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Original Article

Chinese yellow wine inhibits production of matrixmetalloproteinase-2 induced by homocysteine in rat vascular endothelial cells

Zheng Ji[1*], Fei Zhao[2], Liping Meng[2], Changzuan Zhou[2], Weiliang Tang[1], Fukang Xu[1], Longbin Liu[1], Haitao LV[1], Jufang Chi[1], Fang Peng[1], Hangyuan Guo[1*]

[1]*Department of Cardiology, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, Shaoxing 312000, Zhejiang, China;* [2]*The First Clinical Medical College, Wenzhou Medical University, Wenzhou 325000, Zhejiang, China.* [*]*Equal contributors.*

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Abstract: Objective: To investigate the effects of Chinese yellow wine on the synthesis of homocysteine (Hcy)-induced intracellular MMP-2 in rat vascular endothelial cells (VECs). Methods: Isolation, cultivation, purification and identification of vascular endothelial cells of rat thoracic aorta *in vitro* were conducted. The VECs in passages 3 to 4 were used in all studies. HCY was used to induce VECs to over-express MMP-2. Cells were divided into 5 groups: Control, Hcy, Hcy + yellow wine, Hcy + red wine, and Hcy + ethanol. The cells were treated differently for 48 h. The mRNA expression of MMP-2 was detected by FQ-PCR. Western blot and gelatin zymography were used to test the protein levels and enzymatic activity of MMP-2. Results: Compared with the control group, Hcy significantly increased the expression and activity of MMP-2 to a maximum at 500 μmol/L after culturing for 48 h. Compared with those in the Hcy group, the expression and activity of MMP-2 in yellow and red wine groups was significantly decreased. No significant difference was observed between the ethanol and the Hcy groups and no significant discrepancy between the yellow and red wine groups was found. Conclusion: Hcy promotes the expression and activity of MMP-2, which may play an important role in the pathogenesis of atherosclerosis (AS). Treatment with yellow or red wine decreases Hcy-induced MMP-2 production in VECs. Attenuation of MMP-2 activity by yellow and red wines might be cardio-protective.

Keywords: Yellow wine, atherosclerosis, vascular endothelial cells, matrix metalloproteinase-2, homocysteine

Introduction

Coronary heart disease (CHD) is one of the leading causes of death and disability in the cardiovascular diseases (CVD). Clinical and epidemiological studies have demonstrated that hyperhomocysteinemia (HHcy) is a strong predictor of atherosclerosis, independent of classical atherothrombotic risk factors such as hypercholesterolemia or smoking [1, 2]. According to the American Heart Association (AHA), normal homocysteine concentrations range from 5 to 15 μmol/L. In HHcy, plasma homocysteine levels are elevated. The three forms of HHcy include: (1) moderate, with homocysteine serum levels between 15 and 30 μmol/L; (2) intermediate, with homocysteine serum levels between 31 and 100 μmol/L; and (3) severe, with concentrations above 100 μmol/L [3]. Folate and water-soluble B vitamins play a role in homocysteine metabolism. Deficiency of these B vitamins causes HHcy, which is corrected by supplementation of folate, Vitamins B6 and B12 [4].

The vascular endothelial cells (VECs) provide a selectively permeable barrier to blood and regulate the exchange of molecules in response to internal environmental and molecular signals. In humans, endothelial dysfunction correlates with cardiovascular events, and represents one of the first detectable vascular events in the evolution of atherosclerosis. It has been discovered in almost every condition associated with atherosclerosis and CVD [5]. Since endothelial cells are a major source of nitric oxide (NO) in

the vascular bed, endothelial dysfunction results in impaired endothelial nitricoxide synthase (eNOS) function and altered NO synthesis. Endothelial dysfunction most commonly refers to an impaired NO bioavailability due to reduced synthesis of NO by eNOS or increased breakdown by reactive oxygen species (ROS) [5, 6]. NO also prevents leukocyte adhesion and migration into the arterial wall, as well as platelet adhesion and aggregation or inhibition of vascular smooth muscle cells (VSMCs) proliferation, which are key events in the development of atherosclerosis [5]. In vascular endothelium, NO inhibits MMP-2 transcription by interfering with the activating transcription factor-3 (ATF-3)-mediated p53 binding to the promoter region [7]. Atherosclerotic plaque remodeling entails degradation and reorganization of the extracellular matrix (ECM) scaffold of the vessel wall. It is largely dependent on the specialized enzymes called matrix metalloproteinases (MMPs), particularly matrix metalloproteinase-2 (MMP-2). MMP-2 is constitutively expressed in several tissues including the blood vessel wall. As well, VECs and VSMCs continuously produce MMP-2 *in vitro* [8, 9]. MMP-2 is highly expressed in fatty streaks and atherosclerotic plaques compared with normal regions of the vessel [10]. Studies suggest that fatty streaks and fibroatheromas with hemorrhage and calcification, are enriched in MMP-2 [11].

A J-shaped relationship between alcohol consumption and CHD has been reported in apparently healthy people or cardiovascular patients [12-14]. Dose-dependent beneficial and harmful effects have been observed with low-to-moderate wine intake and heavy drinking, respectively, in a study conducted in California involving more than 120000 adults, with different drinking patterns, over a period of 20 years [15]. Epidemiological studies showed an inverse association between wine polyphenol consumption and mortality from cardiovascular events [16]. In the past decade, red wine polyphenolic compounds have been shown to exert numerous biological effects that might participate in cardiovascular protection. These phenolic compounds identified so far include polyphenols such as resveratrol, phenolic acids, flavonoids and anthocyanins [17]. They possess potent antioxidant properties and have been shown to inhibit oxidation of human LDL and platelet aggregation. These compounds has also been shown to have a range of additional cardio-protective and vaso-protective properties including scavenging reactive oxygen and nitrogen species, increasing HDLs, anti-atherosclerotic, anti-arrhythmic, and vaso-relaxing effects [16, 18, 19].

Chinese yellow wine (or rice wine), red wine and beer are the three most ancient wines in the world. It is such a famous wine that has enjoyed a history of over 2400 years [20]. It contributes to the unique features of traditional Chinese alcoholic beverages due to its flavour, subtle aroma and low alcoholicity (< 20%). It is widely consumed all over the country, especially in the city of Shaoxing, which is “China’s Hometown of Wine”. It was honored as the national banquet wine. Therefore, it is also called Shaoxing wine or Shaoxing yellow wine based on its bright brown color. Traditional yellow wine, derived from the city of Shaoxing in China, is brewed from top quality glutinous rice and top quality wheat together with fresh pure water from Shaoxing jianhu Lake. Studies show that the yellow wine contains abundant phenolic compounds similar to red wine, and is also rich in nutrients such as oligosaccharides, vitamins, amino acids, peptides, microelements and organic acids [21, 22]. It has been used in Chinese traditional medicine as a therapeutic component for thousands of years and is especially claimed to have cardio-protective effects. However, the precise mechanisms are still not well known [23, 24]. Our previous study showed that Chinese yellow wine inhibits MMP-2 and atherosclerosis in the LDL receptor knockout (LDLR-/-) mice [25]. Indeed, we previously showed that Chinese yellow wine inhibits the production of homocysteine (Hcy)-induced extracellular MMP-2 in cultured rat vascular smooth muscle cells [26]. Since the VECs are as important as VSMCs in the development of atherosclerosis, both produce MMP-2 constitutively *in vitro*, which is the most important enzyme for degradation of ECM components. Therefore, the purpose of the present study was to determine whether Chinese yellow wine affected Hcy-induced MMP-2 expression and activation in cultured rat VECs.

Materials and methods

Materials

The reagents used in this study were obtained from the following sources: Sprague-Dawley

(SD) rats were obtained from Zhejiang Academy of Medical Sciences (Hangzhou, Zhejiang, China). All animal studies were undertaken in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-27, revised 1996), with the approval of the Institutional Animal Care and Use Committee of Wenzhou Medical University. Trypsin, Medium 199 (M199) and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, NY, USA). Yellow wine (containing 12% alcohol) and ethanol (containing 12% alcohol) was purchased from the Chinese Shaoxing yellow wine Group, red wine (containing 12% alcohol) was obtained from Maison Nicolas Reserve from Languedoc-Roussillon, France. Endothelial Cell Medium (ECM) and Endothelial Cell Growth Supplement (ECGS) were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). DL-Homocysteine ($C_4H_9NO_2S$, purity ≥ 95%) was purchased from Sigma-Aldrich Co (St Louis, MO, USA). Rat VEGF was obtained from PeproTech INC (Rocky Hill, New Jersey, USA). Rat tail tendon collagen type I was purchased from Shengyou Biotechnology (Hangzhou, Zhengjiang, China). All other chemicals were of reagent grade or were of the highest grade commercially available.

Primary cell culture and experimental design

Primary cell cultures of VECs were established by isolating cells from the thoracic and abdominal aortas resected from 2-3-week-old male SD rats (Zhejiang Academy of Medical Sciences, Zhejiang, China). Isolation of aortic VECs was based on the method reported by Kobayashi [27], and was modified and adapted to our system. Briefly, two male SD rats were anesthetized with an intraperitoneal injection before being sacrificed. Their aortas were dissected from the aortic arch to the abdominal aorta. The fat or connective tissue and vessel branches were rapidly dissected from the outer wall of the vessels. The outer membrane was stripped with micro-forceps under a stereoscopic microscope. Any remaining blood residues were removed by perfusing the lumen of the vessels with 2 μL of D-Hank's solution containing 1000 U/μL of heparin. Each segment was bound at one end and the other side was used to fill the lumen with a type I solution containing 0.1% collagenase. The aortas were then incubated in 5 μL D-Hank's solution containing 100 U/μL penicillin and 100 μg/μL streptomycin at 37°C with 5% CO_2 for 30 minutes to release the endothelial monolayer and single cells. The cell suspension was collected and centrifuged at 1000 rpm for 5 min at 4°C. The resulting precipitate was re-suspended by pipette with 2.5 μL of M199 solution containing 20% fetal bovine serum (FBS) and cultured in a rat-tail tendon collagen type I (0.2 μg/μL) coated 6-well cell culture plates. After 3 to 4 days of incubation at 37°C, the medium M199 was removed, the cells were washed with warm D-Hank's, and medium ECM (5% FBS, 100 U/μL penicillin, 100 μg/μL streptomycin, 10 μg/μL apo-transferrin, 5 μg/μL insulin, 1 μg/μL hydrocortisone, 10^{-7} mol/μL retinoic acid,10 μg/μL BSA, 10 ng/μL EGF, 22 ng/μL FGF, 2 ng/Ml VEGF, 2 ng/μL IGF-I, and basal medium) was added. One week later, endothelial monolayers attained confluency and a cell scraper was used to obtain a purer population of VECs. Immunocytochemical staining with VECs-specific anti-von Willebrand factor antibody was used for the identification and characterization of cells. Only VECs from passages 3-4 were used for all studies. All flasks or plates were coated with 2 μg/μL rat-tail tendon collagen type I (BD Biosciences) during the VECs cultures.

Preparation of culture medium

Hcy was added to ECM to obtain final concentrations of 0, 50, 100, 500, 1000 mmol/L. Either 264 μL of yellow wine (12%) or red wine (12%) or ethanol (12%) were diluted in 2 μL ECM medium samples to final concentrations of 14 μl/L. The level of 6.25-12.5 mmol/L Hcy is physiological and >25 mmol/L is pathological. All treatments of yellow wine, red wine, and ethanol were sustained in the 100 mmol/L Hcy-treated media for 48 h.

Division of cultured VECs

The VECs in passages 2 were cultured in a 25-cm^2 cell culture flask coated with rat-tail tendon collagen type I. After the initial culture of 48 h, cells attained 80% confluency. Next, cells were supplemented with serum-free media ECM for further incubation for 24 hours at 37°C and seeded at a density of 2×10^5 cells per well in 6-well culture plates. The death of VECs was assessed with a trypan blue exclusion test. A mixture of trypan blue and cell suspension was incubated for approximately 2 min

at room temperature. A drop of the mixture was applied to a hemacytometer and the stained (nonviable) and unstained (viable) cells were separately counted. The cells were > 98% viable.

In our preliminary experiments, a range of Hcy concentrations from 50 to 1000 mmol/L was used. Based on the results of quantitative real-time PCR, western blot analyses, and gelatin zymography, we subsequently decided that the optimum stimulating concentrations and durations for Hcy were 100 mmol/L and 48 h, respectively. The VECs were divided into the following groups: (1) control group; (2) Hcy group (Hcy 100 mmol/L); (3) yellow wine group (Hcy 100 mmol/L, alcohol 1.4%); (4) red wine group (Hcy 100 mmol/L, alcohol 1.4%); and (5) ethanol group (Hcy 100 mmol/L, alcohol 1.4%) and incubated for 48 h.

MTT assay of cell viability

Cell viability was determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Amresco, OH, USA] method. It is based on the capacity of the mitochondrial succinate dehydrogenase to reduce the yellow MTT tetrazolium salt into purple MTT formazan crystals in living cells. The absorbance of this purple solution was quantified at a specific wavelength using a microplate reader. Hence, the assay allows evaluation of ethanol toxicity based on cell viability. Finally, we selected an appropriate concentration of each type of wine based on the MTT assay results. After the initial 24 h of serum-free culture, cells were seeded at a density of $6\acute{}10^3$ cells/well in ECM containing 10% of FBS, a mixture of Endothelial Cell Growth Supplement (ECGS) and penicillin/streptomycin. Next day, cells were incubated with Hcy or treated with various concentrations of each wine in the following 8 groups: control, Hcy, Hcy + 1.0%, Hcy + 1.2%, Hcy + 1.4%, Hcy + 1.6%, Hcy + 1.8%, and Hcy + 2.0%. The final concentration of Hcy from group 2 to group 8 was 100 mmol/L. After 48 hours, MTT solution (Amresco, OH, USA) was added to each well at a final concentration of 5 μg/μL and incubated at 37°C for 4 h. At the end of the incubation period, the MTT medium was removed and the cells were washed once in D-Hank's. The formazan crystals resulting from MTT reduction were dissolved in 150 μL dimethylsulfoxide (DMSO) per well. The absorbance was measured using an Anthos 2010 microplate reader at a wavelength of 490 nm.

Total RNA extraction

Total RNA from cultured rat vascular endothelial cells was isolated using the TRIzol reagent as instructed (Invitrogen, Carlsbad, CA, USA). The RNA concentration of these samples was determined by recording the absorbance $A_{260/280}$ using an ND 2000 UV spectrophotometer (NanoDrop, Wilmington, DE, USA) and further evaluated by gel electrophoresis. Every sample was dissolved in 20 μL RNase-free water and stored at -80°C until further processing.

Primer design

The forward and reverse sequences of the primers specifically for each gene were designed using Primer 5.0 software and synthesized by Invitrogen. In all PCR reactions, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control for normalization. The reverse primer sets for RT-PCR for MMP-2 and GAPDH were as follows: Forward 5'-ATTTGTTGCCCAGGAAAGTG-3' and Reverse 5'-ACGACCGCGACAAGAAGTAT-3'; Forward 5'-GATGGGTGTGAACCACGAGA-3' and Reverse 5'-TGAGCCCTTCCACGATGC-3', respectively.

Reverse transcription

1 μg of total RNA was reverse transcribed using the PrimeScriptRT Master Mix Kit (TaKaRa, Dalian, China) as instructed. The Real-Time SYBR PCR Master Mix was purchased from TaKaRa. The PCR analysis was performed on an Applied Biosystems 7900 Real-Time PCR System, according to the manufacturer's recommendations. A 1 μL cDNA sample was added to 4 μL of 5×PrimeScriptRT Master Mix and PCR-grade RNase-free water to a volume of 20 μL. The conditions used for reverse transcription-PCR were as follows: 37°C for 15 minutes and 85°C for 5 seconds, followed by cooling to 4°C.

Real-time PCR

Quantitative real-time PCR was performed using SYBRPremix ExTaq TM II (TaKaRa, Dalian, China) on an Applied Biosystems 7900 Real-Time PCR System (Applied Biosystems, Foster,

CA, USA), according to the manufacturer's instructions. The real-time PCR amplifications were carried out in a total volume of 10 μL, including 5 μL SYBRPremix ExTaq TM II, 0.2 μL ROX Reference Dye, 0.2 μL forward primer (20 pmol/μL), 0.2 μL reverse primer (20 pmol/μL), 0.5 μL cDNA template, and 3.9 μL RNase-free water. Triplicate PCR reactions for each of the three independent samples were carried out. SYBR Green mix-free and template-free samples were amplified for each gene as negative controls. The standard amplification conditions were as follows: an initial denaturation at 95°C for 4 min, followed by 35 amplification cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Fluorescence data were acquired at the 72°C step and during the melting-curve program. In all real-time PCR assays, standard curves were created for each primer set to determine their efficiency. Melting curves were generated to detect nonspecific amplification products and their homogeneity was confirmed by dissociation temperature monitoring of SYBR Green fluorescence. Relative expression of real-time PCR products was analyzed by the relative quantification method and expressed as relative RNA levels using the comparative cycle threshold ($2^{-\text{Delta Delta Ct}}$) method. Delta Ct values represent Ct [gene]-Ct [GAPDH], with higher values indicating relatively lower expression levels.

Western blot analysis

After various treatments, the VECs monolayers were washed with D-Hank's twice and scraped from the 6-well plates and transferred into Eppendorf tubes with RIPA Lysis Buffer [50 mmol Tris-HCl (pH 7.4), 150 mmol NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol EDTA, 1 mmol Na_3VO_4] (RIPA Lysis Buffer, Beyotime, China) that was supplemented with a mixture of complete protease inhibitors containing 1 mmol phenylmethylsulfonyl fluoride (PMSF) and 10 mmol leupeptin. The samples were lysed on ice for 20-30 min and centrifuged at 12000 g for 15 min at 4°C. The protein-containing supernatants mixed with 5´ SDS-PAGE loading buffer were boiled in the hot water bath for 5 min. The total protein concentrations were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Meridian Rd, Rockford, USA). Equal amounts of protein (50 μg) were subjected to 10% SDS-polyacrylamide gels (PAGE) and separated by electrophoresis at 70 V for 30 min and 120 V for 90 min using SDS-PAGE electrophoresis buffer (25 mol/L Tris base, 192 mol/L glycine, and 0.1% SDS, pH 8.3). The proteins were subsequently electrophoretically transferred onto Hybond-P polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, England) using transfer buffer that contained 25 mmol/L Tris base, 192 mmol/L glycine, and 10% methanol, pH 8.3. Membranes were blocked with 5% nonfat dry milk in Tris-buffered solution (TBS) for 1 h at room temperature and probed with the appropriate primary monoclonal antibodies overnight at 4°C. After washing three times (15 min each time) with TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) at room temperature, the membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h followed by three washes with TBST for 15 min each. Primary antibodies used were: mouse anti-MMP2 (ab80737; Abcam, UK), and mouse anti-β-Actin (BS6007M; Bioworld Technology, Inc, MN, USA). The HRP-conjugated goat anti-mouse IgG (111-035-003, Jackson ImmunoResearch Laboratories, Inc) was used as the secondary antibody. The membranes were detected using enhanced chemiluminescence (ECL) according to the manufacturer's protocols (BeyoECL Plus, Beyotime, China). Finally, immunoreactivity was visualized using Bio-Rad photo gel imaging systems (ChemiDoc XRS, Bio-Rad) and analyzed by Bio-Rad Quantity One 4.4.

Gelatin gel zymography

After various treatments, secretion of MMP-2 was evaluated by gelatin zymography, as previously described. We collected the supernatant for centrifugation at 1000 rpm for 5 min to remove cell debris. The supernatant (15 μL) was transferred to a Centriprep YM-30 ultrafiltration device (30,000 kDa cut-off, Millipore, Bedford, MA) and centrifuged employing a temperature-controlled centrifuge (Avanti J-30I, Beckman Coulter Inc, CA, USA) with a fixed angle rotor at 1500×g and a temperature of 4°C for 30 min. A 500 μL of ultrafiltrate was obtained. The concentrated proteins were determined using the Bradford method (Bradford Protein assay kit, Beyotime, China).

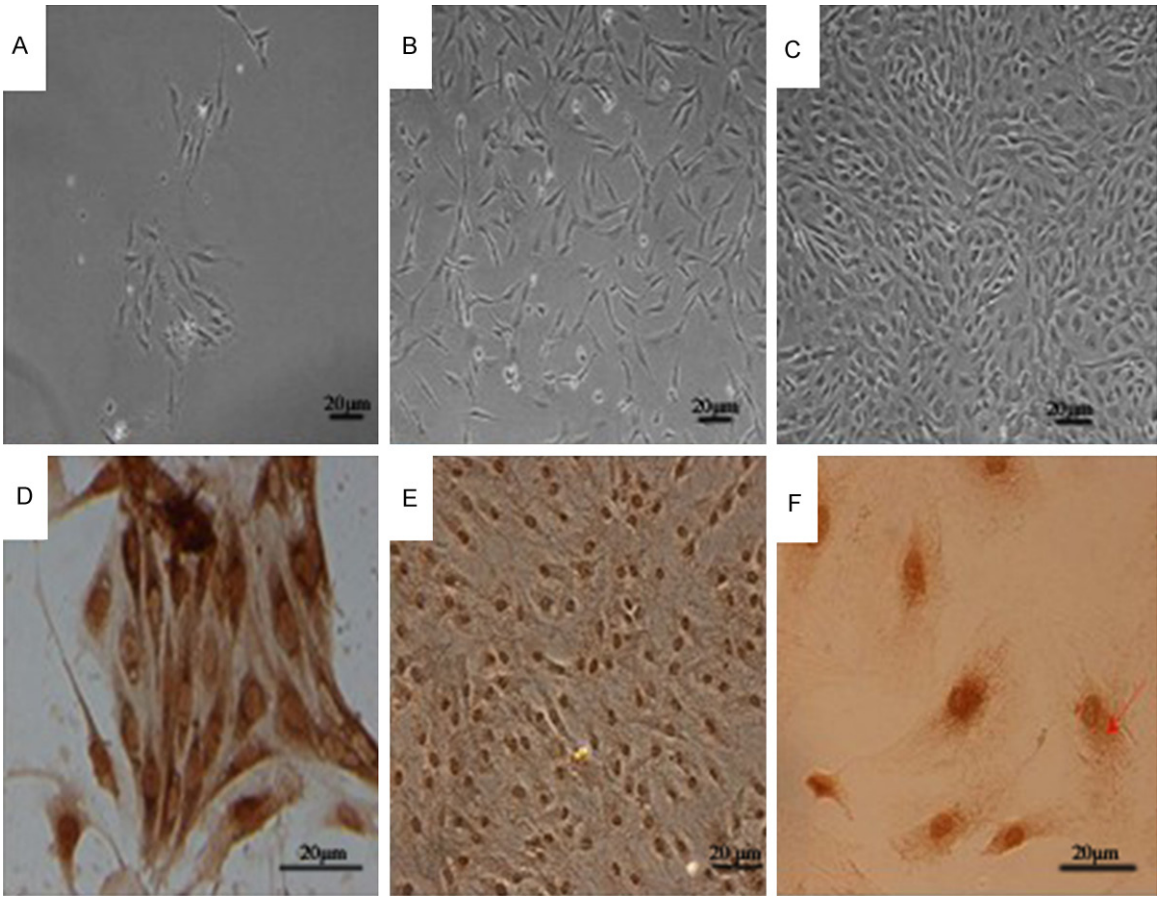

Figure 1. Primary culture of rat aortic vascular endothelial cells (VECs) and immunocytochemical staining. A: 3 days later, a small number of cells were observed (×100); B: 6 days later, after replacing medium ECM, cells grew rapidly (×100); C: 9 days later, when the cells reached 90% confluence VECs showed the characteristic 'cobblestone morphology' (×100); D-F: Cells immune-stained with factor VIII (D, F ×400) antibodies: Factor VIII is a marked antigen of endothelial cells. It demonstrates positive reaction with cytoplasmic brown pigment. No obvious negative cells were seen (E ×200). The factor VIII-related antigens were present as brown granules in the cytoplasm and represented by the red arrow points. D: Primary cells; E and F: Passage 2 cells.

Equal amounts of protein (25 mg) were mixed with 5× sample loading buffer (SDS-PAGE Sample Loading Buffer, Beyotime, China) containing 125 mM Tris-HCl, pH 6.8, 50% glycerol, 8% SDS, 0.02% bromophenol blue. The samples were warmed in a hot water bath (60°C) for 5 min before subjecting to electrophoresis in a 10% SDS-polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin under non-denaturing and non-reducing conditions. After electrophoresis, gels were washed twice for 30 min in 2.5% Triton X-100 (20 mmol Tris-HCl, pH 8.0; 150 mmol NaCl, 5 mmol $CaCl_2$, and 2.5% Triton X-100) at room temperature to remove SDS. The gels were supplemented with 100 μL of zymogram development buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 4 mM $ZnCl_2$) for further incubation at 37°C for 42 h. Following incubation, gels were stained in Coomassie blue R250 (50% methanol, 10% acetic acid, 40% water, and 0.25% Coomassie blue solution) for 4 h. Gelatin enzymes were identified by their ability to digest gelatin as demonstrated by clear zones of digested gelatin. The gels were destained (50% methanol, 10% acetic acid, and 40% water solution) until clear bands of gelatinolysis appeared on a dark background, and transferred to water for rehydration before acquiring images. The relevant band intensities in Bio-Rad Quantity One 4.4 were used to quantify the protease activity by scanning densitometric analysis and normalized to cell number.

Statistical analysis

All values in the figures and text were expressed as mean ± standard deviation (SD) of a minimum of three observations. All data were ana-

Table 1. Effects of Chinese yellow wine, red wine and ethanol on the viability of rat aortic VECs ($\bar{x} \pm s$., $n = 5$)

	A		
Group	Yellow wine	Red wine	Ethanol
Control	1.02 ± 0.11	1.03 ± 0.11	1.01 ± 0.13
Hcy	1.01 ± 0.11	1.02 ± 0.15	1.01 ± 0.12
Hcy + 1.0% (C1)	1.01 ± 0.13	0.99 ± 0.11	1.00 ± 0.10
Hcy + 1.2% (C2)	0.99 ± 0.14	0.98 ± 0.14	0.97 ± 0.15
Hcy + 1.4% (C3)	0.96 ± 0.08	0.96 ± 0.11	0.95 ± 0.09
Hcy + 1.6% (C4)	0.94 ± 0.10	0.95 ± 0.13	0.91 ± 0.12$^{\&\&}$
Hcy + 1.8% (C5)	0.82 ± 0.09**	0.80 ± 0.13$^{\#\#}$	0.79 ± 0.08$^{\&\&}$
Hcy + 2.0% (C6)	0.78 ± 0.06**	0.75 ± 0.09$^{\#\#}$	0.76 ± 0.11$^{\&\&}$

Data were expressed as mean ± S.D. (n = 5 in each group); $^{**}P < 0.01$ vs Hcy; $^{\#\#}P < 0.01$ vs Hcy; $^{\&\&}P < 0.01$ vs Hcy.

lyzed with the SPSS 18.0 statistical package. Differences among all data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by least significant difference *t*-test (LSD *t*-test). Statistical probability of *P* less than 0.05 was considered significant. The reported results were at least representative of three independent experiments.

Results

Identification and characterization of VECs

Rat vascular endothelial cells were isolated from the thoracic aorta and cultured *in vitro*. Nine days after achieving complete confluency, as shown in **Figure 1C**, endothelial monolayers grew with the characteristic cobblestone morphology and contact inhibition. The purity of the VECs was determined by immunocytochemical staining with endothelial-specific anti-Von Willebrand Factor antibody. The Von Willebrand Factor-related antigens were observed as brown granules circulating in cytoplasm as illustrated in **Figure 1D-F**. The purity of the cultured VECs was greater than 97% after 2 passages, as shown in **Figure 1E**. We also used VECs that were passaged second time. Finally, the VECs were visualized and photographed with a Nikon inverted microscope (**Figure 1**).

Cytotoxic effect of alcohol concentration on vascular endothelial cells

To determine the antiproliferative/cytotoxic effect of alcohol concentration on vascular endothelial cells, the optimal alcohol concentration of each wine was selected, and combined with previous studies. Compared with the control group, homocysteine (100 μmol/L) was added to the remaining groups, respectively, and incubated over night. These cells were incubated with or without increasing alcohol concentrations of each wine for 72 h with the cytotoxicity of alcohol measured by a standard MTT assay. As indicated in **Table 1**, a dose-dependent cytotoxicity of alcohol was observed in VECs, In contrast, no significant cytotoxic effects were seen in VECs following exposure to homocysteine (100 μmol/L) for 72 h; No marked differences were seen in the three groups exposed to similar alcohol concentrations in each wine. Compared with the Hcy group, a pronounced and significant reduction in cell viability was observed for VECs incubated with 1.8% alcohol concentration in both yellow wine and red wine groups ($P < 0.01$). In the ethanol group, by contrast, 1.6% of alcohol concentration had a significant effect on the viability of VECs ($P < 0.01$). Based on the experiment results we selected 1.4% alcohol concentration of each wine for the final intervention (**Table 1**).

Effect of Hcy on MMP-2 expression and activation

When VECs were treated with Hcy (0-1000 μmol/L) for 48 h, Hcy (50-500 μmol/L) induced a significant dose-dependent increase in MMP-2 expression, with a maximal response at 500 μmol/L. There were statistically significant differences compared with the control group ($P < 0.05$ or $P < 0.01$) (**Figure 2**).

When VECs were treated with 100 μmol/L of Hcy for 0 h, 24 h, 48 h, and 72 h, there were statistically significant differences between the control group and the Hcy groups (0 h, 24 h, 48 h, and 72 h). Hcy (24-72 h) induced a significant increase in mRNA and protein expression of MMP-2, with a maximal response at 48 h ($P < 0.01$) (**Figure 3**).

When the cells were treated with Hcy (0-1000 μmol/L) for 48 h, the Hcy (50-500 μmol/L) increased the gelatinolytic activity of MMP-2 significantly in a dose-dependent manner.

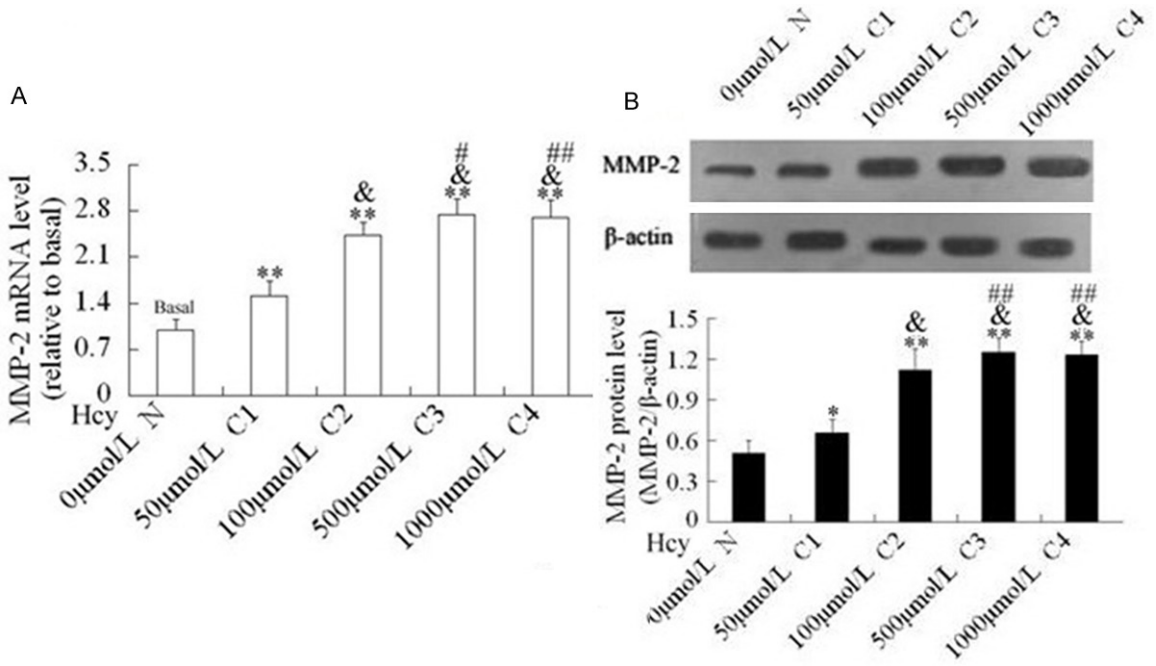

Figure 2. Effects of different concentrations of Hcy on expression of MMP-2 in VECs. VECs were treated with Hcy at different concentrations for 48 h, the mRNA of MMP-2 was tested by real-time PCR (A), and MMP-2 protein was assessed by Western blot (B). (A) Data were expressed as mean ± S.D. (n = 9 in each group); $^{**}P < 0.01$ vs control (0 μmol/L Hcy); $^{\&}P < 0.01$ vs (50 μmol/L Hcy); $^{\#}P < 0.01$ vs (100 μmol/L Hcy); $^{\#\#}P < 0.05$ vs (100 μmol/L Hcy). (B) Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (0 μmol/L Hcy); $^{\&}P < 0.01$ vs (50 μmol/L Hcy); $^{\#\#}P < 0.05$ vs (100 μmol/L Hcy).

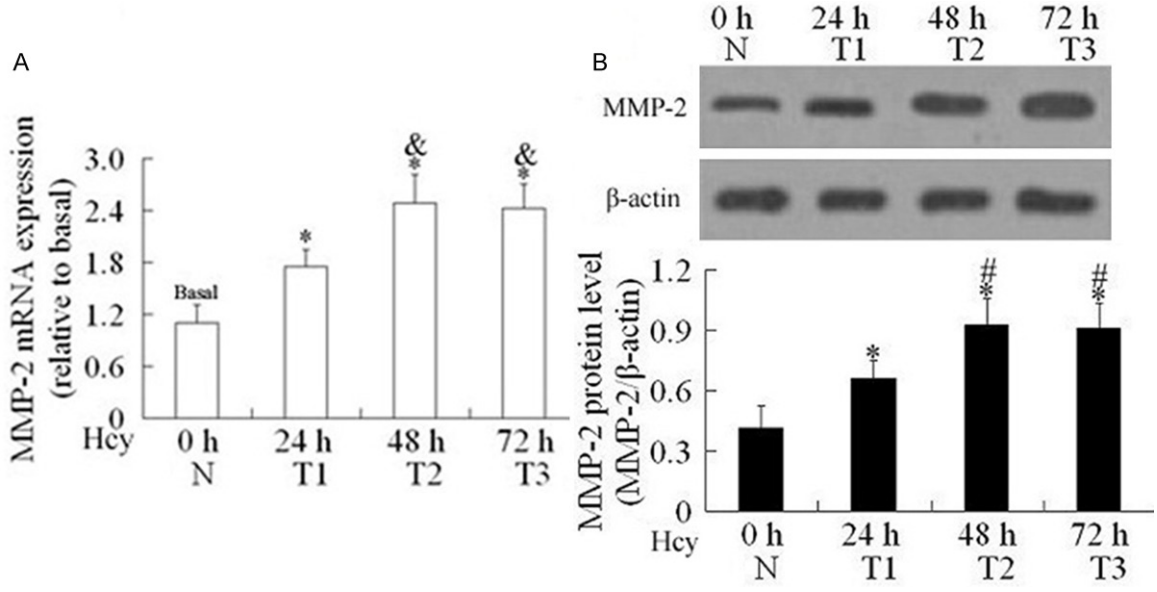

Figure 3. Effects of Hcy treatment on MMP-2 expression: VECs were incubated with 100 μmol/L Hcy for 24 h, 48 h, and 72 h. The mRNA of MMP-2 was tested by real-time PCR (A), and MMP-2 protein was determined by Western blot (B). (A) Data were expressed as mean ± S.D. (n = 9 in each group); $^{*}P < 0.01$ vs control (0 h); $^{\&}P < 0.01$ vs T1 (24 h). (B) Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.01$ vs control (0 h); $^{\#}P < 0.01$ vs T1 (24 h).

There were statistically significant differences between the control group and the Hcy groups (50, 100, 500, and 1000 μmol/L; $P < 0.05$ or $P < 0.01$) (**Figure 5**).

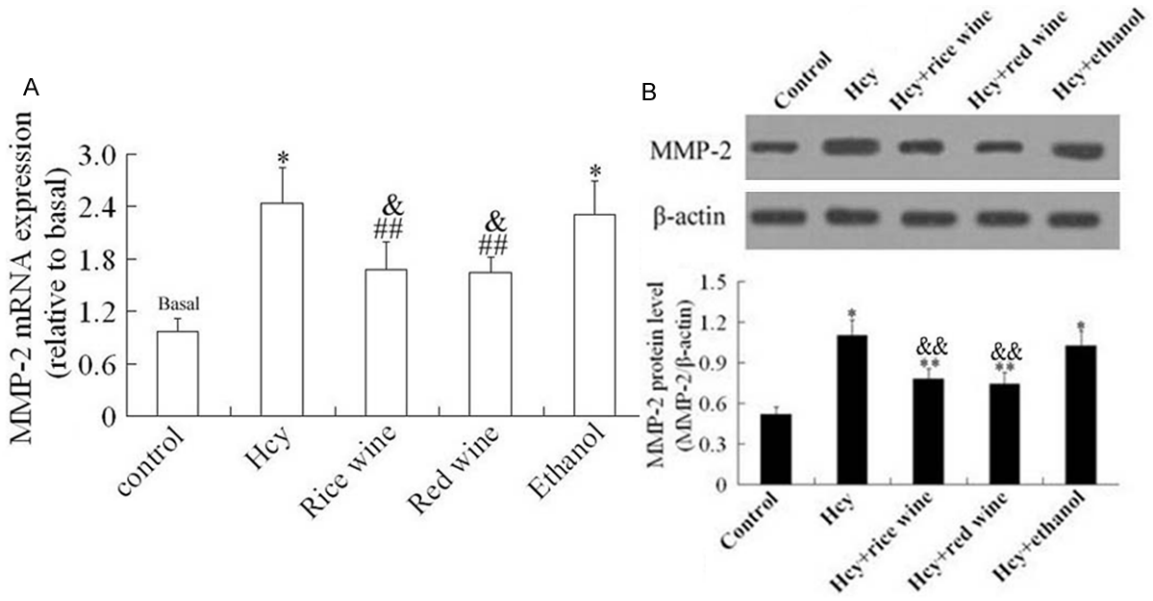

Figure 4. Effects of yellow wine on MMP-2 mRNA expression stimulated by Hcy (100 μmol/L) in cultured VECs (48 h). MMP-2 mRNA was tested by real-time PCR (A), and MMP-2 protein was assessed by Western blot (B). (A) Data were expressed as mean ± S.D. (n = 9 in each group); $^{*}P < 0.01$ vs control; $^{\#\#}P < 0.01$ vs Hcy; $^{\&}P < 0.01$ vs ethanol. (B) Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.01$ vs control; $^{**}P < 0.05$ vs Hcy; $^{\#\#}P < 0.05$ vs Hcy + ethanol.

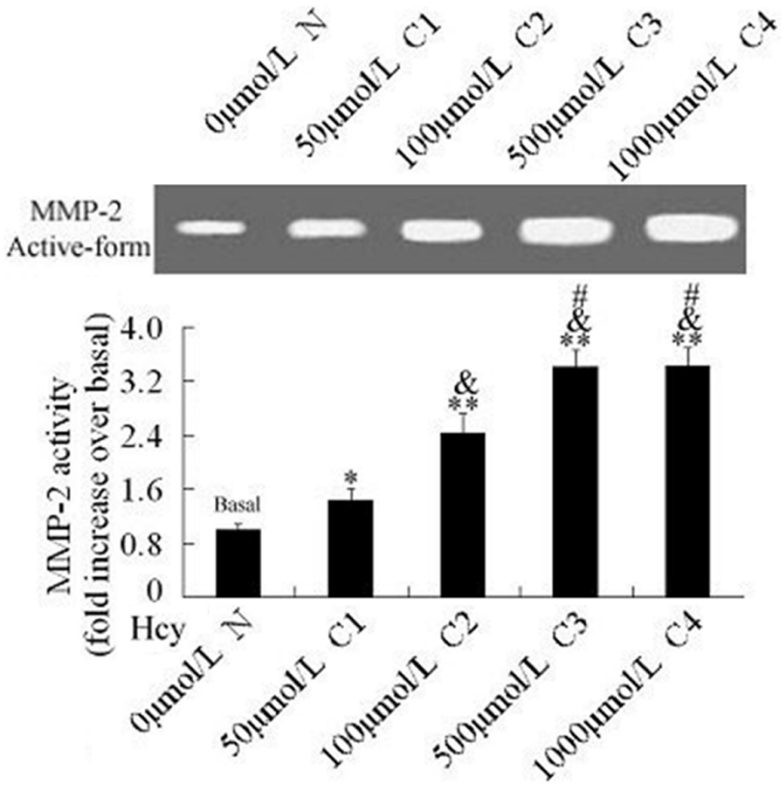

Figure 5. Effects of Hcy on MMP-2 activity: VECs were treated with Hcy at different concentrations for 48 h, and the MMP-2 activity was examined by gelatin zymography assay. Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.05$, $^{**}P < 0.01$ vs control (0 μmol/L Hcy); $^{\&}P < 0.01$ vs (50 μmol/L Hcy); $^{\#}P < 0.01$ vs (100 μmol/L Hcy).

When VECs were treated with 100 μmol/L of Hcy for 0 h, 24 h, 48 h, and 72 h, statistically significant differences existed between the control group and the Hcy groups (0 h, 24 h, 48 h, and 72 h). Hcy (24-72 h) induced a significant increase, with maximal response at 48 h, in gelatinolytic activy of MMP-2 ($P < 0.01$) (**Figure 6**). Representative zymography gels of active MMP2 are shown in **Figures 5**, **6**. In the vascular endothelial cells, gelatinolytic activities at 72 kDa corresponded to the proenzyme forms of MMP2.

Effect of yellow and red wines on MMP-2 expression and activation

VECs were treated with 100 μmol/L of Hcy and 1.4% wine of each kind for 48 h. Compared with the Hcy group, the mRNA expres-

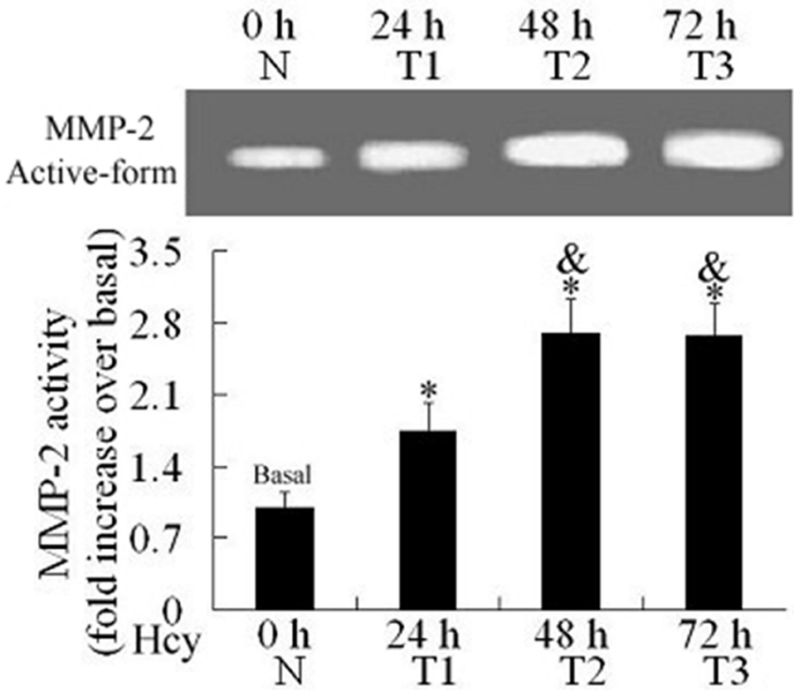

Figure 6. Effects of Hcy treatment duration on MMP-2 activity: VECs were treated with 100 μmol/L Hcy for 24 h, 48 h, and 72 h, respectively. The MMP-2 activity was determined by gelatin zymography assay. Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.01$ vs control (0 h); $^{\&}P < 0.01$ vs T1 (24 h).

sion of MMP-2 in VECs was reduced 26.8% and 25.7% ($P < 0.01$), and the protein expression was reduced 29.1% and 32.6% in the yellow and red wine groups ($P < 0.05$), respectively. However, no statistically significant differences occurred in the expression of mRNA or protein levels of MMP-2 in ethanol group versus Hcy group and yellow versus red wine group ($P > 0.05$) (**Figure 4**).

VECs were treated with 100 μmol/L of Hcy and 1.4% wine of each kind for 48 h. The results suggested that compared with the Hcy group, the gelatinolytic activity of MMP-2 in VECs was reduced to 31.2% and 33.8% in the yellow and red wine groups ($P < 0.01$), respectively. However, no statistically significant differences were seen in the gelatinolytic activity of MMP-2 in ethanol group versus Hcy group and yellow versus red wine group ($P > 0.05$) (**Figure 7**).

Discussion

Homocysteine (Hcy) has been confirmed as one of the independent risk factors of atherosclerosis. Studies confirm that excessive accumulation of Hcy in VECs and ambient environment, damages vascular endothelial function. It stimulated the expression of MMP-2 and promoted atherosclerotic lesions [28-30]. The results indicate that Hcy directly contributestounsteadyremodeling mechanisms of atherosclerotic plaques. Bescond et al. [31] indicated that Hcy directly stimulates pro-MMP-2 resulting in extracellular matrix degradation in the changing process of atherosclerosis. The pro-inflammatory effect of oxidative stress induced by hyperhomocysteine leads to further damage of endothelial cell function [32]. It directly modulates vascular function by reducing NO bioavailability due to the generation of superoxide or reduced NO production by NOS [33]. Studies of blood flow-induced vascular remodeling mechanisms suggested that the biological activity of NO might be mediated via modulation of MMPs expression [34]. *In vitro* experiments involving eNOS gene transfer to VSMCs show reduced MMP-2 and MMP-9 expression and inhibition of migration. However, the biological activity of NO is modified in the presence of other reactive species generated in diseased coronary arteries by activated vascular cells and infiltrating inflammatory cells [35]. Therefore, in this experiment, Hcy was selected as the pro-inflammatory cytokine to stimulate VECs dysfunction, and the foregoing mechanism was used to boost the MMP-2 expression.

Among the 28 known enzymes of matrix metalloproteinase (MMP) family, MMP-2 is the most widely distributed. Further, in normal vessels, VECs and VSMCs continuously produce MMP-2 [11, 26]. MMP-2 is the main enzyme responsible for degradation of type IV collagen, and it mediates ECM remodeling during the atherosclerotic plaque formation [11, 36]. The increased expression of MMP-2 promotes

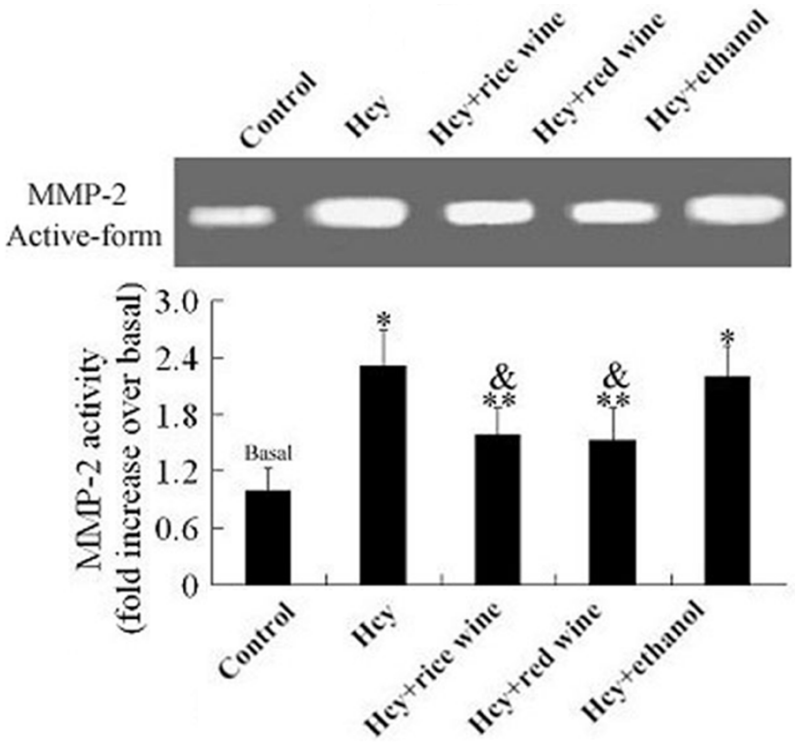

Figure 7. MMP-2 activity in the five groups and the effects of yellow wine on MMP-2 activity stimulated by Hcy (100 μmol/L) in cultured VECs (48 h). Data were expressed as mean ± S.D. (n = 5 in each group); $^{*}P < 0.01$ vs control; $^{**}P < 0.01$ vs Hcy; $^{\&}P < 0.01$ vs Hcy + ethanol.

migration of VSMCs into the vascular intima and their subsequent proliferation is an important mechanism in the pathogenesis of atherosclerosis. MMP-2 is constitutively expressed in several different tissues including the vessel wall, endothelial cells and VSMCs. The studies on human vessels suggest that MMP-2 is highly expressed in fatty streaks and atherosclerotic plaques compared with normal regions of the vessel. In addition, it has been shown that fatty streaks, fibroatheromas with hemorrhage and calcification, and fully occluded lesions are also enriched in MMP-2 [11]. Indeed, MMP-2 plays a significant role in the formation and development of atherosclerotic plaque and in coronary heart disease.

Atherosclerosis is associated with defective secretion and VSMCs proliferation, which is caused by endothelial dysfunction and inflammation. VSMCs rely on the expression of MMP-2 and its translocation from vascular media to intima. VSMCs proliferation is the key pathological event during the initiation and early progression of atherosclerotic plaque. The foregoing studies indicate that the pro-inflammatory effects of Hcy, endothelial dysfunction and related expression and activity of MMP-2 play critical roles during the initiation and progression of atherosclerotic plaque.

Different concentrations of Hcy were used to stimulate the VECs of rats cultured *in vitro.* It was found that 50-500 μmol/L Hcy promoted MMP-2 expression and enhanced its activity in a concentration-dependent manner, while 100 μmol/L of Hcy promoted MMP-2 expression and enhanced its activity in a time-dependent fashion from 24 to 48 h. The effect was most significant in the 48th h suggesting that Hcy enhanced the expression and activity of MMP-2 in VECs, and promoted the evolution of atherosclerotic lesions. The result shows that MMP-2 expression peaked after 2 days as the descending branch after the peak time-concentration curve of MMP-2 mRNA expression met the ascending branch before the peak time-concentration curve of MMP-2 protein expression. In follow-up studies, the smaller time gradients of Hcy stimulation will be further investigated. Based on pathophysiological conditions and the sensitivity of experiment, 100 μmol/L Hcy was selected as the stimulation concentration.

In the last two decades, studies reported that despite a high intake of saturated fat and dietary cholesterol, significantly lower morbidity and mortality rates for coronary heart disease were observed in France compared with other European countries, a phenomenon known as the “French paradox”. Renaud and Lorgeril [37] proposed that the intake of red wine by the French mainly contributed to the low cardiovascular morbidity. However, the detailed mechanism is still unclear. Later studies and epidemiological evidence further illustrated that

most of the beneficial effects of light-to-moderate consumption of red wine on cardiovascular disease were attributed to the presence of red wine polyphenols [38, 39]. Numerous phenolic compounds in red wine including resveratrol, catechin, epicatechin polymers, gallic acid, ferulic acid, caffeic acid, anthocyanin, and other polyphenols [38] contributed to the prevention of endothelial dysfunction, by increasing eNOS expression and bioactive NO in the vasculature, and by reducing ROS or even inhibiting endothelin-1 expression. Red wine polyphenols have also been shown to increase the circulating EPC number and functional activity, anticoagulant, anti-inflammatory, and antioxidant activities, and inhibit LDL synthesis, VSMCs proliferation and migration [16]. A recent study showed stimulation of the other protective factors such as endothelium-derived hyperpolarizing factor (EDHF) [18]. Finally, red wine effectively attenuated the development of atherosclerotic plaque and reduced the incidence of total cardiovascular events [39]. As reported previously [40], researchers demonstrated that red wine polyphenols effectively inhibit matrix invasion of cultured VSMCs, most likely by preventing the expression and activation of MMP-2. The inhibitory effect of polyphenolic compounds present in red wine on the activation of pro-MMP-2 and matrix degradation leads to cardio-protective outcomes. Polyphenols in red wine inhibit the progression of atherosclerosis and prevent cardiovascular disease. Similarly, yellow wine also contains abundant polyphenolic compounds, derived from glutinous rice and wheat [24]. The following 10 phenolic compounds were successfully identified in yellow wine samples: syringic acid, (+)-catechin, and rutin, caffeic acid, (-)-epicatechin, gallicacid, *p*-coumaric acid, vanillic acid, ferulic acid and quercetin. The total phenolic amount was 89.07 μg/μL and 108.73 μg/μL in the two famous Shaoxing yellow wines, the most popular brands in market, Guyuelongshan and Nuomi, respectively. Syringic acid and (+)-catechin were the predominant polyphenols in these yellow wines, contributing to 60% to the total amount. The abundance and diversity of polyphenolic compounds present in yellow wine is similar to that of the red wine [21, 23]. Therefore, both yellow and red wines may inhibit the progression of atherosclerosis mediated by their polyphenolic ingredients.

Shaoxing yellow wine contains functional oligosaccharides in addition to polyphenols, vitamin B, bioactive peptide and other nutritional components [41, 42]. These components in yellow wine contribute to its cardio-protective effects. Yellow wine is abundant in oligosaccharides and vitamins. Oligosaccharides serve many important physiological functions. In human body, oligosaccharides are not easily absorbed. However, they promote the proliferation of *Bifidobacterium*, to improve the intestinal microenvironment and promote the synthesis and absorption of vitamins B6 and B12 [43-45], or folic acid to reduce Hcy [46, 47]. In our previous study investigating the role of Chinese yellow wine in MMP-2 inhibition in atherosclerosis, 32 LDL receptor knockout (LDL-/-) mice were fed a high-fat and L-methionine diet for the entire study. They were randomly divided into four groups (n = 8, each group): yellow wine, red wine, ethanol groups (diluted 1:3 with water and 3% ethanol), and control group (with water only). The results showed that Hcy was significantly reduced in the yellow wine group compared with the other three groups, with no significant difference in the other three groups [25]. The results indicate that light-to-moderate consumption of yellow wine reduced the plasma concentrations of Hcy. Hcy, at concentrations associated with increased risk of cardiovascular events, increases MMP-2 activity, synthesis and secretion in VSMCs via activation of MAPK and P13-K pathways. The direct activation of proMMP-2 by Hcy may be one of the mechanisms mediating extracellular matrix deterioration in HHcy-associated arteriosclerosis [31]. These data suggest that yellow wine may inhibit the expression and activation of MMP-2 via reduction of the plasma concentrations of Hcy. In our study, compared with the Hcy group, the expression and activity of MMP-2 in the yellow and red wine groups were significantly decreased. Despite a tendency for a lower expression and activity of MMP-2 in the ethanol group than the Hcy group, there were no significant differences. Similarly, Escolà-Gil [48] and Tofferi [49] found that ethanol alone did not reduce atherosclerotic plaques, and no significant differences in plaque occurred in the ethanol group compared with the control group. Attenuation of atherosclerosis may be attributed to other ingredients in the red wine and yellow wine. Roland and Ruth found that dealcoholized red wine decreased atherosclerosis

independent of lipid per-oxidation inhibition in the arterial wall [50]. In a series of experiments, researchers found that red wine polyphenols prevented MMP-2 expression significantly in rat, and the inhibition of MMP-2 expression may mediate the protective effect of red wine on coronary heart diseases [40, 51]. It proves that small doses of extracellular yellow wine supplementation reduce the production of Hcy-induced MMP-2 in cultured rat VECs. Our study shows that yellow wine may inhibit the expression of MMP-2 through the mechanism that is similar to red wine, with similar protection. These results suggest that small doses of yellow wine inhibit the expression of MMP-2 and may have anti-atherosclerotic effects. This finding is consistent with our previous findings showing that Chinese yellow wine inhibited the production of Hcy-induced extracellular MMP-2 in cultured rat VSMCs and inhibited MMP-2 and improved atherosclerosis in the LDL receptor knockout (LDLR-/-) mice [25, 26]. Based on previous studies *in vitro* and *in vivo*, we predict that small doses of yellow wine may inhibit atherosclerotic plaques by reducing the level of plasma lipids and Hcy. Finally, the inhibitory effects on the progress of atherosclerosis were realized by inhibiting the expression and activation of MMP-2 in VECs and VSMCs. Further, we concluded that yellow wine inhibited the development of atherosclerosis. The polyphenols present in yellow wine represent critical ingredients that confer cardiovascular benefits. We will conduct additional studies to investigate the anti-atherosclerotic effects of yellow wine *in vivo* and to evaluate whether it was mediated by a single polyphenolic compound or a combination of compounds.

To our knowledge, this is the first report demonstrating that small doses of yellow and red wine inhibit the expression and enzymolytic activity of MMP-2 in cultured VECs, and significantly inhibit the initiation and progression of the pathological process of atherosclerosis. The results indicate that consumption of yellow wine, regularly and in moderate amounts may be an effective and novel approach for primary prevention of atherosclerosis-related diseases. It has significant theoretical and practical implications for studies investigating the role of MMP-2 in the initiation and progression of atherosclerosis and for the development of novel interventions. Further scientific studies of yellow wine are needed to promote the development of Shaoxing yellow wine industry in China.

Conclusions

Hcy promotes the expression and activity of MMP-2, which may play an important role in the pathogenesis of atherosclerosis. In rat VECs, Chinese yellow wine significantly reduces the production of Hcy-induced MMP-2. Our data suggest that the beneficial effect of yellow wine supplementation on cardiovascular disease may be due, at least in part, to the inhibitory effect on MMP-2 synthesis in VECs. The attenuation of MMP-2 activation by yellow and red wines might contribute to their cardio-protective effects.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hangyuan Guo, Department of Cardiology, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, No. 568 Zhongxing North Road, Shaoxing 312000, Zhejiang Province, China. Tel: +86-575-88228888; Fax: +86-575-88228899; E-mail: ghangyuan@hotmail.com

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