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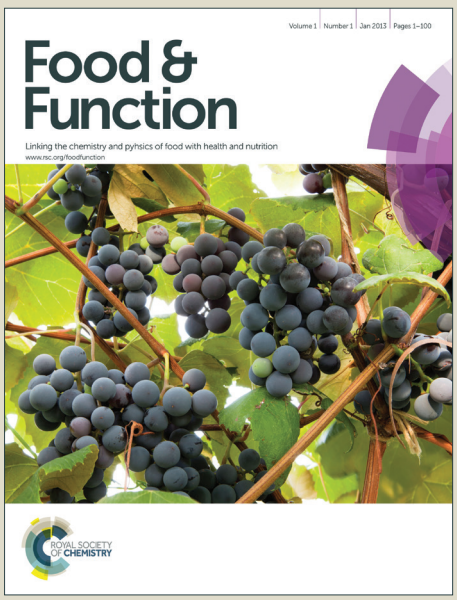

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Protective Effects of Tartary Buckwheat Flavonoids on High TMAO Diet-Induced Vascular Dysfunction and Liver Injury in Mice

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Abstract

This study was to investigate the liver and vascular changes in high trimethylamine-*N*-oxide (TMAO) diet-fed mice, and the possible vasoprotective and hepatoprotective effects of purified tartary buckwheat flavonoids fraction (TBF). HPLC analysis revealed that the content of rutin and quercetin presented in TBF was 53.6% and 37.2%, respectively, accounting for 90.8% of TBF. Mice fed 1.5% TMAO in drinking water for 8 weeks significantly displayed vascular dysfunction and liver damage ($p<0.01$). The administration of TBF at 400 and 800 mg/kg·bw significantly elevated plasma NO and *e*NOS concentrations, and serum HDL-C and PGI_2 levels, and lowered serum TC, TG, LDL-C, ET-1 and TX-A_2 levels of TMAO-fed mice. TBF also reduced serum AST and ALT activities, and hepatic NEFA and MDA levels, and increased the hepatic GSH-P*x* and SOD activities in TMAO-fed mice, which were consistent with the observation of liver histological alteration. This report firstly showed that dietary TMAO might cause liver damage and TBF prevented TMAO-induced vascular dysfunction and hepatic injury.

Keywords: TMAO, Tartary buckwheat, Vascular dysfunction, Liver injury, Protective effects

Introduction

Atherosclerosis is a major cause of cardiovascular disease (CVD), and endothelial dysfunction is an early and independent predictor of most forms of CVD.[1] Damage to the endothelium upsets the balance between vasoconstriction and vasodilation, which triggers or exacerbates several CVD events including atherosclerosis.[2] Recently, plasma trimethylamine-*N*-oxide (TMAO) was identified as a metabolite strongly associated with atherosclerosis in a large case-control cohort for CVD.[1,3] TMAO, an oxidation product of trimethylamine (TMA), is a relatively common metabolite of nutrient choline and other TMA-containing species (e.g. betaine, *L*-carnitine and lecithin) by gut microbiota.[4] However, the mechanism by which TMAO triggers atherosclerosis and increases cardiovascular risk is not completely understood, but it has been reported that dietary supplementation of mice with TMAO, choline or *L*-carnitine promotes up-regulation of multiple macrophage scavenger receptors, such as CD36 and SRA1, both of which are involved in the uptake of modified lipoproteins, which accelerates the build-up of arterial plaque and CVD.[5] Furthermore, mice supplemented with a diet rich in TMAO also exhibited a marked reduction (35%) in reverse cholesterol transport (RCT).[3] TMAO might additionally impact cholesterol metabolism via decreasing bile acid synthesis, evidenced as a reduction of hepatic mRNA expression of the bile acid synthesizing enzymes, Cyp7a1 and Cyp27a1, in mice.[3,5] For this reason, the potential toxicological property of high TMAO diet and its toxic mechanism involved in vascular endothelium damage need be further clarified.

Flavonoids comprise a large family of bioactive polyphenolic compounds found naturally in herbs, fruits and vegetables with the benefits to modern chronic diseases.[6-7] Despite the established antioxidant and anti-inflammatory properties, flavonoids are also found to be beneficial to CVD.[8] Several biological mechanisms have been indicated to support a beneficial effect of flavonoids on vascular endothelial function, suggesting a potential role of flavonoids in improving arterial function and reducing the incidence of cardiovascular events.[9-11] However, to

our best knowledge, there are no available studies regarding the roles of natural dietary flavonoids in the regulation of dietary TMAO-induced CVD, including vascular endothelial dysfunction.

Tartary buckwheat (*Fagopyrum tataricum*) or common buckwheat is traditional crop throughout the world, and has been used as an effective food in the treatment of CVD in the folk, including diabetes, hypertension, hyperglycemia, hyperinsulinemia, and dyslipidemia.[12-15] However, the specific components responsible for these effects and their mechanisms are still not very clear. Interestingly, there is report showing that buckwheat contains multiple flavonoids,[16] and among them, rutin is shown to be the major buckwheat flavonoid responsible for antioxidant, antiplatelet, anti-inflammatory, anti-hyperglycemic, antihypertensive, and vasoprotective properties.[17-20] Besides, the vasoprotective effect of rutin was also involved in the activation of endothelial nitric oxide (NO) synthetic system.[21] Quercetin, as the aglycon of rutin, is another important flavonoid in buckwheat that acts as a vasodilator in the vascular system.[22] Bhaskar et al. demonstrated that quercetin inhibited the formation of the plaques for its antioxidant and anti-inflammatory effects in hypercholesterol diet-induced rabbits.[23] Recent study also shows that intake of quercetin can increase NO production and improve endothelial function, and lower cardiovascular risk due to its vasorelaxant and anti-oxidative properties.[24-25] Interestingly, tartary buckwheat has recently received much attention as a natural flavonoid source since it contains approximately 5-fold higher of rutin than common buckwheat.[26] However, the protective effects of flavonoids in tartary buckwheat on vascular dysfunction and liver damage induced by consumption of a high TMAO diet have not yet been reported.

The present study was therefore designed to purify the flavonoid fraction (TBF) from Chinese tartary buckwheat by AB-8 macroporous resin column, and its profile of compositional rutin and quercetin was identified by HPLC. Furthermore, we also aimed to determine whether the feeding of mice with 1.5% TMAO in tap water for 8 weeks caused the vascular and liver injury in mice, and whether treatment with TBF attenuated TMAO-induced damage, and, if so,

whether the mechanisms underlying the process was involved the antioxidation and *e*NOS/NO signaling. This paper provided a clue for substantiating dietary and therapeutic use of tartary buckwheat in vascular dysfunction and hepatic injury.

Materials and methods

Materials and Reagents

The tartary buckwheat flour from whole seeds was obtained from Liangshan Qiongdu Tartary Buckwheat Products Co. Ltd. (Sichuan, China). AB-8 resin was purchased from Chemical Plant of Nankai University (Tianjin, China). Chlorogenic acid, caffeic acid, rutin, hypericin, quercetin, and phloretin were all obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Trimethylamine-*N*-oxide (TMAO) was purchased from Jinan Shangda Chemical Reagent Co. Ltd. (Jinan, China). Haematoxylin and eosin (H&E) and oil red O were the products of Shanghai Lanji Technological Development Co. Ltd. (Shanghai, China). Assay kits of serum total cholesterol (TC), total triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Changchun Huili Biotechnology Co., Ltd. (Changchun, China). Detection kits for nitric oxide (NO), superoxide dismutase (SOD), glutathione peroxidase (GSH-P*x*), malonaldehyde (MDA) and non-esterified fatty acid (NEFA) were the products of Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits of endothelial nitric oxide synthase (*e*NOS), endothelin 1 (ET-1), prostaglandin I_2 (PGI_2), and thromboxane A_2 ($TX\text{-}A_2$) were also purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Acetonitrile was purchased from Acros-Organic (Geel, Belgium). All other regents and chemicals were of analytical grade.

Extraction of Tartary Buckwheat Flavonoids

Flavonoid fraction of tartary buckwheat was isolated as previously described with some

modifications.[27] Approximately 2.5 kg of the dried tartary buckwheat flour was extracted by 25 L of methanol-water (75:25, v/v) with reflux for 2 h, and repeated three times. The combined extracts were centrifuged at 3000*g* for 10 min, and concentrated at 60°C with a rotary evaporator (RE-52AA, Shanghai, China) under vacuum, and then freeze-dried. The content of rutin and quercetin in crude extract was 162.7 mg/g and 75.6 mg/g, respectively, which was identified by HPLC, and deionized water was added to get rutin and quercetin solution at the concentrations of 0.35 mg/mL and 0.16 mg/mL.

Purification of Flavonoids Fraction from Tartary Buckwheat

The purification of flavonoids-rich tartary buckwheat extract was performed as previously described with some modifications.[28] The purification process was carried out on glass columns (12 mm × 500 mm) wet-packed with 6 g (dry weight) AB-8 resin. Prior to the adsorption experiment, the AB-8 resin was soaked in 95% ethanol, shaken for 24 h and subsequently washed by deionized water thoroughly. The bed volume of resin was 30 mL. For the preliminary test, 0.1 g of crude extract was mixed with 46.5 mL of deionized water (0.35 mg/mL of rutin and 0.16 mg/mL of quercetin), and then the sample solution was loaded on the glass column at a flow rate of 1.5 mL/min. After adsorption, desorption was investigated by comparing the effect of different concentrations of ethanol (40%, 50%, 60%, 70% and 80%) on desorption of flavonoids at a flow rate of 1.5 mL/min, and fractions were collected and the concentrations of rutin and quercetin were analyzed by HPLC. The enrichment of flavonoids was carried out in the glass column with AB-8 resin under the optimized conditions. 160 mL of the sample solution (0.35 mg/mL of rutin and 0.16 mg/mL of quercetin, pH 6.0) was applied to the glass column at the flow rate of 1.5 mL/min. After reaching adsorptive saturation, the column was firstly washed by deionized water to remove the soluble sugar and protein, and then eluted by ethanol-water (pH 6.0) with a flow rate of 1.5 mL/min. The effluent liquid was collected until there was almost no color, and then evaporated to dryness in a rotary evaporator at 60°C and freeze-dried. During adsorption and desorption, aliquots were collected in 8 mL intervals by a Redifrac fraction

collector (BSZ-100, Shanghai, China). The purified tartary buckwheat flavonoid fraction was named as TBF in this study, and this part was further applied in the following experiments for the quantification of flavonoids and its protective effects on vascular endothelium and liver in mice.

HPLC Analysis for Flavonoid Profile in TBF

The quantification of rutin and quercetin as major flavonoids in TBF was carried out using a reversed-phase HPLC column (4.6 mm i.d. × 250 mm, 5 μm, Inertsil ODS-SP, Japan) on a Shimadzu LC-2010 A HPLC system equipped with an UV detector, an autosampler and a Shimadzu Class-VP 6.1 workstation software (SHIMADZU, Kyoto, Japan). The standards and samples were all dissolved in 70% methanol aqueous solution to yield stock solution at the concentration of 0.1 mg/mL. Before injection, all solutions were diluted and subsequently filtered through a 0.45 μm Millipore membrane. A gradient elution was performed by varying the proportion of solvent A (acetonitrile/water, 50:50, v/v) to solvent B (water containing 0.5% formic acid). The gradient program was as follows: 0–30 min with 75% solvent B; 30–45 min from 75% to 50% B; 45–55 min from 50% to 30% B, 55–60 min from 30% to 10% B, 60–65 min from 10% to 75% B. The flow rate of the mobile phase was 1 mL/min, and the UV detection wavelength was 280 nm, and the sample injection volume was 25 μL at a 30°C column temperature. The chromatographic peaks of the analytes were identified by comparison of their retention time (t_R) values and UV spectra with those of known standards and determined by peak areas from the chromatograms.

Animals and Experimental Design

Healthy male Kunming mice (weight 18-22 g) were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). Mice were acclimatized for at least 7 days prior to use and were housed under standard conditions with 12/12 h light-dark cycle at room temperature of 22 ± 2°C and humidity 60 ± 5%. They were allowed free access to tap water and rodent chow (40% corn flour, 26% wheat flour, 10% bran, 10% fish meal, 10% bean

cake, 2% mineral, 1% coarse, and 1% vitamin complex, Qianmin Feed Factory). All experimental protocol used in this study was approved by the Committee on Care and Use of Laboratory Animals of the Fourth Military Medical University, China (SYXK-007-2007). The animals were randomly divided into five groups with 10 mice each: Normal control group, high-TMAO control group (1.5% TMAO water alone), TBF-treated groups (200 mg/kg·bw for low-dose group, 400 mg/kg·bw for middle-dose group, 800 mg/kg·bw for high-dose group, supplemented with 1.5% TMAO water, respectively). The mice were allowed free access to tap water or 1.5% TMAO water. The dosage of 1.5% TMAO was selected according to the previous report,[1] and our pre-experimental results in mice. The capacity of water intake in mice was monitored according to our previous experiments.[29] TBF was suspended in a 1% sodium carboxymethylcellulose (CMC) aqueous solution and administered intragastrically (ig.) at 200, 400 and 800 mg/kg body weight once daily (0.4 mL) for 8 consecutive weeks, where the dosage of TBF was optimized before the study in mice according to the results of our previous experiments.[30] The mice from the normal and TMAO groups were also given the same volume of vehicle, and 1.5% TMAO water was renewed every other day. The mouse body weight of all the groups was measured once a week. Food and water intake was monitored daily, and then the average food and water intake of each mouse in different groups was calculated. All the administrations were conducted between eight and nine o'clock in the morning once daily. After 2 h, all of the animals were fasted but given enough water to drink for 12 h. At the end of the experimental period, all of the animals were fully anesthetized by the inhalation of isofluorane, and then the animals were sacrificed by cervical dislocation. Blood was withdrawn into a syringe from the abdominal aorta, and mouse liver was immediately removed and washed by ice-cold physiological saline.[31] Blood samples were separated for serum aliquots by centrifugation at 3000*g* for 10 min at 4 °C and stored at -20 °C for later biochemical analysis within two weeks, while the isolated livers were refrigerated at -80°C. On the basis of the records of the body weight and corresponding liver weight of every mouse, we calculated the

hepatosomatic index (HI) according to the following formula: HI = liver weight/body weight × 100%. All the experiments were conducted according to the Guidelines of Experimental Animal Administration published by the State Committee of Science and Technology of People's Republic of China.

Assay for Plasma NO and *e*NOS, and Serum ET-1, PGI_2, and TX-A_2 Levels

Plasma NO levels were determined with a commercially available diagnostic kit, and the results were expressed in μmol/L. Plasma *e*NOS and serum ET-1, PGI_2, and TX-A_2 concentrations were measured using competitive inhibition method of enzyme linked immunosorbent ELISA assay kit according to the kit manufacturer's instructions, and the results were expressed as units per litre (U/L), pg/mL, pg/mL, and pg/mL, respectively.

Measurement of Serum Lipid Profile, and ALT and AST Activities

The measurements for fasting serum TC, TG, LDL-C and HDL-C concentrations were conducted by enzymatic colorimetric methods using commercial kits, and the results were all expressed in mmol/L. The serum enzyme activities of ALT and AST were measured by commercially available diagnostic kits. The enzymic activities were expressed as units per litre (U/L).

Assay of Hepatic NEFA, MDA, T-SOD and GSH-P*x* Levels

The liver tissue was homogenized (10%, w/v) in ice-cold 50 mM phosphate buffer (pH 7.4) by an automatic homogenate machine (F6/10-10G, FLUKO Equipment Shanghai Co. Ltd., Shanghai, China). During the preparation, 0.5 g of each hepatic tissue was homogenized in 9-fold frozen normal saline in volume, and centrifuged at 1500*g* for 10 min at 4°C. The supernatant was collected for the measurements of NEFA, MDA, T-SOD and GSH-P*x*. The protein concentration in homogenates was assayed by the method of Coomassie brilliant blue.[31] The analysis for hepatic NEFA and MDA levels was performed with commercially available diagnostic kits, and the results were expressed as μmol/g protein and nmol/mg protein, respectively. T-SOD and GSH-P*x* activities were assessed using common commercial kits, and

the results were expressed as U/mg protein.

Histopathological Observation of Thoracic Aortas and Livers

The thoracic aortas were stripped from any surrounding tissues and put in a buffer solution of 4% paraformaldehyde. Fixed tissues were processed routinely for paraffin embedding, and 5 μm sections were prepared and dyed with hematoxylin and eosin (H&E). For H&E staining of the liver, a portion of the liver from the left lobe was fixed in a 4% paraformaldehyde solution. Fixed tissues were embedded in paraffin, cut into slices (5-6 μm thick), and stained with H&E dye. For oil Red O staining, the liver sample was processed using cryostat (CM1950, Leika, Germany) and then fixed and stained. The stained area was viewed using an optical microscope at 400 ×. Finally, the images were examined and evaluated for pathological change analysis. The cross-sectional areas of the intima, media, and lumen were calculated with a computerized apparatus (VM-30, Olympus, Tokyo, Japan).

Statistical Analysis

All of the experiments were performed at least in triplicate. The data were expressed as means of ± SD (standard deviation), and subjected to an analysis of variance (ANOVA, $p<0.05$) and Duncan's multiple-range tests (SPSS, version 13.0). The p-value <0.05 was considered statistically significant.

Results

Chemical Properties of TBF

The extraction of crude flavonoids from the tartary buckwheat was performed with 75% aqueous methanol. With this method, the extraction yield of crude extracts from tartary buckwheat could reach approximately 9.2% (w/w) of the tartary buckwheat powder. The purified tartary buckwheat flavonoids fraction (TBF) was further obtained from the crude extracts by a separation on a AB-8 resin column. The effects of various concentrations of aqueous ethanol as eluate on the recovery of rutin and quercetin were investigated. As a result,

60% aqueous ethanol was found to be the most effective for the recovery of rutin and quercetin in the various elution systems. The yield of TBF was 1.8% (w/w) of the tartary buckwheat powder.

A routine HPLC chromatographic procedure was performed to further measure the component flavonoids in the flavonoid preparation. A HPLC chromatogram for single flavonoid profile of TBF was illustrated in Fig. 1A, and the tested standard flavonoids were shown in Fig. 1B. The identification of component flavonoids was performed according to the retention time (t_R) obtained from authentic standards under identical HPLC conditions. As depicted in Fig. 1B, six peaks corresponding to authentic standards were identified in the order of chlorogenic acid (12.1 min), caffeic acid (19.2 min), rutin (29.3 min), hypericin (30.5 min), quercetin (47.1 min), and phloretin (53.5 min). In this study, linear regression was assessed for the content calculation, and the assay had excellent linearity between Y (peak area of analyte) and X (concentration of analyte). The regression lines for rutin and quercetin were Y = 1.1E+5X + 8460.2 (R^2 = 0.9999, n = 5), and Y = 1864.2X + 35712 (R^2 = 0.9993, n = 5), respectively. As shown in Fig. 1A, HPLC analysis clearly indicated that the major flavonoids present in purified TBF were rutin and quercetin, and their contents were 536.2 mg/g and 371.6 mg/g, respectively, accounting for up to 90.8% of TBF, suggesting that TBF is a flavonoid fraction with high-purity.

Effects of TBF on body weight, liver weight, and liver index in mice

As can be seen in Table 2, after giving 1.5% TMAO for 8 weeks, the average food and water intake was not significantly different among all the tested groups, where the daily food intake was between 9.77 ± 1.68 g/mice/day and 10.29 ± 2.11 g/mice/day, and water consumption was between 6.32 ± 3.25 mL/mice/day and 6.53 ± 2.16 mL/mice/day. However, the mice fed a high TMAO diet significantly increased the body weight, liver weight and HI from 46.28 ± 2.76 g, 2.04 ± 0.11 g, and 4.41 ± 0.28 g in normal group to 48.97 ± 2.03 g ($p < 0.05$), 2.44 ± 0.15 g ($p < 0.01$), and 4.97 ± 0.33 g ($p < 0.01$), respectively. Interestingly, the increased liver weight could be well decreased by the oral administration of middle- and high- doses of TBF ($p < 0.01$).

Additionally, treatment with TBF at 400 and 800 mg/kg·bw also significantly attenuated the increases in body weight ($p < 0.05$) and dose-dependently decreased the HI ($p < 0.05$, $p < 0.01$) of TMAO-treated mice. However, supplementation with TBF at a low dose led to the slight decreases in body weight, liver weight and HI, but there was no statistical significance ($p > 0.05$). The present result suggests that TBF can inhibit the TMAO-induced weight gain.

Effects of TBF on Plasma NO and *e*NOS, and Serum ET-1 Levels in Mice

Endothelium plays an important role in the regulation of vascular tone, and an imbalance between vasodilator NO and vasoconstrictor endothelin-1 (ET-1) causes endothelial dysfunction.[32-33] As can be seen in Fig. 2A and B, after giving 1.5% high-TMAO water for 8 weeks, plasma NO level and *e*NOS activity in TMAO-fed mice sharply decreased to 3.9 ± 0.7 μmol/L ($p<0.01$) and 12.5 ± 3.3 U/L ($p<0.01$) from 5.3 ± 0.5 μmol/L and 26.5 ± 6.1 U/L of the untreated normal mice, respectively, suggesting that TMAO caused vascular endothelial injury in mice. Interestingly, the tested mice receiving oral administration of TBF at 200, 400 and 800 mg/kg·bw had an increase by 7.9% ($p>0.05$), 27.3% ($p<0.05$) and 42.3% ($p<0.01$) in plasma NO levels, relative to TMAO-treated mice, respectively (Fig. 2A). Similarly, plasma *e*NOS activity was also increased by 17.5% ($p>0.05$), 55.4% ($p<0.01$) and 99.1% ($p<0.01$) following the treatment, respectively (Fig. 2B), indicating the protective effect of TBF on TMAO-induced endothelial injury in mice. As shown in Fig. 2C, application of high TMAO-fed diet in mice caused a severe increase in serum ET-1 concentrations from 64.3 ± 9.5 pg/mL of the normal mice to 91.6 ± 10.1 pg/mL ($p<0.01$), suggesting that 1.5% TMAO feeding might cause endothelial injury in mice. However, this TMAO-induced increase was effectively attenuated by the supplementation of medium-dose or high-dose of TBF with a 38.3% or 50.3% decrease, relative to TMAO-fed mice, respectively ($p<0.01$). However, the co-treatment with TBF at a low dose of 200 mg/kg·bw led to a slight decrease in the level of serum ET-1, but there was no statistical significance ($p>0.05$). The present results suggest that TBF can significantly inhibit the TMAO-induced endothelial injury in mice.

Effects of TBF on Serum PGI_2 and TX-A_2 Levels in Mice

PGI_2 and TX-A_2 are the most common prostanoids in the cardiovascular system, which act as opposite roles in the formation of atherosclerosis.[34] Herein, we further examined the serum PGI_2 and TX-A_2 concentrations in the mice exposed to high TMAO diet in the water. As expected, the mice exposed to 1.5% TMAO water had a 40.3% decrease in serum PGI_2 concentration, and a 24.2% increase in serum TX-A_2 level, respectively (p<0.01, Fig. 2D and E). However, the low PGI_2 and high TX-A_2 levels in TMAO-fed mice could be prevented by TBF administration in a dose-dependent manner, especially when the dosage increased to 400 and 800 mg/kg·bw. As depicted in Fig. 2D and E, serum PGI_2 levels of the mice treated with TBF at 400 and 800 mg/kg·bw were significantly increased by 42.9% (p<0.05) and 95.2% (p<0.01), as compared to TMAO-fed mice, respectively, and serum TX-A_2 levels of the mice treated with middle- and high-doses of TBF were remarkably lowered by 29.2% and 49.6%, (p<0.01 vs TMAO alone group), respectively. However, administration of TBF at a low dose of 200 mg/kg·bw led to a slight increase in the serum PGI_2 and a slight decrease in the serum TX-A_2, but there was no statistical significance (p>0.05).

Effects of TBF on Serum Lipid Profiles in Mice

Excess LDL and TG together with HDL are well known to be targets of therapy during management of CVD.[35] Herein, we further examined the serum lipid profiles in mice fed high TMAO diet that contributed to the CVD risk. As shown in Table 1, the levels of serum TC, TG, and LDL-C were elevated dramatically and HDL-C had an expected decline in the high TMAO-fed mice, where the levels of TC, TG and LDL-C had a significant increase by 34.6% (p<0.01), 86.6% (p<0.01) and 35.2% (p<0.01), and HDL-C had a remarkable decrease by 29.8% (p<0.01), relative to the untreated mice, respectively, indicating that high TMAO diet caused the hyperlipidemia in mice. However, the protective administration of TBF could effectively decrease the serum TC, TG and LDL-C levels and increase the HDL-C concentration. It was also noted that the levels of TC, TG, LDL-C and HDL-C of high doses of TBF-treated mice

were close to that of the normal mice, suggesting that TBF might normalize the dyslipidemia by improving the serum lipid profiles in high TMAO-induced mice.

Aortic Pathology and Protective Effects of TBF

Histopathological observation of the aorta was performed to further support the evidence for the biochemical analysis. As shown in Fig. 3A, the histology of the aortas appeared normal in the untreated normal mice, while the endothelium of the aorta wall of TMAO-induced mice showed extensive vascular injuries, characterized by significant proliferation of the wall, irregular medium and internal elastic lamina in the wall. Interestingly, the administration of TBF protected the vascular endothelium from damage of high TMAO, where the structures of the arteries had a significant reduction of aorta thickness in a dose-dependent manner, when compared with TMAO-fed mice. The administration of TMAO along with TBF at 800 mg/kg·bw showed near normal appearance, suggesting that TBF could protect the blood vessel from TMAO-induced damage.

In addition, the results of intima/media ratio further confirmed that TBF effectively stabilized the vessels against TMAO as seen by the reduction of wall thickness ratio (Fig. 3B). As depicted in Fig. 3B, the TMAO-fed mice had a 0.5-fold increase in intima/media ratio, compared to the untreated normal mice ($p<0.01$). However, the intima/media ratios of the mice treated with 200, 400 and 800 mg/kg·bw TBF was lowered by 13.3% ($p>0.05$), 26.7% ($p<0.05$) and 40.1% ($p<0.01$), respectively, relative to TMAO-treated mice. These results together with biochemical marks demonstrate that TBF can protect vascular tissue from TMAO-induced vessel injury and vascular dysfunction.

Effects of TBF on Serum ALT and AST Levels in Mice

To further test whether TMAO as oxidant would cause oxidative damage of the liver, the activities of serum ALT and AST, an indicator of leakage of hepatocytes into circulation under hepatotoxicity,[36] were evaluated in TMAO-treated mice. As shown in Fig. 4A and B, the enzymatic activities of serum ALT and AST in TMAO-treated mice were remarkably increased

to 103.2 ± 15.0 U/L (p<0.01) and 180.2 ± 26.2 U/L (p<0.01) from 75.0 ± 12.9 U/L and 135.4 ± 14.3 U/L of the normal mice, respectively, indicating that the intake of high TMAO diet caused the hepatotoxicity in mice. However, the protective administration of TBF with a supplement of 1.5% TMAO water decreased the activities of these functional markers, relative to TMAO-treated mice. As illustrated Fig. 4A, the co-treatment of TBF at 400 and 800 mg/kg·bw once daily for 8 consecutive weeks dose-dependently reduced the TMAO-induced elevation of serum ALT activity (p<0.05, p<0.01). Meanwhile, administration of TBF at 400 and 800 mg/kg·bw significantly lowered the AST activities by 31.77% and 39.83% (p < 0.01, Fig. 4B), as compared to HF-fed mice, respectively, suggesting that TBF exhibited strong protective effects against TMAO-induced liver injury in mice.

Effects of TBF on Hepatic NEFA, MDA, T-SOD and GSH-P*x* Levels

As shown in Fig. 5A, hepatic NEFA levels in TMAO-fed mice were significantly increased as compared to the normal mice from 51.7 ± 17.1 μmol/gprot to 116.4 ± 16.5 μmol/gprot (p<0.01). As expected, hepatic NEFA contents were significantly lowered by 21.0% (p<0.01), 34.9% (p<0.01) and 52.4% (p<0.01) in the mice treated with TBF at 200, 400, and 800 mg/kg·bw, when compared to TMAO-fed mice, respectively. In addition, the hepatic MDA, a hallmark of oxidative modification to membrane lipids in liver,[37] was significantly increased from 2.8 ± 1.0 nmol/mgprot of normal group to 6.3 ± 0.6 nmol/mgprot (p<0.01) in the mice fed 1.5% TMAO for 8 weeks (Fig. 5B), indicating that high TMAO diet caused notable liver peroxidation damage in mice. However, this TMAO-induced increase was effectively attenuated by the co-treatment with TBF at the tested dosages of 400 and 800 mg/kg·bw, respectively (p<0.01), but no significant decrease in MDA level was observed in the mice treated with 200 mg/kg·bw of TBF (p>0.05). Furthermore, continuous feeding of TMAO in mice caused characteristic hepatotoxicity in antioxidant parameters of liver tissue, as reflected by 31.4% decrease of hepatic T-SOD activity (p<0.01) and 29.5% decrease of hepatic GSH-P*x* activity (p<0.01) in TMAO-treated mice (Fig. 5C and D). However, TMAO-induced inhibition in antioxidant

enzyme activities was obviously prevented by the supplementation with TBF at 400 ($p<0.05$) and 800 mg/kg·bw ($p<0.01$), and this protection effect could be performed in a dose-dependent manner.

Histopathological Examination of Mouse Liver

Histopathological observations of H&E (Fig. 6) and oil red O staining (Fig. 7) of the livers were investigated to further support the protective effect of TBF against TMAO-induced hepatocyte morphological changes. In the normal group, liver slices showed typical hepatic cells with well-preserved cytoplasm, prominent nucleus and nucleolus, and visible central veins (Fig. 6A). In TMAO-fed mice, the liver sections showed a parenchymal disarrangement, such as hepatocyte necrosis, cytoplasmic vacuolation, and the loss of cellular boundaries (Fig. 6B). However, the hepatic lesions caused by TMAO were markedly ameliorated by the co-treatment with TBF, and TBF at 400 and 800 mg/kg·bw was more effective when compared with 200 mg/kg·bw, showing near normal appearance with well-preserved cytoplasm, prominent nuclei, and legible nucleoli, and this protective effect was dose-dependent (Fig. 6C-E). In addition, the photomicrographs of oil red O staining of the liver specimens were investigated, and compared with the normal mice (Fig. 7A). As shown in Fig. 7B, the liver of TMAO-fed mice showed widespread deposition of lipid droplets inside the parenchyma cells. However, the mice treated with TBF showed scattered droplets of fat when compared with high TMAO-fed mice, and especially, TBF at 800 mg/kg·bw showed near normal appearance as comparable to the normal mice (Fig. 7C-E). These results together with biochemical analysis demonstrated that TBF could protect liver tissue from high TMAO-induced fatty liver and hepatic damage in mice.

Discussion

It is recently known that high ingestion of trimethylamine-containing nutrients, such as choline, *L*-carnitine, lecithin and their metabolite TMAO, is linked to atherosclerosis.[1,3] Recently, tartary buckwheat has been reported to have potential effects in the prevention of CVD.[13-14]

Rutin and quercetin are the main bioactive constituents presented in tartary buckwheat, and play important role in improving endothelial function and attenuating atherosclerosis.[19,24] However, there are no reports in linking the protective effects of flavonoids derived from tartary buckwheat to the endothelial dysfunction and liver injury caused by TMAO. In the present study, the mice fed with 1.5% high TMAO water for continuous 8 weeks was shown to have significant vascular injury, which was consistent with recent findings.[1,3] Besides, our study also firstly found that high TMAO supplementation of mice caused severe liver injury via oxidative damage and lipid peroxidation. Furthermore, it was of interest that the purified tartary buckwheat flavonoids (TBF) were firstly demonstrated to exhibit vasoprotective and hepatoprotective effects against dietary TMAO-induced liver and vascular damage in mice.

NO is produced by *e*NOS, and plays an important role in regulating the diameter of blood vessels and maintaining an anti-proliferative and anti-inflammatory environment in the vessel wall.[38-39] Endothelial dysfunction is thought to arise due to a reduction in the bioavailability/bioactivity of NO, and the *e*NOS dysfunctions can accelerate atherosclerosis.[32] In our study, supplementation of 1.5% high TMAO water markedly decreased the serum NO and *e*NOS levels ($p<0.01$) in mice. However, the mice administrated with TBF at 400 and 800 mg/kg·bw for 8 consecutive weeks remarkably elevated the TMAO-lowered NO and *e*NOS levels, respectively, implying that TBF might effectively prevent endothelial dysfunction by stimulating synthesis of NO. ET-1 is another regulator of vascular tone and an increase in circulating ET-1 levels indicates the dysfunction of vascular endothelium.[40] Consistent with this finding, application of 1.5% high TMAO water in mice also showed a striking increase in ET-1 levels, as compared to the untreated normal mice ($p<0.01$), and TBF was shown to significantly block the increase of serum ET-1 in mice (Fig. 2C). It is well known that NO is a feed-back inhibitor of ET-1 release, and an increase in NO excretion also leads to the decrease in ET-1 levels.[41] The results presented here indicate that TBF exhibits effective protection of vascular cells by up-regulation of NO and inhibition of ET-1 release, thus helping to maintain the balance

between these two vasoactive components. This result agrees with the previous studies showing that rutin exerts endothelium-dependent vasorelaxation action by mediating NO/cGMP pathways,[42] and quercetin administration reduces arterial pressure in hypertensive men by lowering the ratio of circulating ET-1 to NO, and this alteration will improve endothelial function.[43]

PGI_2 and TX-A_2, a pair of common prostanoids, play an opposite role in regulation of cardiovascular homeostasis, which are associated with endothelial dysfunction and are an early marker of atherosclerosis.[34] PGI_2 induces vascular relaxation and potently inhibits platelet activation, and exerts the protective effect on cardiovascular system, whereas TX-A_2 is a potent vasoconstrictor and a strong platelet activator, which works as a factor facilitating the development of atherosclerosis.[34,44] In our study, TBF could significantly dispute the decrease of serum PGI_2 concentration and the increase of serum TX-A_2 level caused by high TMAO feeding (Fig. 2), suggesting that TBF prevented the vascular endothelial impairments induced by TMAO by modulating the balance between serum PGI_2 and TX-A_2. In addition, histopathological examination also showed that the TMAO-induced pathological damage of mouse aortic vessel was markedly reduced by the administration of TBF (Fig. 3A), as reflected by the reduced thickness of the tunica intima of the aorta, relative to TMAO-treated mice (Fig. 3B). This finding obviously demonstrated that TBF produced a protective effect on aortic pathology in the mice fed with high TMAO diet, and the protective effect was consistent with some previous studies, where rutin increased capillary fragility and decreased the permeability of the vessels, and quercetin restored the impaired endothelial function in several animal models.[16,45]

It is widely recognized that abnormal lipid metabolism is an early indication of CVD, and lipids play a key part in the pathogenesis of plaques.[35] In our study, high-TMAO feeding caused obvious dyslipidaemia in the experimental mice, reflected by elevated levels of TC, TG, and LDL-C, and lowered levels of HDL-C (Table 1). The results implied that the intake of high TMAO disordered lipid mechanism of liver that might promote lipid deposition in hepatocytes

and adipocytes, resulting in hyperlipidemia or fatty liver. However, co-treatment of TBF had an efficient enhance in HDL-C levels and a significant decrease in TC, TG and LDL-C levels against high TMAO diet in mice. It was also observed that the high TMAO diet induced the weight gain of the body and liver, and hepatosomatic index, and this effect was prevented by TBF treatment (Table 2). The result was consistent with the previous studies showing that both rutin and quercetin could suppress body weight gain and improve lipid profile in serum or liver, indicating that TBF might interfere with lipid mechanism and decrease the lipid synthesis in mice.[46,47] The oil-red-O staining result of liver tissues further confirmed that TBF could normalize the dyslipidemia induced by TMAO diet and protected the liver from chronic dietary TMAO-induced histopathological alteration (Fig. 7C-E), suggesting that TBF could play a protective role against hyperlipidemia in high TMAO-fed mice.

Nevertheless, considering the typical oxidation property of TMAO, we further assessed the oxidative damage of livers in the mice fed with TMAO. As shown in Fig. 4, application of high TMAO diet markedly raised serum AST and ALT activities in mice, uniquely indicating that the livers in TMAO-fed mice were damaged because the increased ALT activity was an indicator of cell membrane damage, and the elevated AST activity is another indicator of mitochondrial damage.[48] However, it was noted that the alterations of AST and ALT activities were observably mitigated by the co-treatment of TBF, suggesting that TBF not only stabilized the hepatic cellular membrane, but also had a protective effect on mitochondria.[48]

As reported, high NEFA level can lead to hepatic mitochondrial swelling, and permeability increasing, as well as lipid peroxidation,[49] and MDA is the final stage of lipid peroxidation of the polyunsaturated fatty acid of biological membrane,[50] which can result in failure of the antioxidant defense mechanisms to prevent the formation of excessive reactive oxygen species (ROS).[32] In our study, intake of 1.5% TMAO diet caused a significant increase in hepatic NEFA and MDA contents of the mice, as compared to the normal group, respectively ($p<0.01$, Fig. 5A and B). However, protective administration of TBF significantly reduced the hepatic NEFA and

MDA levels against oxidative stress induced by TMAO. These results indicate that TBF can effectively scavenge the non-esterified fatty acid released in the liver and successfully block the oxidative chain reaction, and this may be related to the high radical scavenging activity of rutin and quercetin.[26]

SOD and GSH-P*x* are the major natural antioxidant enzymes which play important roles in the elimination of ROS derived from the redox process in liver tissues.[50] For example, SOD catalyzes the dismutation of superoxide anions into hydrogen peroxide that subsequently converts to water by GSH-P*x* or converts lipid hydroperoxides to nontoxic alcohols.[31] Here, mice treated with TMAO water showed a sharp decrease in antioxidant capacity of the liver as evidenced by inhibiting the enzymic activities of SOD and GSH-P*x* ($p<0.01$, Fig. 5C and D). Interestingly, the inhibitory enzymes were significantly increased by co-treatment with TBF at 400 or 800 mg/kg·bw, suggesting that it could protect the antioxidant enzymes or activate the enzyme activity in TMAO-damaged liver tissue, and these protective effects might be due to the strong antioxidant capacity of rutin and quercetin.[14,17] In further histopathological examination, the mice fed with 1.5% high TMAO water showed distinct necrosis, ballooning degeneration, and inflammatory cell infiltration of the liver, which might be due to prevention of the toxic chemical reactions from the formation of highly ROS induced by TMAO. However, the co-treatment with TBF, especially at dosage of 800 mg/kg·bw (Fig. 6E), showed nearly normal cellular architecture with distinct hepatic cells, suggesting that these histological alterations were observably attenuated by TBF. Taken together, this was the first investigation with unequivocal evidence that TBF could inhibit TMAO-induced hepatic oxidative injury in mice.

In conclusion, data presented in this study for the first time demonstrated that dietary intake of high TMAO was highly associated with liver oxidative damage, and tartary buckwheat flavonoids as TBF exerted systematic protective effect against TMAO-induced endothelial dysfunction and hepatotoxicity in mice through inhibiting TMAO-induced ROS generation and increasing vascular NO production. The present study may provide important evidences in

finding novel TMAO-based nutritional target for intervention in vascular dysfunction and liver diseases in humans. Results of our study also indicate that TBF may play an important role in interfering TMAO-mediated damage mechanism of high methyl-donor diet-caused endothelial dysfunction and liver diseases.

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Figure Captions

Fig. 1. The HPLC chromatogram of purified tartary buckwheat flavonoid fraction (TBF, **A**) and standard flavonoids (**B**). Peaks: 1. Chlorogenic acid, 2. Caffeic acid, 3. Rutin, 4. Hypericin, 5. Quercetin, 6. Phloretin. HPLC analysis was carried out as described in the experimental section.

Fig. 2. Effects of TBF administration on plasma levels of NO (**A**) and *e*NOS (**B**), and serum levels of ET-1 (**C**), PGI_2 (**D**) and TX-A_2 (**E**) in the mice fed 1.5% high TMAO water for 8 consecutive weeks. Data are expressed as means ± SD for 10 mice in each group. $^{\#\#}$ $p<0.01$, vs the normal group. *$p<0.05$, **$p<0.01$, compared to the TMAO group.

Fig. 3. Photomicrographs of aorta morphology. (**A**) Representative histological section of the aorta was stained by H&E (magnification 400 ×). The medial area was determined by the internal elastic lamina and external elastic lamina (arrows) and analyzed with image pro-plus 6.0 software. (**B**) Inhibitory effects of dietary supplementation of TBF on the wall thickness ratio (intimal area/media area) in mouse aorta. Data denoted are means ± SD (n = 10). $^{\#\#}p<0.01$, vs the normal group. *$p<0.05$, **$p<0.01$, compared to the TMAO group.

Fig. 4. Effects of TBF on activities of serum ALT (**A**) and AST (**B**) of high TMAO diet-fed mice for 8 consecutive weeks. Values are expressed as means ± SD of 10 mice in each group. $^{\#\#}p<0.01$, vs the normal mice. *$p<0.05$, **$p<0.01$, compared to TMAO-treated mice.

Fig. 5. Effects of TBF administration on hepatic levels of NEFA (**A**), MDA (**B**), T-SOD (**C**) and GSH-P*x* (**D**) in the mice fed 1.5% TMAO water for 8 consecutive weeks. Data are expressed as means ± SD for 10 mice in each group. $^{\#\#}p<0.01$, vs the normal group. *$p<0.05$, **$p<0.01$, compared to the TMAO-treated mice.

Fig. 6. Effects of TBF on histopathological changes of liver hepatocytes stained with H&E in high TMAO-fed mice (original magnification of 400 ×). (**A**) Normal group, (**B**) TMAO-treated mice, (**C**) TMAO + TBF (200 mg/kg·bw), (**D**) TMAO + TBF (400 mg/kg·bw), (**E**) TMAO + TBF (800 mg/kg·bw). The green arrows indicate normal cellular architecture with clear hepatic cell nucleus. The red arrows indicate the hepatic cell necrosis. The yellow arrows indicate the enlarged sinusoids between the plates of hepatocytes.

Fig. 7. Lipid staining of the liver section in mice (Oil and O staining, 400 ×). (**A**) Normal group, (**B**) TMAO-treated mice, (**C**) TMAO + TBF (200 mg/kg·bw), (**D**) TMAO + TBF (400 mg/kg·bw), (**E**) TMAO + TBF (800 mg/kg·bw).

Table 1

Effects of TBF on serum TC, TG, HDL-C and LDL-C levels of the mice fed high-TMAO diet for consecutive 8 weeks[a]

Groups	TC	TG	HDL-C	LDL-C
	mmol/L			
Normal	3.01 ± 0.31	0.82 ± 0.13	0.94 ± 0.13	0.88 ± 0.14
TMAO	4.05 ± 0.62$^{\#\#}$	1.53 ± 0.25$^{\#\#}$	0.66 ± 0.10$^{\#\#}$	1.19 ± 0.22$^{\#\#}$
TMAO + TBF (200 mg/kg·bw)	3.92 ± 0.43	1.23 ± 0.26*	0.82 ± 0.14	1.03 ± 0.19*
TMAO + TBF (400 mg/kg·bw)	3.47 ± 0.76*	1.02 ± 0.32**	0.94 ± 0.17**	0.96 ± 0.13*
TMAO + TBF (800 mg/kg·bw)	3.27 ± 0.35**	0.84 ± 0.45**	1.02 ± 0.16**	0.90 ± 0.14**

[a] Values are expressed as means ± SD of 10 mice in each group.

$^{\#\#}p<0.01$, as compared with the normal mice. $^{*}p<0.05$, $^{**}p<0.01$, compared to the TMAO-treated mice.

Table 2

Food Consumption, Water intake, Body weight, Liver weight, and Hepatosomatic index (HI) of mice at the end of week 8.

Groups	Food intake (g/d)	Water intake (ml/d)	Initial body wt (g)	Final body wt (g)	Liver wt (g)	HI (%)
Normal	10.29 ± 2.11	6.53 ± 2.16	28.10 ± 1.18	46.28 ± 2.76	2.04 ± 0.11	4.41 ± 0.28
TMAO	9.77 ± 1.68	6.34 ± 3.28	27.62 ± 1.21	48.97 ± 2.03$^{\#}$	2.44 ± 0.15$^{\#\#}$	4.97 ± 0.33$^{\#\#}$
TMAO + TBF (200)	10.03 ± 1.34	6.47 ± 2.31	28.45 ± 0.77	47.74 ± 1.56	2.29 ± 0.17	4.80 ± 1.32
TMAO + TBF (400)	10.07 ± 1.98	6.36 ± 2.45	28.30 ± 1.46	46.61 ± 0.94*	2.09 ± 0.15**	4.48 ± 0.25*
TMAO + TBF (800)	9.89 ± 1.57	6.32 ± 3.25	27.55 ± 0.92	46.39 ± 1.35*	2.05 ± 0.17**	4.42 ± 0.44**

Values are expressed as means ± standard deviation of 10 mice in each group.

$^{\#}p<0.05$, $^{\#\#}p<0.01$, as compared with the normal group. $^{*}p<0.05$, $^{**}p<0.01$, compared to the TMAO-treated mice.

Fig. 1

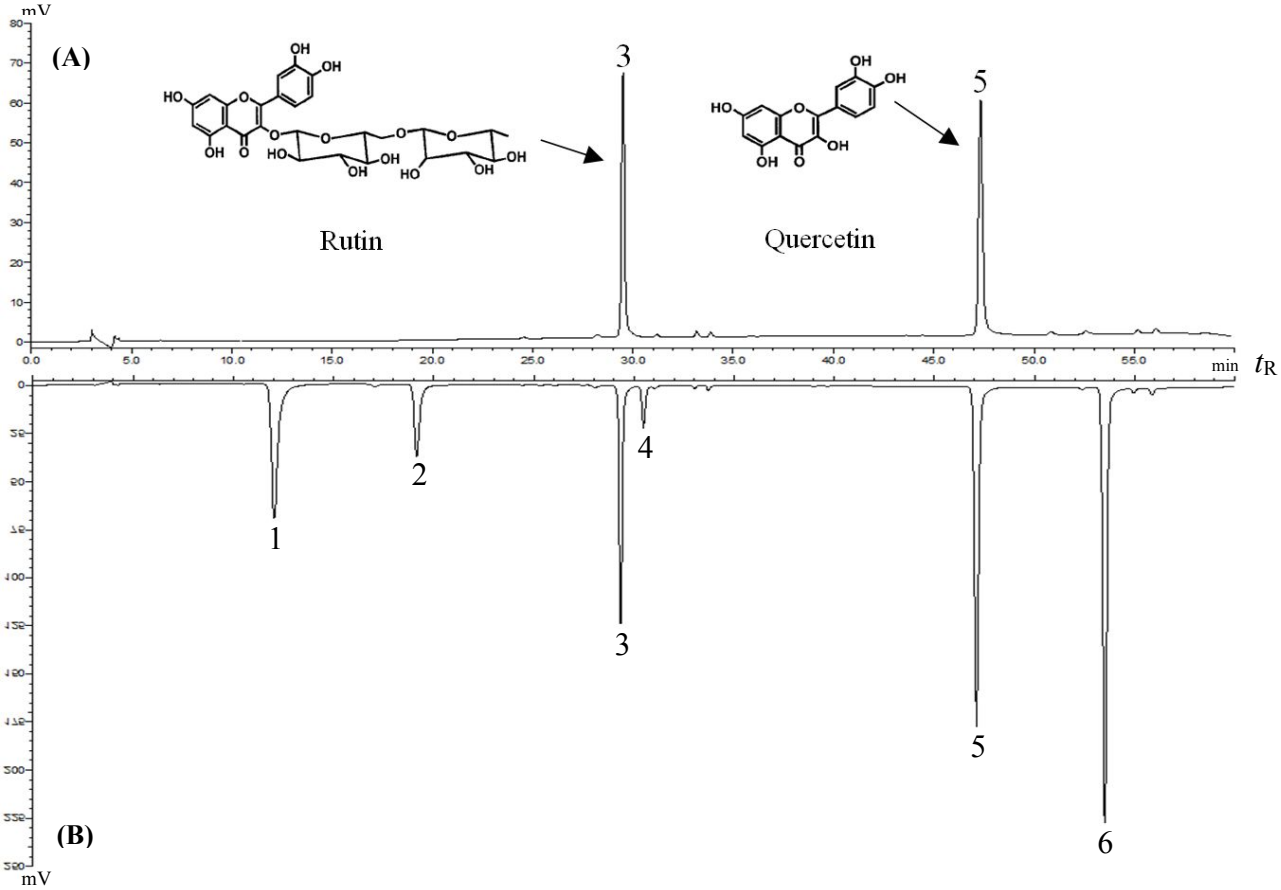


Fig. 2

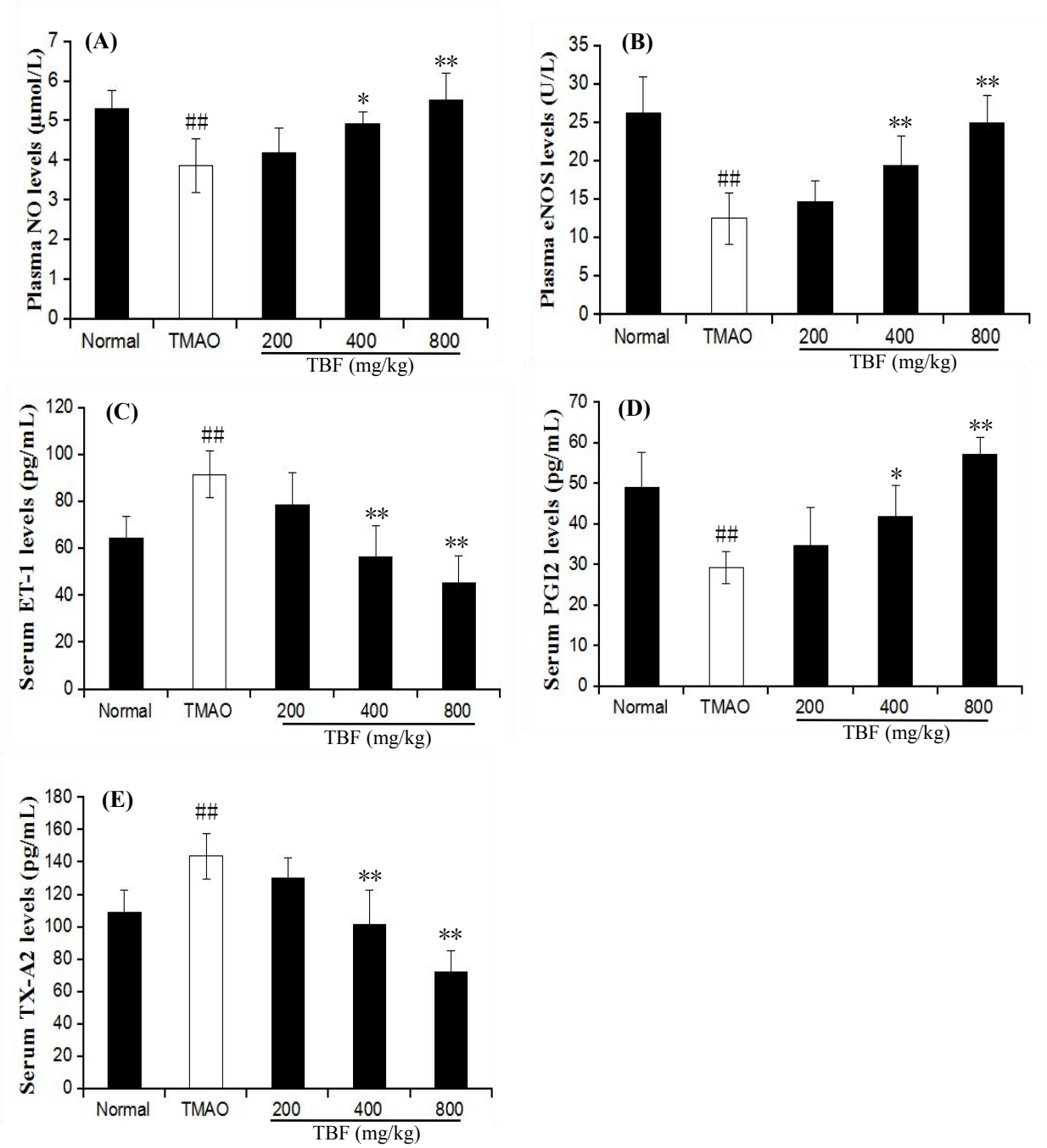
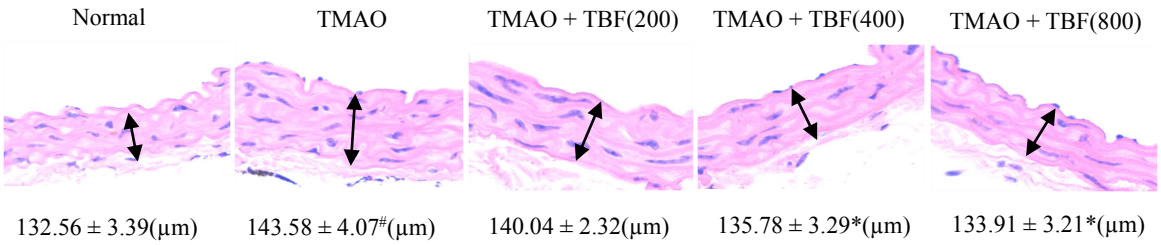


Fig. 3

(A)



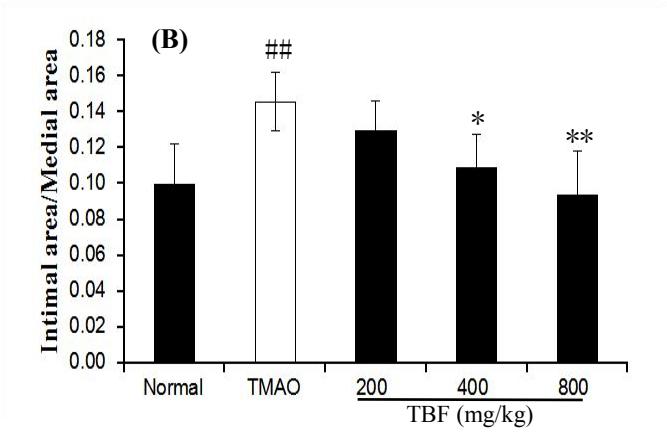


Fig. 4

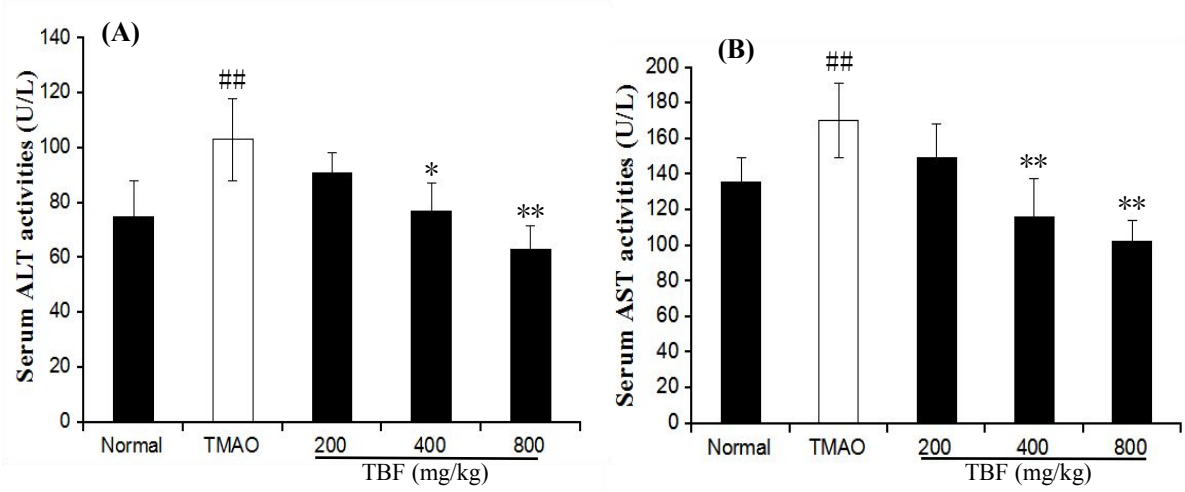


Fig. 5

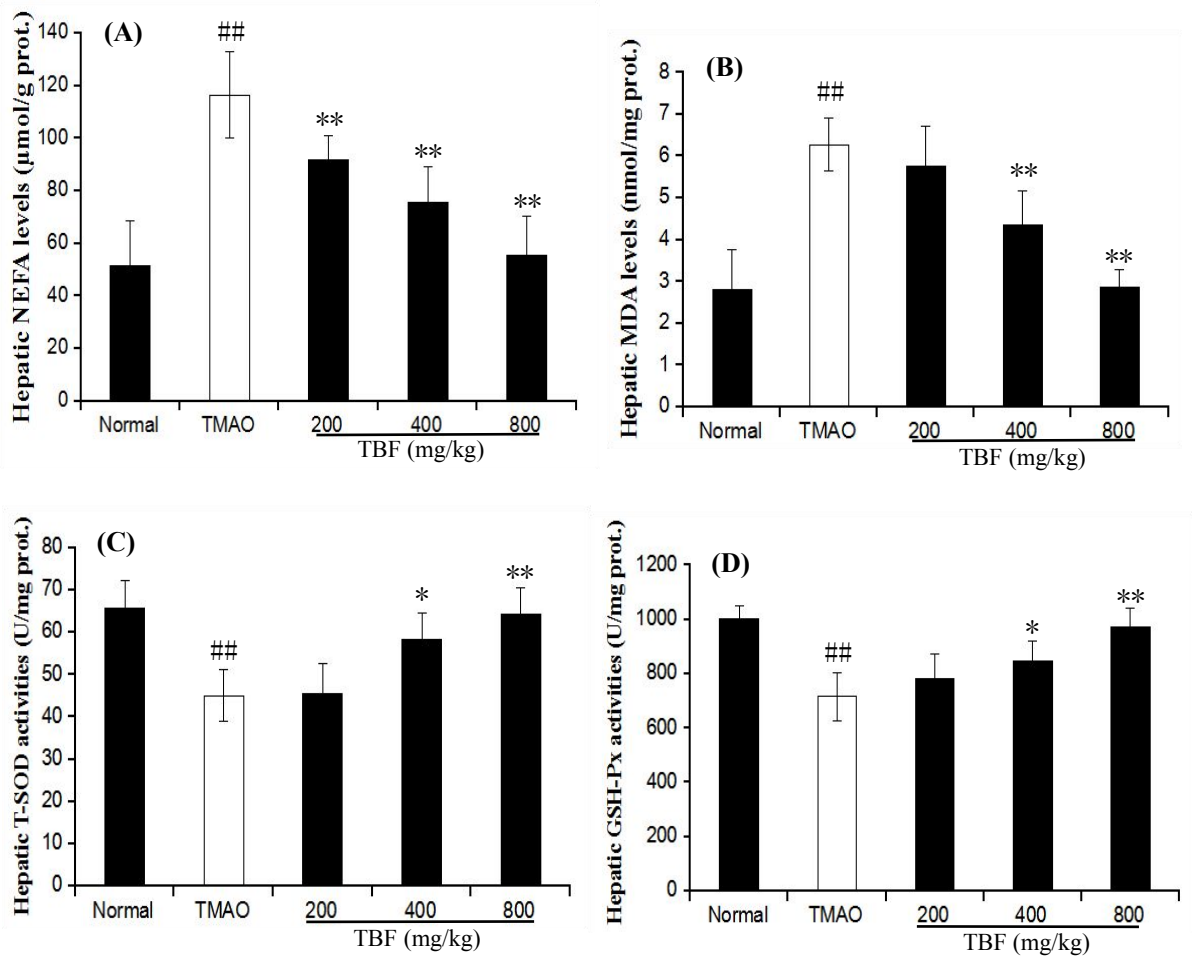


Fig. 6

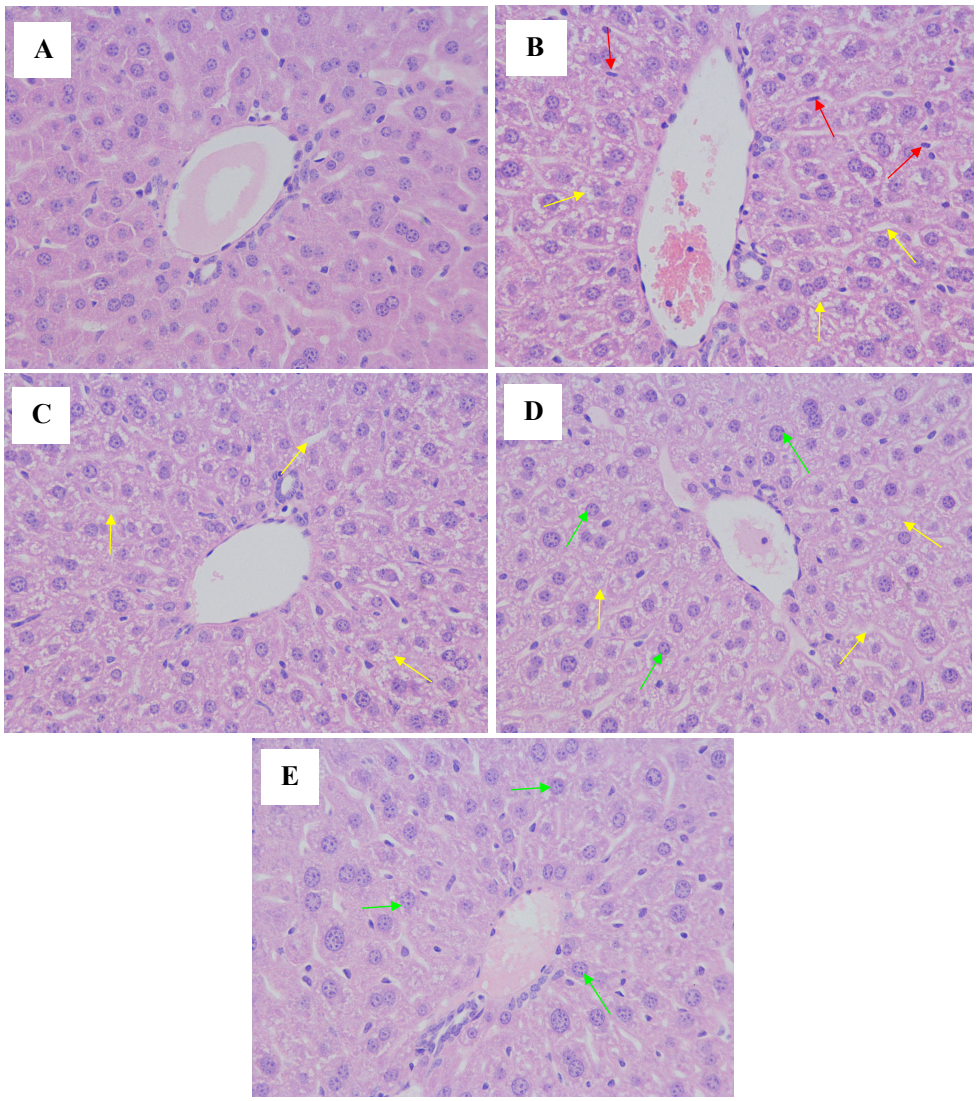
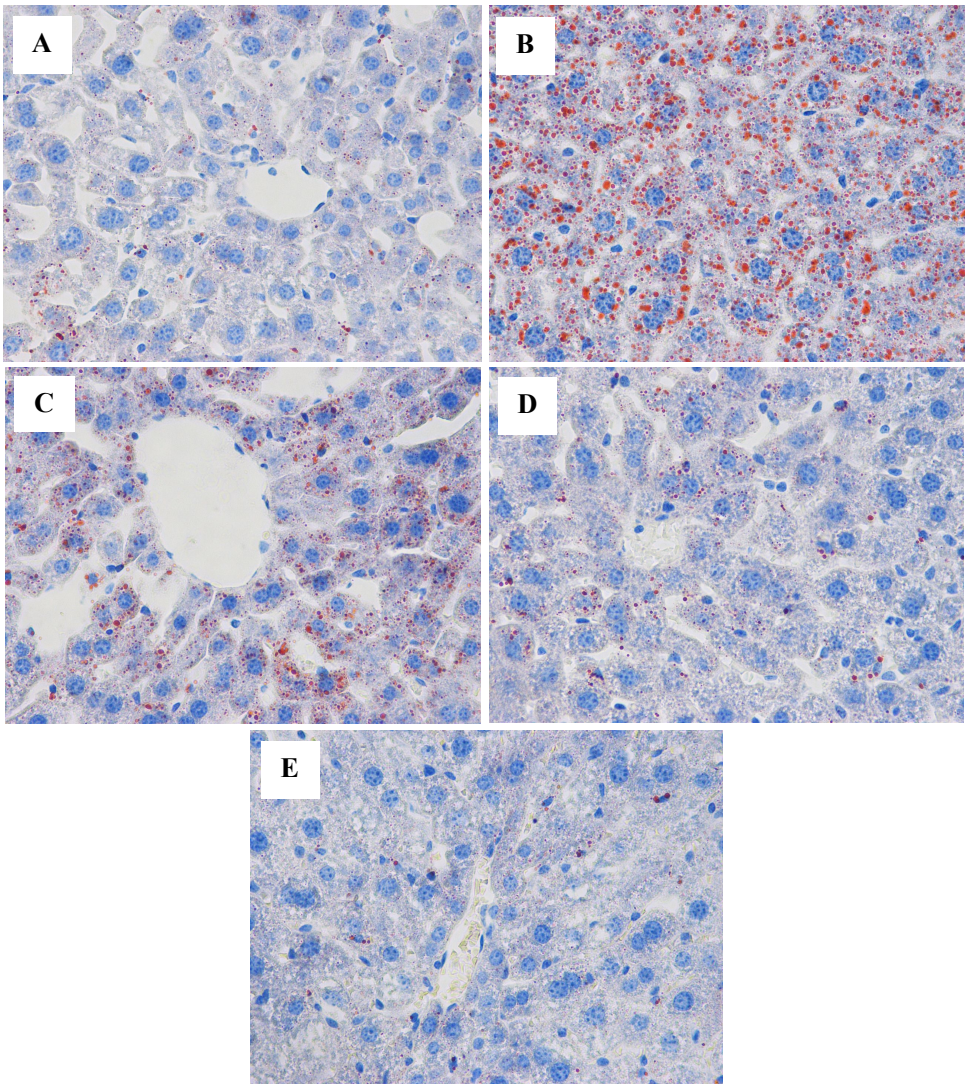


Fig. 7



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