Int J Clin Exp Pathol 2016;9(9):9077-9085
www.ijcep.com /ISSN:1936-2625/IJCEP0031150

Original Article

Polypeptides and polyphenols in Chinese yellow wine inhibitatherosclerosis in LDLR knockout mice

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Received April 22, 2016; Accepted July 19, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Background: Although Chinese yellow wine could prevent the progression of early atherosclerotic lesions in LDLR knockout mice has been proved, which ingredients in it played the key role remained unclear. This study aimed to explore the effective components in Chinese yellow wine that could inhibit atherosclerosis in LDLR-/- mice. Methods: Six weeks old LDLR-/- male mice (n=56) were randomly allocated into 7 groups: normal control group (NC), high fat group (HF), high fat and yellow wine oligosaccharides group (HFYWO), high fat and yellow wine polypeptides group (HFYWPT), high fat and yellow wine polyphenols group (HFYWP), high fat and alcohol group (HFA), high fat and yellow wine group (HFYW). After 16 weeks intervention, mice were sacrificed. The levels of serum lipids were examined. The morphological changes of aorta artery were observed under microscope. The expressions of matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), tissue inhibitor of metalloproteinase 2 (TIMP-2) were determined by Western blot. The activations of MMP-2, MMP-9 were determined by Gelatin Zymography. Results: Compared with HF group, the level of LDL-C and TC in the serum of mice were decreased in HFYWP, HFYWPT and HFYW group, $P<0.05$; the atherosclerosis lesion area in HFYWPT, HFYWP and HFYW groups were significantly reduced, $P<0.05$; the expression and activation of MMP-2 and MMP-9 in HFYWPT, HFYWP and HFYW group were decreased, $P<0.05$. There is no significant difference between each group in the expression of TIMP-2. Conclusion: Polypeptides and polyphenols in the Chinese yellow wine could inhibit the high fat diet induced atherosclerosis in LDLR-/- mice, the mechanisms maybe that these ingredients in the wine could regulate blood lipid, inhibit the expression and activation of MMP-2/9 and keep the balance between MMPs and TIMPs.

Keywords: Chinese yellow wine, polypeptides, polyphenols, MMPs

Introduction

As a specialty of china, Chinese yellow wine is one of the most ancient brewing wine kinds in the world. The wine is made through natural fermentation over 80 days by using yeast, rice and water as raw materials [1]. It is rich in numerous healthful ingredients like amino acids, peptides, oligosaccharides, polyphenols, organic acids, vitamins and minerals [2]. Our previous researches have already proved that Chinese yellow wine could prevent the progression of early atherosclerotic lesions in LDLR knockout mice and inhibit the proliferation and migration induced by homocysteine(HCY) in cultured rat vascular smooth muscle cells (VSMCs) [3, 4]. But exactly which kinds of the ingredients in Chinese yellow wine exhibit the anti-atherosclerosis effect remained unclear.

Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) play an important role in the formation and development of atherosclerotic plaques via degradation and synthesis of extracellular matrix (ECM) [5, 6]. Studies of human vessels showed that MMP-2/9 was highly expressed in fatty streaks and atherosclerotic plaques compared with normal regions of the vessel [7, 8]. Furthermore, abundant MMP-2/9 were detected in fatty streaks or atherosclerotic plaques with hemorrhage and calcification [9, 10].

In present experiment, we use high-fat diet fed LDLR-/- mice as model of atherosclerosis and

explore the anti-atherosclerosis ingredients in Chinese yellow wine by examining mice's serum lipids level, observing morphological changes of aorta artery and detecting the expression and activity of MMP-2, MMP-9 and TIMP-2.

Materials and methods

Materials

Yellow wine peptides and yellow wine polyphenols extracted from yellow wine were provided by national engineering and research center for traditional Chinese medicine (Shanghai, China). The procedures used to extract and analyze polypeptides [11] and polyphenols [12] have been described previously. Because oligosaccharides in yellow wine is mainly composed of isomaltose, panose and isomaltotriose according to previous research [13], yellow wine oligosaccharides used in our experiment were made up by isomaltose, panose and isomaltotriose. The quality ratio of these three components was 3.14:3.96:0.14 respectively.

Animal group and diet

Animal studies conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-27, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical university. Six week-old male LDL-receptor-knockout (LDLR-/-) mice with the genetic background back-crossed ten generations into C57BL/6J (n=56, body weight 20-25 g) were obtained from Model Animal Research Center of Nanjing University (Nanjing, China). After one week of an adaptation, at age 7 weeks, Fifty six mice were randomly divided into 7 groups (n=8, each group): (1) Normal control (NC) group; (2) High-fat (HF) diet group (containing 10% fat and 1.25% cholesterol); (3) High fat and yellow wine oligosaccharides (HFYWO) group (high fat diet plus 0.3 mg/(kg•d) yellow wine oligosaccharides); (4) High fat and yellow wine polypeptides (HFYWPT) group (high fat diet plus 0.5 g/(kg•d) yellow wine polypeptides); (5) High fat and yellow wine polyphenols (HFYWP) group (high fat diet plus 30 mg/(kg•d) yellow wine polyphenols); (6) High fat and alcohol(HFA) group (high fat diet plus 3 ml/(kg•d) alcohol); (7) High fat and yellow wine(HFYW) group (high fat diet plus 25 ml/(kg•d) yellow wine). The general health and activity of the mice were monitored closely, and in the study they were allowed free access to food and water. Liquid consumption, food intake and animal body weight were monitored weekly.

According to The Food and Drug Administration (FDA), moderate alcohol consumption standard is about 12-24 g of alcohol per day. The alcohol concentration in the yellow wine is 12%, so drinking 200 ml yellow wine everyday could be considered as moderate wine consumption. As confirmed by Xie, the oligosaccharides contents in yellow wine was 10 mg/L, polypeptides contents was 20 g/L, and polyphenols content was 1 g/L [2]. For a 70 kg adult drink 200 ml yellow wine everyday, the concentration in the intake of oligosaccharides, polypeptides, polyphenols, alcohol and yellow wine is 0.029 mg/kg, 0.057 g/kg, 2.84 mg/kg, 0.343 ml/kg, 2.86 ml/kg respectively. In 20-5 g mice, according to the coefficient of 9.1 [14], the concentration translates into 0.263 mg/kg of oligosaccharides, 0.513 g/kg of polypetides, 25.8 mg/kg of polyphenols, 3.121 ml/kg of alcohol and 26.026 ml/kg of yellow wine. According to the calculation above, we determined the everyday modulating concentration of oligosaccharides, polypeptides, polyphenols, alcohol and yellow wine as 0.3 mg/kg, 0.5 g/kg, 30 mg/kg, 3 ml/kg, and 25 ml/kg respectively.

Serum lipid analysis

After 16 weeks of intervention, the mice were deprived of food for 12 h and sacrificed after being anaesthetized by inhaling 1.5% isoflurane as previously described. Blood was drawn from the right ventricle into tubes and the serum was isolated by centrifugation at 2000 × g for 15 mins at 4°C. The isolated serum was stored at -80°C for analysis. The low-density lipoprotein cholesterol (LDL-C), triacylglycerol (TG), total serum cholesterol (TC) and high density lipoprotein cholesterol (HDL-C) levels was measured by auto analyzer (Hitachi 7600, Japan).

Atherosclerotic lesion area of the aorta artery

After blood collection, the abdominal cavity of mice were opened and the aorta were removed from the aortic root to the renal artery to a tissue culture dish containing phosphate buffered saline (PBS). The adventitial fat and fascia tissue surrounding the aortas were removed and

the aortas were opened longitudinally under a dissection microscope. The aortas were fixed in formalin (10%) for 24 h, rinsed in 70% alcohol for 30 s, and then stained with Sudan IV for 15 min to make the atherosclerotic lesion clear. After that the aortas were differentiated by 80% alcohol for 20 min, and at last washed by running water for 1 h. Pictures of the aortas were taken by a digital camera which had been connected to the dissection microscope. The atherosclerotic lesion area was calculated and analyzed by Image-pro plus 6.0 as described previously [15].

Western blot analysis

Frozen aorta arteries were homogenized and lysed by radio immunoprecipitation assay buffer (RIPA Lysis Buffer, Beyotime company, China) which contained protease and phosphatase (1 mm inhibitors Phenylmethanesulfonyl fluoride, PMSF, Beyotime company, China). The homogenates were centrifuged at 13, 000 × g for 5 min at 4°C. After that, BCA method (BCA Protein assay kit, Beyotime company, china) were was used to detect the protein concentrations of the supernatant. Then, the supernatant was mixed with 5 × SDS sample buffer(Beyotime company, china) and heated in a boiling water bath for 5 min to make the protein denatured. After that, lysates were separated by SDS-PAGE for electrophoresis and then electrotransferred to the PVDF blotting membranes. The membranes were blocked with blocking buffer for 30 mins at room temperature and then incubated with the primary antibody overnight at 4°C. TBS-T was used to wash the membranes (3 times for 10 min), after that the membranes were incubated in horseradish peroxidase-linked secondary antibody for 1 h at room temperature. The standard chemical luminescence method (Beyotime company, china) was used to detect the antigen by exposing the membranes to Kodak X-Omat AR film. At last, the films were scanned on a gel imaging and analysis system and analyzed by Quantity One 4.4 (Bio-Rad, Hercules, CA, USA).

Gelatin zymography analysis

Zymography was used to measure the activity of MMP-2 and MMP-9 in the supernatants. The supernatant was collected and mixed with 5 × SDS sample buffer without a reducing agent as described in Western blot. Then, equal amounts (30 mg) of the sample were loaded onto the SDS-PAGE gel (8% polyacrylamide gel containing 0.1% gelatin) for electrophoresis. After that the gels were washed for 30 min × 2 in 2.5% Triton X-100 at room temperature to remove the SDS and incubated in the renaturation buffer (pH 7.5, 50 mM Tris-HCl, 10 mM $CaCl_2$, 0.02% NaN_3) for 42 h at 37°C. 0.1% Coomassie blue R-250 in 10% glacial acetic acid/45% methanol were used to stain the gels. Gels were destained (50% methanol, 10% acetic acid, and 40% water solution) until clear bands of gelatinolysis appeared on a dark bacground, then transferred to water for rehydration before acquiring images. At last the images of the bands were analyzed by Quantity One 4.4 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All values in the figures and texts were expressed as mean ± S.D. All data were analyzed by SPSS 20.0. Differences among all data were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Tuk-ey's Studentized Range (HSD) post-hoc test for multiple comparisons. Statistical probability of P less than 0.05 was considered to be sgnificant.

Results

Serum lipid levels

After the mice were treated by each modulation factor for 16 weeks, high fat diet could significantly increase the serum LDLC, TG, TC levels and decrease the HDLC level compared with the NC group, $P<0.05$. Compared with the HF group, the serum LDLC, TG and TC levels of the mice in YWPT, YWP and YW groups were significantly decreased, $P<0.05$, and the decreased level in the YWP group was larger than YWPT group, $P<0.05$. Compared with the HF group, yellow wine oligosaccharides and alcohol had no effect on the serum lipid levels of mice. There was no significant difference of HDLC levels between all these groups (**Table 1**).

Aortic atherosclerotic plaque area

After 16-weeks treatment, compared with the NC group, there were obvious red plaques stained by Sudan IVon the aortic intima wall especially proximal to the aortic arch in the

Table 1. Blood lipid test results of each group (x ± s, n=8)

Group	TG (m mol/L)	TC (m mol/L)	LDLC (m mol/L)	HDLC (m mol/L)
NC	2.75 ± 0.23	15.01 ± 0.93	8.01 ± 0.77	6.61 ± 0.36
HF	5.01 ± 0.43	29.28 ± 1.52	24.82 ± 0.93	4.85 ± 0.33
YWO	6.23 ± 0.42	30.78 ± 1.73	25.82 ± 3.42	4.96 ± 0.34
YWPT	4.35 ± 0.27	26.73 ± 3.34*	16.23 ± 2.43*	4.89 ± 0.25
YWP	3.68 ± 0.38*,#	20.55 ± 2.78*,#	14.67 ± 3.23*,#	5.07 ± 0.28
A	5.56 ± 0.57	28.87 ± 3.39	22.27 ± 1.83	5.23 ± 0.629
YW	3.09 ± 0.12*,#	14.47 ± 2.36*,#	10.36 ± 1.22*,#	5.67 ± 0.33

*P<0.01, VS HF group, #P<0.05, VS YWPT group. NC: Normal control group; HF: high fat diet fed group; YWO: yellow wine oligosaccharides modulation group; YWPT: yellow wine polypeptides modulation group; YWP: yellow wine polyphenols modulation group; A: alcohol modulation group; YW: yellow wine modulation group.

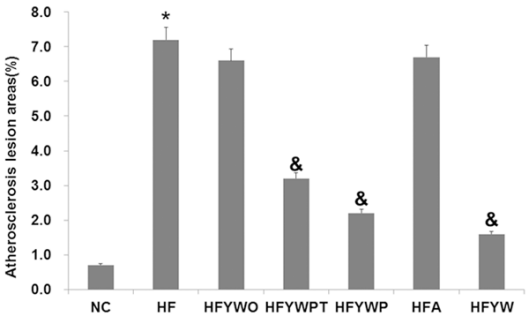

Figure 1. Atherosclerosis lesion areas of aorta artery after treatment for 16 weeks in each group (%). Mean ± SD, N=8. *: P<0.01, vs NC group; #: P<0.05, &: P<0.01, vs HF group; $: P<0.05, vs HFYWPT group. NC: Normal control group; HF: high fat diet group; HFYWO: high fat and yellow wine oligosaccharides group; HFYWPT: high fat and yellow wine polypeptides group; HFYWP: high fat and yellow wine polyphenols group; HFA: high fat and alcohol modulation group; HFYW: high fat and yellow wine group.

mice which were fed by high fat diet. Compared with HF group, the plaque area in the mice of YWPT, YWP and YW group decresed by 33.9%, 52.3% and 62.4% repectively, P<0.05. The effects on reducing the mice's atherosclerosis lesion area of YWP and YW were stronger than YWPT, P<0.05. YWO and alcohol had no significant effect on the atherosclerosis lesion area (**Figure 1**).

The expression of MMP-2, MMP-9 and TIMP-2 in aorta artery

Compared with the NC group, high fat diet could obviously increase the expression of MMP-2 and MMP-9 in the aorta of high fat diet fed mice, P<0.01. Compared with the HF group, the expression of MMP-2 and MMP-9 was significantly decreased of the mice in YWPT, YWP and YW group, P<0.05, and the effects of YWP and YW were much stronger than YWPT, P<0.05. YWO and alcohol had no significant effect on the expression of MMP-2 and MMP-9 (**Figure 2**).

Compared with the NC group, the expression of TIMP-2 was significantly increased in the HF group. YWP tended to increase the expression of TIMP-2 compared with the HF group, but the effect was not statistically significant. There was no significant difference among these groups in which mice were fed with high fat diet (**Figure 3**).

The activity of MMP-2 and MMP-9

After 16-weeks treatment, the activity of MMP-2 was significantly increased in the HF group, compared with the NC group, P<0.01. Compared with the HF group, YWP and YW could distinctly decrease the activity of MMP-2, P<0.01, while YWO, YWPT and alcohol had no effect on the activity of MMP-2 (**Figure 4**).

In addition, compared with NC group, high fat diet could obviously increase the activity of MMP-9 in the aorta of mice, P<0.01. YWPT, YWP and YW could decrease the activity of MMP-9 compared with the HF group, P<0.01, and the effect of YWP was much stronger than YWPT, P<0.05 (**Figure 4**).

Discussion

With concepts like "French paradox" and "Mediterranean Diet" proposed, a lot of researches have confirmed that moderate consumption of red wine had cardiovascular protective effect [16, 17], and it has already been proved that it's the polyphenols in the red wine that plays the key role in this effect [18, 19]. Red wine polyphenols is mainly composed of resveratrol, catechin and anthocyanins. These ingredients protect the cardiovascular systemby improving vascular function [20], modulating blood lipids [21], and regulating Micro RNA expression [22]. Recent studies found that not only the polyphenols in the red wine had cardiovascular protec-

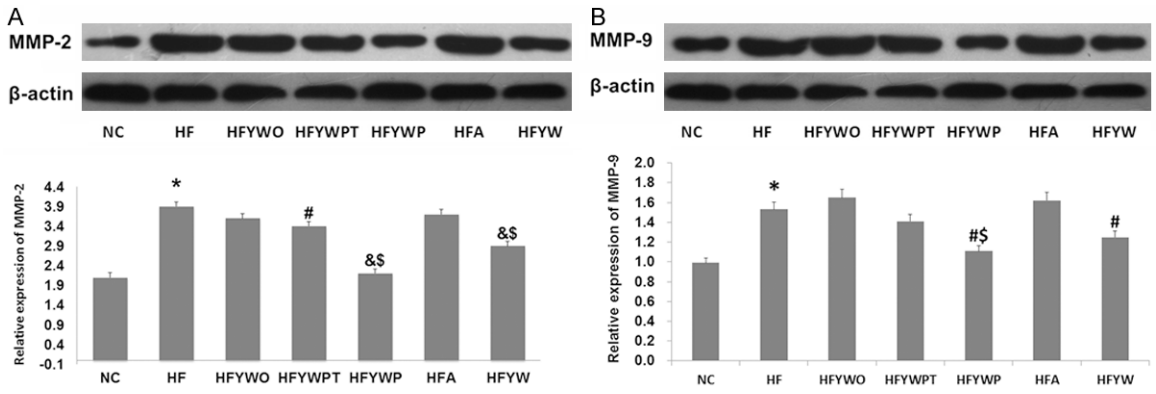

Figure 2. Effects of each treatment on the expression of MMP-2/9. Mean ± SD, N=8. *: *P*<0.01, vs NC group; #: *P*<0.05, &: *P*<0.01, vs HF group; $: *P*<0.05, vs HFYWPT group. NC: Normal control group; HF: high fat diet group; HFYWO: high fat and yellow wine oligosaccharides group; HFYWPT: high fat and yellow wine polypeptides group; HFYWP: high fat and yellow wine polyphenols group; HFA: high fat and alcohol modulation group; HFYW: high fat and yellow wine group.

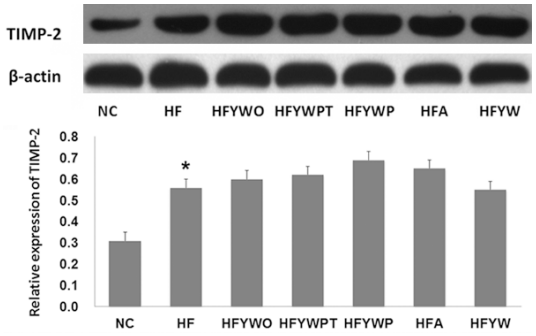

Figure 3. Effects of each treatment on the expression of TIMP-2. Mean ± SD, N=8. *: *P*<0.01, vs NC group. NC: Normal control group; HF: high fat diet group; HFYWO: high fat and yellow wine oligosaccharides group; HFYWPT: high fat and yellow wine polypeptides group; HFYWP: high fat and yellow wine polyphenols group; HFA: high fat and alcohol modulation group; HFYW: high fat and yellow wine group.

tive effect, but also the green tea polyphenols [23, 24], honey polyphenols [25] and polyphenols extracted from herbs [26]. Chinese yellow wine, which is fermented from glutinous rice, is also rich in catechin, gallic acid and other polyphenols [2]. So we hypothesized that the polyphenols in yellow wine may also have the protective effect on cardiovascular system. What's more, Chinese yellow wine contains some other cardiovascular protective ingredients like oligosaccharides and peptides, which are not contained by red wine and recent studies have already confirmed that YWO can promote the body's absorption of VitB6 [2] and YWPT lower the body's blood pressure [13]. As an essential component of wine, alcohol has been intensively studied but have not yet reached an aggrement on its effects on the cardiovascular system [27, 28]. In this experiment, we use high-fat diet fed LDLR-/- mice as the model of atherosclerosis and explore the anti-atherosclerosis active ingredients in Chinese yellow wine by observing morphological changes on the aorta artery. We found that the plaque areas in the mice of YWPT, YWP and YW group were significantly decreased compared with HF group. It was preliminarily confirmed that it were peptides and polyphenols in the yellow wine that have anti-atherosclerotic effect.

The correlation between dyslipidemia and coronary heart disease has already been proven by a lot of studies. It is definite that lower LDLc levels can significantly reduce the risk of coronary artery disease [29], while high levels of HDLc can protect the cardivascular systerm byits anti-inflammatory and antioxidant ingredients, or by taking part in the reverse cholesterol transport (RCT) [30]. Red wine consumption could improve the body's blood lipid profile was proved by studies 30 years ago [31], and the latest research results further elucidated that it was the polyphenols in wine that played the key role in the effect of reducing TC and LDLc levels [32]. Our experimental results also confirmed that Chinese yellow wine and yellow wine polyphenols could reduced TG, LDLc, TC levels and elevated HDLc levels in the serum of LDLR-/- mice. This maybe one of the mechanisms through which Chinese yellow wine protects the

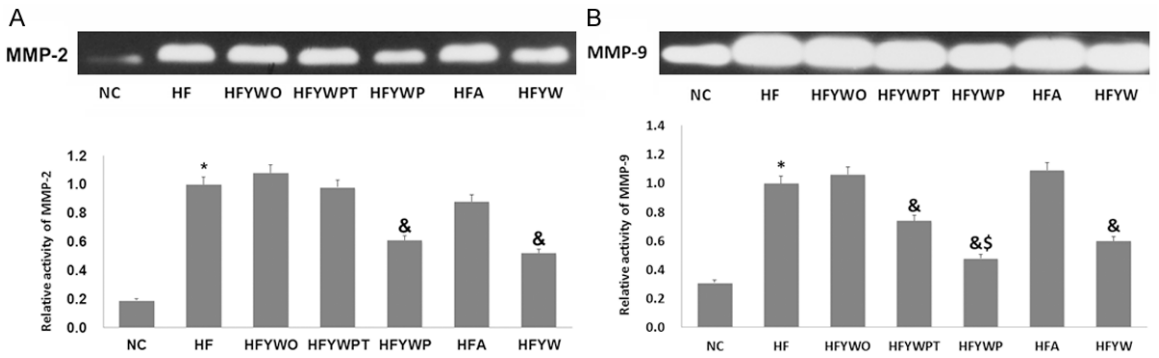

Figure 4. Effects of each treatment on the activity of MMP-2/9. The activity of HF group was set as 1. Mean ± SD, N=8. *: *P*<0.01, vs NC group; &: *P*<0.01, vs HF group; $: *P*<0.05, vs HFYWPT group. NC: Normal control group; HF: high fat diet group; HFYWO: high fat and yellow wine oligosaccharides group; HFYWPT: high fat and yellow wine polypeptides group; HFYWP: high fat and yellow wine polyphenols group; HFA: high fat and alcohol modulation group; HFYW: high fat and yellow wine group.

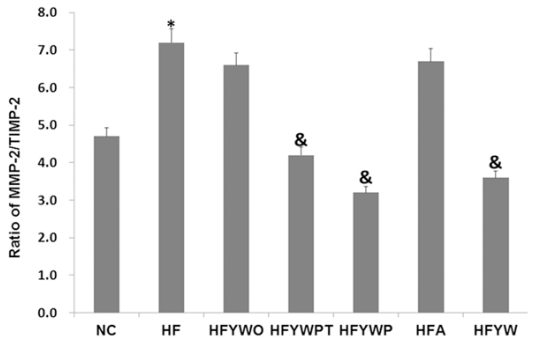

Figure 5. Ratio of MMP-2/TIMP-2 after each treatment Mean ± SD, N=8. *: *P*<0.01, vs NC group; &: *P*<0.01, vs HF group; $: *P*<0.05, vs HFYWPT group. NC: Normal control group; HF: high fat diet group; HFYWO: high fat and yellow wine oligosaccharides group; HFYWPT: high fat and yellow wine polypeptides group; HFYWP: high fat and yellow wine polyphenols group; HFA: high fat and alcohol modulation group; HFYW: high fat and yellow wine group.

cardiovascular system. It should be noted in our experimental results that yellow wine polypeptided could also reduce LDLc and TC level in serum of LDLR-/- mice although it had no significant effect on TG and HDL levels. Polypeptidesin yellow wine was the second effective ingredient in all kinds of wines discovered so far except polyphenols which could improve the body's blood lipid profile.

There are more than ten kinds of MMPs that have been found, among which MMP-2 and MMP-9 have the most close relationship with cardiovascular diseases. The expression and activation of MMPs could result in serious consequences like degradation and rupture of the plaque' fibrous cap, increased local inflammation, progressing ventricular diastolic function failure [33]. Besides degrading extracellular matrix, recent studies have also found that MMPs could play an important role in vascular remodeling by inducing the secretion of growth factors, degrading of intercellular adhesion molecules and activation of other MMPs [6, 34, 35]. Since Oak et al found that red wine polyphenols inhibited VSMCs migration through reducing the expression and activity of MMP-2 [36], it has now been basically acknowledged that inhibiting the expression and activity of MMP-2 and MMP-9 is one of the main mechanisms by which red wine polyphenol protects the cardiovascular system [37]. The results of our experiment showed that YWPT, YWP and YW could inhibit the expression and activation of MMP-2/9 in the aorta of LDLR-/- mice and the effect of YWP was stronger than YWPT. TIMP-2 could inhibit the activation of all the MMPs except MMP-9 by binding with the active structure of MMPs [38]. In the normal organism, MMPs and TIMPs were in a state of dynamic equilibrium, regulating the accumulation and degradation of extracellular matrix together. If this dynamic balance was broken by internal or external factors, the excessive degradation or accumulation of extracellular matrix could cause atherosclerotic plaque rupture or other vascular disease [33]. MMP-2/TIMP-2 was regarded as the indicator of the balance between MMPS and TIMPs, and its correlation with vascular remodeling had already been proved [39]. While Our experimental results showed that the ingredients in yellow wine had no obvious effect on the expression of TIMP-2,

if we put the expression of MMP-2 and TIMP-2 together and compare the ratio of MMP-2 to TIMP-2 in each group, we found that the effect of YWPT, YWP and YW was more significant than just look at the expression of MMP-2 (**Figure 5**). Therefore, we speculate that YWPT and YWP could maintain the balance between MMP-2 and TIMP-2 and inhibit the vascular remodeling in the aorta of LDLR-/- mice and this may be another mechanism by which Chinese yellow wine protected the cardiovascular systerm besides improving blood lipids profile.

Most of the MMPs existed in the extracellular environment as inactive zymogen and would be activated by hemodynamic changing or inflammatory cytokines when diseases like atherosclerosis happened. In our experiment, we found that YWPT, YWP and YW could decrease the expression of MMP-2 and MMP-9, and inhibit the activation of MMP-2 and MMP-9 as well.

Based on our previous research result that Chinese yellow wine could prevent the progression of early atherosclerotic lesions in LDLR knockout mice [3], this expereiment further explored and proved that YWPT and YWP containd in the Chinese yellow wine could inhibit the development of atherosclerosis in LDLR-/- mice and the mechanisms maybe through improving the lipid profile, inhibiting the expression of MMP-2/9 and maintaining the balance between MMPS toTIMPS. As polyphenolsin red wine or other beverages was good for the cardiovascular system had already been comfirmed [40, 41], it's easy to understand that YWP also had this kinds of preventive effects. The most impotant finding in our experiment was that we found YWPT, the unique ingredient of Chinese yellow wine because of its special fermentation by rice, also had the cardiovascular protective effects like polyphenols. YWPT was the second effective ingredient found so far in all kinds of wines except polyphenols which was good for the cardiovascular system. It's worthy for us to make a further study of its functions and identify the exact component which plays the key role.

However, there were some limitations in this study. First, the polypeptides and polyphenols we used in this experiment were a mixture of different monomer components, which monomer component in the polypeptide or polyphenols actually played a role was not clear. Second, we only evaluated the blood lipid and the expression of MMPs and TIMPs at the 16th week, but atherosclerosis was a dynamic developing disease which should be further studied dynamically.

Acknowledgements

The authors thank Dr Weiguang Zhang, Chunjiao Song for their data collection, assistance with statistical analysis and helpful discussions. This study was supported by the Natural Science Foundation of Zhejiang Province, China (No.LY14H020002, LY14H020009). The Science and Technology Plan Project of Zhejiang Province (No. 2016C33227).

Disclosure of conflict of interest

None.

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