



Research Article

Citrus peel flavonoids modulate miRNA-PPAR-mediated signalling pathway, a novel mechanism for improving hepatic lipid metabolism in obesity

Ya-Juan Guo^{1,2}, Li-Hong Dong³, Fei Huang³ (✉), Dong-Xiao Su⁴ (✉)

¹ Perfect (Guangdong) Commodity Co., Ltd., Zhongshan 528451, China

² College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

³ Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences, Key Laboratory of Functional Foods, Ministry of Agriculture and Rural Affairs, Guangdong Key Laboratory of Agricultural Products Processing, Guangzhou 510610, China

⁴ School of Chemistry and Chemical Engineering, Guangzhou University, Guangzhou 510006, China

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Abstract: Citrus flavonoids have demonstrated anti-obesity effects. However, the molecular mechanisms behind citrus flavonoid-induced lipid-lowering regulation have not been well elucidated. The refined citrus peel flavonoids (CPFs) contain hesperidin, nobiletin and tangeretin. CPFs ameliorated high-fat diet (HFD)-induced obesity in KM mice by reducing triglyceride and total cholesterol levels both in serum and liver. The RT-qPCR analysis demonstrated that CPFs have a regulatory effect on the genes associated with lipid metabolism. CPFs significantly up-regulated the gene expression levels of carnitine palmitoyltransferase 1a (*CPT1a*), ATP-binding cassette transporter A1 (*ABCA1*) and peroxisome proliferator-activated receptor α (*PPAR α*). However, the mRNA expression level of fatty acid synthase (*FAS*) was down-regulated by CPFs approximately 39.68%. Supplementation of CPFs resulted in a sharp decrease in the expression levels of miR-33 and miR-122, both of which modulated the mRNA expression levels of *CPT1a*, *ABCA1* and *FAS*. The preventative effects of CPFs against dyslipidemia in mice are likely due to modulation of the miRNA and *PPAR α* signaling pathway.

Keywords: phenolics; flavonoid; lipid metabolism; miRNA; hypolipidemic

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1 Introduction

Citrus fruits are widely consumed for their nutritional and health benefits^[1], including antihepatotoxic, antioxidant, antitumor, antispasmodic and lipid-lowering activities^[2-5]. The above health benefits may be linked to citrus flavonoids. Citrus flavonoids comprise primarily flavanones, polymethoxylated flavones and anthocyanin^[6-8]. Experimental studies conducted both *in vivo* and *in vitro* demonstrate the various mechanisms by which citrus flavonoids modulate lipid metabolism. For instance, citrus depressa hayata extracts containing nobiletin and tangeretin have been found to regulate the expression levels of lipogenesis genes in white adipose tissue in mice^[9]. The polymethoxylated flavones reduced serum triglyceride (TG) and cholesterol levels in hamsters fed with fructose by increasing the protein expression of liver peroxisome proliferator-activated receptor α (*PPAR α*) and *PPAR γ* ^[10]. The mechanisms of action of citrus flavonoids lipid-lowering effects were previously focused around lipid-associated associated proteins and mRNAs. However, miRNAs are now an attractive new appealing target for dietary flavonoids in potentially

reducing the risk of lipid metabolism diseases^[11].

An increasing number of miRNAs are now known to regulate lipid metabolism, including miRNA-27, miRNA-33, miRNA-106b, miRNA-122, miRNA-143, miRNA-144 miRNA-370 and miRNA-758^[12,13]. A previous *in vivo* study found that proanthocyanidins from grape seed decreased the liver expression of miRNA-33 and miRNA-122, with a dose-effect relationship observed in rats after 3 weeks of consumption^[14]. It has been reported that ApoE deficient mice, supplemented with nine phenolics, exhibited regulation of over 29 total miRNAs, either up or down^[15]. Moreover, more than 69 miRNAs, including miR-33, were regulated in the liver of ApoE^{-/-} mice following supplementation with hesperidin or naringin^[15]. The data suggest that citrus flavonoids have the potential to regulate the expression of miRNAs.

The lipid metabolism-associated genes, such as fatty acid synthase (*FAS*) and ATP-binding cassette transporter A1 (*ABCA1*), were regulated by miRNA-122 and miRNA-33^[16,17]. Previous studies found that citrus flavonoids modulated the expression of genes associated with lipid metabolism, which

included PPAR α , carnitine-palmitoyl transferase 1 (CPT-1), and FAS^[18,19]. By modulating the miRNAs' expression levels, citrus flavonoids could introduce a new mechanism of action. Therefore, the objective of this investigation is to examine the hypolipidemic impact of flavonoids found in citrus peel *in vivo* by adjusting the lipid metabolism associated miRNAs and activating the PPAR α signalling pathway.

2 Materials and methods

2.1 Extraction, identification and quantification of citrus peel flavonoids

Commercial mature ponkan mandarin was manually washed and decorticated, and then hot air-dried at 70 °C and smashed. After that, the resulting powder was extracted with 70% aqueous methanol^[20]. The concentrated extract obtained after evaporation was subjected to purification by a column of AB-8 resin (Cangzhou Bonchem Co., Ltd., Cangzhou, China) as previously described^[21]. Finally, the extract was refined using an octadecylsilyl silica gel column with 80% methanol. The citrus peel flavonoids (CPFs) were obtained by concentrating and lyophilizing the collection.

The refined CPFs were identified and quantified by Agilent 1260 Infinity HPLC (Agilent Technologies, CA, USA) with a diode array detector (DAD). Chromatographic separations were carried out as a previously reported method^[22]. Quantification of CPFs using standards was performed with the corresponding standard's calibration equation. The standard substances, hesperidin, nobiletin and tangeretin were available from Sigma-Aldrich.

2.2 Animal experiments

Male KM mice ($n = 30$, 8 weeks) were acquired from Guangdong medical laboratory animal center (Foshan, China). The mice were raised in an environment maintained at (24 ± 2) °C and $(55 \pm 5)\%$ humidity, with a 12 h bright and dark photocycles, and were provided with ad libitum access to water and rodent chow for one week preceding the experiment. Following this, the mice were randomly assigned to one of three groups (10 mice/group). The control diet (CD) group mice were given a normal-fat diet (D12450H diet, Research Diets, Inc.). The obesity model and dose groups of mice were fed a 45% kcal from high-fat diet (HFD) (D12451, Research Diets, Inc.). Animals were maintained on these diets for 10 weeks. During this time, the dose group was administered with 500 mg/kg of CPFs by oral administration consecutively 10 weeks, while the CD and HFD groups were administered with deionized water. The body weights (BW) of the mice were measured at 5-day intervals. Thereafter, the animals were anesthetized after fasting (12 h) and then blood and liver were harvested immediately.

The animal experiments were treated in according to the laboratory animal study basic guidance of national standards (GB 14925–2010) and endorsed by the Ethics Committee on Animal Experiments Committee of Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences (approval number: 00213903).

2.3 Liver histological analysis

Fixed liver specimens were incorporated into paraffin and stained with hematoxylin and eosin (H&E) for histological

analysis. Changes have been observed and photographed using a microscope (Carl Zeiss, Germany).

2.4 Hepatic and serum lipid analysis

The concentrations of TG and total cholesterol (TC) in the liver and serum, as well as the serum concentrations of low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were measured with commercially available test kits purchased from Applygen Technologies Inc. (Beijing, China).

2.5 RNA preparation

The miRNA and RNA from the hepatic tissues were isolated using the mi/mRNA kit SNC50 (Sigma-Aldrich, USA) in accordance with the manufacturer approach. The quality and concentration of RNA were measured using a spectrophotometer (Thermo Scientific, USA).

2.6 RT-qPCR determination of miRNA expression of lipid genes

The levels of miR-33 and miR-122 were measured on a Real-Time PCR (Bio-Rad, USA) with miRNA kit (Thermo Fisher Scientific, USA). The reaction mixtures were added into a 96-well plate and determined according to a previously published procedure^[23]. The relative miRNAs concentrations were normalized to RNA U6 and calculated following the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{\text{treated samples}} - \Delta Ct_{\text{untreated controls}}$ and $\Delta Ct = Ct_{\text{miRNA}} - Ct_{\text{U6}}$.

2.7 mRNA expression of lipogenic genes

The expression of lipogenic mRNA genes, PPAR α , CPT1a, ABCA1 and FAS was determined by RT-qPCR. RT-PCR was applied using specific probes and Universal Master Mix II in accordance with manufacturing protocols. The transcription levels of the above mRNAs were standardized to the endogenous control cyclophilin (PPIA) and calculated with the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = \Delta Ct_{\text{treated samples}} - \Delta Ct_{\text{untreated controls}}$ and $\Delta Ct = Ct_{\text{mRNA}} - Ct_{\text{PPIA}}$.

2.8 Statistical analysis

Data are presented as the means $\pm$ standard deviation. Differences between groups were analysed using a One-way ANOVA and Tukey's test using SPSS 24.0 statistical software with $P < 0.05$ as statistically significant.

3 Results

3.1 CPFs administration improved lipid parameters in HFD-fed mice

The refined CPFs were identified and quantified by HPLC containing hesperidin (49.33%), nobiletin (16.96%) and tangeretin (12.05%) (Fig. 1). Refined CPFs (500 mg/kg) were administered orally to mice for 10 consecutive weeks.

The average BW of the HFD group significantly surpassed that of the CD group after 4 weeks ($P < 0.05$) (Fig. 2). Following a 10-week intervention involving CPFs supplementation, the CPFs group's weight gain resulting from HFD consumption reduced significantly in comparison to the HFD group. The groups exposed to HFD, with or without CPFs, exhibited similar food intake levels.

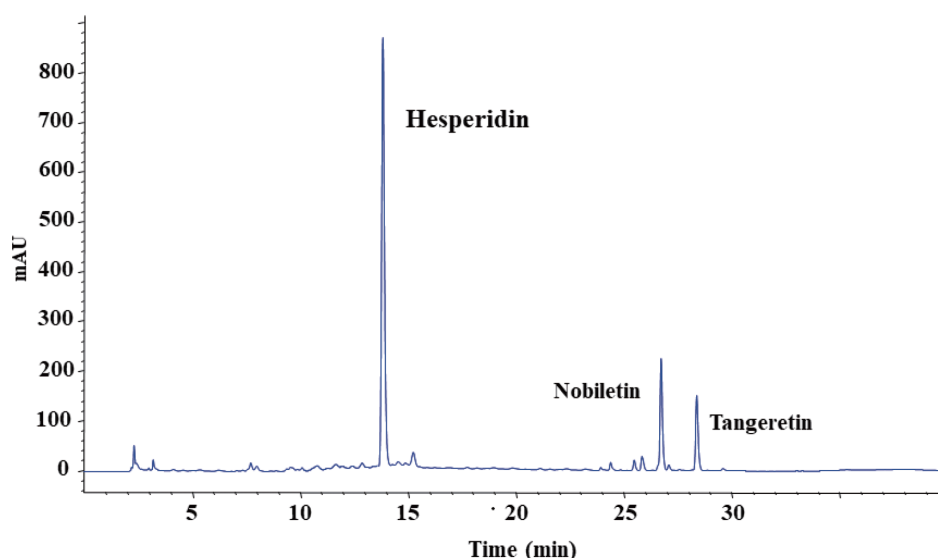


Figure 1 Chromatogram for analysis of flavonoids in citrus peel from China using HPLC.

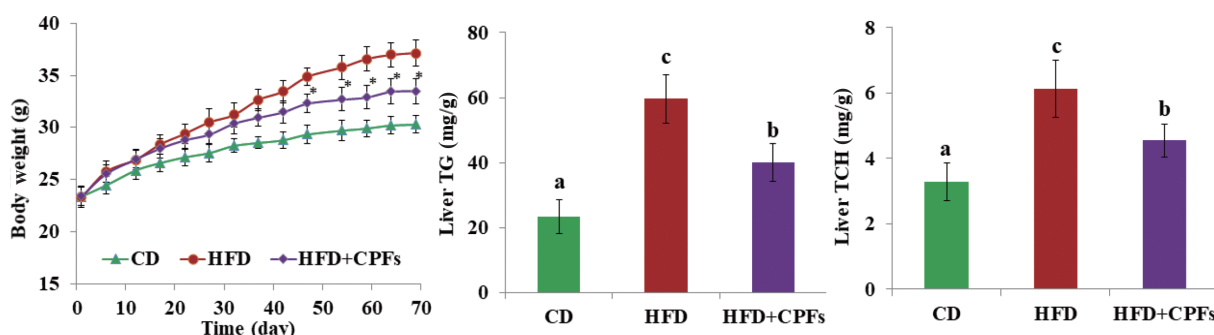


Figure 2 Effects of CPFs administration on lipid content of mice liver. Data is provided in mean $\pm$ SD ($n = 10$). The bars with various letters are noticeably different ($P < 0.05$) according to Tukey's test.

Lipid levels in serum and hepatic tissue are indicated in Table 1 and Fig. 2. After administration of HFD 10 weeks, significantly higher concentrations of TG, TC, LDL-C and HDL-C in plasma were found in the plasma of the HFD group compared to the CD group. However, the administration of CPFs greatly decreased dyslipidemia in the HFD-fed mice, resulting in decreased concentrations of TC and TG and LDL-C in the serum (Table 1). Additionally, the HDL-C level was considerably increased by CPFs ($P < 0.05$) when compared to the CD group. These findings demonstrate that CPFs could effectively ameliorate diet-induced obesity.

Table 1 Effects of CPFs on serum lipid contents (mmol/L) of KM mice fed an HFD.

Indicator	CD	HFD	HFD+CPFs
TG	0.73 ± 0.06^a	1.32 ± 0.05^c	1.03 ± 0.06^b
TCH	2.79 ± 0.14^a	3.87 ± 0.24^c	3.18 ± 0.12^b
HDL-C	1.34 ± 0.15^a	1.68 ± 0.27^b	1.83 ± 0.25^b
LDL-C	0.97 ± 0.15^a	1.53 ± 0.19^c	1.21 ± 0.11^b

Note: Data are presented as the mean $\pm$ SD ($n = 10$); the values within each column with different letters are significantly different ($P < 0.05$).

3.2 CPFs administration alleviated steatosis in the liver of mice fed an HFD

Representative histopathological observation of H&E stained

hepatic tissue sections was conducted to further confirm the above biochemical analysis outcomes (Fig. 3). Normal hepatic architecture was visible during microscope observation in CD-fed mice with regular cellular morphology and no pathologic change. However, cytoplasmic vacuolation, fatty degeneration and hepatocellular hypertrophy were observed after a 10-week HFD intake treatment. Numerous large fat vacuoles were scattered throughout the cytoplasm, along with small diffuse fat vacuoles. The administration of CPFs helped alleviate the hepatic histopathological changes caused by HFD. Although small vacuoles were still present in many hepatic cells, no large lipid vacuoles were observed, and hepatocyte hypertrophy was reduced. This indicates that CPFs have a beneficial impact on liver steatosis. The quantitative analysis of liver lipids revealed that HFD-fed mice who received CPFs had lower TG and TC levels than those who did not receive them (Fig. 2). The administration of CPFs significantly suppressed lipid accumulation in the liver.

3.3 CPFs suppressed miR-33 and miR-122 implicated in PPAR α signaling pathway

To better comprehend the molecular mechanisms underlying the hypolipidemic effects of citrus peel flavonoid compounds' hypolipidemic effects *in vivo*, we evaluated the miR-33 and miR-122 expression in the liver *via* qPCR (Fig. 4). A 10-week HFD supplementation increased miR-33 expression by roughly 61.17% ($P < 0.05$) compared to the CD group. Moreover, miR-122

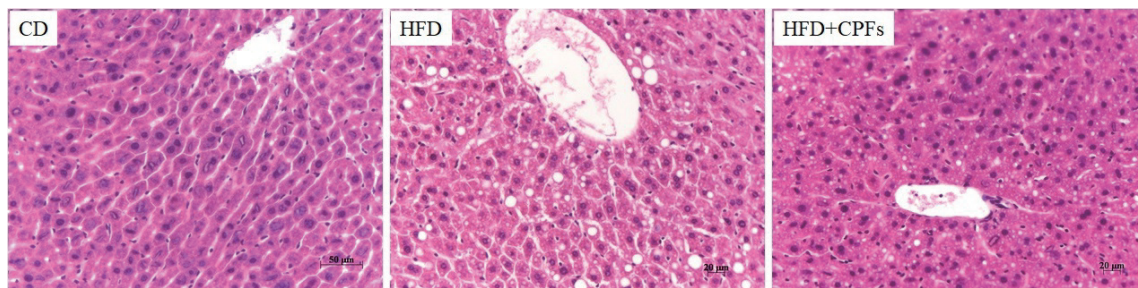


Figure 3 Effect of CPFs treatment on liver histopathological alterations.

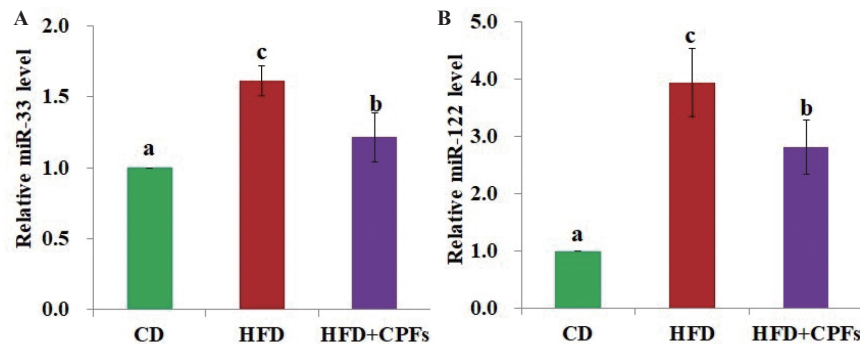


Figure 4 Effects of CPFs supplement on the expression levels of miR-33 and miR-122 genes involved in hepatic lipid metabolism. Bars with different letters are significantly different ($P < 0.05$).

expression levels in HFD-fed mice increased notably by 2.93-fold. Nevertheless, once supplemented with CPFs, the levels of both miR-33 and miR-122 declined. The level of miR-122 significantly decreased by 28.37% when compared to the HFD group. Additionally, the CPFs caused a significant decrease in the miR-33 expression level, which decreased by 24.71% in comparison with the HFD group.

3.4 CPFs regulated hepatic lipid metabolic genes expression

In order to ascertain whether the lipid-lowering impact of CPFs was linked to the regulation outcome of lipid-related metabolic mRNA expression, an assessment was carried out to determine the expression level of *ABCA1*, *CPT1a* and *FAS* in the livers of mice *via* qPCR. The HFD substantially increased *FAS* and *ABCA1* hepatic mRNA expression levels, while the *CPT1a* mRNA expression level remained unchanged ($P > 0.05$) as illustrated in Fig. 5. However, following a 10-week administration of CPFs the *CPT1a* was obviously up-regulated ($P < 0.05$). But CPFs led to a decrease of approximately 39.68% in *FAS* mRNA expression compared to the HFD group, although the level of *FAS* remained higher than in the CD group. Regarding the expression level of *ABCA1*, it was repressed by HFD; however, CPFs supplementation could activate this gene expression, and the effect was significant ($P < 0.05$). Furthermore, it was found that by supplementing HFD, the expression of the *PPARα* gene was significantly activated, up-regulated by approximately 41.03% compared to the CD group. In addition, administration of CPFs further improved this gene expression, up-regulating by approximately 33.97% when compared to the group of HFD fed mice. The above observations indicate that the preventive effects of citrus peel flavonoid compounds against dyslipidemia induced by HFD in mice are likely to occur with improved *PPARα* signaling.

4 Discussion

4.1 Citrus flavonoids improve parameters related to obesity

Dietary citrus flavonoids have demonstrated positive effects on cardiovascular diseases^[24]. The lipid-lowering capacity was determined based on their molecular structures. Nobiletin, tangeretin and 5,7,3',4'-tetramethoxyflavone were identified as the prominent polymethoxylated flavonoids (PMFs)^[25]. These PMFs are known to inhibit TC and TG synthesis^[26]. Citrus peel extract, which contains over 27% of nobiletin, reduced serum lipid content and inhibited hepatic histopathology in mice^[27]. Citrus flavonoids, nobiletin, tangeretin and hesperidin, which were used in the present study, are accountable for regulating lipid metabolism. These findings are consistent with previous studies^[9].

4.2 Citrus flavonoids modulate lipid metabolism related mRNA genes expression

Multiple mRNA genes are involved in regulating lipid metabolism. Previous *in vivo* studies have confirmed that citrus flavonoids could modulate gene expression related to lipid metabolism-induced by diet. Citrus polymethoxylated flavones, mainly tangeretin and nobiletin, could ameliorate the fructose-induced hypertriglyceridemia in hamsters^[10]. reported that these polymethoxylated flavones exerted lipid-lowering activity by significantly increasing *PPARα* and *PPARγ* expression in the liver. The current study used a mixture of nobiletin, tangeretin and hesperidin. The expressions of *ABCA1*, *CPT1a* and *PPARα* were raised and *FAS* was reduced simultaneously by citrus flavonoids supplements, which is consistent with the above results^[10,18]. A previous study found that improving the expression level of *PPARα* can enhance lipid clearance through elevated β -oxidation^[19]. Additionally, hormonal expression, is also

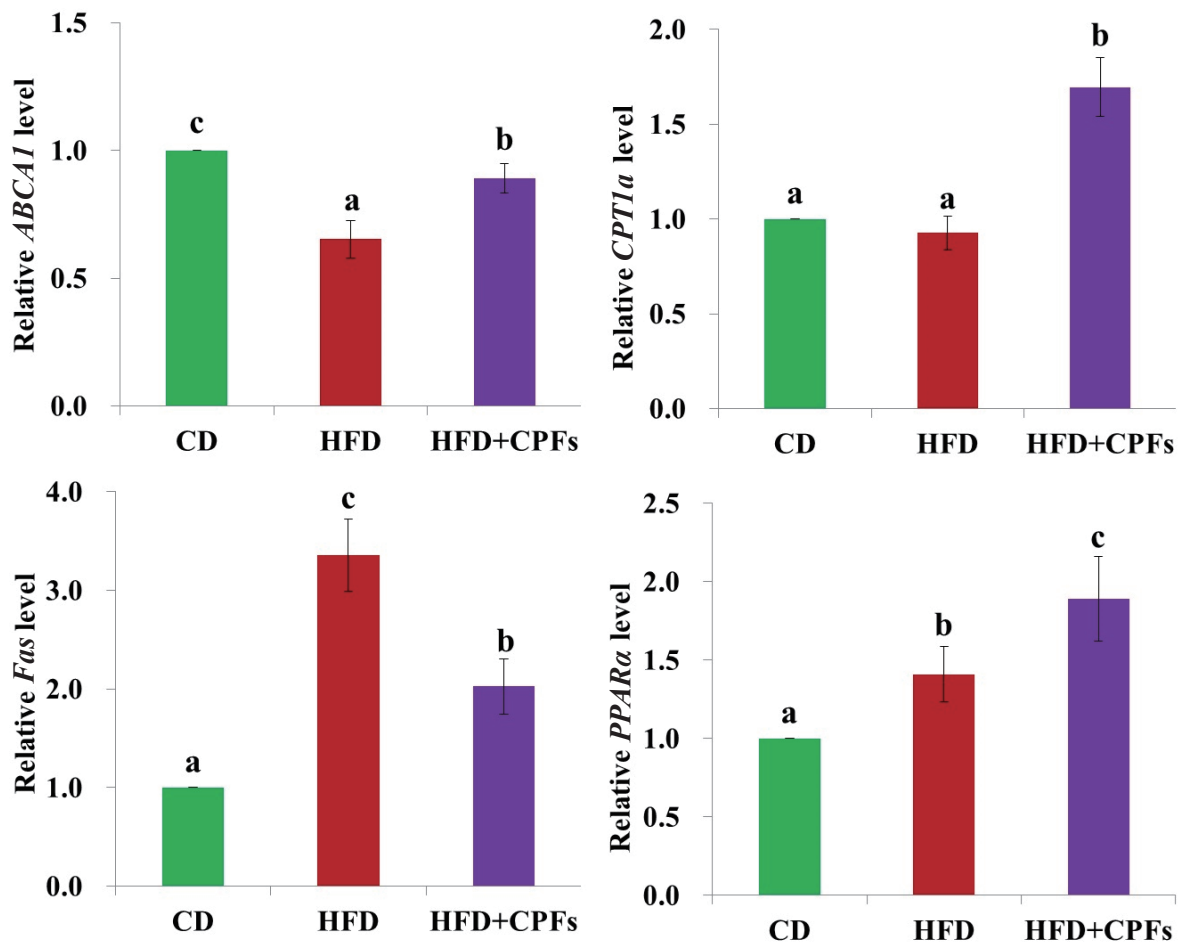


Figure 5 Effects of CPFs supplementation on the expression levels of mRNAs involved in hepatic lipid metabolism. Bars with different letters are significantly different ($P < 0.05$).

associated with lipid metabolism regulation. However, a research reported that citrus extracts from unshiu peel, specifically hesperidin at a dosage of 300 mg/kg for 8 weeks, can improve lipid metabolism without affecting hormone levels^[28]. This suggests that citrus flavonoids may exert lipid-lowering effects through the PPAR α pathway by regulating the expression of genes involved in lipid metabolism, rather than through hormone mechanisms.

4.3 Citrus flavonoids modulate lipid metabolism related miRNA genes expression

Phenolic compounds regulate the expression of microRNAs. miR-122 modulated FAS, and miR-33 regulated CPT1a, which is involved in fatty acid β -oxidation^[29]. In hamsters, citrus PMFs decreased the expression of SREBP1c, FAS, and PPAR α ^[30]. In the present study, miR-33 and miR-122 expressions were reduced compared to model group in mice after treatment with CPFs. Grape seed proanthocyanidin extracts were found to repress the expression of miR-122 and its indirect target FAS mRNA^[31]. A previous study confirmed that low miR-33 expression in mouse liver cells increases the expression of both ABCA1 and CPT1a expression^[29]. While ABCA1 mRNA is directly modulated by miR-33, CPT1a is adversely correlated with miR-33^[32]. Similar results were produced by lychee pulp flavonoids^[23]. The modulation of miR-122 and miR-33 may be responsible for the *in vivo* hypolipidemic activity of CPFs.

Lipid metabolism is a complex network involving multiple genes and proteins. PPAR α expression is regulated by different

miRNAs^[33], and ABCA1 is also regulated by other miRNAs^[34]. Additionally, the molecular structure of methylation should also be concerned. Research has shown that methylated catechins (0.2% for 6 weeks) are more effective in lowering lipids in mice than non-methylated catechins^[35]. Further *in vivo* investigations are necessary to take into consideration the bioavailability of citrus flavonoids.

5 Conclusions

Citrus flavonoids, including nobiletin, tangeretin and hesperidin, have been shown to modulate lipid metabolism in mice. The expression of miR-33 was down-regulated after CPFs supplementation, which changed the expression of its direct target ABCA1 mRNA and its indirect target CPT1a mRNA. These genes are involved in cholesterol transport and fatty acid β -oxidation, respectively. PPAR α increases lipid clearance through elevated β -oxidation and its gene expression level was markedly up-regulated by CPFs. Additionally, the down-regulation of miR-122 affected FAS, which is associated with fatty acid synthesis. Thus, the modulation of miR-122 and miR-33, which are involved in the PPAR α signaling pathway, represents a novel hypolipidemic mechanism of citrus flavonoids.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

Acknowledgments

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