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Chinese Yellow Wine Could Inhibit Production of Matrixmetalloproteinase-2 Induced by Homocysteine in Cultured Rat Vascular Endothelial Cells

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Abstract

Background: The effects of Chinese yellow wine on the production of homocysteine (Hcy) induced intracellular MMP-2 in cultured rats vascular endothelial cells (VECs) has not been investigated.

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 expressing MMP-2. Cells were divided into 5 groups: Control, Hcy, Hcy+yellow wine, Hcy+red wine, Hcy+ethanol and the cells were given different treatment for 48 h. The mRNA expression of MMP-2 was detected by FQ-PCR. The western blotting and gelatin zymography were applied to test the protein levels and the enzymatic activity of MMP-2.

Results: Hcy could significantly increase the expression and activity of MMP-2 compared with the control group, and could reach the maximum at 500 μ mol/L, cultured for 48 h. Compared with those in Hcy group, the expression and activity of MMP-2 in yellow wine and red wine groups was significantly decreased. No significant difference was shown as between the ethanol group and the Hcy group and no significant discrepancy between the yellow wine and red wine group was found.

Conclusions: The result suggest that Hcy promotes the expression and activity of MMP-2, which may play an important role in pathogenesis of atherosclerosis (AS). Treatment with yellow wine or red wine decreases Hcy-induced MMP-2 production in VECs. The attenuation of MMP-2 activation by yellow wine and red wine might contribute to their beneficial effects on the cardiovascular system.

Keywords: Yellow wine; Atherosclerosis; Vascular endothelial cells (VECs); Matrix metalloproteinase-2(MMP-2); Homocysteine

Introduction

Coronary heart disease (CHD) is one of the leading cause 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 from classical atherothrombotic risk factors such as hypercholesterolemia or smoking [1,2]. According to the American Heart Association (AHA), normal homocysteine concentrations are included between 5 and 15 μmol/L and HHcy corresponds to an elevation of plasma homocysteine level and exists including above three forms: (1) moderately homocysteine serum levels are between 15 and 30 μmol/L; (2) intermediately homocysteine serum levels are between 31 and 100 μmol/L; (3) severely elevated concentrations are above 100 μ mol/L [3]. As is known to all folate and water-soluble B vitamins are both involved in homocysteine metabolism. Deficiency of these B vitamins will cause hyperhomocysteinemia, which can be corrected by vitamins supplementation of folate VitB6 and VitB_{12} [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. Moreover, in humans endothelial dysfunction correlates well with future cardiovascular events, and its presence is also one of the first detectable vascular alterations in the evolution of atherosclerosis. So far 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 would result in impaired eNOS function and altered NO synthesis. It can refer to disruption of any processes that require healthy endothelial cells, it is most commonly used to describe an impaired NO bioavailability due to reduced production of NO by eNOS or increased breakdown by reactive oxygen species (ROS) [5,6]. In the process of further research, NO also prevents leukocyte adhesion and migration into the arterial wall, as well as platelet adhesion and aggregation or inhibits vascular smooth muscle cells (VSMCs) proliferation, all key events in the development of atherosclerosis [5]. In vascular endothelium, NO is reported to inhibit MMP-2 transcription through activating transcription factor-3 (ATF-3) interference with p53 binding to the promoter region [6,7]. Atherosclerotic plaque remodeling entails degradation and reorganization of the extracellular matrix (ECM) scaffold of the vessel wall. It is largely dependent on the specialized

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enzymes called matrix metalloproteinases (MMPs), particularly on matrix metalloproteinases-2(MMP-2). MMP-2 is constitutively expressed in several different tissues including the blood vessel wall, also, VECs and VSMCs continuously produce MMP-2 *in vitro* [8,9]. Studies of human vessels showed that MMP-2 was highly expressed in fatty streaks and atherosclerotic plaques compared to normal regions of the vessel [10]. Furthermore research suggested that fatty streaks, fibroatheromas with hemorrhage and calcification, were enriched in the gelatinases MMP-2 [11].

A J-shaped relationship between alcohol consumption and CHD has been reported in apparently healthy people or cardiovascular patients [12-14]. A dose-related combination of beneficial and harmful effects has been showed from low and moderate wine intake to heavy drinking, as shown by a research program conducted in California involving more than 120000 adults, with different drinking patterns, followed during 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 all 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 cardioprotective and vasoprotective properties including scavenging reactive oxygen and nitrogen species, increasing HDLs, antiatherosclerotic, antiarrhythmic, and enhancing vasorelaxation actions [16,18,19]. Dose the Chinese yellow wine play the same effect as the red wine?

As is well known Chinese yellow 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]. Chinese yellow wine contributes to the unique characteristic features of traditional Chinese alcoholic beverages due to its unique flavour, subtle aroma and low alcoholicity (<20%). It is widely consumed all over the country, especially in the city of Shaoxing-"China's Hometown of Wine" and was honored as the national banquet wine. So it is also called Shaoxing wine or Shaoxing yellow wine for the sake of its bright brown color. Traditionally 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 shows the yellow wine contains abundant phenolic compounds similar to the red wine, and it 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 especially claimed to have beneficial effects on the prevention of cardiovascular disease, but the exact mechanisms involved are still not well known [23,24]. Our previous study found that Chinese yellow wine can inhibit MMP-2 and atherosclerosis in the LDL Receptor knockout (LDLR−/−) mice [25]. Indeed, we have previously shown that Chinese yellow wine could inhibit the production of homocysteine (Hcy) induced extracellular MMP-2 in cultured rat vascular smooth muscle cells [26]. Because the VECs is just likely equally important as VSMCs in the development of atherosclerosis, both produce constitutively *in vitro* MMP-2, the most important enzyme for degradation of ECM components. No previous studies have indicated that Chinese yellow wine could inhibit production of Hcy induced MMP-2 in cultured rat VECs. Therefore, the purpose of the present study was to determine whether Chinese yellow wine could affect MMP-2 expression and activation in VECs.

Materials and Methods

The detailed Methods section is available as previously described [25,26], or see our Supplemental Material.

Primary cell culture and experimental design

Isolation of aortic VECs followed the method reported by Kobayashi et al. [27], and it was modified and adapted to our system.

Division of cultured VECs

In our preliminary experiments, a range of Hcy concentration from 50 to 1000 μmol/L was used and in regards to our first data. According to our result, however, we subsequently decided that the optimum stimulating concentrations and time durations for Hcy were 100 μmol/L and 48 hours. Then the VECs were divided into groups as follows: (1) Control group; (2) Hcy group (Hcy 100 μmol/L); (3) Yellow wine group (Hcy 100 μmol/L, Alcohol 1.4%); (4) Red wine group (Hcy 100 μmol/L, Alcohol 1.4%); (5) Ethanol group (Hcy 100 μmol/L, Alcohol 1.4%) and incubating for durations of 48 hours.

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 had grown 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, and the Von Willebrand Factor related antigen were presented as brown granules circulating in cytoplasm as shown 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 had been passaged up to second

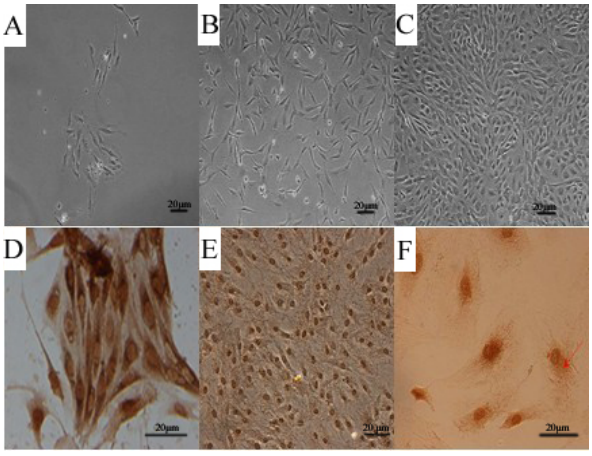

Figure 1: The primary culture of rat aortic vascular endothelial cells (VECs) and Immunocytochemical staining. Panel A shows 3 days later, a small amount of cells are observable (×100). Panel B shows 6 days later, after replaceing medium ECM, cells are growing rapidly(×100). Panel C shows 9 days later, when the cells reached 90% confluence it shows that VECs grew with the characteristic 'cobblestone morphology' (×100). Panel D, E and F shows these cells were immunostained with factor VIII (D, F×400) antibodies, which is marked antigen of endothelial cells. It demonstrates positive reaction with cytoplasmic brown pigment. No obvious negative cells were seen (E×200). And the factor VIII related antigens were presented as brown granules in the cytoplasm as the red arrow points. D: primary cells; E and F: passage 2 cells.

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, then select the optimal alcohol concentration of each wine, and combined with previous studies for the experimental. In comparison with control group, homocysteine (100 μmol/L) was respectively added in the remaining groups for incubating over-night. Then these cells were incubated in the absence or presence of increasing alcohol concentrations of each wine for 72 hours with the cytotoxicity of alcohol measured by a standard MTT assay. As indicated in Table 1, the result showed the dose-dependent cytotoxicity of alcohol in VECs, In contrast, no significant cytotoxic effects were seen in VECs under the exposure of homocysteine (100 μmol/L) for 72 hours; No marked differences were seen in the three groups under the intervention of same alcohol concentration of each wine; Compared with Hcy group, a pronounced and significant reduction in cell viability was observed for VECs incubated with 1.8% alcohol concentration in both yellow wine group and red wine group(P<0.01). In ethanol group, by contrast, 1.6% of alcohol concentration had a significant effect on the viability of VECs (P0.01). Based on the experiment result, we select 1.4% alcohol concentration of each wine for the final intervention (Table 1).

Effect of Hcy on the mRNA, protein expression of matrix metalloproteinase-2 and its activion

The mRNA and protein expression of MMP-2 in VECs treated with various concentrations of Hcy: VECs were treated with Hcy (0–1000 μmol/L) for 48 hours. Hcy (50-5 L) induced a significant dose-dependent increase, maximal response at 500 μmol/L, in mRNA and protein expressions of MMP-2. There were statistically significant differences compared to the control group (P<0.01) (Figure 2).

Group	A		
	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$^{\&\&}$

**P<0.01 vs. Hcy, $^{\#\#}$P<0.01 vs. Hcy, $^{\&\&}$P<0.01 vs. Hcy

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

The mRNA and protein expression of MMP-2 in VECs treated with different time period of Hcy: VECs were treated with 100 μmol/L Hcy for 0, 24, 48, and 72 hours. There were statistically significant differences between the control group and the Hcy groups (0, 24, 48, and 72 hours; Figure 3). Hcy (24-72 hours) induced a significant increase, maximal response at 48 hours, in mRNA and protein expressions of MMP-2 (P<0.01) (Figure 4).

The gelatin lytic activity of MMP-2 in VECs treated with different concentrations of Hcy: The cells were treated with Hcy (0–1000 μmol/L) for 48 hours. Hcy (50–500 μmol/L) increased the activity of MMP-2 significantly in a dose-dependent manner. There were statistically significant differences between the control group and the Hcy groups (50, 100, 500, and 1000 μmol/L; P<0.01) (Figure 4). The gelatinolytic activity of MMP-2 in VECs treated with different time period of Hcy: VECs were treated with 100 μmol/L Hcy for 0,24,48 and 72 hours. There were statistically significant differences between the control group and the Hcy groups (0,24,48 and 72 hours). Hcy (24-72 hours) induced a significant increase, maximal response at 48 hours, in gelatinolytic activity of MMP-2 (P<0.01) (Figure 5). Representative zymography

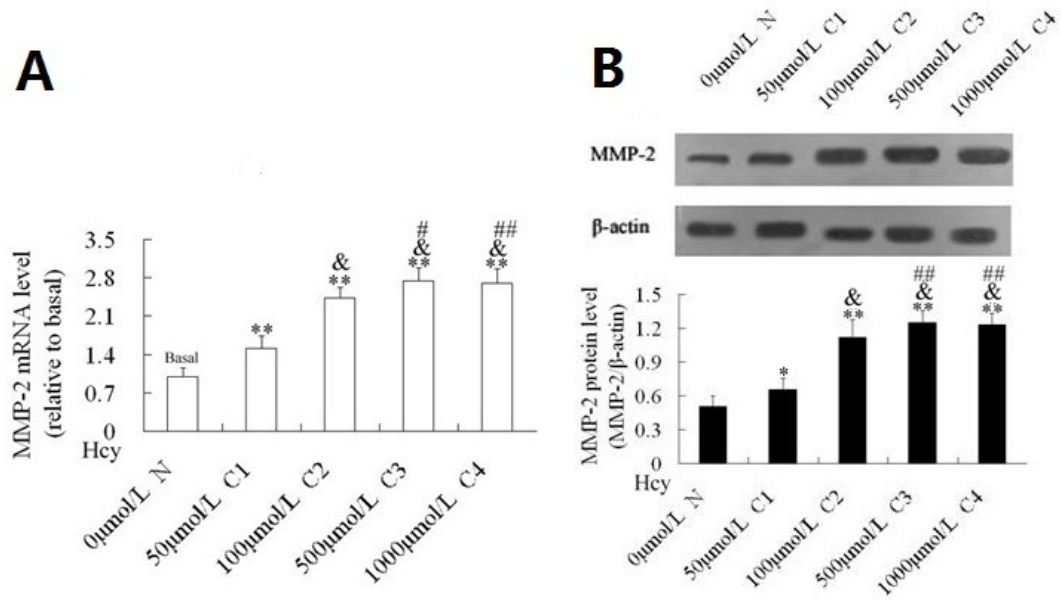

Figure 2: (A) Effects of different concentrations of Hcy on mRNA and protein expression of MMP-2 in VECs. VECs were treated with Hcy at different concentration for 48 h, the mRNA of MMP-2 was tested by Real-time PCR (Figure 2A), and MMP-2 protein was examined by western blot assay. (B) A: $\bar{x}$ ±s, n=9, **P<0.01 vs. (0 μmol/L Hcy); and 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: $\bar{x}$ ± s, n=5, *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).

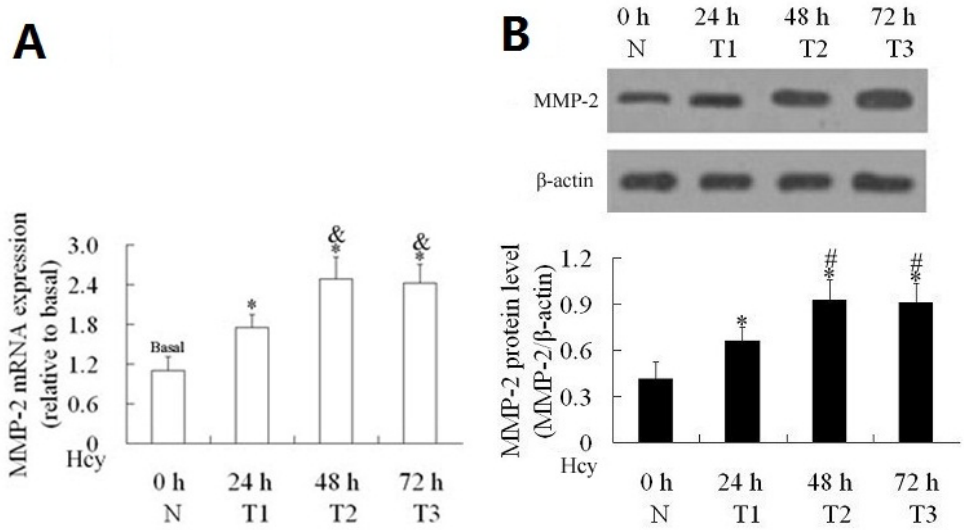

Figure 3: (A) Effects of Hcy treatment for different time lengths on mRNA and protein expression of MMP-2. VECs were incubated with 100 mmol/L Hcy for 24 h, 48 h, 72 h. The mRNA of MMP-2 was tested by Real-time PCR (Figure 3A), and MMP-2 protein was examined by western blot assay. (B) A: χ ±s, n=9, *P<0.01 vs. N (0 h); and P<0.01 vs. T1 (24 h), B: χ ±s, n=5, *P<0.01 vs. control N (0 h); #P<0.01 vs. T1 (24 h).

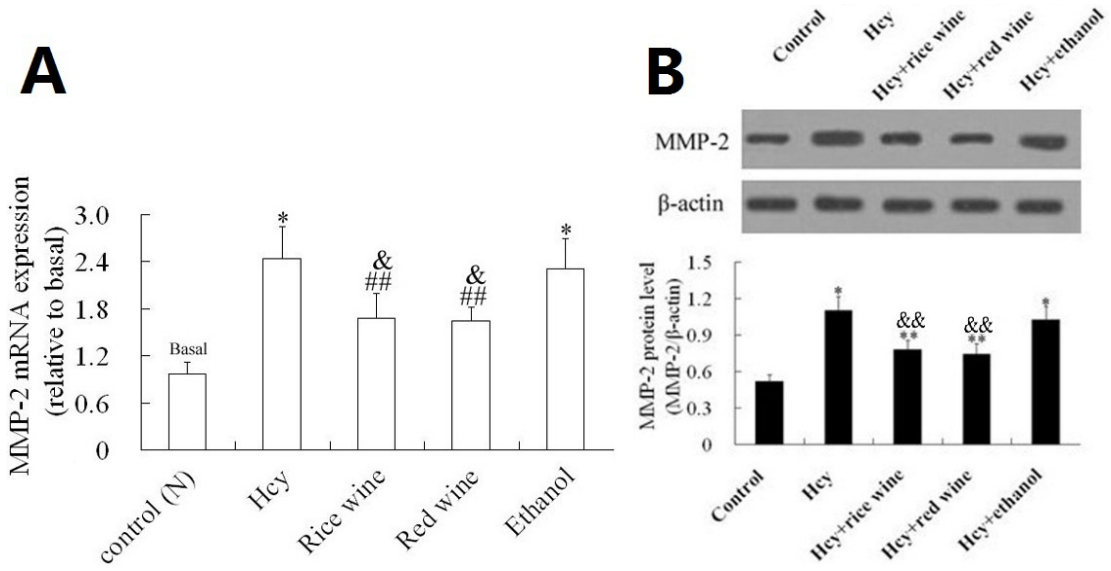

Figure 4: (A) The mRNA and protein expression levels of MMP-2 in the five groups and the effects of Yellow wine on mRNA expression of MMP-2 stimulated by Hcy (100 μmol/L) in cultured VECs (48 h). The mRNA of MMP-2 was tested by Real-time PCR (Figure 4A), and MMP-2 protein was examined by western blot assay. (B) A: χ ±s, n=9, *P<0.01 vs. N (control); ## P<0.01 vs. Hcy; and P< 0.01 vs. Ethanol B: χ ±s, n=5, *P<0.01 vs. control; **P<0.05 vs. Hcy; ## P<0.05 vs. Hcy+Ethanol.

gels of active MMP-2 were shown in Figures 5 and 6. In the vascular endothelial cells, gelatinolytic activities at 72 kDa corresponded to the proenzyme forms of MMP-2.

Effect of yellow wine and red wine on the mRNA and protein expression of matrix metalloproteinase-2 and its activion

VECs were treated with 100 μmol/L Hcy and 1.4% each kind of wine for 48 hours. Compared to the Hcy group, the mRNA expression of MMP-2 in VECs reduced 26.8% and 25.7% (P<0.01), and the protein expression reduced 29.1% and 32.6% of the yellow wine group and red wine group (P<0.05), respectively. However, there is no statistically significant differences with the expression of mRNA or protein level of MMP-2 in ethanol group versus Hcy group and yellow wine group versus red wine group (P>0.05) (Figure 6).

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

Discussion

Homocysteine (Hcy) has been confirmed as one of independent

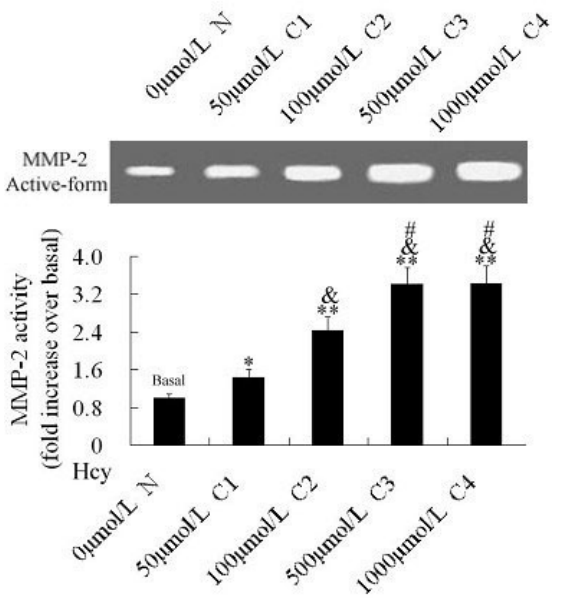

Figure 5: Effects of different concentrations of Hcy on the activity of MMP-2. VECs were treated with Hcy at different concentration for 48 hr, the MMP-2 activity was examined by gelatin zymography assay. $\bar{x}$ ±s, n=5, *P< 0.05, **P< 0.01 vs. control (0 μmol/L Hcy); and P< 0.01 vs. 50 μmol/L Hcy; #P<0.01 vs. 100 μmol/L Hcy.

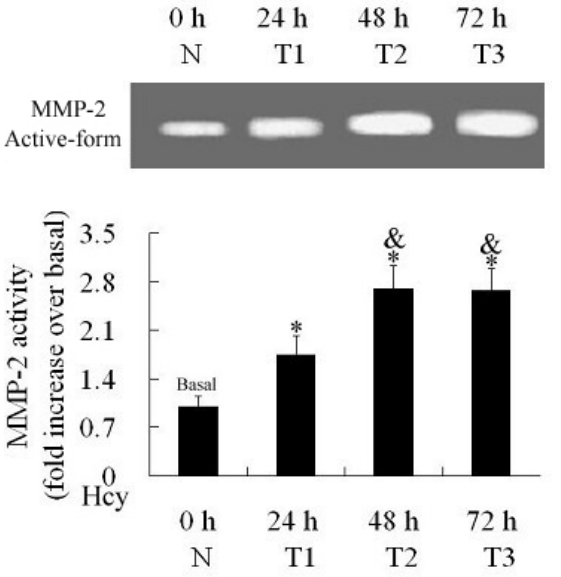

Figure 6: Effects of Hcy treatment for different time on the activity of MMP-2. VECs were treated with 100 μmol/L Hcy for 24 hr, 48 hr, 72 hr. The MMP-2 activity was determined by gelatin zymography assay. $\bar{x}$ ±s, n=5, *P<0.01 vs. control (0 h); and P<0.01 vs. 24 h.

risk factors of atherosclerosis. The researches have confirmed that when Hcy is largely accumulated in VECs and ambient environment, it may damage vascular endothelial function through different mechanisms. It can stimulate the expression of MMP-2 and promote the lesion of atherosclerosis [28-30]. This indicates that Hcy directly leads to the unsteady and remodeling mechanism of atherosclerotic plaque. Researches of Bescond et al. [31] indicate that Hcy directly stimulating pro MMP-2 may be one of the mechanisms that participate in extracellular matrix degradation in the changing process of atherosclerosis. The pro-inflammatory effect of hyperhomocysteine that induced oxidative stress will lead 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 production of NO by NOS [33]. Studies of blood flow-induced vascular remodeling mechanism suggested that the biological activity of NO might be exerted via modulation of MMPs expression [34]. *In vitro* experiment, eNOS gene transfer to VSMCs was shown to reduce MMP-2 and MMP-9 expression and impair their migration. However, it has become apparent that the biological activity of NO are 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 pro-inflammatory cytokine to stimulate VECs to be dysfunctional, and the mechanism above was used to adjust the increase of the MMP-2 expression.

Among the already known 28 enzymes of Matrix metalloproteinase (MMP) family, MMP-2 is most widely distributed. Also in normal vessels, VECs and VSMCs continuously produce MMP-2 [11,26]. MMP-2 is the main enzymes responsible for degradation of type IV collagen, and it participates in the ECM remodeling when the atherosclerotic plaque is formed [11,36]. The increasing expression of MMP-2 promotes migration of VSMCs into the vascular intima and their subsequent proliferation is important mechanisms in the pathogenesis of atherosclerosis. MMP-2 is constitutively expressed in several different tissues including the vessel wall, endothelial cells and VSMCs. The researches 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 furthermore been shown that fatty streaks, fibroatheromas with hemorrhage and calcification, and fully occluded lesions are also enriched in MMP-2 [11]. It shows that MMP-2 plays a significant role in the formation and

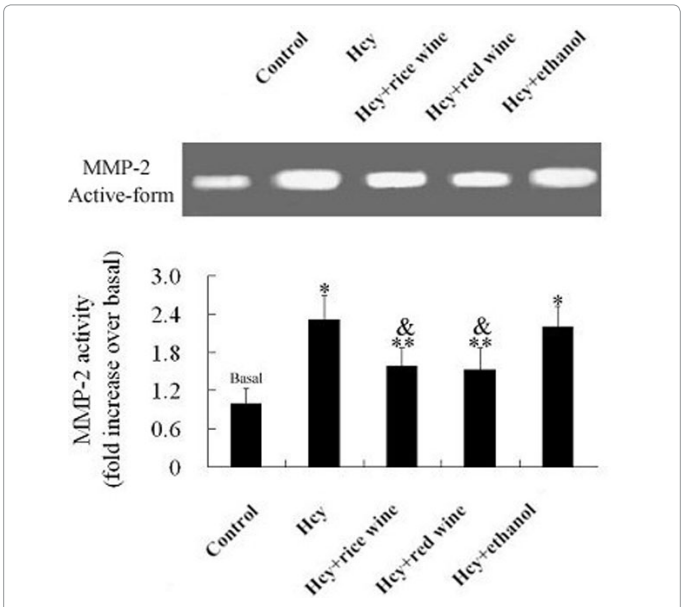

Figure 7: The activity of MMP-2 in the five groups and the effects of yellow wine on the activity of MMP-2 stimulated by Hcy (100 μmol/L) in cultured VECs (48 h). They were analyzed by gelatin zymography. $\bar{x}$ ±s, n=5, *P<0.01 vs. control; **P<0.01 vs. Hcy; and P<0.01 vs. Hcy+Ethanol.

development of atherosclerotic plaque. Moreover, this also proves its position in coronary heart disease.

Atherosclerosis has much to do with secretion dysfunction and VSMCs proliferation, which is caused by endothelial dysfunction and inflammation damage. VSMC relies on the expression of MMP-2 and its movement from vascular media to intima, and its proliferation is the key pathological link and important characteristic during the initiation and early progression of atherosclerotic plaque. The above researches all indicate that proinflammatory effects of Hcy, endothelial dysfunction and the relevant expression and activity of MMP-2 all play important roles during the initiation and progression of atherosclerotic plaque. So in order to know more about atherosclerosis, it is very important to research the interaction of the above three.

Different concentrations of Hcy were used to stimulate the VECs of rats cultured *in vitro*, and it was found that 50-500 μmol/L Hcy promoted MMP-2 expression (both mRNA and protein) and enhanced the activity in a concentration dependent manner, while 100μmol/L Hcy promoted MMP-2 expression (both mRNA and protein) and enhanced its activity in a time dependent manner from 24-48 hours. The effect was most significant at the 48th hour. This indicated that Hcy can enhance the expression and activity of MMP-2 in VECs, and can promote the evolution of atherosclerosis lesions. The result shows that, at the 48th hour, both MMP-2 mRNA and protein expression were at their peak, the reason may be that the descending branch after the peak of the time-concentration curve of MMP-2 mRNA expression met the ascending branch before the peak of the time-concentration curve of MMP-2 protein expression. In follow-up studies, further discussion will be made with smaller time gradients of Hcy stimulation. In normal blood plasma, the homocysteine concentrations are included between 5-15 μmol/L, intermediately homocysteine serum levels are between 31-100μmol/L, and severely elevated concentrations are >100μmol/L (3). Considering actual human pathphysological conditions and the sensitivity of experiment, 100 μmol/L Hcy was selected as stimulation concentration.

In the last two decades, scholars found that despite a high intake of saturated fat and dietary cholesterol, a significantly lower morbidity and mortality rate for coronary heart disease was observed in France compared with other European countries, a phenomenon known as the so-called famous "French paradox". Then Renaud and Lorgeril [37] put forward through their researches that French people's love for red wine is mainly due to the low morbidity of cardiovascular event. However, the detailed mechanism is still unclear. From then on, a lot of studies and epidemiological evidences further illustrated that most of the beneficial effects of light-to-moderate consumption of red wine on cardiovascular disease have been attributed to the presence of red wine polyphenols [38,39]. The numerous phenolic compounds in red wine is the main effective constituent and also a complex mixture. It includes resveratrol, catechin, epicatechin polymers, gallic acid, ferulic acid, caffeic acid, anthocyanin, and other polyphenols [38]. These compounds contribute to the prevention of endothelial dysfunction, by increasing endothelial nitric oxide synthase expression and bioactive NO in the vasculature, and by reducing ROS or even by inhibiting endothelin-1 expression. At the same time, red wine polyphenols have been shown to increase circulating EPC number and functional activity, anticoagulant, anti-inflammatory, antioxidant, inhibition of LDL; inhibition of VSMCs proliferation and migration. Moreover, a recent study showed also the other protective factors such as endothelium-derived hyperpolarizing factor (EDHF) are stimulated. Finally, red wine can effectively attenuate the development of atherosclerotic plaque and reduce the incidences of total cardiovascular events [39]. In a previous issue of Circulation [40], it was 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 might contribute to its beneficial effects on the cardiovascular system. Polyphenols in red wine can inhibit the process of atherosclerosis progression and prevent the cardiovascular disease. Similarly, yellow wine also contains abundant polyphenolic compounds, which stems from glutinous rice and wheat [24] successfully identified 10 phenolic compounds in yellow wine samples, including syringic acid, (+)-catechin, and rutin, caffeic acid, (-)-epicatechin, gallic acid, p-coumaric acid, vanillic acid, ferulic acid and quercetin with a total phenolic amount of 89.07 μg/ml and 108.73 μg/ml for two famous Shaoxing yellow wine, the most popular brands in market, Guyuelongshan and Nuomi, respectively. Syringic acid and (+)-catechin were detected to be the predominant polyphenols in these yellow wines, contributing about 60% to the total amount. The amount and diversity of_polyphenolic compounds present in yellow wine is similar with that in the red wine [21,23]. So we deduce that both yellow wine and red wine may inhibit the progression of atherosclerosis through their polyphenolic compounds.

Shaoxing yellow wine contains not only the above polyphenols but also functional oligosaccharides, vitamin B, bioactive peptide and other nutritional components [41,42]. These components in yellow wine contribute to its cardioprotective effects. Yellow wine is abundant with oligosaccharides and vitamins, and also oligosaccharide has many important physiological functions. In human body, oligosaccharides are difficult to be absorbed, however, it can promote the bifidobacterium multiplication, benefit the intestinal microecological environment and promote the synthesis and absorption of VitB (especially VitB6 and VitB12) [43-45], and VitB6, VitB12, or folic acid can reduce Hcy [46,47]. In our previous research about whether Chinese yellow wine can inhibit MMP-2 and improve atherosclerosis in the LDL Receptor knockout (LDLR−/−) mice. Thirty-two LDL Receptor knockout 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 group, red wine group, ethanol group (yellow wine, red wine, and ethanol were 1:3 given into the water and diluted to 3% ethanol), control group (given water only). The results showed that Hcy was significantly reduced in the yellow wine group compared to the other three groups, and there was no significant difference in the other three groups [25]. This indicates that light-to-moderate consumption of yellow wine can reduce the plasma concentrations of Hcy. Hcy, at concentrations associated with increased risk of cardiovascular events, increases MMP-2 activity, synthesis and secretion in VSMC through a mechanism involving the activation of MAPK and P13-K pathways. And the direct activation of proMMP2 by Hcy could be one of the mechanisms involved in the extracellular matrix deterioration in hyperhomocysteinemia-associated arteriosclerosis [31]. These data suggest that yellow wine may inhibit the expression and activation of MMP-2 through the reduction of the plasma concentrations of Hcy. In our study, compared with Hcy group, the expression and activity of MMP-2 in yellow wine group and red wine group were significantly decreased. Although a tendency with a lower expression and activity of MMP-2 in the ethanol group than the Hcy group, there were no significant differences. Similarly, As Escolà-Gil [48] and Tofferi [49] found that ethanol alone did not reduce atherosclerotic plaque, there was no significant difference with atherosclerotic plaque in ethanol group compared with control group. The improving of atherosclerosis

may be some other ingredients in the red wine and yellow wine. Roland and Ruth found that dealcoholize red wine decreased atherosclerosis independently of inhibition of lipid peroxidation in the artery wall [50]. Also, in a series of experiments, the researchers found red wine polyphenols prevented MMP-2 expression significantly in rat, the inhibiting of MMP-2 expression maybe involved in the protective effect of red wine on coronary heart diseases [40,51]. It proves that small doses of extracellular yellow wine supplementation reduced 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 (both protective component besides alcohol). On the basis of these results, they all support that small doses of yellow wine can inhibit the expression of MMP-2 and may have anti-atherosclerotic effects. This finding is in agreement with our previous findings showing that Chinese yellow wine could inhibit the production of Hcy induced extracellular MMP-2 in cultured rat VSMCs and could inhibit MMP-2 and improve atherosclerosis in the LDL Receptor knockout (LDLR−/−) mice [25,26]. Through the previous experiments both *in vitro* and *in vivo*, we can predict that small doses of yellow wine may inhibit atherosclerotic plaques by reducing the level of plasma lipids and Hcy. Finally, its inhibition effects on the progress of atherosclerosis were realized by inhibiting the expression and activation of MMP-2 in VECs and VSMCs. Moreover, we bravely deduced that yellow wine can inhibit the development of atherosclerosis process, and the polyphenols present in it has been thought as a critical constituent that contributes to the cardiovascular health effects. It is probably similar to the red wine. We will make some researches in the next step of the experiment to study whether many *in vivo* anti- atherosclerotic effects of yellow wine are likely to be attributable to a single polyphenolic compound or in concert with a number of polyphenolic compounds mixture.

To our knowledge, this is the first report found that both small doses of yellow wine and red wine can inhibit the expression and enzymolysis activity of MMP-2 in the cultured VECs, and may significantly inhibit the initiation and progression of the pathological process of atherosclerosis through the mechanism above. The result indicates that like taking red wine, the regular and moderate consumption of yellow wine in daily life may be effective and novel strategies for primary prevention of atherosclerosis-related diseases. This new finding could be of considerable significance: It has theoretical and practical significance for researches to prove the significance of MMP-2 in the initiation and progression of atherosclerosis lesion. For seeking a new anti-atherosclerosis and implementing new treatment strategies; And further scientific studies on yellow wine will benefit the development of Shaoxing yellow wine industry as well as China's national products.

Conclusions

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

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