

Effect of Maotai liquor on the liver: an experimental study

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BACKGROUND: Epidemiology investigation showed that no worker drunk Maotai liquor for nearly 30 years died of hepatic diseases, and no obvious hepatic fibrosis and cirrhosis were found in 99 workers who had drunk Maotai liquor for a long period by epidemiology investigation and needle biopsy. The same finding was detected in rats that were drunk by Maotai liquor continued for 56 days. This study was to investigate the effects of Maotai liquor on the liver and its mechanism of preventing hepatic fibrosis.

METHODS: After ingestion of Maotai for 56 consecutive days, male SD rats were killed for detecting the levels of metallothionein and malondialdehyde (MDA) in liver tissues. Rat hepatic stellate cells (HSCs) and human HSCs were cultured in vitro to observe the effect of Maotai on HSCs proliferation and collagen synthesis. After ingestion of Maotai for 14 consecutive weeks, the livers of male SD rats were harvested for pathohistological examination.

RESULTS: The level of metallothionein in the liver of Maotai-induced rats increased by 22 folds, whereas the levels of hepatic lipid peroxide and MDA was decreased significantly ($P < 0.05$) in Maotai-induced animals suffering from CCl_4 . Maotai demonstrated obvious inhibitory effect on proliferation of HSCs and the inhibition was concentration-dependent. Gene expression and protein secretion of collagens could also be inhibited by Maotai. In alcoholic group, typical liver cirrhosis was observed. In Maotai group, however, though fatty degeneration of hepatocytes and mild fibrosis of the interstitium were observed, no obvious hepatic fibrosis and cirrhosis were found.

CONCLUSION: It might be an important mechanism of interfering the progress of hepatic fibrosis that Maotai increases the level of metallothionein in the liver and inhibits the activation of HSCs and the synthesis of collagen proteins.

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KEY WORDS: liver cirrhosis; metallothionein; hepatocytes; Maotai liquor

Introduction

During 1997-2001 we analyzed the nutritional ingredients of Maotai liquor and also epidemiologically studied on our workers who had drunk Maotai liquor for a long period.^[1,2] Basic and clinical studies were focused on whether Maotai liquor causes hepatic fibrosis. In this experimental study, we observed the effect of Maotai liquor in inducing metallothionein (MT) and its relations to CCl_4 hepatic injury, hepatic stellate cell proliferation in vitro, collagen generation, gene expression, and growth of human hepatic stellate cells. In order to explore the possible effect of Maotai liquor on hepatic fibrosis, liver needle biopsies were performed in rats fed with Maotai liquor for 14 weeks.

Methods

Materials

Male SD rats weighing 300 ± 20 g were purchased from the Experimental Animal Center of the Third Military University, Chongqing. Maotai liquor ($53\% \pm 2\%$, v/v) was produced by Guizhou Maotai Distillery Factory with bar code 6902952880026) provided by Section 4 of Guizhou Oil & Foodstuff Import and Export Company, Guiyang, China. Analysis alcohol was produced by No. 1 Factory of Shanghai Zhenxing Chemical Factory, batch No. 280065913.

Reagents and solutions included; MT standard provided by Dr. Jie Liu of National Institute of Environmental Health Sciences, USA; 5-diphenyltetrazolium bromide (MTT) bought from Sigma Co., USA; trypsin purchased from Difco., USA; 199 culture medium and MEM culture medium without calcium of Gibco Co., USA; newborn bovine serum (NBS) produced by Shanghai Huamei Co., China; type I rat tail collagen standard diluted slowly with $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ to 10^{-}

200 ng/L, and rabbit anti-rat type I collagen antibody diluted 1:500 with 0.01 mol/L PBS, which were produced by Cambiolem Co.; horseradish peroxidase marker labeled goat anti-rabbit antibody (IgG-HRP, diluted 1:1000 with PBS containing 10% NBS) purchased from Huamei Company, China; total protein determination agent from Biorad Co., USA; RT-PCR reaction agent and PCR marker purchased from Promega Co., USA; and diethylpyrocarbonate, guanidine sulfocyanate saturated mixture of phenol and agarose bought from Shengong Engineering Co., Shanghai, China.

Equipments

Freezing high-speed centrifuge (1.0R, 22R), CO₂ incubator and ultra low temperature freezer were purchased from Heraeus Co., Germany. Inverted microscope was produced by Olympus Co., Japan. Thermostat water bath, thermostat water bath vibrator were produced by Shanghai Medical Equipment Factory, Shanghai, China. LabSystems Multiskan Ms Enzyme Marker Device was produced in Finland Danbury CT ultrasonic membrane breaker was the product of Sonicsmaterials Co., USA. 90 mm culture plate, 60 mm culture plate, and 6-well, 24-well, and 96-well culture plates were the products of Nunc Co., Denmark. Beck wallac 1410 liquid scintillation counter was the product of Beckman Co., USA. Watson-Marlow 101U constant current pump was produced in USA.

Effect of Maotai liquor in inducing MT

Forty SD male rats were divided into 4 groups randomly, 10 rats in each group. Two groups were fed with Maotai liquor at 2 ml/kg diluted by distilled water in a ratio of 1:1 once a day for 56 days and the other 2 groups were fed with saline at the same volume. After all rats were fed for the last time, one of the two groups was given a mixture of CCl₄ and olive oil in a ratio of 1:1 at 2.5 ml/kg, the rest groups were fed with saline at the same volume. All rats were sacrificed at 20 hours after last feeding to get blood for biological test, calculate liver indexes by liver quantity, and determine the content of MT in the liver by saturation of Cd-hemoglobin^[3,4] and the lipid peroxidation product of aldehyde measured by thiobarbituration.

Effect of rats' hepatic stellate cell (HSC) by Maotai liquor of different concentration

Isolation and culture of hepatic stellate cell

The reported method of Lie-Ming Xu was used.^[5]

Test of cell proliferation MTT^[6]

In experiment group, Maotai liquor (53% v/v) supposed to have a concentration of 100% diluted by dulbecco MEM (medium) (DMEM) solution containing 5% NBS to the concentration 0.2%, 2%, 10%,

20%, and 40%, respectively. 5% NBS solution served as control group. When monolayer hepatic stellate cells (HSCs) appeared in the 96-well culture plate, they were cultured for 18 hours in the medium containing Maotai liquor with different concentration of 5% NBS, then 20 ml MTT was added into each well and the HSCs were cultured again for 4 hours. After the removal of suspension, the plate was aired and 100 µl acid isopropanol with 0.05 mol/L HCl was added to each well. Little black crystals were dissolved by agitating to form a steady purple solution. The absorbance (A) in each well was determined by ELISA at the wave-length of 570 nm. There were 4 wells in each sample.

Primer synthesis

The primer of precollagen α1(I)^[7,8] was purified and evaluated by Shanghai Shengong Company, Shanghai, China, with the following sequence: α1(I) upstream primer: 5'-CACCCCTCAAGAGCCTGAGTC-3'; downstream primer: 5'-GTTCGGGCTGATGTACCAGT-3', product 253bp; β-actin: upstream primer: 5'-ACATCTGCTGGAAGGTGGAC-3'; downstream primer: GGTACCACCATGTACCCAGG-3', product 163bp.

Determination of type I collagen^[9]

When the subculturing HSCs grew into full monolayer in the 24-well culture plate, they were cultured for 24 hours in the medium containing Maotai liquor with a concentration of 0.2%–40% centrifuged at 450 r/min for 20 minutes at 4 °C. Both the suspension and cell layer were collected. The collected cells were dissolved by 0.2 mol/L NaOH 0.5 ml in each well and washed with 0.5 ml double distilled water, followed by ultrasonic membrane-breaking for 10 seconds at 4 °C. The suspension was determined by ELISA, and cell layer by DC protein assay to detect total protein. The content of collagen was expressed by cell layer total protein.

Total RNA isolated from HSCs by phenol-chloroform extraction and isopropanol precipitation^[10]

According to the reference book of RT-PCR kit, complementary DNA (cDNA) was synthesised and proliferated in samples by adding 1 µg RNA, 50 pmol/L α1(I) primer precollagen or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) into the plate (1 µ/L 10 mmol/L dNTPs, 2 µ/L 25 mmol/L MgSO₄, 5 units avian myeloblastosis virus (AMV) reverse transcriptase, 5 units Tfl DNA polymerase and 10 ml AMV/Tfl buffer). Then the following reactions were performed on a PCR proliferation device: 48 °C, 45 minutes, 1 cycle; 94 °C, 2 minutes, 1 cycle; 94 °C, 30 seconds, 60 °C, 1 minute, 38 °C, 2 minutes, 30 cycles; and 68 °C, 7 minutes, 1 cycle. The PCR product or DNA marker mixed with 5 µl loading buffer was electrophoresed on a

1.5% agarose gel, visualized by ultraviolet (UV) and quantified densitometrically, 4 v/cm, 20 mA for 1–1.5 hours. The expression precollagen [I], prealbumin, or hydroxy-proline was calculated by the gray levels of precollagen [I] and β -actin mRNA.

Effect of Maotai liquor with different concentration on human HSCs

Culturing of human HSCs

80 000 human HSCs were inoculated into each well in the 96-well plate, and they were cultured for 24 hours in the DMEM medium with 10% NBS at 37 °C in an environment containing 95% air, and 5% CO₂. Afterwards, they were continuously cultured in the DMEM medium with 2% NBS for 24 hours.

Test of cell proliferation

There were 3 groups: blank control group, control group, and experimental group. The experimental group took Maotai liquor (53%, v/v) supposed to have a concentration of 100% density, which was diluted with 2% NBS DMEM into 0.05%, 0.1%, 0.5%, and 1%, respectively. The control group, analysis alcohol was diluted with a concentration of 53%, then diluted to a concentration of 1% according to the dilution of Maotai liquor. Lie Xu-1 (LX-1) was cultured with Maotai liquor of different concentration and 1% alcohol, respectively. 2% NBS DMEM cultured cells which served as blank control group were cultured for 20 hours and added with 10 μ l 5 mg/ml MTT into each well, and cultured continuously for 4 hours. After abandoning the solution, 100 μ l acid isopropanol was added to dissolve black crystals into each cell. Value A was obtained at a wavelength of 570 nm by the lab system ELISA equipment; suppression rate of cell proliferation = $(1 - \text{value A in experimental group} / \text{value A in control group}) \times 100\%$.

Effect of Maotai liquor on rats glutathione

Malondialdehyde (MDA) and biopsy: 100 rats were divided into 5 groups (20 in each group): control group, high-fat group (saline 10 ml/kg), Maotai liquor high dosage group, low dosage group (10 ml/kg, 20 ml/kg Maotai liquor diluted with distilled water at a ratio of 1:1), and alcohol control group (10 ml/kg 30% alcohol) fed once every morning 6 times every week for 14 successive weeks. During the experiment, rats in the control group were fed with common food, and the other groups were fed with high-fat-low-protein food (79% corn, 20% grease, 0.5% cholesterol). All rats were sacrificed after the last feeding to get the liver to weigh. Liver variables were calculated, and a piece of the right liver lobe was cut for pathological examination, and the rest for the test of glutathione (GSH) and MDA.

Statistical analysis

All data were expressed by mean \pm SD, and analyzed by the *q* test.

Results

Effect of Maotai liquor on induction of the content of rat MT (Table 1)

Maotai liquor increased the level of MT in rat liver 22 times over its original level. In the control group, the level of MDA in the liver was obviously increased after intoxication by CCl₄, Maotai liquor, however, did not influence the level of MDA in rat's liver. The level of MT in rats fed with Maotai liquor when intoxicated by CCl₄ was increased more obviously than that in those fed with no Maotai liquor, but MDA was decreased. There was a negative relationship between MT and MDA in rat liver intoxicated by CCl₄ in the control and experimental groups ($r = -0.8023$, $P < 0.01$).

Effect of Maotai liquor on rat HSCs

Obtainment and type of HSCs

Three- 5×10^7 HSCs were obtained from one rat lipid droplet in obvious primary HSCs, but in the course of culture, lipid droplets decreased and as they were passaged, HSCs became extended and looked like myofibroblasts.

Effect on the proliferation of HSC (Table 2)

Maotai liquor of different concentration had no

Table 1. Effect of Maotai liquor on MT and MDA in rat liver (mean \pm SD)

Group	Rats	MT (μ g/g)	MDA (nmol/g)
Control	10	10.0 \pm 2.8	60.2 \pm 3.1
Maotai liquor	10	216.0 \pm 10.8 *	60.1 \pm 2.4
CCl ₄	10	126.4 \pm 4.8 *	150.8 \pm 6.7 *
Maotai liquor + CCl ₄	10	304.8 \pm 12.1 * Δ	102.0 \pm 3.4 * Δ

*: Vs control group, $P < 0.01$; Δ : Maotai liquor group + CCl₄ vs CCl₄ group, $P < 0.01$.

Table 2. Effect of Maotai liquor on the proliferation of rat HSCs, type I collagen and its gene expression (mean \pm SD)

Group	Value A (n=4)	Level of type I collagen (μ g/g total protein) (n=4)	mRNA in type I precollagen (%) (n=3)
Control	0.818 \pm 0.062	61.4 \pm 4.5	87.9 \pm 2.8
0.2% Maotai liquor	0.742 \pm 0.027	59.9 \pm 10.3	0
2% Maotai liquor	0.736 \pm 0.024 *	49.2 \pm 16.2	0
10% Maotai liquor	0.720 \pm 0.036 *	50.1 \pm 19.7	74.4 \pm 8.5
20% Maotai liquor	0.682 \pm 0.046 *	48.7 \pm 8.4 *	58.5 \pm 2.2 *
40% Maotai liquor	0.604 \pm 0.020 **	34.3 \pm 5.9 **	0

Vs control group, *: $P < 0.05$; **: $P < 0.01$.

Table 3. Effect of Maotai liquor on the growth of HSCs (mean \pm SD)

Group	Well	Value A	Depression ratio of the proliferation of HSC
Control	5	0.323 \pm 0.022	0
0.05% Maotai liquor	3	0.338 \pm 0.001	-4.5 \pm 0.3
0.1% Maotai liquor	3	0.283 \pm 0.034	12.4 \pm 10.4 *
0.5% Maotai liquor	3	0.267 \pm 0.005	17.4 \pm 1.6 *
1% Maotai liquor	3	0.270 \pm 0.008	16.4 \pm 2.3 *
1% alcohol	3	0.351 \pm 0.007	-8.4 \pm 2.3

*: Vs alcohol group, $P < 0.001$.

Table 4. Effect of Maotai liquor on rats GSH and MDA (mean \pm SD)

Group	Rats	Liver index (g/kg)	GSH (value A)	MDA (nmol/L)
Normal control	20	37.4 \pm 4.0	0.21 \pm 0.04	3.9 \pm 1.8
High-fat	20	56.9 \pm 6.4 *	0.18 \pm 0.08	7.4 \pm 1.5 *
Low-dose	20	53.3 \pm 5.4 * Δ	0.20 \pm 0.10	5.0 \pm 1.8
High-dose	20	50.9 \pm 8.1 * Δ	0.15 \pm 0.06	5.8 \pm 1.6
Alcohol	20	58.9 \pm 6.1 *	0.14 \pm 0.07	5.9 \pm 2.3

*: Vs alcohol group, $P < 0.05$; Δ : vs normal control group, $P < 0.1$.

effect on the shape of HSCs. From a concentration of 2%, the proliferation of hepatic stellate cells was obviously suppressed by Maotai liquor and the effect was concentration dependent (2%, 10%, 20% concentration, $P < 0.05$; 40% concentration, $P < 0.01$).

Effect on the secretion of type I collagen of HSCs

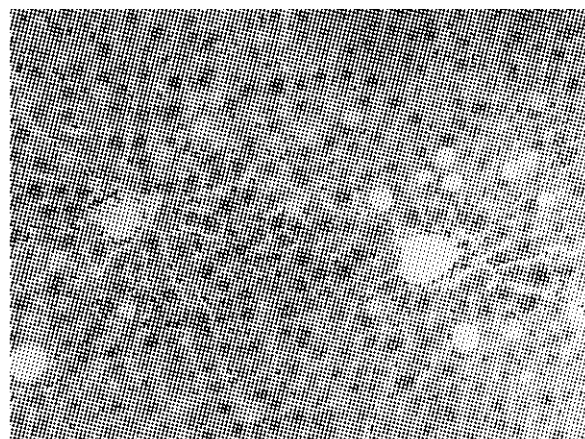
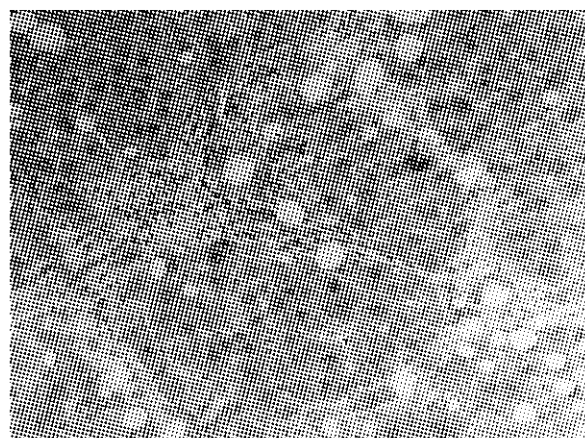
Maotai liquor with a concentration of 20%–40% obviously suppressed the secretion of type I collagen of HSC ($P < 0.05$). The analysis of gene expression of HSCs in Maotai liquor with a concentration of 10% and 20% showed that the 20% concentration of Maotai liquor could obviously suppressed the gene expression of type I precollagen, but the 10% concentration of Maotai liquor did not show such effect ($P > 0.05$).

Effect of Maotai liquor on the growth of HSC (Table 3)

Maotai liquor with different concentration showed different suppression effect on HSCs. Significant difference was seen in the suppression rate on HSCs between the 1% alcohol group and 1% Maotai liquor ($P < 0.01$). Maotai liquor obviously suppressed the growth of HSCs but alcohol did not have such effect.

Effect of Maotai liquor on rats GSH, MDA and biopsy

Liver variables, GSH and MDA of rats fed with Maotai liquor for 14 weeks were measured (Table 4). The liver variables of the rats fed with a diet of high fat

**Fig. 1.** Fourteen weeks after MT had been administered, regenerated fibrotic tissues, fat drop accumulation, and fat degeneration were observed.**Fig. 2.** Fourteen weeks after MT had been administered, fibrotic tissues regenerated widely in liver, fat degeneration of hepatocytes and formation of pseudo-lobuli appeared as the typical expression of liver cirrhosis.

but low protein were higher than those of normal control group ($P < 0.01$), but the increase of liver variables in Maotai liquor group was obviously lower than that of alcohol group ($P < 0.05$). There was no significant difference in GSH between each group. The level of MDA in high fat group was markedly increased, but less markedly in Maotai liquor and alcohol groups. Pathologically, fatty changes were seen in Maotai liquor group, mild proliferation of fibrotic tissues in liver interstitial cells (Fig. 1), and extensive proliferation of fibrotic tissues in alcohol group (Fig. 2).

Discussion

The relation of Maotai liquor increasing liver MT to anti-CCl₄ injury

In recent years, many researches^[11-13] have indicated that free radical and its induced bioactive function are closely related to liver injury, and that bioactive substance may result in hepatic fibrosis through the metabolism of collagen. MT is more superior than SOD in removing free radicals, particularly the hydroxy free radicals.^[14-18] Extracor-poreal experiment also showed that MT may clean oxygen free radical, prevent lipid-peroxids and injury to DNA.^[19-25] After the rats were fed with Maotai liquor for 8 weeks in this experiment, the increased MT in rat liver was able to decrease the lipid peroxidation product of MDA in the liver intoxicated by CCl₄. Our results showed that there is a negative correlation between MT and MDA in the liver and that Maotai liquor is able to induce MT to antagonize the effect of poisoning caused by CCl₄, and MT as one of internal free radicals has the function of anti-peroxidation. However, whether Maotai liquor has positive influence on chronic liver injury and hepatic fibrosis through this mechanism needs further study.

Effect of Maotai liquor on the proliferation of HSC and the formation of collagen

Many investigations^[26-34] have indicated that HSC is the key point in the pathological process of hepatic fibrosis. The proliferation of cells and the formation of collagens in the extracellular matrix are the main characteristics of activation of HSC. Thymidine incorporation is able to reflect the cell division and proliferation. Suppression experiments of different concentration of Maotai liquor in HSCs from rats and human beings showed that Maotai liquor has a concentration-dependent suppression effect on the proliferation of HSCs, but there was no such effect in alcohol group. The generation of collagen was that first the collagen gene was transcribed and then translated into precollagen and the product secreted in the extracellular matrix. Our experiment showed the effect of Maotai liquor on the expression of the collagen gene and that Maotai liquor could suppress the activation of HSC in many ways. The activation of HSC and the depressed formation of collagen protein may be the key factors for slowing down the development of hepatic fibrosis, for which substance takes effect in Maotai liquor awaits further study.

Effect of Maotai liquor on high-fat-low-protein-fed rat liver

The rats given high-fat-low-protein-food fed were subjected to Maotai liquor and alcohol for 14 weeks, respectively. The results showed the decreased level of MDA insignificant change of GSH level, and fat change in the liver in the Maotai liquor group, but typical hepatic cirrhosis in the alcohol group. These results provide a scientific basis for the effect of Maotai liquor on the liver by long-term consumption.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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