ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF MASSON PINE POLLEN AQUEOUS EXTRACT ON ACTIVATED HUMAN HEPATIC STELLATE CELLS

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ABSTRACT

Background and Objectives: The activation and proliferation of human hepatic stellate cells (HSC) are believed to be the central mechanism in liver fibrosis. The aim of the study was to observe the effects of Masson pine pollen aqueous extracts (MPPAE) on activated human hepatic stellate cells (HSC).

Methods: Via in vitro cell culture, a human HSC line LX-2 activated model stimulated by conditional medium was developed to observe the inhibitory effects and efficacy of MPPAE on HSC proliferation activity, cell morphology, apoptosis, and functional changes.

Results: After being stimulated by L-02 conditioned medium, the LX-2 cell was activated, and the contents of type I and type III collagen increased. The expression of matrix metalloproteinases MMP-1 and MMP-2 did not change significantly, but the expression of TIMP-1 significantly increased (about 85.2%). MPPAE can reduce collagen content and upregulate the expression of MMP-1 and MMP-2. At the same time, MPPAE played a significant regulatory role on the unusually high expression of TIMP-1. Therefore, MPPAE regulates the contents of type I and III collagen through downregulation of TIMP-1 expression to reduce the inhibitory effect of TIMP-1 on MMPs and indirectly enhance extracellular matrix catabolism.

Conclusion: MPPAE effectively inhibits activated HSC in vitro. The mechanism is the combination of regulation of the expression of extracellular matrix – related protein and promotion of apoptosis of activated HSC, among other factors. Thus, it might be an ideal nutritional supplement for antifibrosis of the liver.

Keywords: Aqueous extract, hepatic stellate cells, Masson pine pollen.

INTRODUCTION

Hepatic fibrosis, the repair response of the liver to serial chronic stimulation or damage, is characterized mainly by excessive deposition of extracellular matrix (ECM). The activation and proliferation of human hepatic stellate cells (HSC) are believed to be the central mechanism in liver fibrosis. Therefore, research and discovery of the activation mechanism of HSC and the inhibition of the activation pathway are key factors in the prevention and treatment of hepatic fibrosis.[1,2] To look for molecular targets in the pathogenetic mechanism of hepatic fibrosis, namely the mechanism of HSC activation, the activation of HSC under different physiological and pathological conditions is simulated in vitro. This provides an effective tool for the development of hepatic fibrosis drugs, and the inhibition of HSC activation is the key point in antifibrosis treatment. Therefore, HSC are widely used in vitro models for the study of hepatic fibrosis.[3] The human hepatic cell line LX-2 is detected with a gene chip; its genetic similarity with the original generation of HSC reaches 98.7% therefore, it is widely used in cell models for the study of activation and inhibition of HSC.

Masson pine pollen has a large number of biological elements and rich nutritional ingredients. Its effects include immunity enhancement, antiaging and antifatigue properties, regulation of metabolism, reduction of blood fat and blood sugar, hepatic protection, and beauty – enhancing properties. It is a highly efficient and versatile nutritional product and enjoys the reputation of being a natural health food.[4] In recent years, protective effects of Masson pine pollen on the liver have been reported,[6-8] and some studies have identified a protective effect against acute liver injury, which is conducive to liver function recovery and ascites absorption. Seventy percent of Masson pine pollen is lignin, which cannot be absorbed by the human body. Purification and processing can greatly improve the functional components in Masson pine pollen; therefore, Masson pine pollen extracts can be used as func-

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tional and nutritional components in the clinical and rehabilitative treatment of patients with hepatopathy.

According to the pathogenetic mechanism of hepatic fibrosis, the inhibition of hepatic fibrosis is mainly achieved through three pathways: inhibition or reduction of the damaging effects of harmful factors on hepatic cells; regulation of the expression of cytokines and related signaling proteins, inhibition of the activation of the immune cells or stellate cells, or promotion of apoptosis; and inhibition of collagen hyperplasia or enhancement of collagen degradation.[9,10]

We selected the human normal hepatic cell line L-02 and the human normal HSC line LX-2 as study parameters. By carrying out in vitro experiments in 3 steps, we visually observed the effects of MPPAE on proliferation activity, morphology, and function of human hepatocytes and stellate cells using morphological and immunohistochemical methods. Earlier, we had observed the protective effect of MPPAE on oxidative damage of human normal hepatic cell line L-02, and we continued to observe the effect of MPPAE on the activation of human normal HSC line LX-2 in this study.

METHODS

This study was approved by the ethic committee of the Institute of Health and Environmental Medicine and done in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Experimental Subjects

Freeze – dried MPPAE powder. Wall – breaking treatments using low – temperature, high – speed airflow grinding technology was performed for 20 minutes, followed by water immersion at room temperature and atmospheric pressure for 30 minutes and ultrasound extraction for 30 minutes. The MPPAE was centrifuged for 15 minutes at 10,000 – 16,000 rpm at low temperature. The supernatant was collected, and the solid substance was removed. Water-soluble molecules with a molecular weight of less than 3000 D were separated by ultra filtration using a molecular sieve at 1-5°C before lyophilization to obtain lyophilized powder and storage at -20°C for later use. When used, 0.100 g lyophilized powder was weighed and dissolved in 100 mL high – purity water and formulated into a solution of 1.0 mg/mL for standby application.

Human normal hepatic cell line L-02 was purchased from Shanghai Fuxiang Biological Science and Technology Co., Ltd.

Human normal HSC line LX-2 was purchased from Shanghai Fuxiang Biological Science and Technology Co., Ltd.

Main Equipment

Multiskan MK3 Microplate Reader: Thermo Fisher Scientific Inc, USA.

Lab systems Series Constant Temperature Biochemical Incubator: Thermo Company, USA.

Muse Cell Analyzer: Merck Company, USA.

Nikon Tisi Inverted Microscope: Nikon Corporation, Japan.

Sn – 69513 Type Immune Counter: Shanghai Nucleus Research Institute Rihuan Photoelectric Instrument Co., Ltd., China.

5810R/5415D Centrifuge: Eppendorf, Germany.

Sartorius Electronic Balance: Sartorins AG, Germany.

Labsystems Clean Bench: Thermo Company, USA.

LDZ5-2 Automatic Balance Centrifuge: Beijing Medical Centrifuge Factory, China.

Ultra – low Temperature Freezer: SANYO Company, Japan.

Main Reagents

AR CCl_4: Beijing Chemical Reagent Company, China.

DMEM Medium (high glucose): Gibco Company, USA.

0.25% Trypsin: Gibco Company, USA.

PBS Buffer: Beyotime Institute of Biotechnology Co. Ltd. China.

Penicillin – Streptomycin Mixture: Gibco Company, USA.

Hematoxylin – Eosin Dye: Beyotime Institute of Biotechnology Co. Ltd. China.

Immunohistochemical Goat Anti-Rabbit Monoclonal Antibodies: Tianjin Sungene Biotech Co., Ltd., China.

Cell Culture Grade DMSO: Beyotime Institute of Biotechnology Co. Ltd. China.

Acridine Orange Fluorescent Agent: Beyotime Institute of Biotechnology Co. Ltd. China.

Cell Proliferation and Cytotoxicity Assay Kit: Beyotime Institute of Biotechnology Co. Ltd. China.

20%CCl_4- prepared with ethanol working solution

Because CCl4 cannot be dissolved in water, the CCl_4 working solution was prepared with ethanol as the solvent. 20 mLAR CCl_4 was dissolved and mixed into 80 mL absolute ethyl alcohol, and the mixture was filtrated with a 0.22-μm sterile filter for use. The molar concentration was 4 mol/L.

Preparation of CCl4 Intervention culture Medium.

Under sterile conditions, 2 mL 20% CCl4 – ethanol solution was added to 48 mL serum – free DMEM high – glucose culture medium to formulate into 50 mL intervention culture medium with a concentration of 80 mmol/L CCl4, which was stored at 4°C for later use.

Preparation of MPPAE application culture medium.

100 mg lyophilized MPPAE powder was weighed accurately and dissolved in 10 mL PBS for a concentration of 10 mg/mL (10,000 mg/L), which was filtered with a sterile filter and stored at 4°C for later use. When used, it was diluted to the desired concentration with serum – free DMEM.

Experimental Methods

Exploration of the Activation Conditions of LX-2 Cells

Preparation of L-02 cell injury conditioned medium.

Normal L-02 cells were cultured in a flask for 24 hours and then were continuously cultured for 24 hours with 20 mL serum – free DMEM containing 60 mmol/L CCl4 – ethanol. The morphologies of L – 02 cells were observed under a microscope to have significantly changed (cytoplasmic shrinkage, nuclear decomposition). The supernatant was collected and centrifuged at 800 rpm for 5 minutes (the cellular debris was removed). Finally, the supernatant was collected as the conditioned medium, which must be prepared immediately before use and stored at 4°C for less than 48 hours.

L – 02 Exploration of conditions for stimulation of the conditioned medium on LX-2 cell activation.

The normal LX-2 cells were seeded into 2.96-well plates and cultured in 5% CO2 at 37°C for 12 hours. The seeding density was 4×10^3/well. The culture solutions were changed with serum – free DMEM containing different volumes of L – 02 cell conditioned culture medium, and the culture continued for 24 hours. The WST – 1 solutions were added to the media after color development was carried out for 3 hours at 37°C in a dark place. The microplate reader was used to detect the optical density (OD) values at 450 nm, and the cell activity was calculated.

The effect of MPPAE on Activated LX – 2 Cells

The Effect of MPPAE on the Morphologies of Activated LX – 2 Cells

Adherent cell growth was good 12 hours later, and the supernatant was discarded. The normal control group was cultured in serum-free DMEM, and the experimental groups were added with 1.2 mL L – 02 conditioned medium and 1.8 mL MPPAE, for a final concentration of 0.100 and 400 mg/L. The supernatant was discarded 24 hours later, and the cells were washed with PBS 3 times. The cells were fixed with precooled 95% ethanol for 15 minutes, washed with PBS 3 times for 2 minutes each, and stained with hematoxylin and eosin. The cells were taken out for cell growth on the glass slide, and the cellular morphologies were observed and photographed under a microscope.

Effects of MPPAE on the Proliferation Activities of Normal LX-2 Cells and Activated or Stimulated LX – 2 Cells

The normally grown LX-2 cells were seeded into 2 96–well plates and cultured in 5% CO2 at 37°C for 12 hours. The seeding density was about 4×10^3/well. The medium in one plate was replaced with MPPAE with different concentrations. The medium in the other plate was replaced with L – 02 cell injury conditioned medium and different concentrations of MPPAE. The cultures were continued for 24 hours, and WST – 1 solution was added to the plates. After color development was carried out for 2 hours at 37° in a dark place, the microplate reader was used to detect the OD values at 450 nm, and the cell activity was calculated.

Effect of MPPAE on Synthesis of Type I and III Collagen in Activated LX – 2 Cells

The normally cultured LX-2 cells were seeded and cultured into a 24 – well plate for 4 hours. The cells were divided into 6 groups, each with 4 replicate wells. The normal control group was continuously cultured with 1.0 mL DMEM. The medium in each well of the experimental group was replaced with 0.4 mL L – 02 cell conditioned medium and mixed with 0.6 mL DMEM culture medium containing, respectively, 0, 100, 200, 400, and 800 mg/L MPPAE (the system final concentrations were 0, 50, 100, 200, and 400 mg/L), which were cultured at 37°C for 24 hours. Then the cell supernatant was collected, and the contents of type I and III collagen were detected using a double – antibody sandwich ELISA.

Effect of MPPAE on the Apoptosis of Activated LX – 2 Cells

The normally cultured LX – 2 cells were seeded and cultured into flasks with slides for 4 hours. After the cells adhered to the wall completely, they were divided into four groups. The normal control group was continuously cultured with DMEM, and 1.2 mL L – 02 conditioned medium and 1.8 mL DMEM containing MPPAE (final concentrations were 0, 100, and 400 mg/L, respectively) were added to the experimental groups. The supernatant was removed 24 hours after culture, and the cells were washed with PBS 3 times and fixed with precooled 95% ethanol for 15 minutes. The cells were washed with PBS 3 times and stained with acridine orange solution at room temperature in a dark place for 30 minutes and then washed with PBS 3 times. After the slides were mounted with glycerine to prevent fluorescence quenching, they were observed and photographed under a microscope using a 520-nm excitation light, and the apoptosis of the cells was observed.

Expression Levels of MMPs and TIMP – 1 in LX – 2 Cell Supernatants

ELISA method: the microwell titer plate was coated with purified antibodies, and the solid – phase carrier was made. The sample or standard substance, biotinylated anti-MMPs antibodies, and avidin – HRP were added successively to the microwells coated with anti-MMPs antibodies. After thorough washing, TMB substrate solution was added to each well for color development. The depth of color was positively correlated with MMPs in samples. A microplate reader was used to detect the absorbance (OD value) at the

450 nm wavelength, and the concentrations of the samples were calculated according to the standard curve.

2.5 Statistical methods

Experimental data were expressed as mean ± standard deviation (x ± s), and SPSS 11.0 software was used for statistical analysis. The data were tested by normality and homogeneity of a variance test. The data approximated normal distribution, and homogeneity of variance was analyzed with a multifactor analysis of variance (ANOVA). The data with variance between groups or at least one group that did not approximate the normal distribution were analyzed using the Kruskal - Wallis rank sum test. $P < 0.05$ was considered statistically significant. The top right corner of the data were marked with different letters, which indicated a significant difference between groups ($P < 0.05$).

RESULTS

Activation Conditions for Human HSC LX - 2

As seen from the dose-effect curve between the L - 02 cell conditioned medium and the proliferation activity of LX - 2 cells (Table 1, Figure 1), the increase in the dose of L - 02 conditioned medium also stimulated the proliferation activity of LX - 2 cells. When the dose reached 40 μl (total systemic volume, 100 μl, or 40%), the proliferation activity of LX – 2 cells was maximized. Along with the increase of L - 02 cell conditioned medium, the proliferation activity of LX - 2 cells gradually decreased. Therefore, in our experiment, the intervention amount of 40% L - 02 cell conditioned medium was selected as the stimulation condition for LX - 2 cell activation.

Table 1: *Dose – response relationship between L-02 cell injury conditioned medium and the proliferation activity of human hepatic stellate cells LX-2.*

Column No. of Culture Plate	*n*	*Volume of L-02 Conditioned Medium (μl)*	*OD Value*
1.	6	0	0.399 ± 0.024
2.	6	10	0.409 ± 0.033
3.	6	20	0.429 ± 0.022
4.	6	30	0.435 ± 0.028
5.	6	40	0.454 ± 0.020
6.	6	50	0.427 ± 0.035
7.	6	60	0.419 ± 0.024
8.	6	70	0.409 ± 0.012
9.	6	80	0.395 ± 0.021
10.	6	100	0.366 ± 0.015

Inhibiting Effect of MPPAE on the Activation of LX - 2 Cells

Effect of MPPAE on the proliferation activity of activeted LX – 2 cells.

The appropriate dose of L – 02 cell conditioned medium stimulated the LX – 2 cells, which were also affected by MPPAE. The proliferation activity of LX – 2 cells was gradually decreased, along with the increase in the concentration of MPPAE, which clearly indicated an inhibiting effect of MPPAE on the activation of LX – 2 cells (Table 2, Figure 2).

Effect of MPPAE on the collagen content in the supernatant of activated LX - 2 cells.

The current study suggests that ECM is the material basis for the formation of fibrosis, while collagen is the main component of the ECM. HSC is the main source of ECM in normal and fibrotic livers, and collagens synthesized by HSC in the normal liver mainly include type I, III, and IV collagens (Table 3, Figure 3). Its synthetic amount is 10 times that of hepatic cells and 20 times that of endothelial cells. MMPs are the main degrading enzymes of ECM in liver and contribute to ECM degradation and the reversal of hepatic fib- rosis. The MMP – 1 in the human body and MMP – 13 in rats are the degrading enzymes of type I and III interstitial collagens; the natural substrates of MMP – 2 are the components of type IV collagen (gelatin) molecules and other ECM.[5,10] TIMPs are regulators of MMPs expression, of which TIMP – 1 is an inhibitor of MMP–1 and MMP – 13. The degradation of type I and III collagens can be promoted through the inhibition of the expression of TIMP – 1.[11]

The study results showed that the contents of collagen I and III in the supernatant of activated LX – 2 cells stimulated by L – 02 cell conditioned medium were slightly higher (Table 4). There was no significant change in the expression level of MMPs, while the expression level of TIMP – 1 increased by 85.2% compared with the normal group ($P < 0.05$). MPPAE had a tendency to reduce the collagen content and increase the expression levels of MMP – 1 and MMP – 2, but there was no statistical significance between the groups ($P > 0.05$). MPPE played a significant regulatory role in the abnormally high expression of TIMP - 1, thus reducing its inhibiting effect on MMPs and indirectly enhancing the catabolism of ECM, which may also be the reason for the regulation of MPPAE on the contents of type I and III collagens in cell supernatant.

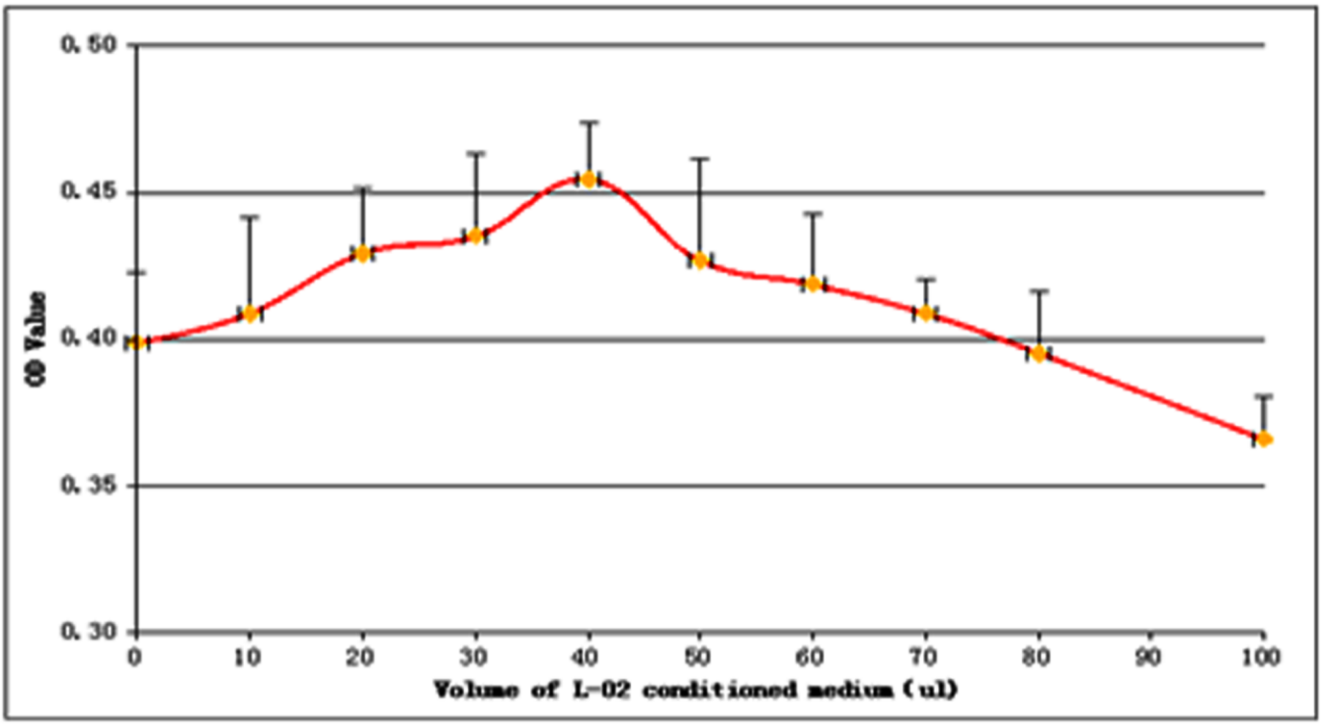

Figure 1: *Dose - effect curve between L - 02 cell conditioned medium and the proliferation activity of LX - 2 cells.*

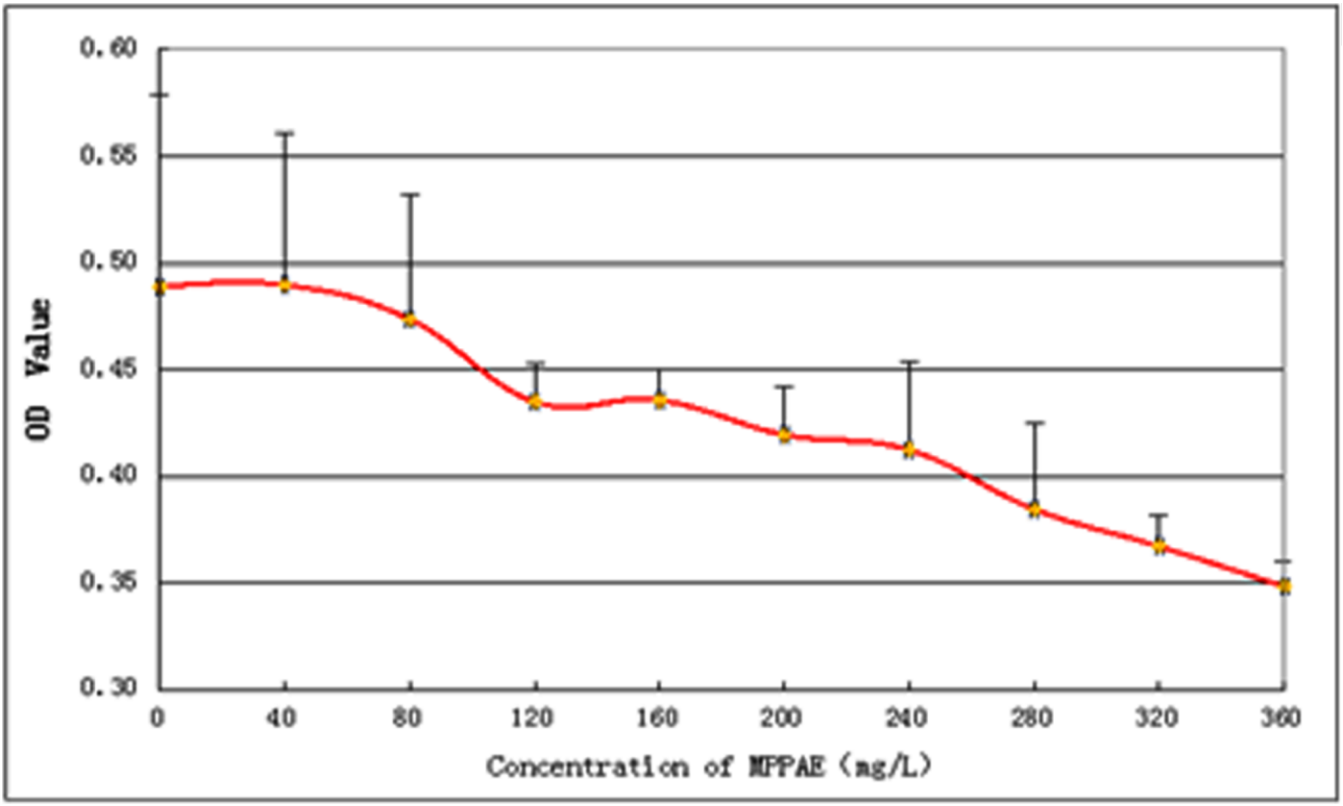

Figure 2: *Effect of MPPAE on the activation and proliferation of LX-2 cells stimulated by the conditioned medium.*

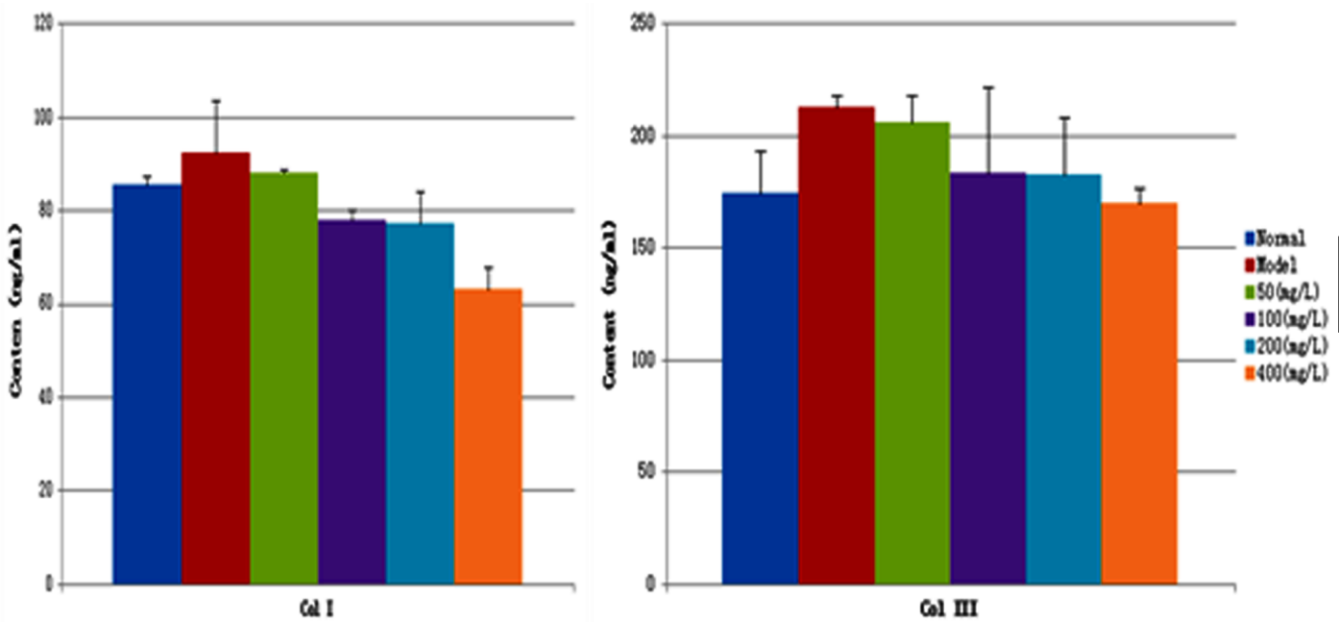

Figure 3: *Collagen I and III levels in the supernatant of LX-2 cells.*

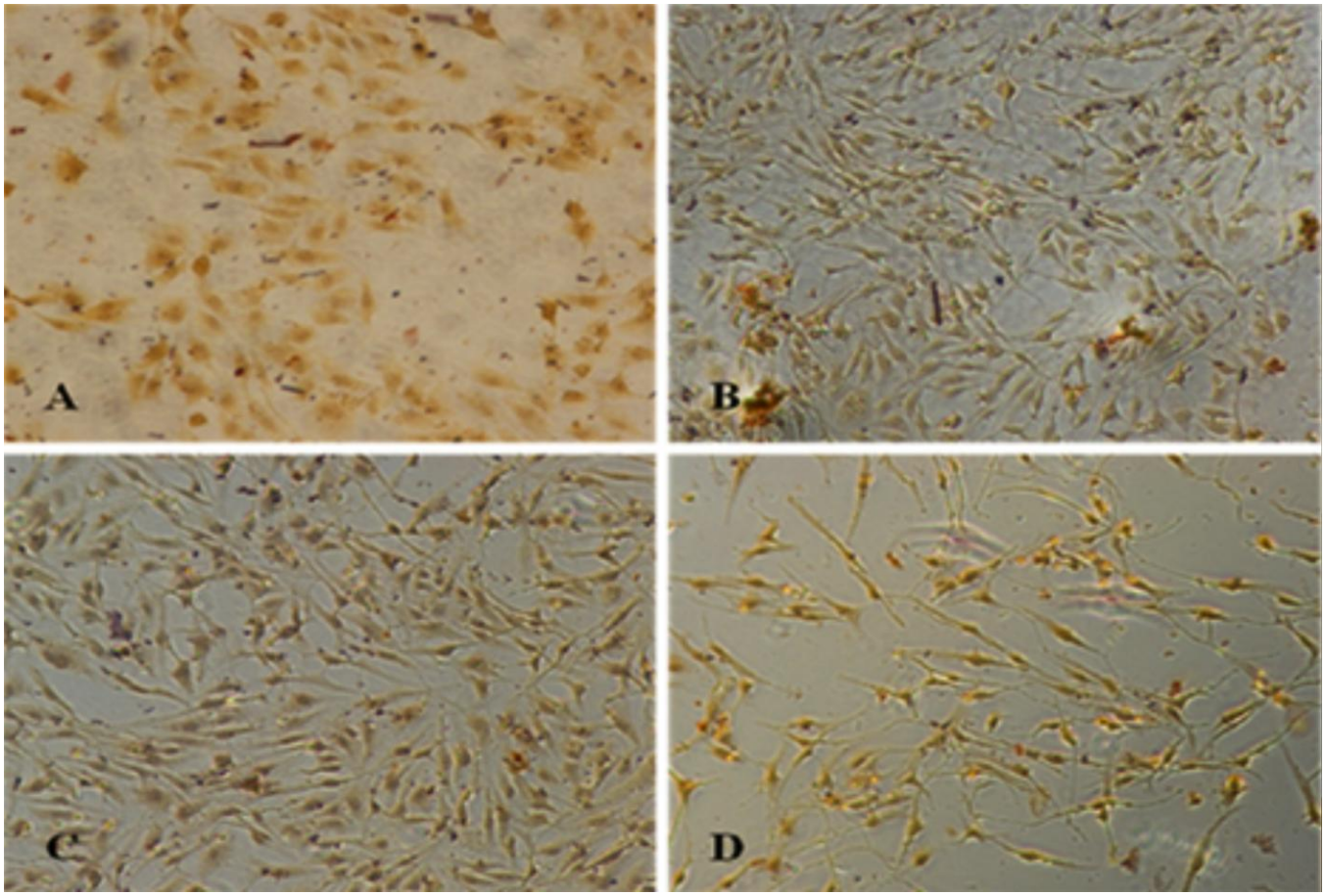

Figure 4: *Morphologic changes of LX-2 cells after stimulation with conditioned medium and the intervention effect of MPPAE (100X). (A) Normal control group, (B) Model group, (C)MPPAE 100 mg / L, (D)MPPAE 400 mg/L.*

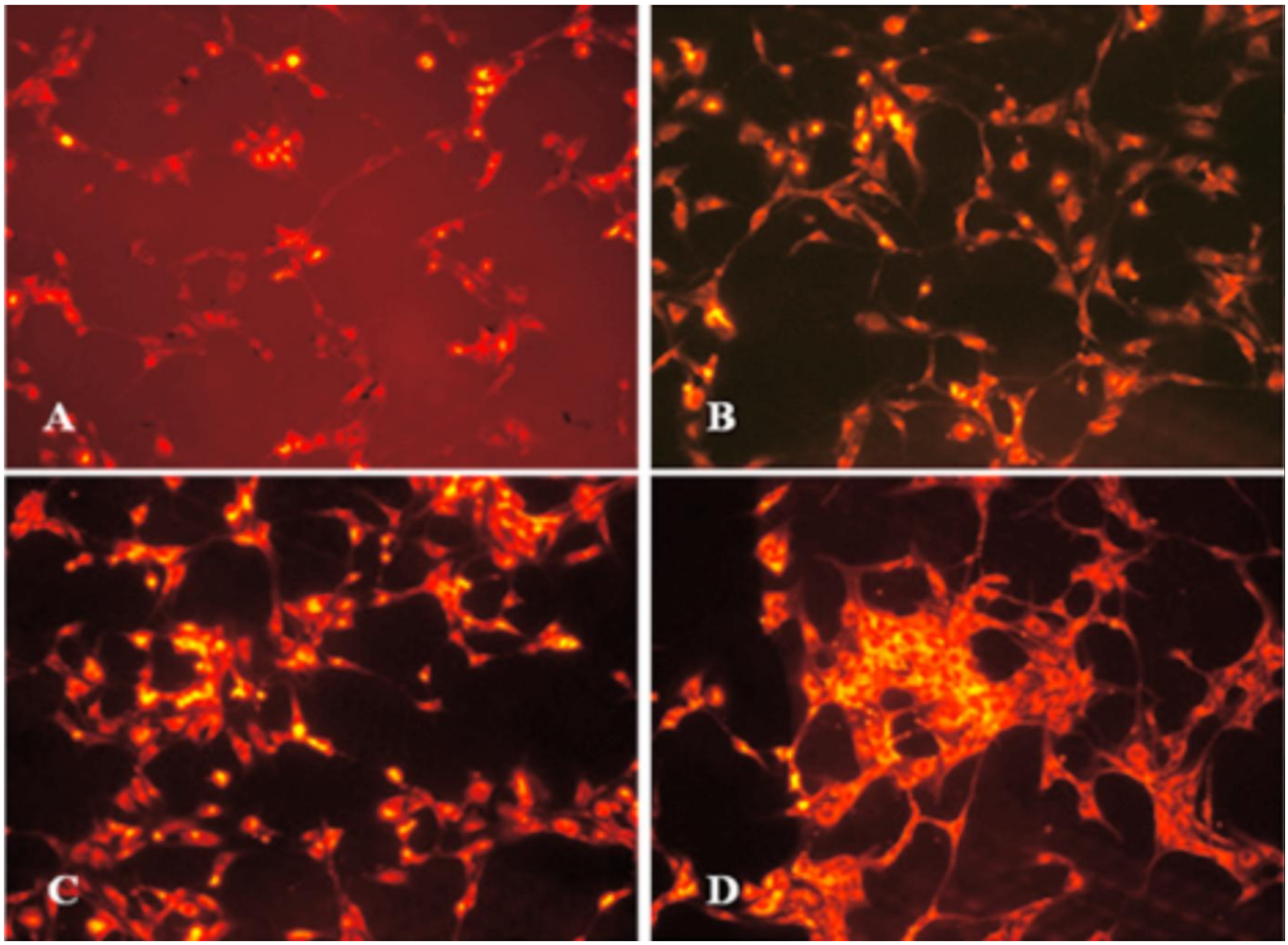

Figure 5: *Acridine orange staining of LX-2 cells (100X).*
(A) Normal control group, (B) Model group, (C) MPPAE 100 mg/L, (D) MPPAE 400 mg/L).

Pro-apoptotic effect of MPPAE on activated LX - 2 cells.

The normal morphology of LX - 2 cells in the resting phase was fusiform (Figure 4). The cytoplasm and nucleus were rich, and all grew while adhering to the wall. The LX - 2 cells activated by the L - 02 cell conditioned medium shrunk the cell body and long cytoplasmic processes. Some long cytoplasmic processes were filamentous and entangled with each other. There was collagen fiber deposition in the surrounding area. The cells were arranged densely and chaotically; the nucleus was larger, and two nuclei were occasionally observed. After the MPPAE intervention, the cytomembrane was intact, but the cytoplasm shrunk and still presented as long, thin fibers. The numbers of filamentous fibers and cells were significantly reduced, and the dense, stained nuclei were highlighted.

Table 2: *Effect of MPPAE on the proliferation of LX-2 cells activated by the conditioned medium.*

Volume of L-02 Conditioned Medium (μl)	*MPPAE Final Concentration (mg/L)*	*24-Hour OD Value*
0	0	0.422 ± 0.027
40	0	0.488 ± 0.090
40	40	0.490 ± 0.071
40	80	0.473 ± 0.058
40	120	0.435 ± 0.017
40	160	0.435 ± 0.015
40	200	0.419 ± 0.023
40	240	0.413 ± 0.041
40	280	0.384 ± 0.041
40	320	0.367 ± 0.015
40	360	0.349 ± 0.011

Table 3: *Effects of different concentrations of MPPAE on the collagen content in the supernatant of LX-2 cells.*

Groups	n	*Volume of L-02 Conditioned Medium (mL)*	*MPPAE Final Concentration (mg/L)*	*Col I (ng/mL)*	*Col III (ng/mL)*
Normal	4	0	0	85.57 ± 1.92[b]	174.45 ± 18.64
Model	4	0.4	0	92.29 ± 11.43[b]	212.40 ± 5.42
MPPAE Treated	4	0.4	50	88.02 ± 0.78[b]	205.72 ± 12.16
	4	0.4	100	78.08 ± 1.86[ab]	183.07 ± 38.77
	4	0.4	200	77.34 ± 6.68[ab]	182.97 ± 25.39
	4	0.4	400	63.08 ± 4.87[a]	169.72 ± 6.64

Note: Different letters marked in the top right corner of the data indicate a significant difference between groups ($P < 0.05$).

Table 4: *Effect of MPPAE on the expression levels of MMPs and TIMP-1 in the supernatant of activated LX-2 cells.*

Groups	*n*	*Volume of L-02 Conditioned Medium (mL)*	*MPPAE Final Concentration (mg/L)*	*MMP-2 (ng/mL)*	*MMP-1 (ng/mL)*	*TIMP-1 (ng/mL)*
Normal	4	0	0	1.022 ± 0.015	2.241 ± 0.056	1.28 ± 0.23[a]
Model	4	0.4	0	1.046 ± 0.062	2.247 ± 0.065	2.37 ± 0.26[b]
MPPAE Treated	4	0.4	50	1.066 ± 0.043	2.260 ± 0.083	2.14 ± 0.06[b]
	4	0.4	100	1.076 ± 0.077	2.313 ± 0.102	2.10 ± 0.26 [b]
	4	0.4	200	1.107 ± 0.005	2.365 ± 0.009	1.84 ± 0.21[b]
	4	0.4	400	1.103 ± 0.019	2.478 ± 0.132	1.76 ± 0.03[b]

Note: Different letters marked in the top right corner of the data indicate a significant difference between groups ($P < 0.05$).

DISCUSSION

Apoptosis, or programmed cell death, is the process of a cell ending its life under certain physiological or pathological conditions. It is an active, highly ordered process controlled by genes and involving a series of enzymes. Apoptosis, which plays a key role in ensuring the healthy survival of the multicellular organisms, is a biological process regulated by intracellular genes and some extracellular factors. The cell has unique morphological changes when apoptosis occurs; its cell membrane generally remains intact and is not accompanied by the release of cell contents. The cell eventually breaks down into apoptotic bodies, which are devoured by macrophages or tissues. The specific process is as follows: the cell is shrunken, and apoptotic bodies appear; the dead cells and apoptotic bodies are quickly devoured by macrophages with no inflammatory response; the nucleus is concentrated; and the chromatin aggregates along the nuclear membrane.[12]

LX-2 cells were stained by acridine orange (Figure 5). Nonactivated cells were observed under a microscope to be normally fusiform shaped. The cytoplasm and nucleus were intact, the fluorescent staining was pale orange – red, and strong fluorescences were rare. The cytoplasm was shrunken and lightly stained into the jacinth after cell activation. The cell membranes of apoptotic cells were intact, and there were strong orange fluorescences in the nucleus. The cytoplasm was pale orange – red, and the fluorescences of nuclear chromations were concentrated on the inner side of the nuclear membrane. Some apoptotic bodies formed. The number of apoptosis cells in LX – 2 cells with the MPPAE intervention was significantly increased, and some cytoplasms were fused.

Recent studies on the signaling pathway of HSC activation and related inhibition conditions have provided new approaches for the clinical treatment of hepatic fibrosis.[13,14] However, because the activation of HSC is the result of the influence of a variety of cytokines and multiple signaling pathways, their complexity and unpredictability result in the specificity and diversity of the inhibiting methods. Because of these difficulties, this study is still confined to the laboratory.

It is ***concluded*** that in vitro activated stellate cell models, MPPAE downregulates the expression of TIMP-1 and thus reduces its inhibiting effect on MMPs, indirectly enhances the catabolism of ECM, and has a regulating effect on the contents of type I and III collagens. It also effectively inhibits the proliferation activities of stellate cells and promotes the apoptoses of acti-

vated LX-2 cells, thereby reducing the generation of ECM. It is the ideal functional component to inhibit fibrosis. Next, it's in vivo efficacy validation will be carried out in animal models.

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Conflict of Interest
None.

Authorship
T. C. conceptulized the paper; X. J. performed the study; L. Z. collected and analysed data; P. Z. and C. G. prepared the manuscript.

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