菜素、根皮素、橙皮素和查耳酮对血清、巨噬细胞上清和血小板源生生长因子刺激的 HSC-T6 细胞增殖的影响. 方法：结晶紫染色法测定细胞增殖. 结果：黄颜木素、槲皮素、芹菜素、根皮素、橙皮素和查耳酮(6.25 – 50 μmol/L)以剂量依赖方式显著抑制血清、巨噬细胞上清和血小板源生生长因子诱导的 HSC-T6 细胞增殖. 结论：黄颜木素、槲皮素、芹菜素、根皮素、橙皮素和查耳酮具有抑制肝储脂细胞增殖的作用.

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