Persimmon Tannin accounts for the anti-hyperlipidemic effects of persimmon through activating of AMPK and suppressing NF-κB activation and inflammatory responses in High-Fat Diet Rats

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Tannin → AMPK

- SREBP1C down
- CPT1 down
- PPARα down
- PGC-1α up
- FAS down
- ACC1 down
- β-oxidation up
- NFκB down

TG down → Inflammation down

Preventing liver steatosis and its progression to NASH
High molecular weight persimmon tannin (HMWPT) was a central component accounting for the anti-hyperlipidemic effects of consuming persimmon fruits. HMWPT attenuate high-fat diet induced hepatic lipid accumulation through activating of the AMPK in liver and suppressed NF-κB activation and inflammatory responses.
Persimmon Tannin accounts for the anti-hyperlipidemic effects of persimmon through activating of AMPK and suppressing NF-κB activation and inflammatory responses in High-Fat Diet Rats

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Abstract

The present study was to investigate whether high molecular weight persimmon tannin (HMWPT) is the main component associated with the anti-hyperlipidemic effect of consuming persimmon and its underlying mechanism. Male wistar rats were given a basic diet (control), a high-fat diet, a high-fat diet plus 0.5% of HMWPT or 4.2% of lyophilized fresh persimmon fruit (with the same diet HMWPT content in the two groups) for 9 weeks. Administration of HMWPT or persimmon fruit significantly (p<0.05) lowered serum triglyceride and free fatty acid, enhanced the excretion of triglyceride, cholesterol and bile acids, and improved hepatic steatosis in rats fed a high-fat diet. Dietary HMWPT or persimmon fruit significantly decreased the protein levels of fatty acid synthase (FAS), and stimulated AMP-activated protein kinase (AMPK) phosphorylation and down-regulated genes involved in lipogenesis, including transcriptional factor sterol regulatory element binding protein 1 (SREBP1) and Acetyl CoA carboxylase (ACC). In addition, the expression of proteins involved in fatty acid oxidation, such as carnitine palmitoyltransferase-1 (CPT-1), were notably up-regulated. Furthermore, HMWPT and persimmon fruit suppressed inflammatory cytokines such as tumor necrosis factor α (TNFα) and C-reactive protein (CRP) and protein level of nuclear factor- kappa B (NFκB) in liver. Taken together, our findings demonstrated that HMWPT reproduced the anti-hyperlipidemic effects of persimmon fruit, and was a pivotal constituent of persimmon fruit accounting for preventing liver steatosis and its progression to nonalcoholic steatohepatitis (NASH) by activation of the AMPK and regulation of its downstream targets, suppressing NF-κB activation and inflammatory responses, inhibiting lipids and bile acids absorption.
Keywords

High molecular weight persimmon tannin (HMWPT); AMP-Activated Protein Kinase; Hepatic steatosis; Fatty acid oxidation; Inflammatory response

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic fat accumulation in the absence of significant ethanol consumption, virus infection or other specific causes of liver disease. NAFLD is closely related to obesity and insulin resistance.\(^1\)\(^2\) It progresses from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis.\(^3\) A long-term excessive intake of fat results in steatosis which increases the susceptibility of the liver to oxidative stress and proinflammatory cytokines, thus triggering the progression to NASH.\(^1\)\(^3\)\(^4\) Currently, there are no valid therapies for NAFLD except for weight loss, which is difficult to achieve and maintain.\(^2\)\(^5\) The novel dietary therapies for the prevention of liver steatosis and its progression to NASH have attracted great attention among researchers.

Persimmon fruit, which contains a large number of components such as condensed tannin, dietary fiber, carotenoids, gallic acid, catechins and flavonoids etc, is reported to exert hyperlipidemia effects in some animal models.\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) But the main components accounting for the hypolipidemic effects of persimmon have not been confirmed yet. Recently, Matsumoto et al.\(^11\) showed that a highly polymerized tannin extracted from persimmon fruit had bile acid binding ability \textit{in vitro} and could promote fecal bile acid excretion \textit{in vivo}. Our previous study\(^12\) also showed that high molecular weight persimmon tannin (HMWPT) could prevent hyperlipidemia and fatty liver in animals fed a high-cholesterol diet. However, to our knowledge, comparison of the hypolipidemic effect between HMWPT and persimmon fruit to confirm whether HMWPT
reproduces the anti-hyperlipidemic effect of persimmon fruit is not available. Moreover, the underlying mechanisms by which persimmon fruits or persimmon tannin exert the anti-hyperlipidemic effect are not clear.

AMP-activated protein kinase (AMPK), a phylogenetically conserved intracellular energy sensor, has been known to be involved in the regulation of lipid metabolism.\(^2\)\(^,\)\(^13\)\(^,\)\(^14\) Once activated, AMPK inhibits lipogenic enzyme activities, such as Acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS).\(^15\)\(^,\)\(^16\) AMPK also mediates lipid metabolism by down-regulating mRNA levels of the transcriptional factor sterol regulatory element binding protein 1 (SREBP1), SREBP-1c regulates the lipogenic process by activating genes involved in fatty acid and triglyceride synthesis.\(^2\)\(^,\)\(^13\)\(^,\)\(^17\) The inhibition of ACC leads to enhancement of mitochondrial fatty acid oxidation.\(^2\)\(^,\)\(^13\) In addition, AMPK activation is known to increase the expression of genes involved in fatty acid oxidation, including peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), peroxisome proliferator-activated receptor α (PPARα) and carnitine palmitoyltransferase-1 (CPT-1).\(^18\)\(^-\)\(^20\) Furthermore, a number of studies demonstrated that AMPK signaling could inhibit the inflammatory responses induced by the nuclear factor-kappa B (NFκB)\(^21\) which is a transcription factor that centrally regulates inflammatory gene expression, and acts as a contributing factor of development of NASH progression.\(^4\) The NFκB can be activated by elevated free fatty acid (FFA), reactive oxygen species (ROS) and inactivated by intracellular antioxidant.\(^4\)\(^,\)\(^7\) It was demonstrated that accumulation of FFA activated NFκB which subsequently increased tumor necrosis factor α (TNFα) and resulted in further liver injury.\(^22\) Rats fed high-fat diet led to an increase of NFκB activity and up-regulating the expression of TNFα in liver and epididymal adipose, thus increasing stepwise from steatosis to NASH.\(^1\)\(^,\)\(^4\) Jou et al.\(^23\) suggested that the steatosis, oxidative stresses and inflammation are interwined and result in
hepatocyte death. Therefore, AMPK activator has been thought to be a novel therapeutic approach for multiple metabolic disorders, such as NAFLD. Some polyphenols, such as (epi)gallocatechin-3-O-gallate (EGCG), resveratrol and theaflavins have been demonstrated to attenuate hepatic lipid accumulation via activation of AMPK. So we presumed that AMPK might be also a central target for anti-hyperlipidemic effect of persimmon fruit or persimmon tannin. Therefore, one aim of the present study is to evaluate if HMWPT is responsible for the anti-hyperlipidemic effect of consuming persimmon, and the other is to investigate whether the effect of HMWPT or persimmon fruit on lipid metabolism are mediated by AMPK and its downstream targets.

2. Materials and methods

2.1 Chemicals and reagents.

Rabbit polyclonal antibodies against AMPKα (Cat. #2532), phospho-AMPKα (p-AMPKα, Thr-172, Cat. #2531) were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody against FAS (Cat. #sc-55580) and rabbit polyclonal antibodies against β-actin (Cat. #sc-1616-R) and Histone H3 (Cat. #sc-8654-R) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against NFκB p65 (Cat. #ab72555) was purchased from Abcam Inc. (Hongkong, China). Commercial kits used for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) and enzyme-linked immunosorbent assay (ELISA) kit for measuring free fatty acids (FFA), insulin, leptin, adiponectin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), C-reactive protein (CRP) and BCA protein were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Commercial kits for determination of serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein
cholesterol (LDL-C) and triglyceride (TG) were from Shanghai Mind Bioengineering Co., Ltd. (Shanghai, China). All solvents and reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and were of analytical grade.

2.2 Samples preparation.

Mature and fully colored fruit of the astringent persimmon (*Diospyros kaki* Niuxin) was harvested in late November from an orchard in Shan’xi province (China). After harvest, fruit was held at 100 °C for about 5 min to inactivate polyphenol oxidase, and then stored deep frozen at -20 °C. The freezing persimmon fruit was cut into slices, lyophilized and then powdered. High molecular weight persimmon tannin (HMWPT) was prepared according to our previous reports.\textsuperscript{12, 27} It was characterized by MALDI-TOF, thiolysis-HPLC-ESI-MS and NMR.\textsuperscript{28} The mean degree of polymerization of HMWPT was estimated to be 26 by thiolysis. The extension units were epicatechin, epigallocatechin, (epi) gallocatechin-3-\textit{O}-gallate, and (epi) catechin-3-\textit{O}-gallate with the relative moles of 2.78, 3.95, 11.0 and 7.58, respectively, and the terminal units were catechin, (epi) gallocatechin-3-\textit{O}-gallate, and myricetin with the relative moles of 0.29, 0.26, and 0.45. The proposed structure was elucidated in our earlier papers.\textsuperscript{28, 29} The content of total polyphenols in HMWPT was 98.7% on a mass basis by Folin–Denis method\textsuperscript{30} using gallic acid as a standard.

2.3 Animals and diets.

All experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the Huazhong Agricultural University of Science and Technology Committee on Animal Care and Use. Forty male wistar rats, weighing 190-230 g, were purchased from the Experimental Animal Center of Disease Prevention and Control of Hubei Province (Wuhan, China). The animals were housed in the temperature and humidity
controlled room (temperature 24±2 °C and humidity 50±10%) with a 12 h light-dark cycle and
given free access to diet and water. After one week of acclimation, the rats were randomly
divided into four groups and were fed different diets: the basic diet group (normal control ,NC;
20.34% protein, 4.14% fat, 50.75% carbohydrates, 12% calories from fat); the high–fat diet
group (HF; 17.70% protein, 16.60% fat, 44.15% carbohydrates, 38% calories from fat), the high–fat
diet plus 0.5% of persimmon tannin group (HF+T), the high-fat diet plus 4.2% of lyophilized
persimmon fruit powder group (HF+P), each group consisting of 10 animals. The 4.2%
persimmon powder was replaced by 3.7% (4.2%) cellulose in HF+T (HF) group. The basic diet
and HF diet was purchased from Chunlong Animal Feed Co., Ltd. (Wuhan China). Rats were
given free access to food and water during the experimental period. Food consumption and body
weight were recorded daily and weekly. Feces were collected at the final 72 h and lyophilized for
analysis. The experiment was terminated after 9 weeks. All animals were fasted 14 h before
anesthetized with diethyl ether and sacrificed. Blood samples were drawn from the ophthalmic
venous plexus. After centrifugation (5000 g, 15 min, 4 °C), the serum samples were collected
and stored at -80 °C. The whole organs were excised, rinsed in ice-cold 9 g/L sodium chloride,
gently blotted on filter paper, weighed and then stored at -80 °C. Portions of liver were collected
into 10% formalin for histologic examination.

2.4 Determination of lipids in serum, liver and feces.

The serum TC, HDL-C and TG were determined by enzymatic colorimetric methods with
commercial kits (Ming, Shanghai, China). The serum free fatty acid was measured using ELISA
kits (Jiancheng, Nanjing, China). Liver tissue and feces (0.5 g) were subjected to lipid extraction
with 10 mL of cold chloroform-methanol (2:1, v/v) by the method of Folch et al., The lipid
extraction was dried under nitrogen at 30 °C on Nitrogen Concentration and the residue was
dissolved in isopropanol. The TC, HDL-C, LDL-C, TG and FFA concentrations of liver and the
TC and TG of feces were analyzed with the same kits as described above. The total lipid of feces
was determined gravimetrically. The fecal cholic acid was extracted with 90% ethanol at 65 °C
for 4 h, and the ethanol extract was evaporated under reduced pressure, neutral lipids were
removed by extracting with n-hexane and the residue was dissolved in 60% acetic acid to
determine the cholic acid concentration according to the method of Duan.

2.5 Hepatic injury, serum insulin, leptin, adiponectin and inflammatory cytokines analysis.
Serum AST, ALT activities were measured by the method of Reitman and Frankel and ALP
activity was measured by the method of King and Armstrong using commercial enzymatic kits
(Jiancheng, Nanjing, China). The concentrations of serum insulin, leptin, adiponectin, TNF-α,
IL-6 and CRP were measured by ELISA according to the manufacturer’s instructions (Jiancheng,
Nanjing, China). Liver tissues were homogenized on ice in a Triton X-100 cell lysis buffer and
centrifuged for 10 min at 4 °C, the supernatant was used for determining TNF-α, IL-6 and
CRP levels using the same kits as described above. The protein concentrations were determined
by BCA protein assay kit (Jiancheng, Nanjing, China).

2.6 Histologic examination of liver.
Small portions of liver were immediately fixed with 10% formalin in water at the time of killing,
dehydrated gradually in a graded series of ethanol, and then clarified in xylene and embedded in
paraffin wax. The morphology of liver was observed with the method of Hematoxylin and Eosin
staining by Nikon Eclipse 80i advanced research microscope (Tokyo, Japan).

2.7 Western Blot Analysis.
Each liver (500 mg) was homogenized in a lysis buffer (5ml, 20 mM Tris-HCl, pH7.4, 150 mM
NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate,
1 mM sodium orthovanadate, 1 mM β-glycerolphosphate, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 2 µg/mL pepstatin, and 1 mM PMSF) and kept for 30 min on ice. After centrifugation at 12000 g for 15 min at 4 °C, the supernatant of the same group was mixed for western blotting. Nuclear extracts from liver were isolated using a separate nuclear extraction kit (Beyotime, Shanghai, China) and the extracts of the same group were combined for determining p65 subunit of NFκB levels by western blotting. Protein concentrations were determined by BCA protein assay kit (Jiancheng, Nanjing, China). For Western blotting, 40 µg protein were separated by 7.5% (FAS, the marker molecular weight from 10 kDa to 250 kDa) or 10% (the marker molecular weight from 14 kDa to 120 kDa) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 25 mM Tris buffer, containing 250 mM glycine and 0.1% SDS and then electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) in 25 mM Tris buffer, containing 192 mM glycine and 20% methanol. The membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies in TBST (AMPKα, 1:1000; p-AMPKα, 1:1000; FAS, 1:1000; NFκB p65, 1:500; β-actin, 1:1000, Histone H3, 1:3000), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:10000) for 45min at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescent reagents (Beyotime, Shanghai, China). The intensity of bands was quantified using Image Lab software (Bio-Rad). The band density of phosphorylated AMPK was normalized to total AMPK protein, the levels of FAS and NFκB were normalized to β-actin and Histone H3.

2.8 Quantitative Real-Time RT-PCR.
Total RNA was isolated from each liver (100 mg) using Trizol reagent (1 ml, Invitrogen; Carlsbad, CA, USA). Reverse transcription was performed with a first-strand cDNA synthesis kit (Toyobo, Osaka, Japan). After cDNA synthesis, quantitative realtime PCR was performed on SLAN PCR system (Hongshi, Shanghai, China) using the SYBR Green PCR Master Mix (Toyobo) according to the manufacturer’s instructions. Reaction mixtures were incubated for an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s. Primers were designed according to the GenBank database using Primer Express software (supplemental table). Expression of mRNA values was calculated using the threshold cycle (C_T) value. For each sample, the ΔC_T sample value was determined by calculating the difference between the C_T value of the target gene and the C_T value of β–actin reference gene. The expression levels relative to control were estimated by calculating ΔΔC_T (ΔC_T sample − ΔC_T control) and subsequently using the 2^{−ΔΔC_T} method.  

2.9 Statistical analysis.

All data were presented as means ± standard deviation (means±SD) and calculated using one-way ANOVA of SPSS 17.0 followed by Tukey’s multiple-range test. Differences were considered significant at P-values<0.05.

3. Results

3.1 Effect of tannin and persimmon fruit on body weight, energy intake and tissues weights.

In order to evaluate if HMWPT is responsible for the anti-hyperlipidemic effects of consuming persimmon, we compared the effect of HMWPT and persimmon fruit on lipid homeostasis of high fat diet rats. As shown in table 1, food intake of rats was reduced in the HF+T and HF+P groups compared with that of the NC group, while no difference was observed among the three high-fat diet groups (HF, HF+T and HF+P). Nevertheless, there was no difference in energy
intake among the four groups during the experiment. Food efficiency ratio was significantly higher in the HF group than that in the NC group, while no significant difference was observed among HF, HF+T and HF+P groups. Besides, high-fat diet induced significant increase in weights of the liver, epididymal adipose and retroperitoneal adipose compared to that of the NC group (Table 1). In contrast, feeding HMWPT or persimmon fruit significantly (P<0.05) decreased the liver mass and slightly lowered adipose tissue weight compared to the HF group (Table 1).

3.2 Effect of HMWPT and persimmon fruit on morphological changes in hepatocytes.

At the end of the experimental period, we examined the effect of HMWPT and persimmon fruit on hepatic morphology. It was indicated that excess of high-fat intake induced a significant high degree of steatosis and inflammatory cell infiltration, accompanied by an apparent accumulation of fat in comparison with normal rats. However, administration of HMWPT or persimmon fruit could markedly prevent the high fat diet induced hepatic steatosis and fat accumulation (Fig. 1). The inflammatory cell infiltration was also alleviated in the livers from rats fed HMWPT or persimmon fruit compared with the animals in HF group (Fig. 1). These results indicated that HMWPT maybe a dominated component in persimmon fruit to exert hypolipidemic effect and inhibit hepatic steatosis.

3.3 Effect of tannin and persimmon fruit on serum and hepatic lipids.

Fig. 2 showed the effect of persimmon fruit or HMWPT on serum and hepatic lipids in rats fed a high-fat diet. It was indicated that high-fat diet induced a significant increase in serum TC, TG and FFA levels, accompanied by a notably decrease in HDL-C level in comparison with the normal rats (p < 0.05). Administration of HMWPT or persimmon fruit reduced serum levels of TG in high-fat diet fed rats by 22.79%, and 24.05%, respectively, compared to the animals in HF
group (Fig. 2A). In addition, treatment with HMWPT or persimmon fruit also significantly
decreased (P<0.05) the serum FFA content and markedly elevated (P<0.05) the levels of serum
HDL-C by 38.46% and 57.14%, separately, compared to the HF group (Fig. 2A).

To test the effects of HMWPT or persimmon fruit on hepatic lipid homeostasis, we next
determined hepatic lipid contents. The rats in HF group had higher levels of TG (P<0.01), TC
(P<0.01) and LDL-C (P<0.01) in liver tissue compared with rats in the NC group (Fig. 2B). As
compared with the HF group, the hepatic TG, TC and LDL-C levels in the HF+T (HF+P) group
decreased significantly (P<0.01) by 36.71% (46.64%), 44.71% (46.53%) and 37.48% (38.76%),
while HDL-C level increased significantly (P<0.01) by 23.99% (46.26%).

3.4 Effect of HMWPT and persimmon fruit on lipids and cholic acid excretion.
The effect of HMWPT or persimmon fruit on lipids and bile acids excretion was analyzed. As
indicated in Fig. 3, total lipids, TG, TC and cholic acid excretion in rats fed a high-fat diet with
HMWPT (persimmon fruit) increased by 1.59 (1.20), 1.73 (1.48), 2.25 (1.50) and 1.49 (1.20)
fold, respectively (P < 0.05), compared to rats fed a high-fat diet.

3.5 Effect of HMWPT or persimmon fruit on hepatic lipid metabolism.
To investigate whether AMPK activation is involved in the effect of HMWPT or persimmon
fruit on lipid metabolism, we examined the total AMPK and the Thr172 phosphorylation of
AMPK in livers of rats through western blotting. As shown in Fig. 4, HMWPT or persimmon
fruit significantly (P<0.05) increased levels of phosphorylated AMPK and the protein level of
phosphorylated AMPK was higher in HF+P group than that in HF+T group. Meanwhile, the
level of FAS was significantly decreased (P<0.05) in HF+T and HF+P groups compared with
that in the HF group (Fig. 4). Gene expression of FAS was also significantly decreased in HF+P
(Fig. 5A). Although the mRNA expression of FAS in HF+T and HF+P groups was higher than
that in NC group, the protein level of FAS was decreased in rats treatment of HMWPT and persimmon fruit.

The effects of HMWPT or persimmon fruit supplementation on the mRNA levels of genes involved in lipid metabolism in the liver of rats were determined by real-time RT-PCR. The lipogenic genes such as SREBP-1C, ACC1 and SCD1 were significantly (P<0.05) suppressed by treatment with HMWPT or persimmon fruit (Fig. 5A). However, the expression of genes involved in fatty acid oxidation including PGC1α, CPT1a, PPARα, CYP4A10 and CYP4A14 in liver were markedly elevated by 1.84, 3.98, 1.80, 1.38 and 1.41 fold in HMWPT fed rats, respectively, compared with the animals in the HF group (Fig. 5B). Persimmon fruit had a tendency of increased the mRNA levels of PGC1α and PPARα, but did not reach the statistical significance. The expression of CPT1a was notably increased by 6.02 fold in persimmon fruit fed rats, compared to those of HF group (Fig. 5B). In addition, the mRNA levels of cholesterol-7α-hydroxylase (CYP7A1) was also up-regulated by HMWPT (Fig. 5C).

3.6 Effect of HMWPT or persimmon fruit on hepatic injury and inflammation.

It was seen that high fat diet induced a significant (P<0.05) elevation in serum AST and ALT levels compared with the normal control group (Table 2). However, administration of HMWPT or persimmon fruit notably (P<0.05) lowered the elevated AST level by 26.30% and 24.80%, respectively, compared with rats in HF group (Table 2). Meanwhile, HMWPT could also significantly decreased serum ALT level (P<0.05). The ALP activity was unaffected by HMWPT or persimmon fruit (Table 2). Additionally, high-fat diet induced greatly increases in serum insulin, leptin, CRP, and hepatic CRP, IL-6, TNF-α levels of rats (Table 2), suggesting inflammation was triggered. HMWPT or persimmon fruit treatment remarkably (P<0.05) decreased the hepatic CRP and TNF-α levels, compared to rats in HF group (Table 2).
Meanwhile, hepatic adiponectin concentration significantly (P<0.05) increased in HF+T or HF+P groups (Table 2). Furthermore, the protein levels of NFκB (p65 subunit) were significantly decreased by treatment with HMWPT or persimmon fruit (Fig. 6). A quantitative real-time RT-PCR analysis showed the expression of Kupffer cell receptor (Clec4f), and Kupffer cells marker genes Cd68, F4/80 (Emr) were significantly increased in the livers of rats fed high-fat diet in comparison to those observed in the livers of the animals fed normal control diet (Fig. 7). However, HMWPT or persimmon fruit treatment was associated with a significant reduction in expression of F4/80 (Emr) and CD68 (Fig. 7).

4. Discussion

The present study was designed to investigate whether HMWPT is the main component associated with the anti-hyperlipidemic effect of consuming persimmon, and the possible underlying mechanisms were also explored. We directly compared the hypolipidemic effect of HMWPT and persimmon fruit (with the same tannin levels) on high fat diet induced NASH rats. Some studies indicated that Wistar rats fed a HF diet for 8 weeks could induce NASH successfully. In our preliminary study, we found that rats fed a high-cholesterol diet for 9 weeks could induced hepatic steatosis and increase alanine aminotransferase activity. Therefore, in present study, we fed the rats with HF diet for 9 weeks. In line with previous observations in other animal models and our previous report, our results demonstrated that both persimmon fruit and HMWPT could significantly (P<0.05) reduce the liver mass, notably (P<0.05) lower the serum TG and FFA concentrations and hepatic TG, TC, LDL-C levels, and remarkably elevate the serum and liver HDL-C levels. Our data also indicated that persimmon fruit and HMWPT could decrease lipogenesis, increase fatty acid oxidation and enhance fecal lipids and bile acids excretion, thus suppressing hepatic steatosis. Furthermore, our data provided
a direct and clear evidence for that HMWPT was responsible for protecting effect of persimmon fruit against NASH for the first time.

In our study, high-fat diet feeding to rats slightly increased body weight gain and significantly raised liver and white adipose tissues compared to the those in NC group, these results maybe due to a higher food efficiency ratio and a lower feces excretion. Interference with intestinal absorption of triglyceride, cholesterol and bile acids leading to an increase in fecal lipids and bile acids excretion is thought to be an important mechanism for the hypolipidemic effect of many compounds.\(^{39}\) Grape seed polymeric tannins were proved to exert hypocholesterolemic effect by impairing the intestinal absorption of cholesterol and bile acids.\(^{40}\) Recently, persimmon tannin has been reported to bind bile acids \textit{in vitro} and \textit{in vivo}.\(^{6, 11}\) In our study, we observed a significantly increases in cholesterol and bile acids excretion in the HF+T and HF+P groups. Real time RT-PCR analysis revealed that HMWPT treatment significantly up-regulated hepatic CYP7A1 gene in rats (Fig.5C), which is the rate-limiting enzyme in the synthesis of bile acid in liver, indicating that elimination of cholesterol from the body by HMWPT could be partially due to the conversion of cholesterol to bile acids.\(^{41}\) Similar results were reported in young persimmon fruit fed mice\(^{8}\) and hamsters administrated with grape seed proanthocyanidin.\(^{41}\) Interestingly, we also observed that HMWPT enhanced fecal triglyceride and total lipids excretion in rats. The results were in agree with persimmon leaf\(^{9}\) and EGCG.\(^{42}\) An increased excretion of dietary lipids by HMWPT could be possibly achieved by inhibition of pancreatic lipase in the intestine, because polyphenols have been reported to interact with proteins such as pancreatic lipase.\(^{43, 44}\) In our study, we found that HMWPT showed stronger pancreatic lipase inhibition than that of grape seed proanthocyanidins \textit{in vitro} (Data not shown).
AMPK is an enzyme that controls lipid metabolism in liver, adipose and muscle. Activation of AMPK switches off fatty acids synthesis by a direct phosphorylation of ACC, and by inhibition of SREBP1c. To further investigate whether the effects of HMWPT and persimmon fruit on lipid metabolism are mediated by AMPK, we first determined the AMPK and Thr172 phosphorylation AMPK in livers in all groups via western blotting. Our results clearly stated that Thr172 phosphorylation AMPK in livers was significantly activated by HMWPT or persimmon fruit treatment (Fig. 4). Although high-fat feeding was reported to suppression of endogenous fatty acid synthesis in liver in some references, some studies showed that high fat diet could enhanced expression of lipogenic genes in liver such as Srebp-1c, FAS and ACC, our results were consistent with these studies. Treatment with persimmon fruit or HMWPT down-regulated the expression of SREBP-1c, ACC1 and SCD1 and decreased protein level of FAS in the liver. Similar results were found in diet-induced obese mice treated with Hibiscus sabdariffa L. aqueous extract. Although the animals fed a high-fat diet with HMWPT and persimmon fruit increased gene expression of FAS compared with that of the NC group, the protein level in HF+T and HF+P groups was lower than rats fed a basic diet. FAS was also regulated by miR-122 at the posttranscriptional level. Grape seed proanthocyanidins was reported to mediate the expression of miR-122 and its target gene FAS. Therefore, the mRNA expression of FAS may be regulated by miR-122 at the posttranscriptional level which result in less protein level in HF+T and HF+P groups than that in NC group. Furthermore, AMPK activation may also lead to increases in fatty acid oxidation and mitochondrial biogenesis. CPT1, the rate-limiting step in the import and oxidation of fatty acids in mitochondria, was regulated by AMPK. In addition, PGC1α (a master regulator of mitochondrial biogenesis and function) and PPARα are known to be mediated by AMPK. Our present study indicated that mRNA levels of CPT1,
PGC1α and PPARα were up-regulated by HMWPT. Similar effects of mangiferin\textsuperscript{20} and resveratrol\textsuperscript{19} were also reported. Furthermore, we determined mRNA expression of CYP4A10 and CYP4A14 (two targets of PPARα) in liver, and observed that they were also up-regulated by HMWPT compared with that in the HF group. Persimmon fruit had a tendency of increased the mRNA levels of PPARα, CYP4A10 and CYP4A14, but did not reach the statistical significance. It has been reported that treating mice with persimmon tannin up-regulated gene expression of PPARα in skeletal muscle and brown adipose tissues.\textsuperscript{11} Some studies suggested that PPARγ is involved in the regulation of liver energy metabolism.\textsuperscript{51, 52} PPARγ agonist rosiglitazone also promotes AMPK activity.\textsuperscript{53} Ethanolic chamomile flowers extract was reported to activate PPARγ and PPARα to prevent high-fat diet fed mice.\textsuperscript{54} The pathophysiology of NAFLD as well as HMWPT and persimmon fruit intervention are based on complicated networks. In this study, one of our goal is to investigate whether the hypolipidemic effect of HMWPT and persimmon fruit are based on AMPK, thus we focused on AMPK and its downstream results of AMPK activation. We did not determine the PPARγ level in the present study, whether PPARγ directly involved in the action of HMWPT needs further research.

Although the role of AMPK in lipid metabolism has been highlighted in recent years and several polyphenolic compounds such as EGCG, resveratrol, theaflavins and S17834 were reported to exert their anti-hyperlipidemic effects by activating AMPK. To our best knowledge, the relationship between the structure of polyphenols and the AMPK activating effect is very limited. In general, plant polyphenols which have been shown to activate AMPK all contain flavone or isoflavone moieties in their structures. Way et al.\textsuperscript{16} compared the hypolipidemic potential of different fractions of Pu-erh tea and found that the fraction enriched with EGCG and ECG significantly activated AMPK of human hepatoma HepG2 cells. Lin et al.\textsuperscript{26} revealed that
among theaflavins, the gallate forms of tea polyphenols such as EGCG and ECG and the non-
gallate forms of tea polyphenols such as C and EC, theaflavins excreted the strongest effects on
reducing the accumulation of triglycerides in human HepG2 cells, and gallate forms of tea
polyphenols showed secondary effect while non-gallate EC and C exhibited no effects against
cellular lipid accumulation. In addition, compared to EGCG, theaflavins, particularly theaflavin-
3-gallate and theaflavin-3,3-digallate had greater effect on accelerating AMPK phosphorylation
in human HepG2 cells. These results suggested that the presence of galloyl moieties within the
structure of proanthocyanidins and the polymerization of flavan-3-ols might be very important
for activating of AMPK. The high content of EGCG and ECG in the extension units of HMWPT
may attribute to its effect on AMPK activation in vivo. Although it is thought that large
polymeric proanthocyanidin (mean degree of polymerization > 3) cannot be absorbed in the
small intestine in their native forms, it would be metabolized by colonic microbiota. Because
the structure of HMWPT is very complex, which structural features are pivotal for the AMPK
activation and its downstream metabolic targets effects remains unclear. In order to fully
understand the in vivo mechanisms associated with AMPK activation effect of HMWPT, the
digestion and absorption mechanism of HMWPT, especially the possible absorbable biologically
active compounds derived from it and the structure-specific activities of HMWPT on AMPK
need further study.

It has been confirmed that exposure to a high-fat diet leads to overproduction of
proinflammatory cytokines such as CRP, TNF-α 1 and IL-6. Jour et al. suggested that steatosis
and inflammation are interlaced in a round feed-forward relationship, which probably increase
the rate of hepatocyte death. Our data suggested that prominent hepatic inflammatory cell
infiltration was observed in rats fed a high-fat diet for 9 weeks (Fig. 1), together with significant
increases of pro-inflammatory mediators such as CRP and hepatic TNFα, IL-6 and CRP in liver tissues (table 2). Since the disordered production of proinflammatory cytokines and the activation of the inflammatory signaling pathways are tightly connected with obesity related metabolic diseases, components that can inhibit pro-inflammatory cytokine production are regarded to be an effective approach for alleviating excess fat induced metabolic disorders.1, 2 Grape seed procyanidins were demonstrated to decrease rat plasma CRP levels, down-regulate CRP mRNA expression in the liver and mesenteric white adipose tissue, and decrease the expression of TNFα and IL-6 in the mesenteric white adipose tissue.56 Our results indicated that treatment of HMWPT decreased the contents of serum free fatty acid and IL-6, as well as hepatic TNFα and CRP levels. Accumulation of lipids, especially FFA, leads to lipotoxicity and imbalance in the production of reactive oxygen species.57 It is known that elevated FFA and ROS can activate NFκB, which has a pivotal role in the transcriptional regulation of gene expression related to tissue injury and infection.4 Activation of NFκB can up-regulate the expression of its downstream proinflammatory genes such as TNFα, IL-6 and CRP,58 hence enhancing inflammatory injury and related lipid metabolic disorder. Many polyphenolic compounds such as green tea extract4 and persimmon oligomeric proanthocyanidin,7 were reported to alleviate inflammatory responses through NFκB signaling pathway. Our data provided convincing evidence for the first time, to our knowledge, that HMWPT mitigated the high fat diet induced NASH through suppression of NFκB (Fig. 6). Multiple studies demonstrated that AMPK signaling can inhibit the inflammatory responses induced by NFκB system.21 However, the NFκB subunits are not direct phosphorylation targets of AMPK, the inhibitory effects of AMPK on NFκB signaling are likely to be mediated by several downstream targets such as PGC1α13, 21 which is associated with inhibition of TNFα induced NFκB activation.59 Our results showed that
the activation of AMPK by HMWPT and followed by up-regulating its downstream PGC1α gene expression may be partially attributed to the inhibition of NFκB.

5. Conclusion

In summary, the present study demonstrated that HMWPT was a central component accounting for the anti-hyperlipidemic effects of consuming persimmon fruit. HMWPT attenuated high-fat diet induced hepatic lipid accumulation and NASF through activating the AMPK and modulation of the downstream protein levels in liver, inhibition of lipids and bile acids absorption in intestine and suppressing NFκB activation and inflammatory responses. Although the molecular mechanisms of HMWPT-mediated activation of AMPK remain to be elucidated, HMWPT shows potential as a natural agent for the prevention and treatment of NASH.

Acknowledgements

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Abbreviations

HMWPT, High molecular weight persimmon tannin; AMPK, AMP-Activated Protein Kinase; NAFLD, Nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ACC, Acetyl CoA carboxylase; SCD1, stearoyl-Coenzyme A desaturase 1; FAS, fatty acid synthase; SREBP1, transcriptional factor sterol regulatory element binding protein 1; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; PPARα, peroxisome proliferator-activated receptor α; CPT-1, carnitine palmitoyltransferase-1; NFκB, nuclear factor- kappa B; FFA, free
fatty acid; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; EGCG, (epi)gallocatechin-3-O-gallate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; IL-6, interleukin-6; CRP, C- reactive protein; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; NC, normal control group; HF, high–fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group; BSA, bovine serum albumin; CYP7A1; cholesterol-7α-hydroxylase; Cyp4a10, cytochrome P450 4A10; Cyp4a14, cytochrome P450 4A14

References


562 **Figures’ Legends**

564 **Fig. 1** Effect of persimmon fruit or HMWPT on hepatic tissue morphology in rats fed a high-fat diet. Tissues were stained with hematoxylin and eosin (10×40). A representative photo from three independent experiments is shown. NC, normal control group; HF, high–fat diet group;
HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group.

The “I” indicates inflammatory cell infiltration, the “L” indicates lipid droplet.

Fig. 2. Effect of persimmon fruit or HMWPT on serum and liver lipids in rats fed a high-fat diet.
(A) Serum lipid concentration. (B) Liver lipid concentration. Results are means ± SD from ten rats per group. NC, normal control group; HF, high–fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; #p<0.05, ##p<0.01 versus HF group.

Fig. 3. Effect of persimmon fruit or HMWPT on fecal lipids excretion in rats fed a high-fat diet. Results are means ± SD from ten rats per group. NC, normal control group; HF, high–fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; #p<0.05, ##p<0.01 versus HF group.

Fig. 4. AMPK Thr-172 phosphorylation (p-AMPK) and FAS status in liver of rats fed a high-fat diet alone with or without persimmon fruit or HMWPT. The total protein extracts of 10 rats in the same group were combined. Equal amounts of liver protein extracts of each group (40 µg)
were separated by 7.5% (FAS) or 10% (AMPK and p-AMPK) SDS-PAGE. Results are means ± SD. A representative blot from three independent experiments is shown. NC, normal control group; HF, high-fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; ##p<0.01 versus HF group.

Fig. 5. Gene expression of lipid metabolism in liver of rats fed a high-fat diet alone with or without persimmon fruit or HMWPT. (A) Expression in liver of SREBP1C and ACC1 of lipogenic genes. (B) Expression in liver of PGC1α, CPT1, PPARα, Cyp4a10 and Cyp4a14 of fatty acid oxidation. (C) Expression of CYP7A1. Results are means ± SD from ten rats per group. NC, normal control group; HF, high-fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; #p<0.05, ##p<0.01 versus HF group.

Fig. 6. The protein level of NFκB in liver of rats fed a high-fat diet alone with or without persimmon fruit or HMWPT. The total protein extracts of 10 rats in the same group were combined. Equal amounts of liver protein extracts of each group (40 µg) were separated by 10% SDS-PAGE. Results are means ± SD. A representative blot from three independent experiments is shown. NC, normal control group; HF, high-fat diet group; HF+T, high-fat plus 0.5%
Fig. 7. Gene expression of Kupffer cell marker genes Cd68, F4/80, and Clec4f in liver of rats fed a high-fat diet alone with or without persimmon fruit or HMWPT. Results are means ± SD from ten rats per group. NC, normal control group; HF, high-fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; #p<0.05 versus HF group.

Table 1. Effect of persimmon fruit or HMWPT on body weight, composition and food intake in rats fed a high-fat diet.

<table>
<thead>
<tr>
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<th>HF+T</th>
<th>HF+P</th>
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<tr>
<td>Initial body weight (g)</td>
<td>251±15</td>
<td>253±12</td>
<td>250±18</td>
<td>257±13</td>
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<td>Final body weight (g)</td>
<td>427±41</td>
<td>452±32</td>
<td>437±39</td>
<td>432±38</td>
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<td>Body weight gain (g)</td>
<td>176±34</td>
<td>199±29</td>
<td>187±31</td>
<td>175±30</td>
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<td>Food intake (g/d)</td>
<td>30.6±5.9</td>
<td>24.1±2.4*</td>
<td>25.3±4.2</td>
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<td>Energy intake (kcal/day/rat)</td>
<td>98.5±19.0</td>
<td>95.6±9.7</td>
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<td>100.4±14.4</td>
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<td>Food efficiency ratio</td>
<td>0.091±0.017</td>
<td>0.131±0.019*</td>
<td>0.117±0.019</td>
<td>0.110±0.019</td>
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<tr>
<td>Liver (g)</td>
<td>11.0±1.3</td>
<td>15.3±2.7*</td>
<td>12.0±1.7#</td>
<td>12.1±1.9#</td>
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<td></td>
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<tr>
<td>Heart (g)</td>
<td>1.11±0.10</td>
<td>1.26±0.14</td>
<td>1.29±0.17*</td>
<td>1.23±0.15</td>
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<td>Kidney (g)</td>
<td>2.60±0.30</td>
<td>2.57±0.17</td>
<td>2.33±0.26</td>
<td>2.40±0.22</td>
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<td>Spleen (g)</td>
<td>0.76±0.12</td>
<td>0.82±0.14</td>
<td>0.82±0.14</td>
<td>0.74±0.11</td>
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<tr>
<td>Epididymal adipose (g)</td>
<td>5.78±1.20</td>
<td>7.67±1.66*</td>
<td>7.04±1.71</td>
<td>6.63±1.61</td>
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<tr>
<td>Retroperitoneal adipose (g)</td>
<td>3.36±0.76</td>
<td>6.09±1.44*</td>
<td>4.67±1.51</td>
<td>5.37±1.81*</td>
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</table>

Results are means ± SD from ten rats per group. NC, normal control group; HF, high–fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. Food efficiency ratio = body weight gain / (food intake × 63d), × 63d because the experiment lasted nine weeks (63d). *p<0.05 versus NC group; # p<0.05 versus HF group.

Table 2. Effect of persimmon fruit or HMWPT on insulin, leptin, adiponectin, Inflammatory cytokines and hepatic injury in rats fed a high-fat diet.

<table>
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<tr>
<td>Serum</td>
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<tr>
<td>Insulin (mU/L)</td>
<td>13.7±2.4</td>
<td>16.6±1.4*</td>
<td>14.9±0.7</td>
<td>13.8±2.0#</td>
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<tr>
<td>Leptin (ng/mL)</td>
<td>7.41±0.73</td>
<td>8.95±0.73*</td>
<td>8.11±0.67</td>
<td>7.73±0.82#</td>
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<tr>
<td>Adiponectin (µg/mL)</td>
<td>6.43±0.79</td>
<td>5.81±0.53</td>
<td>6.00±0.35</td>
<td>7.31±0.54*#</td>
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<td>Parameter</td>
<td>NC</td>
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<td>HF+P</td>
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<tr>
<td>CRP (ng/mL)</td>
<td>843±102</td>
<td>1037±82</td>
<td>938±76</td>
<td>980±93*</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>79.7±7.4</td>
<td>86.6±9.4</td>
<td>77.5±6.0#</td>
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<td>TNF-α (pg/mL)</td>
<td>116±13</td>
<td>121±13</td>
<td>112±8</td>
<td>104±8#</td>
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<tr>
<td>AST (karmen unit)</td>
<td>26.7±6.0</td>
<td>53.6±6.4**</td>
<td>39.5±7.9*#</td>
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<td>ALT (karmen unit)</td>
<td>15.8±3.4</td>
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<td>ALP (King unit/100 mL)</td>
<td>2.97±0.47</td>
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<td>3.74±0.41</td>
<td>3.65±0.60</td>
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<td>liver Adiponectin (µg/mg prot)</td>
<td>1.07±0.13</td>
<td>1.26±0.27</td>
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<tr>
<td>CRP (ng/mg prot)</td>
<td>129±23</td>
<td>248±44 **</td>
<td>177±11*#</td>
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<tr>
<td>IL-6 (pg/mg prot)</td>
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<td>12.12±2.73*</td>
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<td>10.92±2.01</td>
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<td>TNF-α (pg/mg prot)</td>
<td>13.5±1.6</td>
<td>24.5±1.5**</td>
<td>21.4±1.3*#</td>
<td>19.8±1.5*#</td>
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Results are means ± SD from ten rats per group. NC, normal control group; HF, high-fat diet group; HF+T, high-fat plus 0.5% HMWPT group; HF+P, high-fat plus 4.2% persimmon fruit group. *p<0.05, **p<0.01 versus NC group; #p<0.05, ##p<0.01 versus HF group.
Fig. 1.
Fig. 2

A

Serum lipid level (mmol/L)

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<td>FFA</td>
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B

Liver lipid level (µmol/g protein)

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<td>FFA</td>
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NC: Normal Control; HF: High Fat; HF+T: High Fat + Treatment; HF+P: High Fat + Placebo
Fig. 3
Fig. 4.
Fig. 5
Fig. 6
Fig. 7