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Chrysophanol ameliorates high-fat diet-induced obesity and inflammation in neonatal rats

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Chrysophanol is a member of the anthraquinone family abundant in rhubarb, a widely used herb for obesity treatment in Traditional Chinese Medicine. Though several studies have indicated numerous features of chrysophanol, no study has yet reported the effect of chrysophanol on juvenile obesity. In this study, we tried to identify the anti-obesity effects of chrysophanol by using high-fat diet (HFD)-induced rats as *in vivo* models. In HFD rats, chrysophanol treatment decreased body weight, blood glucose and the blood level of triglyceride (TG), and enhanced the level of high-density lipoprotein-cholesterol (HDL-C). In addition, chrysophanol markedly reduced lipid accumulation in HFD rats-derived primary hepatocytes. Moreover, chrysophanol effectively relieved HFD-induced inflammation, as demonstrated by the reduction of interleukin (IL)-6 and IL-1β and the elevation of IL-10. Furthermore, chrysophanol markedly increased the levels of lipolytic genes and decreased the expressions of lipogenic genes in HFD rats, which was probably benefited from the activation of AMP-activated protein kinase (AMPK)/ Sirtuin 1 (SIRT1). Taken together our study has demonstrated that chrysophanol could improve the HFD-induced obesity and provided a molecular basis for chrysophanol potential applications in the treatment of juvenile obesity and other metabolic diseases.

1. Introduction

Overweight and obesity in childhood and adolescence are among the the greatest challenges of healthcare systems worldwide. Metabolic syndrome (MetS), which can be understood as a combination of obesity and metabolic disorder, occurs in 3.3% of the general population but 11.9% in overweight children (Cárdenas Villarreal et al. 2009; Friend et al. 2013). It seems that reduction of juvenile MetS is the most crucial task in the prevention of juvenile obesity. In a wider sense, therapy of juvenile obesity can in turn act as prevention for the resulting MetS. Until today, there does not exist a straightforward or infallible approach to treat obesity because the integrated network of obesity is influenced not only by genetics but also by circadian rhythms, as well as physical and social environments (Pospisilik et al. 2010). Fortunately, in recent years, more and more natural products from Traditional Chinese Medicine have been reported to have anti-obesity effects, such as berberine, resveratrol and magnolol (Guo et al. 2008; Jeon et al. 2012; Zhang et al. 2014). This provides a new perspective in prevention and treatment of juvenile obesity.

The AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine protein kinase. It acts to simultaneously shutdown ATP-consuming biosynthetic processes and facilitate ATP-producing catabolic processes during periods of metabolic stress, leading to rapid changes in the control of fatty acid metabolism. Thus, AMPK is a key player in energy homeostasis (Kim and Choung 2012). In addition, AMPK-mediated activation of sirtuin 1 (SIRT1) regulates lipid metabolism and inflammation (Chen et al. 2012). It is reported that AMPKα deficient mice displayed enriched ROS generation and accelerated degradation of I-κB, resulting in excessive upregulation of NF-κB and consequent NADPH oxidase activation (Wang et al. 2010).

Chrysophanol (1,8-dihydroxy-3-methyl-9,10-anthraquinone) is an active compound originally extracted from rhubarb and belongs to the anthraquinone family. According to previous studies, chrysophanol exerts a number of pharmacological effects, including anticancer, anti-diabetic, anti-deppressive, anti-inflammatory and hepatoprotective activities (Lee and Sohn 2008; Zhang et al. 2014; Kai et al. 2015; Choi 2016), for which the underlying mechanisms remain to be elucidated. As several studies have indicated that obesity is associated with a low-grade pro-inflammatory state (Weisberg et al. 2003; Hajer et al. 2008), the anti-inflammatory feature of chrysophanol suggests its potential use of in obesity-related issues. In addition, several studies reported the use of rhubarb in obesity care (Amin and Nagy 2009; Tseng et al. 2010; Zhou et al. 2014). However, the anti-obesity characters of chrysophanol are not well explored.

In this study, we investigated the anti-obesity effect of chrysophanol using HFD-induced obese rat models. We specially selected neonatal Sprague Dawley (SD) rats to imitate juvenile obesity as much as possible. In addition, the role of inflammation and AMPK signaling in chrysophanol-mediated protective effect was further explored.

Abbreviations: HFD, High-fat diet; TG, Triglyceride; HDL-C, High-density lipoprotein-cholesterol; IFN-γ, Interferon-γ; IL-17, interleukin-17; IL-4, interleukin-4; AMPK, AMP-activated protein kinase; SIRT1, Sirtuin 1; MetS, Metabolic syndrome; ACC , Acetyl-CoA carboxylase; FAS, Fatty acid synthase; SREBP-1, Sterol regulatory element-binding protein-1; PPARα, Peroxisome proliferators-activated receptor α; CPT-1, Carnitine palmitoyl transterase-1; ACOX1, Acyl-coenzyme A oxidase 1; PGC-1α, Peroxisome proliferator-activated receptor γ coactivator 1α.

2. Investigations and results

2.1. Chrysophanol improves HFD-induced obesity in neonatal SD rats

A HFD rat model was used to investigate the potential benefits of chrysophanol. The results showed that HFD significantly

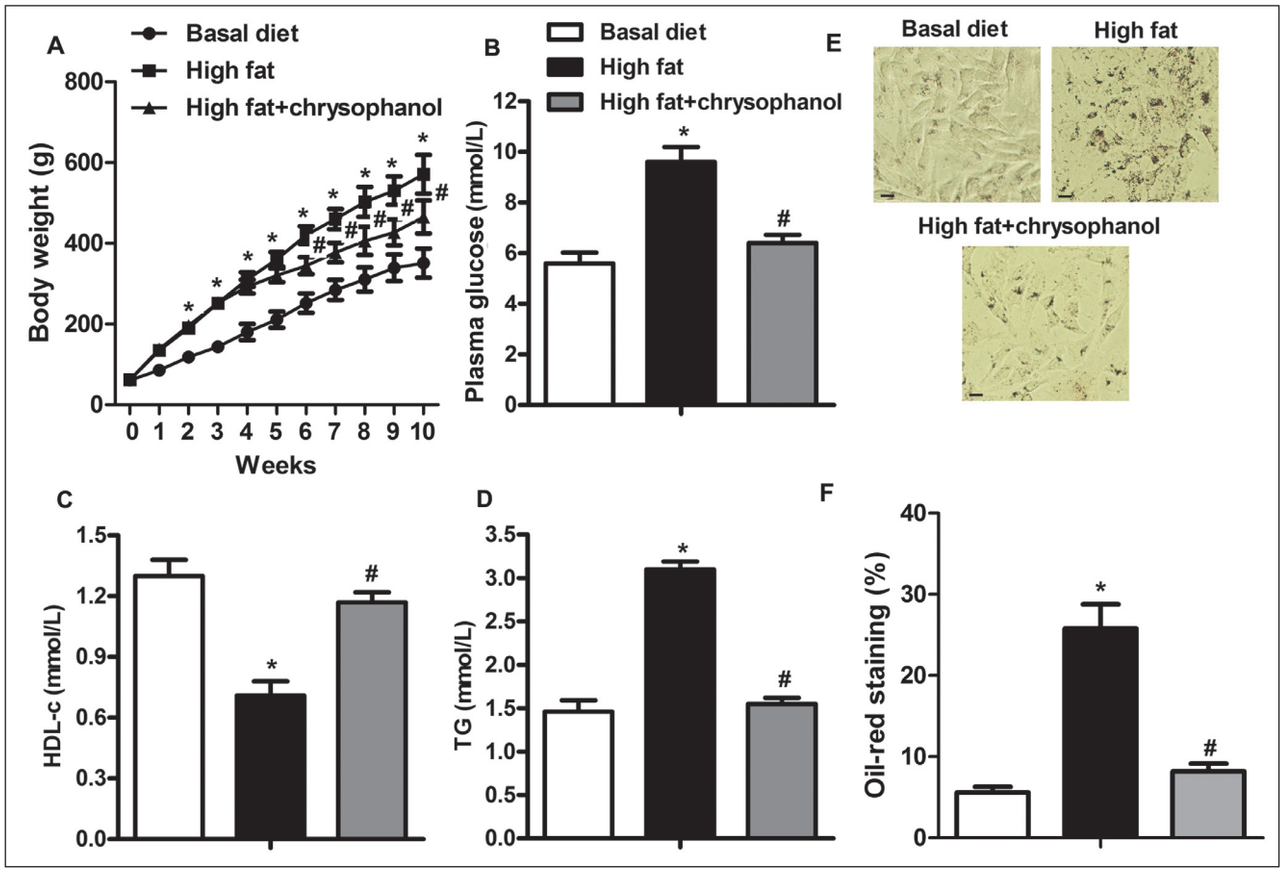

Fig. 1: Chrysophanol improves HFD-induced obesity in neonatal SD rats. SD rats were divided into three groups: basal diet, high fat diet, and high fat diet complemented with chrysophanol. (A) Weight changes for 10 weeks. (B) Plasma glucose was measured. (C) Levels of HDL-C in serum. (D) Levels of TG in serum. (E) Representative micrographs of Oil Red O analysis (magnification 200×). (F) Quantification of Fig.1E. All the experiments were repeated at least three times. n=6. $^{*}P < 0.05$ versus basal diet, $^{\#}P < 0.05$ versus high fat diet.

increased the body weight of experimental rats, indicating that the obesity model in this study was successfully established. However, chrysophanol treatment efficiently decreased body weight ($P < 0.05$, Fig. 1A). After the 10-weeks experiment, plasma parameter analysis was conducted. As shown in Fig. 1B, chrysophanol significantly decreased HFD-induced elevation of plasma glucose. In addition, the level of HDL-C in serum was markedly decreased in HFD treated rats, whereas TG was elevated. Interestingly, chrysophanol treatment notably corrected these abnormalities ($P < 0.05$, Fig. 1C and 1D). To further verify the effect of chrysophanol in adipocyte development, the lipid accumulation in primary hepatocyte was measured by Oil Red O staining. As shown in Fig. 1E-1F, cells from HFD rats displayed a much higher lipid accumulation compared to the basal group. However, chrysophanol administration significantly relieved this alteration. These results indicate that chrysophanol may improve HFD-induced obesity in neonatal SD rats.

2.2. Chrysophanol regulates lipid metabolism

After 10 weeks of HFD, the expressions of lipogenic proteins (ACC, FAS and SREBP-1) were significantly increased, whereas the lipolytic genes (PPARα, CPT-1 and ACOX1) were decreased. Chrysophanol administration notably corrected these abnormalities ($P < 0.05$, Fig. 2). These results indicate that chrysophanol improves HFD-induced obesity by regulating lipid metabolism.

2.3. Chrysophanol mitigates HFD-associated inflammation

Since immune response plays a crucial role in lipid metabolism, the expression of inflammation-related cytokines was further investigated. The results showed that the expression of pro-inflammatory cytokines IL-6 and IL-1β was markedly enhanced in HFD groups compared to normal control ($P < 0.05$, Fig. 3A-3B). Meanwhile the expression of regulatory factor IL-10 was significantly decreased ($P < 0.05$, Fig. 3A-3B). As expected, chrysophanol treatment effectively relieved these abnormalities ($P < 0.05$, Fig. 3A-F). Collectively, these results indicate that chrysophanol possesses a strong anti-inflammation function in HFD mice.

2.4. Chrysophanol restored HFD-induced inhibition of AMPK signaling

AMPK-derived stimulation of fatty acid metabolism occurs as a result of AMPK phosphorylation (Kim and Choung 2012). As shown in Fig. 4, HFD significantly reduced the expression of phosphorylated AMPKα and SIRT1. However, chrysophanol treatment partially restored the expression of these proteins ($P < 0.05$), implying that AMPK activation may contribute to the anti-obesity effect of chrysophanol.

3. Discussion

Obesity is considered as a chronic disease that can induce a series of comorbidities and complications. SD rats fed with HFD are a widely used experimental model for obesity research (Cao et al. 2011; de Andrade et al. 2017). Although genetically engineered mice models such as *ob*/*ob* (mice unable to produce leptin) or *db*/*db* (mice unable to respond to leptin) are available for obesity research, they lack convincing evidence for whether leptin-related mutation can represent typical obesity in humans (Wang et al. 2014). Long-term HFD administration in SD rats results in obesity development, showing increased body weight gain and fat accu-

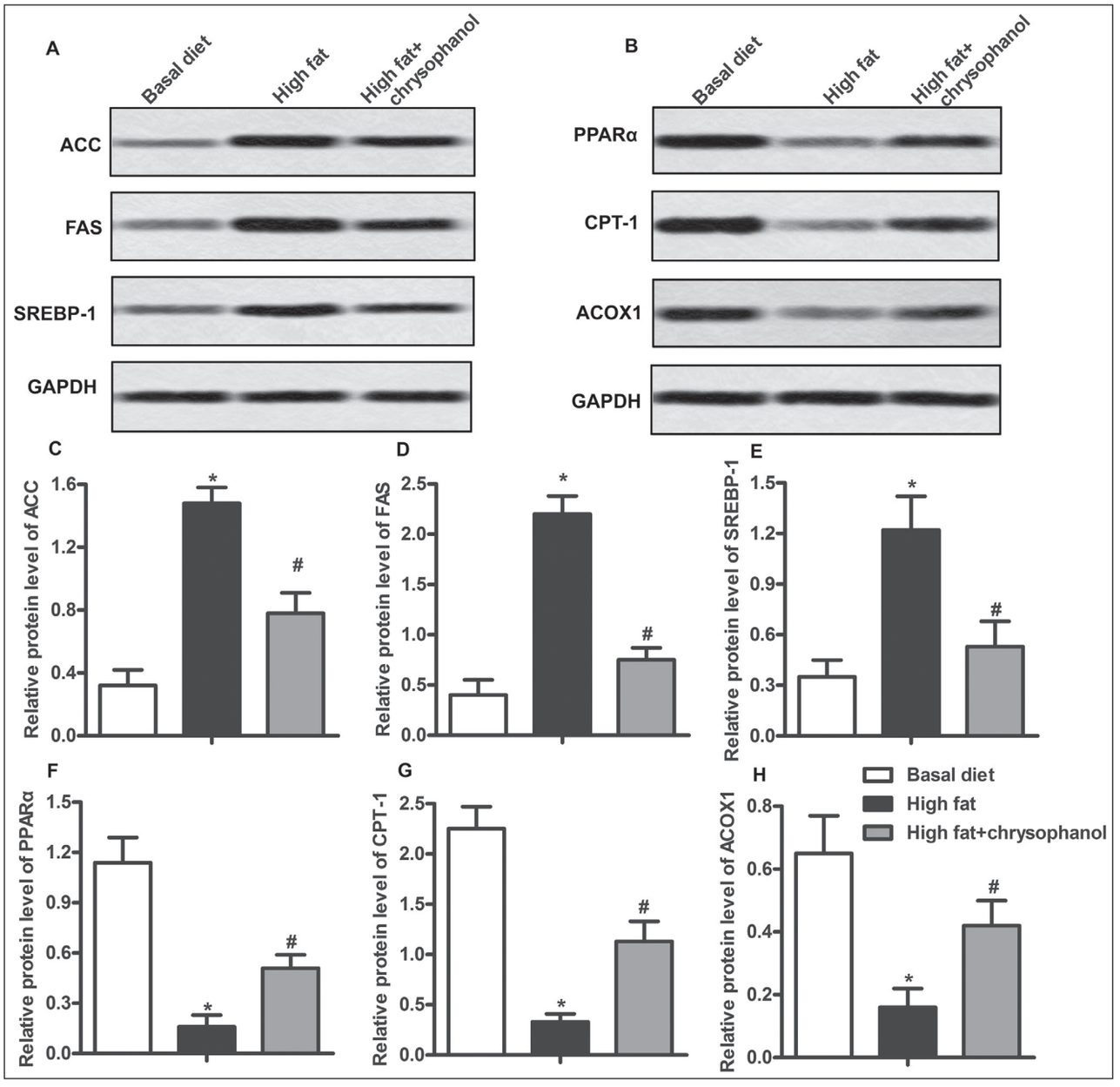

Fig. 2: Chrysophanol regulates lipid metabolism. (A) The expression levels of ACC, FAS and SREBP-1 were detected by Western blot. (B) The expression levels of PPARα, CPT-1 and ACOX1 were detected by Western blot. (C-H) Quantification of 2A and 2B. All the experiments were repeated at least three times. n=6. $^{*}P < 0.05$ versus basal diet, $^{\#}P < 0.05$ versus high fat diet.

mulation (Ahn et al. 2017; Rahman et al. 2017). We conducted an *in vivo* model referring this report, in order to imitate the pathogenesis of juvenile obesity. The results showed that neonatal rats with 10 weeks of HFD administration displayed high levels of body weight, lipid production and accumulation, implying that the rat model used in this study was successfully constructed.

Traditional Chinese Medicine has long clinical experiences in the treatment of obesity. Chinese crude drugs are generally containing various active ingredients, in which the identified anti-obesity ingredients can be divided into saponins, polysaccharides, alkaloids, polyphenols and others (Zhang et al. 2014). Chrysophanol is a yellow crystalline substance extracted from rhubarb, which is frequently used in Traditional Chinese Medicine for various purposes including treatment for obesity. Despite previous studies have shown numerous functions of chrysophanol, none of them mentioned the effect of chrysophanol on obesity, especially, on juvenile obesity. In the present study, we found that chrysophanol, as an anthraquinones, also has an anti-obesity effect. Chrysophanol significantly relieved HFD-induced elevation of body weight and blood sugar in neonatal SD rats. Likewise, chrysophanol effectively corrected the anomalous variation of HDL-C and TG in HFD rats and decreased HFD-induced lipid accumulation in primary hepatocytes.

The fatty acid biosynthetic pathway, composed of some 25 enzymes, has been elucidated in detail (Goodridge 1991). Among these enzymes, FAS, the main synthetic enzyme that catalyzes the condensation of malonyl-CoA to produce the 16 carbon saturated fatty acid palmitate, and ACC, which synthesizes malonyl-CoA from acetyl-CoA, are of particular importance. In liver, these genes share a regulatory sequence in their promoters that interacts with SREBP-1 (Shimomura et al. 1998; Shimano et al. 1999). PPARα is a nuclear receptor highly expressed in the liver, which is able to exert its transcriptional effects on genes involved in fatty acid oxidation, uptake as well as inflammation (Ferré 2004; Tailleux et al. 2012). PPARα activated by ligand can enhance transcription of ACOX1 and CPT1. CPT1 regulates uptake of fatty acid and is

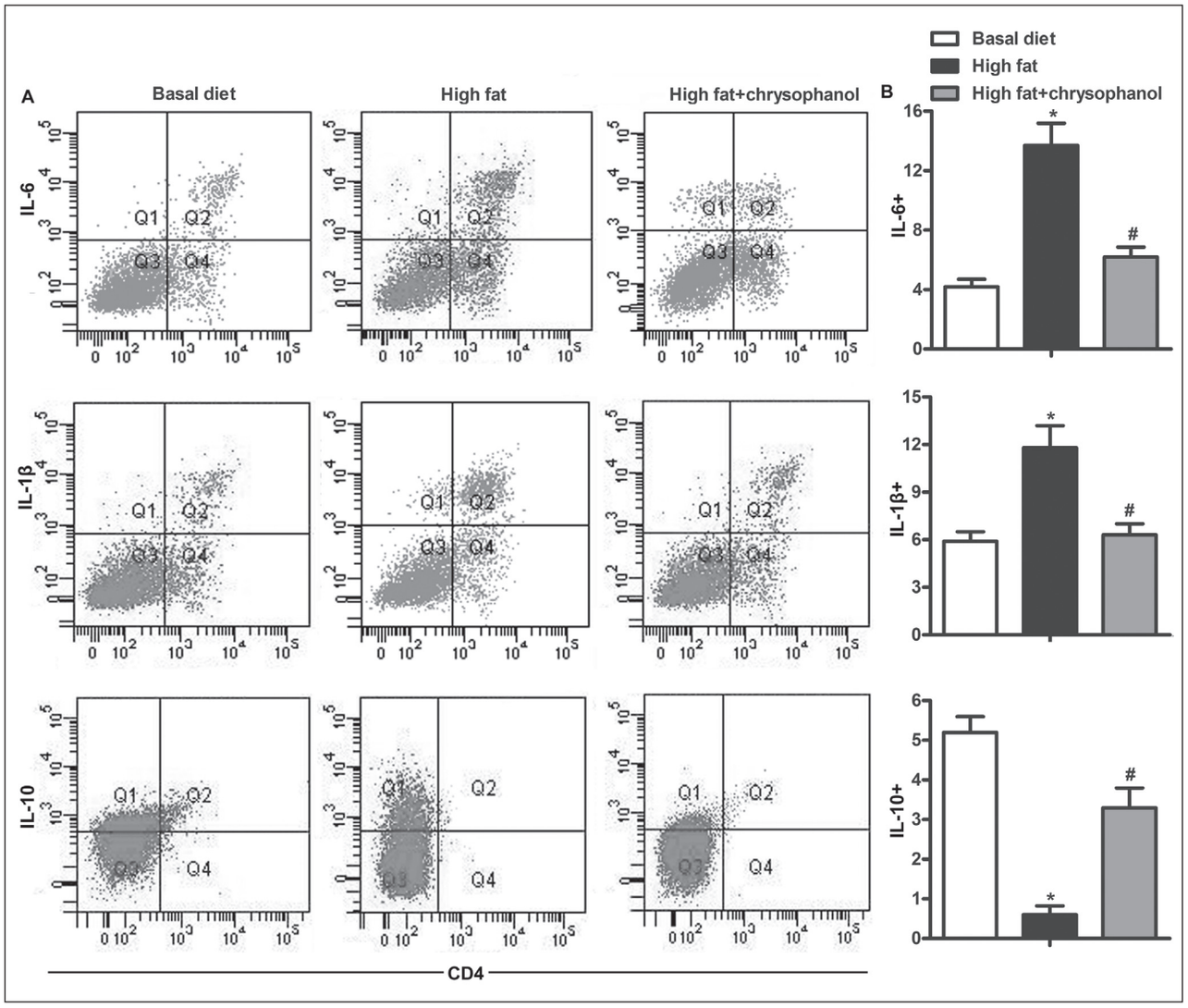

Fig. 3: Chrysophanol mitigates HFD-associated inflammation. (A) The expressions of IL-6, IL-1β and IL-10 in serums were detected using a Cytometric Bead Array TM kit. (B) Quantification of Fig.3A. All the experiments were repeated at least three times. n=6. $^{*}P < 0.05$ versus basal diet, $^{\#}P < 0.05$ versus high fat diet.

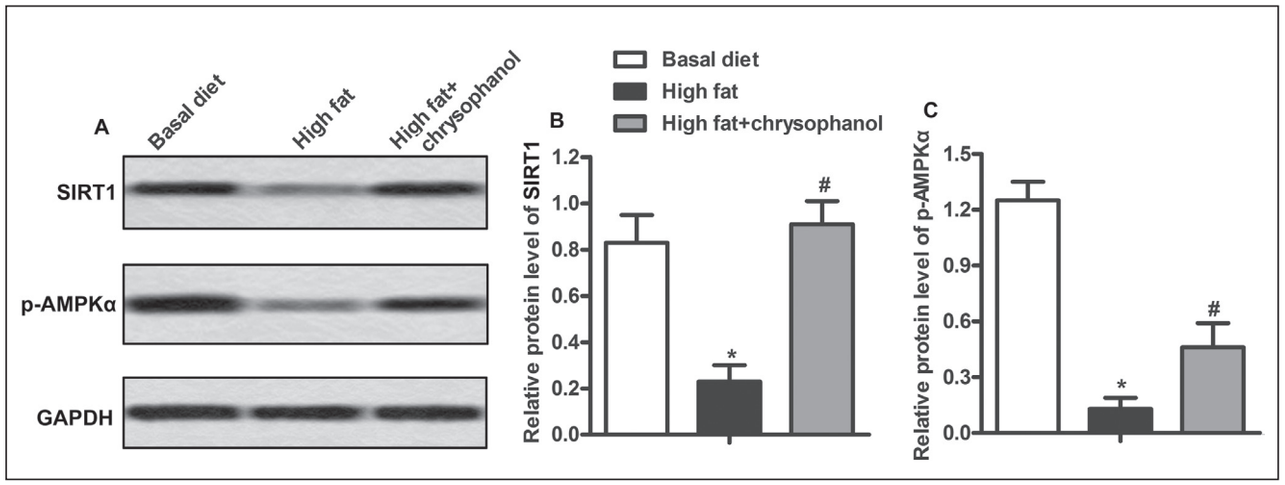

Fig. 4: Chrysophanol restored HFD-induced inhibition of AMPK signaling. (A) The expression levels of SIRT1 and p-AMPKα were detected by Western blot. (B-C) Quantification of 4A. All the experiments were repeated at least three times. n=6. $^{*}P < 0.05$ versus basal diet, $^{\#}P < 0.05$ versus high fat diet.

considered the rate-limiting enzyme in fatty acid oxidation in the mitochondria. ACOX1 is the key enzyme of peroxisomal β- oxidation, and lack of the gene results in hindered oxidation of long chain fatty acids and causes hepatic steatosis (Minnich et al. 2001; Zeng et al. 2016). Our data showed that the expression of FAS and ACC were significantly reduced by chrysophanol in HFD-induced obese rats. In addition, chrysophanol markedly enhanced the levels of ACOX1 and CPT1. These findings confirm that chrysophanol could promote lipolysis at the cellular and molecular levels.

As a major regulator of energy expenditure, AMPK has been shown to coordinate metabolic programs that increase energy expenditure and decrease energy storage by modulating the activities of the key transcriptional regulators such as SIRT1 and ADD1/SREBP1 (Zhou et al. 2001; Xue et al. 2012). It is reported that AMPK interact with SIRT1 and regulate macrophage inflammation (Yang et al. 2010). Indeed, AMPK activation deacetylates NF-κB, which acts through SIRT1, and therefore leads to inhibition of NF-κB signaling and cytokine expression (Yang et al. 2010). In addition, SIRT1 and AMPK form a close relationship in the energy metabolic mechanism (Ruderman et al. 2010). In differentiated fat cells, upregulation of SRT1 triggers lipolysis and loss of fat (Picard et al. 2004). On the other hand, phosphorylated AMPK downregulate the expression of SREBP1 directly, resulting in the reduction of lipogenesis. Metformin has been shown to block the expression of lipogenic genes by suppressing the activity of ADD1/SREBP1 through AMPK activation in primary hepatocytes (Zhou et al. 2001). Some AMPK activators, such as 5-aminoimidazole-4-carboxamide, improve fatty liver by stimulating PGC-1α expression, a crucial factor in the transcriptional regulation of mitochondrial biogenesis and fatty acid oxidation (Suwa et al. 2006; Kim et al. 2009). Our data indicate that HFD significantly decreased the expression of phosphorylated AMPKα and SIRT1, which lead to an elevation of SREBP-1 and pro-inflammation cytokines. Chrysophanol administration effectively corrected these abnormalities. Therefore, it is likely that chrysophanol improves HFD-induced obesity and inflammation through the AMPK/SIRT pathway.

Taken together, our study demonstrates that as the major active component of rhubarb extract, chrysophanol, is a promising compound for preventing HFD-induced obesity and inflammation. Further experimental and clinical investigations are required to explore the additional mechanisms involved and establish the potential clinical applications.

4. Experimental

4.1. Reagents

Chrysophanol (purity≥ 99.9%) was purchased from Rochen Pharma Co.Ltd (Shanghai, China).

4.2. Animal work and experimental protocols

Twenty-four neonatal SD rats (20-day-old) were purchased from the Animal Center of Xi'an Jiaotong University Medicine School (Xi'an, China). Rats were housed under controlled conditions (25±2 °C, 70% humidity and 12-hlight-dark periods) and provided with laboratory diet and water *ad libitum*. All rats were randomly divided into three groups. The control group was fed with commercial standard chow diet. The model group was fed with high fat diet (HFD, containing 1% cholesterol, 18% lipid, 40% sucrose, 1% AIN-93G vitamins, and 19% casein, with equal quantities of fiber and minerals as in the rat maintenance diet). The HFD plus chrysophanol group received chrysophanol (10 mg/kg/day) intraperitoneally after 4 weeks of HFD. After 10 weeks of feeding, rats were fasted overnight and euthanized. Blood samples and liver tissues were collected. All the animal experiments were performed according to relevant national and international guidelines and were approved by the Animal Experimental Ethical Committee.

4.3. Plasma parameter analysis

Plasma glucose, triglyceride (TG) and high density lipoprotein-cholesterol (HDL-C) levels were measured using enzymatic commercial kits (Beyotime Biotechnology, Shanghai, China) implemented in an automated clinical analyser.

4.4. Oil red O staining

Rat liver cells were collected and cultured basically as described (Wu and Dickson 2010). Cells were fixed with 4% formalin for 30 min and then stained by oil red O for 15 min. Finally, the cells' morphology was observed by a light microscope (Olympus, Tokyo, Japan) equipped with an imaging system at 200 magnification.

4.5. Flow cytometry

Quantitation of IL-6, IL-1β and IL-10 in serum was determined using a Cytometric Bead Array TM kit (CBA; BD Biosciences, USA). Serum samples were mixed to capture specific beads for each cytokine. After incubated with antibodies and conjugated with phycoerythrin, tubes were then centrifuged and the supernatant was carefully discarded. The pellets containing beads were resuspended and the samples were analysed on the BD LSR Fortessa cytometer.

4.6. Western blot

Total protein samples from liver tissues were prepared according to a standard protocol. Equivalent amounts of protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, USA). Membranes were then incubated at room temperature with 5% non-fat dry milk dissolved in T-BST. The blots were probed overnight with respectively primary antibodies (all purchased from Santa Cruz Biotechnology, CA, USA) at 4⊠ and then incubated with HRP-conjugated secondary antibodies (Beyotime Biotechnology, Shanghai, China) at room temperature. Membranes were extensively washed several times. Proteins were detected using a ChemiDoc XRS imaging system and Quantity One analysis software (Bio-Rad, USA). GAPDH were used as endogenous reference.

4.7. Statistical analysis

All results were presented as mean±SD. The statistical significance of the studies was analyzed using one way ANOVA. The difference was considered statistically significant at $P < 0.05$.

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