PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Effect of Maotai liquor in inducing metallothioneins and on hepatic stellate cells

Ming-Liang Cheng, Jun Wu, Hai-Qin Wang, Lie-Ming Xue, Ying-Zhi Tan, Liu Ping, Cheng-Xiu Li, Neng-Hui Huang, Yu-Mei Yao, Lan-Zheng Ren, Lan Ye, Ling Li, Mei-Lin Jia

Ming-Liang Cheng, Jun Wu, Hai-Qin Wang, Yu-Mei Yao, Department of Infectious Diseases, Affiliated Hospital, Guiyang Medical College, Guiyang 550004, Guizhou Province, China
Lie-Ming Xue, Ying-Zhi Tan, Liu Ping, Shanghai University of Traditional Chinese Medicine. Shanghai 200020, China
Cheng-Xiu Li, Neng-Hui Huang, Lan-Zheng Ren, Lan Ye, Ling Li, Mei-Lin Jia, Department of pharmacology, Guiyang Medical College, Guiyang 550004, Guizhou Province, China
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Correspondence to: Ming-Liang Cheng, Professor Department of Infectious Diseases, Affiliated Hospital, Guiyang Medical College, Guizyang 550004, Guizhou Province, China. chengml@21cn.com
Telephone: +86-851-6782199(H), +86-851-6855119 Ext. 3263 (O)
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Abstract

AIM: **To explore the possible mechanism why drinking Maotai liquor dose not cause hepatic fibrosis.**

METHODS: **After being fed with Maotai for 56 days consecutively, the male SD rats were decollated for detecting the biological indexes, and the livers were harvested to examine the liver indexes and the level of hepatic metallothioneins (MT). Hepatic stellate cells (HSC) proliferation and collagen generation were also observed.**

RESULTS: **Hepatic MT contents were 216.0ng·g^{-1}±10.8 ng·g^{-1} in the rats of Maotai group and 10.0ng·g^{-1}±2.8 ng·g^{-1} in the normal control group, which was increased obviously in Maotain group (P<0.05). In the rats with grade CCL_2 poisoning induced by Maotai, hepatic MT content was 304.8ng·g^{-1}±12.1ng·g^{-1} whereas in the controls with grade CCL_4 poisoning, it was 126.4ng·g^{-1} ±4.8ng·g^{-1} (P<0.05). MDA was 102.0nmol·g^{-1}±3.4 nmol·g^{-1} in Maotai group and 150.8nmol·g^{-1}±6.7 nmol·g^{-1} in the control group (P<0.05). When both of the groups were suffering from grade CCL_4 poisoning, hepatic MT contents was negatively correlated with MDA (r=-0.8023, n=20, P<0.01). The 570nmA values of each tube with HSC regeneration at concentrations of 0, 10, 50, 100, and 200g·L^{-1} of Maotai were 0.818, 0.742, 0.736, 0.72, 0.682, and 0.604, respectively. From the concentration of 10g·L^{-1}, Maotai began to show obvious inhibitory effects against HSC, and the inhibition was concentration-dependent (P<0.05, P<0.01). Type I collagen contents in HSC were 61.4, 59.9, 50.1, 49. 2, 48.7, 34.4μg·g^{-1} at concentrations of 0, 10, 50, 100, and 200g·L^{-1} of Maotai. At the concentration of 100-200g·L^{-1}, Maotai had obvious inhibitory effect against the secretion of type I collagen (P<0.05). Gene expression analysis was conducted on cells with Maotai concentrations of 0, 50, 100g·L^{-1} respectively and the ash values of β-actin gene expression were 0.88, 0.74, and 0.59, respectively,suggesting that at the concentration of 100g·L^{-1}, Maotai could obviously inhibit gene expression of type I procollagen (P<0.05), but the effect was not obvious at the concentration of 50g·L^{-1} (P>0.05). At the concentration of 10g·L^{-1}, HSC growth *in vitro* inhibition rates were 16.4±2.3 in Maotai group and -8.4±2.3 in the control group (P<0.05).**

CONCLUSION: **Maotai liquor can increase metallothioneins in the liver and inhibit the activation of HSC and the synthesis of collagen in many aspects, which might be the mechanism that Maotai liquor interferes in the hepatic fibrosis.**

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INTRODUCTION

Long term alcohol abusing may result in alcoholic liver diseases, and the volume and duration of drinking has a close relationship with alcoholic liver diseases[1-5]. According to recent studies, the main cause of alcoholic hepatic injury[6-12] is due to acetaldehyde and hydroxy free radicals oxidized from alcohol which can injure the hepatocytes and activate the lipid peroxidation. The necrosis, inflammation, alcohol, its metabolites and lipid peroxidation are all able to activate Kupffer cells to secrete many cytokines which in turn activate hepatic stellate cells to produce various components of extracelluar matrix (ECM). When a large amount of ECM is deposited in the liver, it will lead to hepatic fibrosis[13-18]. Now many studies have demonstrated that metallothionein (MT) has the cytoprotective effect of clearing away the oxygen-derived free radicals[19-24]. It is found[25,26] that alcohol can induce the increase of metallothionein in rats liver, but the mechanism has not been elucidated. MT is endogenous anti-injury substance and plays a role in the defence of stress reaction[27]. Maotai liquor has its unique brewing technique, at the same time, there are multiple microorganisms in the special geographical situations which are able to absorb abundant amino acids, vitamins and many essential microelements [28]. It was reported by Li Xinyan *et al* that drinking Maotai liquor 150g for ten years daily did not result in significant damage to the liver, moreover, it could help protect one's health. Epidemiological study showed that no one died of liver disease in those workers who had drunk Maotai liquor for about 30 years. No obvious hepatic fibrosis or cirrhosis of liver was found in the epidemiological study of 99 workers who had a long history of drinking, or in the pathological examination of their liver needle biopsies nor were they seen in rats fed with Maotai liquor for a successive 56 days[28]. In order to explore the effect of Maotai liquor on the liver, we observed its effect on hepatic stellate cell proliferation *in vitro*, collagen generation,gene expression and growth of human hepatic stellate cells, and the effect of Maotai liquor in inducing metallothioneins in the rats liver and the relationship between it and CCL_4 hepatic injury were also studied in order to demonstrate the possible mechanism in the inhibition on the hepatic fibrosis.

MATERIALS AND METHODS

Materials

Male SD rats, weighing (300±20)g, were purchased from the

Experimental Animal Center of the Third Military Medical University, Chongqing, China. Maotai liquor (530±2)g·L^{-1}, was produced by Guizhou Maotai Distillery with bar code 6902952880026 provided by Section 4 of Guizhou Oil & Foodstuff Export and Import Company. MT standard was provided by Dr. Jie Liu in National Institute of Environmental Health Sciences, U. S. A. 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide. MTT were bought from Sigma Co., U. S.A; trypsin was purchased from Difco. U. S. A and; 199 culture medium and MEM culture medium without calcium are the products of Gibco Co., U. S. A; newborn bovine serum (NBS) was produced by Shanghai Huamei Co.; type I rat tail collagen standard (diluted slowly with Na^a-2CO_3/$NaHCO_3$ to 10 -200ng.L^{-1}) and rabbit anti-rat type I collagen antibody (diluted 1∶500 with 0.01mol.L^{-1} PBS) were products of Cambiolem Co.; horseradish peroxidase marker labeled goat anti-rabbit antibody (IgG-HRP, diluted 1∶1000 with PBS containing 100ml.L^{-1} NBS) was purchased from Hua Mei Company; Total protein determination agent (dcproteinassag) was the product of Biorad Co., U. S. A.; RT-PCR reaction agent and PCR marker were purchased from Promega Co.; diethylpyrocarbonate, guanidine sulfocyanate, saturated mixture of phenol and chloroform, and agarose were bought from Shanghai Sangon Co. Freezing High-speed Centrifuge (1.0R, 22R), CO_2 incubator and ultra low temperature freezer were purchased from German Heraeus Co.; inverted microscope was produced by Japanese Olympus Co.; thermostat water bath, thermostat water bath vibrator were produced by Shanghai Medical Equipment Factory; Labsystems Multiskan MS Enzyme Marker Device, was produced in Finland; Danbury CT ultrasonic membrane breaker was the product of Sonicsmaterials Co.; 90mm culture plate, 60mm culture plate, 6-well, 24-well, and 96-well culture plate were the products of Danish Nunc Co.; Beck Wallac 1410 Liquid Scintillation Counter was the product of Beckman Co. U. S. A; Watson-Marlow 101U Constant current pump was produced in U. S. A.

Effect of maotai liquor on liver MT of rats

Forty SD male rats were divided into two groups averagely. 20 rats were fed with Maotai liquor at 2mL·kg^1 diluted 1∶1 by distilled water once everyday for 56 days. The other 20 rats in the control group were fed with saline at the same volume. After all rats had been fed for the last time, 10 rats of each group were given mixture of CCl_4 and olive oil in a volume ratio of 1∶1 at 2.5mL·kg^1. All rats were sacrificed after the last feed to get blood for testing biological indexes, to calculate liver indexes by liver quantity, and to determine the content of MT in the liver by saturation method of Cd- hemoglobin, and the lipid peroxidation product of aldehyde measured by the method of thiobarbiturate.

Isolation and culture of hepatic stellate cells (HSC)

HSC were isolated by in situ perfusion. The test of cell proliferation was done by MTT. When monolayer HSC appeared in the 96-well culture plate, they were cultured in the medium containing Maotai liquor with different concentration of 1-400mg·L^{-1} and 50g·L^{-1} NBS for 18 hours, then 20μL MTT (5g·L^{-1}) was added in each well and continued the culture for 4 hours. After that, suspension was removed and the plate was aired, then 100μL acid isopropanol with 0.05mol·L^{-1} HCl was added to each well, little black crystals were dissovled by agitating which formed the steady purple solution. The absorbance (A) in each well was determined by ELSIA at the wave-length of 570nm. There were 4 well in each sample. When the subculturing HSC grew into full monolayer in the 24-well culture plate, then they were cultured in the medium containing Maotai liquor with different concentration from 1mg·L^{-1} to 400mg·L^{-1} and 50g·L^{-1} NBS for 24 hours.

Determination of type I collagen and total protein

After the culture was ended, it was centrifugated at 450×g at 4℃ for 20 minutes. Both the suspension and cell layer were collected separately. The collected cells were dissolved by 0.2mol.L^{-1} NaOH 0.5ml in each well and washed with 0.5ml double distilled water. Ultrasonic membrane-breaking was done for 10s at 40℃. Type I collagen in the suspension was determined by ELISA (the enzyme labelling plate was coated with type I collagen standard and samples at different concentration were kept overnight at 4℃; then IgG-HRP was used for incubation after they bound to type I collagen antibody; pyrocatechol oxidation was used for staining, and value A was obtained at 492nm wavelength by Labsystem ELISA equipment, which automatically calculated the standard curve and contents of each specimen.). Total protein in the cell layer was determined by DC protein assay kit.

Semiquantitative RT-PCR

Total RNA was isolated from HSC by phenol-chloroform extraction and isopropanol precipitation. The primer of procollagenβ1(I) was synthesized, purificated and evaluated by Shanghai Sangon Company (See Table 1). One μg of total RNA was reversely transcribed according to the instructions of the RT-PCR Kit at 48℃ for 45min. The PCR mixture contained 50pmo1·L^{-1} primer of procollagen β1[I] or GAPDH, 1μL 10mmol·L^{-1} dNTPs, 2μL 25mmol·L^{-1} $MgSO_4$, 5 units AMV reverse transcriptase, 5 units Tfl DNA polymerase and 10mL AMV/Tfl buffer. The PCR conditions included an initial denaturation-2min at 94℃, 30 cycles consisting of (a) 30s denaturation at 94℃;(b)1 min primer annealing at 60℃; (c) 2min elongation at 38℃; and one final step of 7min at 68℃. The PCR product or DNA marker mixed with 5μL loading buffer were elcectrophoresed on a 1.5% agarose gel, visualized by UV and quantified densitometrically. Procollagenβ1[I], pre-albumin, or hydroxyproline expression was calculated by determining the ratio of Procollagen α1[I], relative to β-actin mRNA.

Table 1 Primer sequence and expected PCR product length

Primer designation	Sequence	Product length
α1(I)upstream	CACCCTCAAGAGCCTGAGTC	253bp
α1(I)downstream	GTT CGGGCTGATGTACCAGT	
β-actin upstream	ACATCTGCTGGAAGGTGGAC	163bp
β-actin downstream	GGTACCACCATGTACCCAGG	

The effect of Maotai liquor on human HSC

80000 human HSC cells were inoculated in each well in the 96-well plate, and they were cultured in the DMEM medium with 100mL·L^{-1} FBS at 37℃ in a humidified atmosphere containing 5% CO_2 for 24 hours. Then they were continuously cultured in the DMEM medium with 20mL·L^{-1} FBS for 24hours. At last they were cultured in the medium containing different concentration of Maotai liquor (Maotai liquor was diluted to 0.5g·L^{-1}, 1g·L^{-1}, 5g·L^{-1}, 10g·L^{-1}, 50g·L^{-1} by DMEM medium with 20mL·L^{-1} FBS or alcohol). Cells cultured in the DMEM medium with 20mL·L^{-1} FBS was used as the control. After they were cultured for 20 hours, 20μL MTT (5g·L^{-1}) was added in each well and the culture continued for 4 hours more. Then the medium was removed, 100μL acid isopropanol with 0.05mol·L^{-1} HCl was added in each well, after little black crystals were dissovled, value A was obtained at 570nm wavelength by Labsystem ELISA equipment and the inhibiting rate of cell proliferation was calculated.

Statistics analysis

Data were analyzed by *t* test and *q* test.

RESULTS

Effect of Maotai liquor on rats liver MT content

Maotai liquor could induce the MT in rats liver to increase to 22 times of its original level (See Table 2). It is thought that lipid peroxidation of cell memb rane and the intracellular accumulation of Ca^{2+} are the important links of hepatocellular damage. In the control group, MDA in the liver was obviously increased after they were intoxicated by CCl_4, merely Maotai liquor did not influence MDA in rats' liver. MT in rats fed with Maotai liquor when intoxicated by CCl_4 was increased obviously more than those rats they were not fed with Maotai liquor, but MDA was decreased. There was a negative relationship between MT and MDA in rats liver intoxicated by CCl_4 in the control and trial group (r=-0.8023, n=20, P<0.01).

Table 2 The effect of Maotai liquor on MT and MDA in rats liver (n=10,$\bar{x}\pm s$)

Group	MT(ng·g^{-1})	MDA(ng·g^{-1})
Control group	10.0±2.8	60.2±3.1
Maotai liquor group	216.0±10.8^{b}	60.1±2.4
CCL_4 Group	126.4±4.8^{b}	150.8±6.7^{b}
CCL_4 + Maotai liquor group	304.8±12.1^{bd}	102.0±3.44^{bd}

^{b}P<0.01,*vs* control group;^{d}P<0.01,*vs* CCL_4 group(analysis of variance and q test)

Effect of Maotai liquor on rats' HSC

We normally obtained (3-5)×10^7 HSC from one rat. Lipid droplets in the primary HSC were obvious, but during the course of culture, lipid droplets decreased. And as they were passaged, HSC became extended, and looked like myofibroblasts. Each concentration of Maotai liquor had no effect on the shape of HSC. The absorbance (A) at 570nm of HSC in the medium with 0mg·L^{-1}, 10mg·L^{-1}, 50mg·L^{-1}, 100mg·L^{-1} and 200mg·L^{-1} Maotia liquor were 0.818, 0.742, 0.736, 0.72, 0.682 and 0.604, respectively. From the concentration of 10g·L^{-1}, Maotai liquor had obvious inhibiting effect on the proliferation of HSC, and the effect was enhanced with the increase of the concentration of Maotai liquor (P<0.05, P<0.01). The content of type I collagen of HSC in the medium with 0mg·L^{-1}, 10mg·L^{-1},50mg·L^{-1},100mg·L^{-1} and 200mg·L^{-1} Maotia liquor were 61.4μg·g^{-1},59.9μg·g^{-1},49.2μg·g^{-1},50.1μg·g^{-1},48.7μg·g^{-1} and 34.4μg·g^{-1}, respectively. The 100-200g·L^{-1} Maotai liquor had obvious inhibiting effect on the secretion of type I collagen (P<0.05). The analysis of gene expression of HSC in the 0mg·L^{-1}, 50mg·L^{-1} and 100mg·L^{-1} Maotia liquor group showed that the relative density of β-actin (n=3) analyzed by computer were 0.88,0.74 and 0.59, respectively. The result showed that 100g·L^{-1} Maotia liquor could significantly inhibit the gene expression of type I procollagen (P<0.05), but 50g·L^{-1} Maotia liquor did not have such effect (P>0.05).

Table 3 The effect of Maotai liquor on HSC of human (n=3, $\bar{x}\pm s$)

Group	Inhibiting Rate of HSC
Alcohol group (10g·L^{-1})	-8.4±2.3
Maotai liquor group (0.5g·L^{-1})	-4.52±0.3
Maotai liquor group (1g·L^{-1})	12.4±10.4^{b}
Maotai liquor group (5g·L^{-1})	17.4±1.6^{b}
Maotai liquor group (10g·L^{-1})	16.4±2.3^{b}

^{b}P<0.01,*vs* alcohol group

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

There were three experiment groups in our study: blank control group, control group and trial group. First alcohol was diluted to 530 g·L^{-1} (the same concentration as Maotai liquor) and then it was dispensed to different concentrations which was the same as Maotai liquor. We used 0.5g·L^{-1},1g·L^{-1}, 5g·L^{-1} and 10g·L^{-1} Maotai liquor to study the different inhibiting effect on HSC. Our result showed that there was a significant difference between the 10g·L^{-1} alcohol group and 10g·L^{-1} Maotai group (P<0.01). That was Maotai liquor had a significant inhibiting effect on the proliferation of HSC, but alcohol did not have such effect.

DISCUSSION

MT is a low molecular weight metal-binding protein with rich cysteine existing widely in the biosphere and can be induced *in vivo* by many factors[29-32]. MT is also a non-enzyme protein which has bioactive functions of binding heavy metals, clearing free radicals, anti-oxidation and cytoprotection. It is now the most powerful bioactive substance which can remove free radicals. In recent years, a lot of research studies show that MT can protect hepatic cells from injury and be helpful in repairing hepatocytes without inducing hepatic fibrosis[33-38]. Induced by Maotai liquor, the increased MT in rats' liver was able to decrease the lipid peroxidation product of MDA in the liver intoxicated by CCl_4. Our result showed that there was a negative correlationship between MT and MDA in the liver and it proved that Maotai liquor was able to induce MT to antagonize the effect poisoning by CCl_4. It may be one of the possible mechanisms in explaining why long term drinking proper volume of Maotai would not cause hepatic fibrosis or cirrhosis.

Hepatic fibrosis is an important pathologic process resulted from many chronic liver diseases and may process to liver cirrhosis. It is also the key point that many chronic liver diseases are hard to cure completely. Many investigations showed that the activation of HSC is the key point in the pathological process of hepatic fibrosis. HSC was activated by many pathological factors so that alterations in the phenotype and function of HSC happened. HSC was highly proliferated and secreted a large amount of extracellular matrix deposited in the liver which would result in hepatic fibrosis. Collagen was the most important component in the extracellular matrix[46-48]. The degree of increase of collagen was type I>type III>type IV. The cell proliferation and the enhanced generation of collagen were the main characteristics of activation of HSC. [^{3}H]thymidine incorperation was able to reflect the cell division and proliferation. In the inhibiting experiments of different concentration of Maotai liquor in both HSC from rats and HSC from human beings showed that Maotai liquor had a concentration-dependent inhibiting effect on the proliferation of HSC. The generation of collagen was that first the collagen gene was transcribed and then translated into procollagen and the product secreted in the extracellular matrix. Our experiment showed that Maotai liquor had inhibiting effect on the expression of collagen gene and the secretion of collagen protein, but 10g·L^{-1} alcohol had no such inhibiting effect. All those showed that Maotai liquor could inhibit the activation of HSC in many links. The inhibiting effect of Maotai liquor in the activation of HSC and the generation of collagen may be the possible reasons why Maotai liquor can interfere with the process of hepatic fibrosis.

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