Differential Effects Between Maotai and Ethanol on Hepatic Gene Expression in Mice: Possible Role of Metallothionein and Heme Oxygenase-1 Induction by Maotai

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Alcohol is a risk factor for liver fibrosis and hepatocellular carcinoma. On the other hand, light alcoholic beverage consumption is believed to be beneficial because of the effects of both alcohol and nonalcoholic components of the beverage. Maotai is a commonly consumed beverage in China containing 53% alcohol. Epidemiological and experimental studies show that Maotai is less toxic to the liver than ethanol alone. To examine the differential effects of Maotai and ethanol, a low dose of Maotai or an equal amount of ethanol (53%, v/v in water, 5 ml/kg) were given to male mice daily for 1 week, and hepatic RNA was extracted for microarray analysis. Approximately 10% of genes on the liver-selective custom array (588 genes) were altered following Maotai or ethanol administration, but Maotai treated livers had fewer alterations compared with ethanol alone. Real-time reverse transcription–polymerase chain reaction confirmed and extended microarray results on selected genes. An induction of metallothionein and heme oxygenase-1 occurred with Maotai, which could not be explained by alcohol consumption alone, whereas the attenuation of ethanol responsive genes such as quinone dehydrogenase, DNA-ligase 1, IGFBP1, and IL-1β suggests less liver injury occurred with Maotai. The expression of genes related to liver fibrosis, such as cytokeratin-18, was slightly increased by the high dose of ethanol, but was unchanged in the Maotai group. In summary, gene expression analysis indicates that Maotai induces a different response than ethanol alone. The dramatic induction of metallothionein and heme oxygenase-1 with Maotai could be important adaptive responses to reduce alcoholic liver injury.
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Introduction

Alcoholic beverage consumption has long been recognized as a risk factor for hepatocellular carcinoma (1). There is compelling epidemiological data indicating increased cancer risk associated with alcohol abuse (2). Hepatocellular carcinoma, which is associated with alcohol abuse, is one of the most common malignant tumors worldwide, and malignant transformation of hepatocytes may occur as a consequence of various factors, including chronic liver injury, development of liver fibrosis and cirrhosis, metabolic disorders, oncogene activation, genomic instability, and overexpression of growth and angiogenic factors (3). On the other hand, light alcohol consumption, including the nonalcohol factors contained in alcoholic beverages, is believed to be beneficial to health, and can reduce cardiovascular diseases, risk of diabetes, and may have protective effects toward carcinogenesis and osteoporosis (4, 5).

An epidemiological study was conducted on Maotai production workers to examine the health effects of light ethanol consumption (6). Maotai is a common alcoholic beverage in China containing 53% alcohol (v/v). Workers typically consume a small amount of Maotai to taste its quality during the production process. A retrospective survey revealed none of the workers died from hepatocellular carcinoma in the past 30 years, and among 99 workers investigated, liver function was basically normal, and there was no evidence of hepatic fibrosis or cirrhosis in 23 biopsies (6). These results suggest that Maotai could be different from ordinary ethanol and could be less toxic to the liver than alcohol from other beverage sources.

Experimental studies on animals showed that Maotai

Table 1. Sequences for Real-Time RT-PCR

Gene	Accession No.	Forward	Reverse
β-actin	M12481	GTATGACTCCACTCACGGCAAA	GGTCTCGCTCCTGGAAGATG
Cytokeratin-18	M11686	GGATGTGGAGGCCCGATAC	CGAGTTTGTGCCAGCTCTGA
DNA ligase-1	U19604	GCCTCCACCTCCACTTACTATTTC	CATGGAAGCACTGCCAGTGA
GST pi	D30687	TGGGCATCTGAAGCCTTTTG	GATCTGGTCACCCACGATGAA
HO-1	M33203	CCTCACTGGCAGGAAATCATC	CCTCGTGGAGACGCTTTACATA
IGFBP-1	X81579	TGGACAGCTTCCACCTGATG	TGATGGCGTTCCACAGGAT
IL-1β	NM_008361	CTGGTGTGTGACGTTCCCATTA	CCGACAGCACGAGGCTTT
MT-1	BC027262	AATGTGCCCAGGGCTGTGT	GCTGGGTTGGTCCGATACTATT
NQO1	BC004579	TATCCTTCCGAGTCATCTCTAGCA	TCTGCAGCTTCCAGCTTCTTG

induced hepatic metallothionein and increased hepatic glutathione levels compared with ethanol alone (7). In comparing chronic oral administration of Maotai to equivalent amounts of ethanol alone, Maotai led to significantly less hepatic oxidative damage and liver pathology (6, 7). Maotai can also inhibit alcohol-induced hepatic stellate cell proliferation and reduces overexpression of hepatic collagen-I (7, 8). However, the basis for such a difference remains unclear, and little is known about the effects of Maotai and ethanol on hepatic expression of genes relevant to liver injury, fibrosis, and hepatocarcinogenesis.

To further examine the risk and beneficial effects of Maotai compared with ethanol, a low dose of Maotai or the equal amount of ethanol in water (53%, v/v) were given to mice daily for 1 week; hepatic RNA was then extracted for microarray and real-time reverse transcription–polymerase chain reaction (RT-PCR) analysis to profile changes in liver gene expression. The results showed the induction of metallothionein and heme oxygenase-1 by Maotai could be important adaptive responses that reduce alcohol-induced liver injury.

Materials and Methods

Animals and Treatment. Male CD-1 mice weighing 25–30 g were housed in the animal facilities of Guiyang Medical College in a 12-hr light-dark cycle. Animals were allowed free access to tap water and rodent chow at 20–22°C. All procedures involving the use of laboratory animals were in accordance with National Institutes of Health guidelines and were approved by the institutional animal use and care committee. Maotai (53% ethanol, v/v) was obtained from Maotai Company (Guizhou, China) and absolute ethanol was obtained from Chongqing Chemical Company (Chongqing, China). Mice were divided into five groups and given Maotai or 53% alcohol (v/v) in water at doses of 5 ml/kg and 10 ml/kg by intragastric incubation daily for 7 days. Untreated animals were used as controls. Twenty-four hours after the last dose livers were removed and total RNA was extracted, purified, and subjected to microarray and real-time RT-PCR analysis.

Microarray Analysis. The custom-designed mouse liver arrays (588 genes, Clontech, Palo Alto, CA) were used for cDNA microarray analysis (9). Total RNA was isolated from liver samples with TRIzol agent (Invitrogen, Carlsbad, CA), followed by purification and DNase-I digestion with RNeasy columns (Qiagen, Valencia, CA). Approximately 5 mg of total RNA was converted to [a-^{32}P]dATP-labeled cDNA probe using Moloney murine leukemia virus (MMLV) reverse transcriptase and the Atlas customer array-specific cDNA synthesis primer mix, and then purified with a NucleoSpin column. The membranes were prehybridized with Expresshyb (Clontech) for 2 hrs at 68°C, followed by hybridization with the cDNA probe overnight at 68°C. The membranes were then washed four times in 2× saline-sodium citrate (SSC)/1% sodium dodecyl sulfate (SDS), 30 mins each, and two times in 0.1× SSC/0.5% SDS for 30 mins. The membranes were then sealed with plastic wrap and exposed to a Molecular Dynamics Phosphoimage Screen. The images were analyzed densitometrically using AtlasImage software (version 2.01). The gene expression intensities were first corrected with the external background and then globally normalized.

Real-time RT-PCR Analysis. The levels of expression of the selected genes were quantified using real-time RT-PCR analysis (9). Briefly, total RNA was reverse transcribed with MuLV reverse transcriptase and oligo-dT primers. The forward and reverse primer sequences for selected genes were designed with the ABI Primer Express software (Applied Biosystems, Inc., Foster City, CA) and are listed in Table 1. The SYBR green PCR master mix (Applied Biosystems, Cheshire, UK) was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values as follows: the Ct values of the interested genes were first normalized with β-actin of the same sample, and then the relative differences between control and treatment groups were calculated and expressed as relative increases, setting the control as 100%.

Statistics. Liver samples were pooled and microarray analysis was performed in triplicate, and individual samples ($n = 5$) were used for real time RT-PCR analysis. Data are expressed as the mean ± SEM. For comparisons of gene expression between two groups, the Student's t-test was performed. The level of significance was set at $P < 0.05$ in all cases.

Table 2. Increased Expression of Genes Following Administration of Maotai or 53% Ethanol[a]

Accession No.	Protein/gene	Baseline control	Ethanol ratio	Maotai ratio
U19604	Ligase I, DNA, ATP-dependent	64	36.9	7.2
AB008553	CD36 antigen (collagen type I receptor)	74	12.4	8.0
M32502	WNT-3 proto-oncogene	85	9.0	6.2
X00195	c-myc proto-oncogene	329	4.1	1.7
D26186	src-related kinase	139	3.9	3.9
Y00082	Lung carcinoma myc-related oncogene 1	717	3.7	1.8
U36277	I-κB (I-kappa B) alpha chain	553	3.6	2.0
U10871	CDC2-related kinase 1	2208	3.0	2.1
AF011908	Apoptosis-associated tyrosine kinase	452	2.9	1.6
J00423	Hypoxanthine-guanine phosphoribosyltransferase	472	2.8	1.0
U88908	Apoptosis inhibitor 1	389	2.6	1.7
V00741	Epidermal growth factor	1382	2.2	1.3
X53476	HMG-14 nonhistone chromosomal protein	1731	2.2	1.1
X81579	IGF-binding protein 1	6577	2.1	1.3
D90176	NF-1B protein (transcription factor)	3158	2.0	1.8

[a] Maotai or 53% ethanol were administered v/v in water, 5 ml/kg, po × 7 days.

Results

Microarray Analysis. Total RNA was isolated and purified from liver samples of mice given Maotai orally (5 ml/kg, daily for 7 days), or ethanol (53%, v/v in water, 5 ml/kg, daily for 7 days) or left untreated (control), and subsequently subjected to microarray analysis. Under the criteria of a >2-fold difference and $P < 0.05$ as significant, expression of approximately 10% of the genes assessed were altered out of the total of 588 genes on the liver-selective custom array. Ethanol treatment produced 21 instances in which genes were up-regulated and 57 that were significantly down-regulated. Maotai produced 28 genes that were up-regulated and 55 that were down-regulated. In general, ethanol induced more pronounced gene expression alterations than Maotai.

Table 2 lists the increased expression of genes based on treatment. Ethanol alone increased gene expressions relevant to the acute-phase response and tissue damage that were distinctly less pronounced in the Maotai group. For example, ethanol alone significantly increased the expression of DNA ligase 1, CD36 (collagen type 1 receptor), Wnt-3, c-myc, and L-myc proto-oncogenes, IκB and kinases related to Src and CDC-2 signaling, apoptosis-associated kinases and proteins, epidermal growth factor, and IGF-binding protein 1. All these increases were less pronounced in Maotai-treated mouse livers.

Table 3 lists the decreased expression of selected genes, such as the tumor suppressor Wilms tumor protein, glutathione S-transferases, cytochrome P450 enzymes, calmodulin, DNA damage repair protein Rad50, and 7,8-dihydro-8-oxoguanine triphosphatase, and phospholipase C gamma. These decreases were less pronounced in the Maotai group except for the decreased expression of CYP2A4 and early growth response protein 1, which were similar in animals treated with ethanol alone and in those treated with Maotai.

Real-Time RT-PCR Analysis of Aberrant Expressed Genes. To confirm and to extend microarray

Table 3. Decreased Expression of Genes Following Administration of Maotai or 53% Ethanol[a]

Accession No.	Protein/gene	Baseline control	Ethanol ratio	Maotai ratio
M55512	Wilms tumor protein; tumor suppressor	1,089	−42.4	−1.7
J03958	Glutathione S-transferase-alpha	1,382	−24.3	−4.3
V00727	c-Fos proto-oncogene	618	−22.8	−2.3
X78445	Cytochrome P450 1B1	1,759	−6.1	−2.4
X61432	Calmodulin	2,084	−6.0	−1.0
U66887	Rad50; DNA repair protein	1,059	−5.0	−1.0
D49956	7,8-Dihydro-8-oxoguanine triphosphatase	1,023	−3.7	−0.7
X95346	Phospholipase C gamma	1,887	−2.9	−1.5
M33324	Growth hormone receptor	1,107	−2.8	−1.0
M20157	Early growth response protein 1	13,512	−2.6	−2.6
U36993	Cytochrome P450 7B1	9,668	−2.6	−0.9
M13226	Granzyme A	2,607	−2.5	−1.2
U24428	Glutathione S-transferase-mu	1,253	−2.2	−1.0
J03549	Cytochrome P450 2A4	18,662	−2.1	−2.1

[a] Maotai or 53% ethanol were administered v/v in water, 5 ml/kg, po × 7 days.

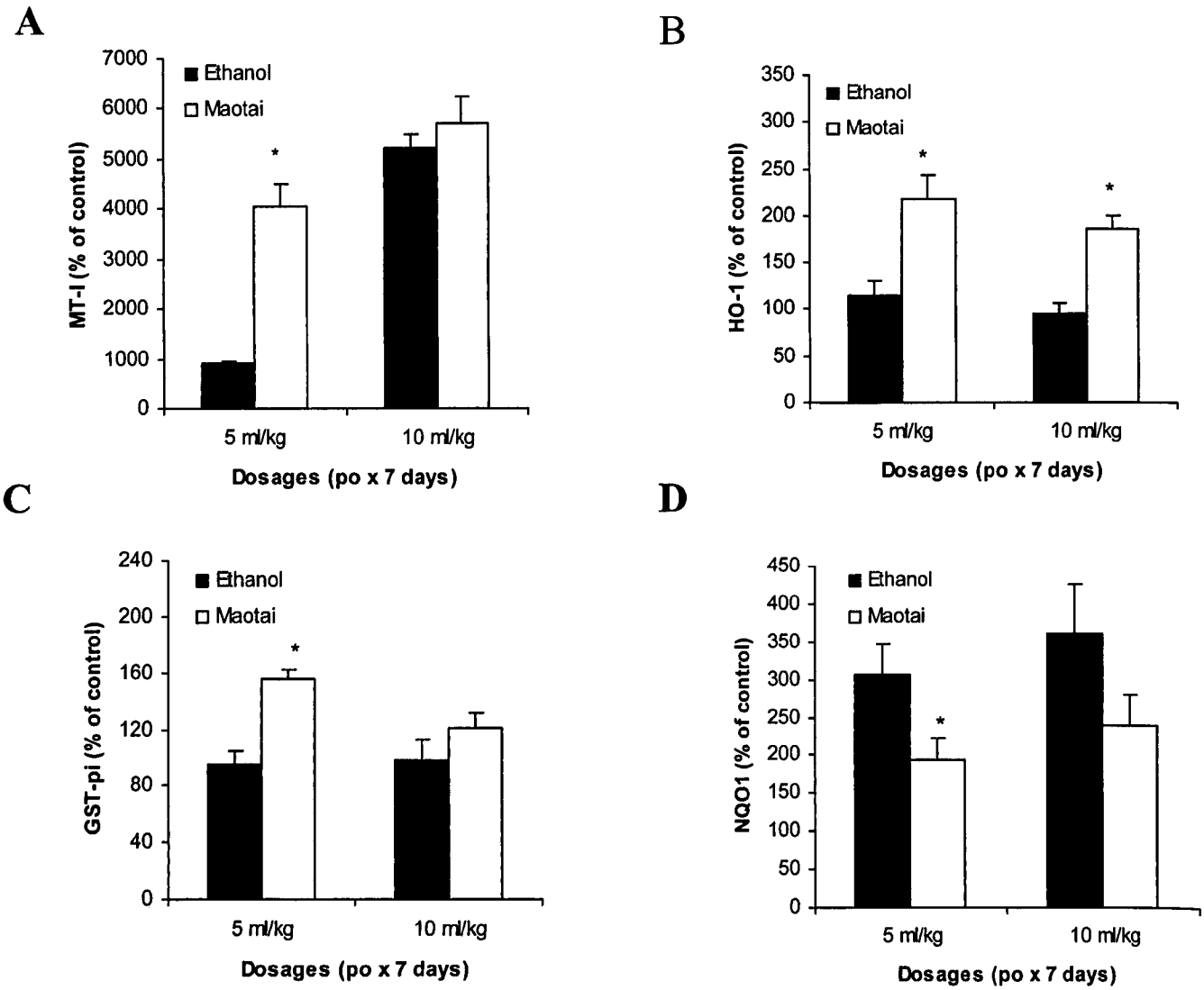

Figure 1. Real-time RT-PCR analysis of hepatic gene expression related to adaptive response. Mice were given Maotai (5–10 ml/kg po × 7days) or an equal amount of 53% alcohol, and compared with untreated controls. Data are mean ± SEM of five animals. *Significantly different from controls, $P < 0.05$.

results, real-time RT-PCR analysis of selected genes was performed using individual liver samples ($n = 5$) from mice treated with a low dose (5 ml/kg) and a high dose (10 ml/kg) of Maotai or ethanol (53%, v/v). In general, real-time RT-PCR confirmed the microarray results, but they appeared more sensitive. Figure 1A shows the expression of the metallothionein-1 gene (which is not included in the array). The low dose of Maotai increased the expression of metallothionein 40-fold compared with a 10-fold increase in the ethanol group. However, at the higher doses of 10 ml/kg, both ethanol and Maotai appeared to increase metallothionein expression to a maximal 50-fold, and no difference between the two groups was evident. Figure 1B shows the expression of the heme oxygenase-1. Maotai was more efficient than ethanol alone in inducing heme oxygenase-1 at both doses. In addition, the low dose of Maotai was more efficient in inducing the expression of GST-pi than ethanol alone (Fig. 1C). Ethanol alone induced the expression NADPH quinone reductase (NQO1), an effect not observed with the low dose of Maotai (Fig. 1D).

The expression of genes associated with a response to tissue damage is listed in Figure 2. Ethanol-alone treatment increased the expression of DNA ligase-1, IGFBP-1, and IL-1β, genes responsible for repair of tissue damage (Fig. 2A–C). The expression of genes related to liver fibrosis, such as cytokeratin–18 (K-18), was also increased by ethanol alone (Fig. 2D). The expression of all these genes was much less after treatment at the low dose of Maotai, but with the higher dose, the differences were not significant except for the expression of DNA ligase 1. The expressions of genes related to hepatocarcinogenesis, such as alpha-fetoprotein, PAI-1, c-myc, and c-met were unaltered by Maotai or ethanol alone with the experiment conditions (data not shown).

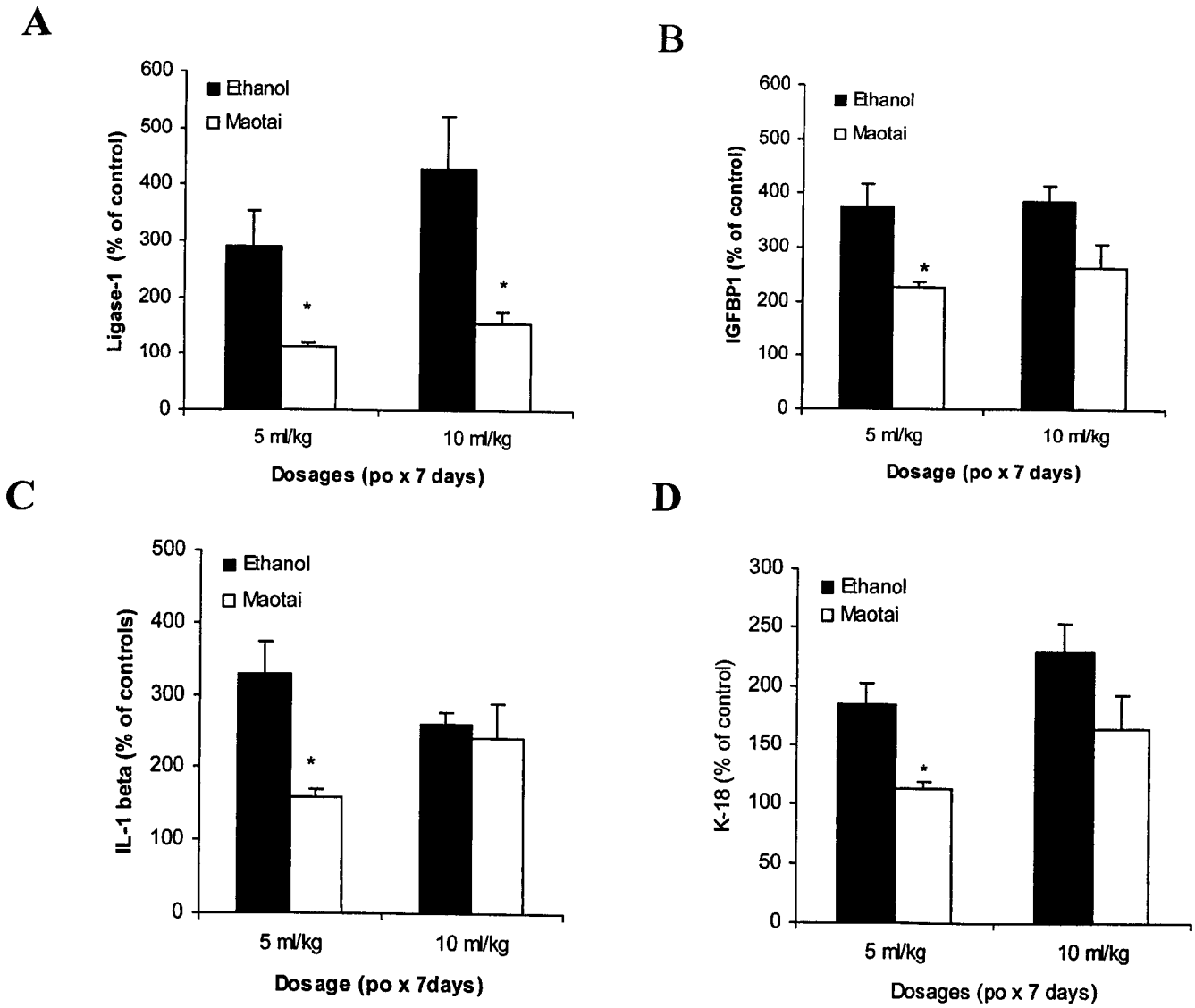

Figure 2. Real-time RT-PCR analysis of hepatic gene expression related to oxidative stress and liver fibrosis. Mice were given Maotai (5–10 ml/kg po × 7days) or an equal amount of 53% alcohol, and compared with untreated controls. Data are mean ± SEM of five animals. *Significantly different from controls, $P < 0.05$.

Discussion

The current study demonstrated that ethanol alone and the alcoholic beverage Maotai could both induce significant alterations in key gene expression in mouse liver, but that there were critical differences between the altered expressions with Maotai and ethanol alone, particularly at the lower dose (5 ml/kg). At the higher dose (10 ml/kg), these differences were frequently lost. These results support the notion that light consumption of an alcohol-containing beverage induces a dramatically different response compared with heavy alcohol abuse. It has been shown that light consumption of ethanol increases panaoxonase activity, an enzyme important for lipid metabolism and cardioprotection, whereas alcohol abuse decreased panaoxonase activity (10). Similarly, light ethanol consumption protects against the hepatotoxicity of D-galactosamine, while moderate to heavy alcohol consumption exacerbates D-galactosamine–induced liver damage (11). This is possibly due to the stimulation of liver regeneration with light ethanol consumption, an effect not observed after heavy ethanol intake (12). Thus, the beneficial and harmful effects of alcohol containing beverage consumption are dependent on the dose of alcoholic beverage intake.

In the present study, Maotai produced a dramatic induction of metallothionein (40-fold vs. 10-fold in the ethanol-alone group at the low dose), even though equal amounts of ethanol were given. Thus, this effect cannot be explained by alcohol content alone, suggesting that non-alcoholic components in Maotai could be responsible for the beneficial effect. Induction of hepatic metallothionein is an important adaptive mechanism affecting the magnitude and progression of toxic insults to the liver (13). Indeed, ethanol is an effective inducer of hepatic metallothionein (14), and

induction of metallothionein by ethanol decreases cadmium hepatotoxicity in rats (15). It has been recently reported that metallothionein plays an important role in protection against alcoholic liver injury (16). Thus, a dramatic induction of metallothionein with a low dose of Maotai could be an important adaptive mechanism to reduce liver injury as a result of alcoholism.

Maotai was more effective than ethanol in inducing heme oxygenase-1 (HO-1) and GST-pi. Both HO-1 and GST-pi are hepatoprotective when induced at appropriate levels. Consistent with a previous observation that Maotai increased hepatic glutathione levels (7), the expression of GST-pi by Maotai would suggest the activation of hepatic glutathione systems in favor of hepatoprotection. HO-1 is the rate-limiting enzyme in heme degradation. In various model systems, HO-1 induction confers protection of tissue from further damage, whereas the blockage of HO-1 induction accelerates cellular injuries (17). For example, the preinduction of HO-1 protects against alcohol hepatotoxicity, whereas inhibition of HO-1 increases liver injury due to alcohol (18). Inhibition of HO-1 also exacerbates carbon tetrachloride induced hepatotoxicity (19). Induction of HO-1 may protect cells against oxidant injury by reducing cellular free heme (a pro-oxidant) and by producing biliverdin as an antioxidant. HO-1 also improves nutritive perfusion via carbon monoxide release, and fosters the synthesis of the iron binding protein ferritin (20). However, high levels of HO-1 may sensitize the cell to oxidative stress. Thus, the moderate induction of HO-1 by Maotai observed in this study could protect the liver from alcohol injury.

Expression of NADPH quinone dehydrogenase 1, a sensitive redox indicator (21), was induced by ethanol, whereas low-dose Maotai attenuated its induction. The expression of DNA ligase 1, IL-1β, and IGF-binding protein 1 could be envisioned as a cellular response to liver injury from alcohol (22, 23), and their induction was not evident with Maotai at the low dose. The expression of genes related to liver fibrogenesis, such as cytokeratin-18 (18), was also increased by ethanol alone but was unchanged by a low dose of Maotai. It is noteworthy that nonalcoholic components contained in Maotai could be responsible for the attenuation of alcohol responsive gene expressions. Maotai contains zinc and at least 68 flavor components, including alcohols, organic acids, esters, acetals, carbonyl, and heterocyclic compounds identified with fine-tuning separation followed by gas chromatography/mass spectrometry (24). The biological effects of these nonalcoholic components warrant further investigation.

Similar studies using microarray to profile gene expression patterns following ethanol administration are available (25, 26). The effects of ethanol on the alteration of metabolic genes are similar to those observed in the present study, corresponding to alcohol induced liver toxicity (25). Expression of genes related to overt liver carcinogenesis is not evident even after 15 weeks of ethanol administration (26), consistent with the present results. It should be kept in mind that the gene expression profiles following ethanol consumption are dependent on dosage, duration, alcohol formulation, and other experimental conditions. It should also be noted that Maotai as an alcoholic beverage is different from pure ethanol, and other alcoholic beverages of the same kind may have the same benefit effects as Maotai.

In summary, the present study compared the gene expression profiles after a low dose of ethanol or Maotai administration. Ethanol responsive gene expression changes are generally less pronounced with Maotai, and a marked induction of metallothionein and heme oxygenase-1 could be part of adaptive mechanisms affecting the progression and magnitude of liver injury due to alcohol beverage consumption.

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