

Antifibrotic effects of matrine on *in vitro* and *in vivo* models of liver fibrosis in rats¹

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KEY WORDS matrine; liver cirrhosis; cell division; collagen; platelet-derived growth factor; transforming growth factor beta

ABSTRACT

AIM: To study the antifibrotic effects of matrine *in vitro* and *in vivo*. **METHODS:** Rat hepatic stellate cell HSC-T6 and mouse fibroblast cell NIH3T3 proliferation stimulated with serum and platelet-derived growth factor (PDGF) was measured by crystal violet staining assay. Collagen synthesis stimulated with serum and transforming growth factor β_1 (TGF- β_1) was determined by [³H]proline incorporation. Liver fibrosis was induced by carbon tetrachloride (CCl₄) in rats and evaluated with plasma hyaluronic acid level and hepatic hydroxyproline content. **RESULTS:** Matrine (1 ~ 2 mmol · L⁻¹) markedly reduced serum-driven proliferation and collagen synthesis of HSC-T6 cells as well as NIH3T3 cells. PDGF-driven proliferative activity and TGF- β_1 -driven collagen synthesis in HSC-T6 cells were attenuated by matrine (0.25 ~ 2 mmol · L⁻¹) in a concentration-dependent manner. *In vivo* matrine (50 mg · kg⁻¹ and 100 mg · kg⁻¹) significantly decreased serum hyaluronic acid levels and hepatic hydroxyproline contents in rats treated with CCl₄. **CONCLUSION:** Inhibition of PDGF and TGF- β_1 actions on hepatic stellate cell by matrine might provide a possible mechanism of its antifibrotic activities.

INTRODUCTION

Matrine (Mat), an alkaloid found in kinds of *Sophora* plants in *Leguminosae*, shows pharmacological

effects such as anti-inflammation, immuno-inhibition, and anti-arrhythmia, and has been used in treatment of chronic liver disease^[1]. We have previously reported the favorable effects of Mat on liver damage in experimental models were induced by lipopolysaccharide/*D*-galactosamine and *Propionibacterium acnes*^[2,3]. However, the protective effect of Mat on the development of liver fibrosis and its possible mechanism remain unknown.

Hepatic stellate cells (HSC) and the derived myofibroblasts play a central role in liver fibrogenesis. HSC are the major collagen-producing cells of the liver and are transformed into proliferative myofibroblasts during fibrosis^[4]. Here, we studied effects of Mat on cell proliferation and collagen synthesis of rat myofibroblast cell line, HSC-T6 cells^[5] and mouse fibroblast cell NIH3T3 cells in response to serum, platelet derived growth factor (PDGF) or transforming growth factor beta-1 (TGF- β_1), as well as its antifibrotic properties in a model of liver fibrosis induced by carbon tetrachloride (CCl₄) in rats.

MATERIALS AND METHODS

Reagents Mat (mp 75.5 °C ~ 77.5 °C, purity > 99 %) was obtained from Yanchi Pharmaceutical Factory, Ningxia, China. PDGF and TGF- β_1 were from Sigma. Dulbecco's modified Eagle's medium (DMEM) was from Gibco. [³H]proline was from Beijing Institute of Nuclear Research, Chinese Academy of Science. Hyaluronic acid RIA kit was purchased from Shanghai Navy Medical Research Institute.

Animal and cell line Sprague-Dawley rats, ♂, weighing 164 ± s 30 g, were from the Animal Center of Second Military Medical University (Grade II, Certificate No 02-25-3).

HSC-T6 cell, an immortalized rat hepatic stellate cell line, which had the stable phenotype and biochemical characters, was kindly provided by Dr Friedman SL (Liver Center Laboratory, San Francisco General Hospital, USA). NIH3T3 fibroblast was from Department of

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Immunology of this university. All the cells were cultured in DMEM with 10 % calf serum at 37 °C in a humidified atmosphere of 5 % CO₂ + 95 % air.

Cell proliferation HSC-T6 cells or NIH3T3 cells (1 × 10⁴/well) were seeded in 96-well microplate for 24 h. Cells were then incubated in DMEM with 10 % calf serum with or without matrine. After a 48-h incubation, cell density was measured by crystal violet assay and expressed as A₅₉₅⁽⁷⁾.

To test the effect of matrine on PDGF-driven proliferation of HSC-T6 cells, the culture medium was replaced with DMEM supplemented with 0.4 % calf serum and incubated for 48 h. PDGF (10 μg · L⁻¹) and matrine were then added simultaneously and incubated for another 24 h. The inhibitory rate was calculated;

$$\text{Inhibition \% (serum)} = (A_{\text{Control}} - A_{\text{Drug}}) / A_{\text{Control}} \times 100 \%$$

$$\text{Inhibition \% (cytokine)} = (A_{\text{Control}} - A_{\text{Drug}}) / (A_{\text{Control}} - A_{\text{Medium}}) \times 100 \%$$

Collagen synthesis HSC-T6 cells or NIH3T3 cells (3.5 × 10⁴/well) were seeded in 96-well microplate for 24 h. Cells were then incubated in DMEM with 10 % calf serum, ascorbic acid 50 mg · L⁻¹, together with or without matrine for 24 h, and labeled with [³H] proline 7.4 kBq/well for an additional 24 h. The cells were treated with trypsin and harvested onto glass fibers. The radioactivities were counted in a liquid scintillation counter. Collagen synthesis was expressed as dpm and inhibitory rate was calculated.

To test the effect of matrine on TGF-β₁-driven collagen synthesis of HSC-T6 cells, the culture medium was replaced with DMEM supplemented with 2 % calf serum, TGF-β₁ (2 μg · L⁻¹), ascorbic acid 50 mg · L⁻¹, and matrine.

Animal model and drug treatment Twenty four Sprague-Dawley rats were divided into four groups (six rats in each group): the first and second matrine groups were treated with subcutaneous injection of 1 mL · kg⁻¹ of 50 % CCl₄ diluted in olive oil twice a week for 12 weeks. These rats were also intragastrically given matrine at a dose of 50 or 100 mg · kg⁻¹ respectively, once a day till the end of experiment after the first injection of CCl₄. A control group was treated with CCl₄ as described above. The rats were given equal volumes of normal saline instead of matrine. A model control (normal group) was not treated with either CCl₄ or matrine but received equal volume of olive oil.

At the end of the experiment, blood samples were centrifuged and plasma was kept at -20 °C until the as-

says were performed. The liver was collected at the time the animals were killed. Plasma hyaluronic acid (HA) concentration was determined using HA RIA analysis kit. Hepatic hydroxyproline content was measured by a colorimetric method^[8].

Statistics Data were analyzed by ANOVA and student *t*-test, and data were expressed as $\bar{x} \pm s$.

RESULTS

Effect of matrine on serum- and PDGF-driven cell proliferation HSC-T6 cell proliferation was concentration-dependently decreased by matrine at the concentration of 0.5, 1, and 2 mmol · L⁻¹ with an inhibitory rate of 15 %, 24 %, and 43 % respectively when stimulated with serum (*P* < 0.01), while NIH3T3 fibroblast cell proliferation was markedly inhibited at the concentration of 2 mmol · L⁻¹ with an inhibitory rate of 14.4 % (Tab 1). These inhibitory effects were not attributed to the unspecific cytotoxic effects of matrine.

Tab 1. Effect of matrine on serum-driven proliferation of HSC-T6 cells and NIH3T3 cells. *n* = 6. $\bar{x} \pm s$. **P* > 0.05, ***P* < 0.01 vs matrine 0 mmol · L⁻¹.

Matrine/mmole · L ⁻¹	A ₅₉₅	
	HSC-T6	NIH3T3
0	1.00 ± 0.05	1.04 ± 0.06
0.25	0.89 ± 0.14 ^a	1.03 ± 0.01 ^a
0.50	0.85 ± 0.07 ^c	1.01 ± 0.06 ^a
1.00	0.76 ± 0.12 ^c	0.96 ± 0.06 ^a
2.00	0.57 ± 0.10 ^c	0.89 ± 0.06 ^c

PDGF (10 μg · L⁻¹) effectively stimulated HSC-T6 cell proliferation. Matrine (0.5 ~ 2 mmol · L⁻¹) decreased PDGF-driven proliferation in a concentration-dependent manner (*P* < 0.01) such that matrine 2 mmol · L⁻¹ almost completely blocked the proliferative effect of PDGF (Tab 2).

Effect of matrine on collagen synthesis stimulated by serum and TGFβ₁ Incubation of HSC-T6 cells or NIH3T3 cells with matrine for 48 h resulted in a suppression of collagen synthesis in a concentration-dependent manner. Inhibitory rate was 20.2 %, 29.5 % for HSC-T6 cells, and 35.2 %, 53.9 % for NIH3T3 cells at a matrine concentration of 1 mmol · L⁻¹ and 2 mmol · L⁻¹, respectively (*P* < 0.01). All the results were normalized with respect to cell density (Tab 3).

Tab 2. Effect of matrine on PDGF-driven proliferation of HSC-T6 cells. Baseline proliferation was assessed in DMEM supplemented with 0.4 % calf serum. Cells were stimulated with PDGF ($10 \mu\text{g} \cdot \text{L}^{-1}$) in the presence or absence of serial concentrations of matrine. $n=6$. $\bar{x} \pm s$. $^*P > 0.05$, $^cP < 0.01$ vs control.

Matrine/ $\text{mmol} \cdot \text{L}^{-1}$	A_{595}	Inhibition/%
Medium	0.63 ± 0.03	-
Control	0.89 ± 0.07	-
0.25	0.82 ± 0.06^a	26.9
0.50	0.74 ± 0.06^c	57.6
1.00	0.68 ± 0.06^c	80.7
2.00	0.64 ± 0.07^c	96.1

Tab 3. Effect of matrine on collagen synthesis of HSC-T6 cells and NIH3T3 cells stimulated with serum. $n=4$. $\bar{x} \pm s$. $^*P > 0.05$, $^cP < 0.01$ vs matrine $0 \text{ mmol} \cdot \text{L}^{-1}$.

Matrine/ $\text{mmol} \cdot \text{L}^{-1}$	$[^3\text{H}]$ proline incorporation/dpm	
	HSC-T6	NIH3T3
0	3058 ± 525	3008 ± 103
0.25	3377 ± 574^a	2401 ± 968^a
0.50	2641 ± 282^a	2043 ± 973^a
1.00	2439 ± 384^c	1948 ± 101^c
2.00	2157 ± 212^c	1386 ± 530^c

TGF- β_1 significantly enhanced the collagen synthesis of HSC-T6 cells ($P < 0.01$). Matrine ($0.5 \sim 2 \text{ mmol} \cdot \text{L}^{-1}$) reduced the TGF- β_1 -driven collagen synthesis and matrine ($2 \text{ mmol} \cdot \text{L}^{-1}$) totally blocked the action of TGF- β_1 (Tab 4).

Effect of *in vivo* matrine on hepatic fibrogenesis Serum HA levels as well as hepatic hydroxyproline contents were approximately 2.6 times and 5 times higher in CCl_4 group than that in normal group. There was a significant decrease in serum HA levels and hepatic hydroxyproline contents in matrine both $50 \text{ mg} \cdot \text{kg}^{-1}$ and $100 \text{ mg} \cdot \text{kg}^{-1}$ treated group (Tab 5).

DISCUSSION

Liver fibrosis is a complex process characterized by increased synthesis and deposition of extracellular matrix, particularly collagen, proteoglycan, and hyaluronic acid. In this study, we used a hepatotoxic model of fibrogenesis, chronic CCl_4 -induced fibrosis in rat, to investigate the antifibrotic effect of matrine *in vivo*. Our results

Tab 4. Effect of matrine on collagen synthesis of HSC-T6 cells stimulated with TGF- β_1 . Collagen synthesis was assessed in DMEM supplemented with 2 % calf serum, TGF- β_1 ($2 \mu\text{g} \cdot \text{L}^{-1}$), ascorbic acid $50 \text{ mg} \cdot \text{L}^{-1}$, and matrine. $n=4$. $\bar{x} \pm s$. $^*P > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs control.

Matrine/ $\text{mmol} \cdot \text{L}^{-1}$	$[^3\text{H}]$ proline incorporation/dpm	Inhibition/%
Medium	3688 ± 255	-
Control	6717 ± 699	-
0.25	6576 ± 555^a	4.6
0.50	5526 ± 274^b	39.3
1.00	4445 ± 450^b	75.0
2.00	3256 ± 112^c	114.2

Tab 5. Effect of matrine on serum hyaluronic acid (HA) and liver hydroxyproline (HyP) in rats treated with CCl_4 to induce fibrosis. $n=6$. $\bar{x} \pm s$. $^cP < 0.01$ vs normal group. $^*P < 0.05$, $^fP < 0.01$ vs CCl_4 group.

Group	HA level/ $\text{mg} \cdot \text{L}^{-1}$	HyP content/ $\mu\text{g} \cdot \text{mg}^{-1}$
Normal	169 ± 33	1.33 ± 0.51
CCl_4	448 ± 78^c	6.28 ± 2.27^c
$\text{CCl}_4 + \text{Matrine } 50 \text{ mg} \cdot \text{kg}^{-1}$	313 ± 26^f	3.35 ± 0.96^c
$\text{CCl}_4 + \text{Matrine } 100 \text{ mg} \cdot \text{kg}^{-1}$	201 ± 65^f	3.27 ± 0.55^c

demonstrated that rats receiving CCl_4 caused a significant elevation in serum hyaluronic acid and liver hydroxyproline, two important indices of fibrosis. Treatment with matrine decreased the elevation of serum hyaluronic acid and liver hydroxyproline. These *in vivo* results indicate that matrine has a potential antifibrotic effect.

The precise antifibrotic mechanism of matrine is unknown. It has been well documented that hepatic stellate cells play a central role in liver fibrogenesis, in experimental models of liver fibrosis, as well as in human chronic liver disease^(4,8). Hepatic stellate cells undergo a phenotypic change to highly proliferative myofibroblasts and synthesize most extracellular matrix components once they are activated during liver fibrosis. Therefore, in this work, we focused on rat myofibroblast cell line, HSC-T6 cell and mouse fibroblast NIH3T3 cell as the target cells to investigate the *in vitro* antifibrotic effects of matrine. We showed that exposure of HSC-T6 cells to matrine at higher concentration resulted in decrease in proliferative activity and collagen synthesis capacity when stimulated with serum. Moreover, matrine attenuated

PDGF-driven proliferative activity and TGF- β_1 -driven collagen synthesis in a concentration-dependent manner. The actions of PDGF and TGF- β_1 were completely blocked by matrine at the highest concentration. And these inhibitory effects are not attributed to its unspecific toxic effects. These results suggest that matrine could reduce the pool of HSC and deposit of extracellular matrix such as collagen in fibrotic liver. The number of cytokines involved in the pathogenesis of fibrosis remains to be established, but PDGF mainly stimulates HSC proliferation and TGF- β_1 primarily enhances the synthesis of extracellular matrix^(9,10). Thus, the inhibition of PDGF and TGF- β_1 actions by matrine might provide a possible mechanism for its antifibrotic activities.

Exposure of fibroblast NIH3T3 cells to the maximal concentration of matrine significantly inhibited cell proliferation, but had a greater effect on collagen synthesis compared to the results obtained with HSC-T6 cells. These different reactions may arise from the different physiological properties of hepatic stellate cell and fibroblast cell.

Taken together, these data provide evidence that matrine may prevent liver fibrogenesis *in vitro* and *in vivo*, indicating that one important mechanism of antifibrotic effects of matrine might be blocking the actions of PDGF and TGF- β_1 .

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苦参碱体内外抗大鼠肝纤维化的作用¹

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关键词 苦参碱; 肝硬化; 细胞分裂; 胶原; 血小板源生长因子; 转化生长因子 β

目的: 研究体外苦参碱对 HSC-T6 大鼠储脂细胞和 NIH3T3 成纤维细胞增殖和胶原合成的影响, 以及体内对四氯化碳诱导的大鼠肝纤维化的影响. 方法: 细胞增殖和胶原合成分别采用结晶紫染色法和 [³H] 脯氨酸掺入法. 肝纤维化评价以血清透明质酸和肝中羟脯氨酸含量为指标. 结果: 苦参碱 (1~2 mmol·L⁻¹) 显著减少血清刺激的 HSC-T6 细胞以及 NIH3T3 细胞增殖和胶原合成; 苦参碱 (0.25~2 mmol·L⁻¹) 浓度依赖地抑制血小板源生长因子 (PDGF) 促 HSC-T6 细胞增殖以及抑制转化生长因子 β_1 (TGF- β_1) 促胶原合成的作用. 体内苦参碱 (50, 100 mg·kg⁻¹) 均能显著降低血清透明质酸和肝脏羟脯氨酸水平. 结论: 苦参碱阻断 PDGF 和 TGF- β_1 的作用, 抑制储脂细胞增殖和胶原合成可能是其抗肝纤维化作用的机制之一.

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