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Original Article

Uraria crinita ameliorates high-fat diet-induced prediabetes in mice

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Abstract: Objectives: *Uraria crinita* (UC) is widely used as a food supplement. This study was performed to investigate the effects and potential mechanism of UC on prediabetic mice. Materials: A prediabetic mouse model was established after feeding male C57BL/6J mice with a high-fat diet (HFD) for 27 continuous weeks. The modeled mice were then fed with HFD containing UC extract, and assessed for phenotypes, liver damage and expression of genes involved in lipid metabolism. Results: HFD containing UC extract significantly reduced the level of fasting blood glucose (FBG), improved impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and the profiles of serum lipids. Molecular analysis showed that the expression of PPAR-γ and SREBP-1c associated with lipid metabolism was downregulated in the liver. H&E analysis indicated that HFD supplemented with UC extract ameliorated the liver damage induced by HFD diet. Conclusions: UC effectively prevents HFD-induced hyperglycemia and dyslipidemia and may be potentially useful to prevent the development and progression of diabetes.

Keywords: *Uraria crinita,* prediabetes, lipogenesis, oxidative stress

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disease. Its occurrence and development have been associated with a number of genetic and environmental factors. It often leads to disorders in glycometabolism, protein metabolism and fat metabolism. Prediabetes is considered a precursor for the development of impaired glucose regulation (IGR) in the blood, leading to an increase in blood glucose, although the level is not high enough to reach the diagnostic criteria set for diabetes. The common characteristics of prediabetes include impaired fasting blood glucose (IFG) and impaired glucose tolerance (IGT), due to insulin resistance (IR) and deficiency of insulin secretion [1]. IGT is a "sub-healthy state" in the early stage of diabetes [2, 3]. Although patients with prediabetes do not have typical clinical signs of diabetes, these patients may progress to T2DM, with increased risk of having cardiovascular diseases, cerebrovascular diseases, microvascular diseases, tumors and dementia. Therefore, timely diagnosis and effective management of prediabetes are the keys to prevent the onset of diabetes. In addition, insufficient insulin secretion and insulin resistance will lead to reduced fat synthesis and enhanced fat decomposition, which would increase free fatty acids in plasma, leading to blood lipid disorders [4-6].

At present, the main preventive measures for prediabetic patients are diet management and lifestyle interventions. In addition, the use of various drugs, including metformin, acarbose [7], and thiazolidinediones (DTZDs) [8], such as rosiglitazone, are shown to reduce the risk of prediabetes progressing to diabetes. However, regardless of the type of drugs used, the adverse reactions of these drugs are often significant. Although a large number of IGT/IFG patients will not develop T2DM, an assessment of the risks and benefits of early intervention with oral hypoglycemic drugs is still needed and desirable [9]. In the search for drugs with high efficacy and low side effects, antioxidants from natural products are increasingly attractive for the treatment of prediabetes or the prevention of T2DM [10].

Table 1. Composition of experimental diets

Ingredients	Content (%, wt/wt)				
	ND	HFD	HFD+2% UC Powder	HFD+4% UC Powder	HFD+4% GT Powder
Sucrose	10	10	10	10	10
cornstarch	46.57	17.57	17.57	17.57	17.57
Maltodextrin	15.5	15.5	15.5	15.5	15.5
Lard	0	29	29	29	29
Soybean oil	4	4	4	4	4
Cellulose powder	5	5	3	1	1
Casein	14	14	14	14	14
Choline chloride	0.25	0.25	0.25	0.25	0.25
l-cystine	0.18	0.18	0.18	0.18	0.18
AIN-93M vitamin mix	1	1	1	1	1
AIN-93M mineral mix	3.5	3.5	3.5	3.5	3.5
UC powder			2		
UC powder				4	
GT powder					4

Uraria crinita (UC), an edible herb in traditional Chinese medicine, is widely presented throughout India, Thailand, Indonesia, southern China and Taiwan [11]. UC has been demonstrated to have anti-oxidative activity and nitric oxide-scavenging activity. It is also able to repel and kill blowfly larvae [12]. This herb is shown to inhibit the formation of stress-induced ulcers and have anti-oxidative activity [13].

Our previous study has revealed that the UC water extract (UCWE) has hypoglycemic and hypolipidemic activity, indicating its potential for treating diabetes [14]. In this study, we further investigated the hypoglycemic effects of HFD containing UC extract on C57BL/6J mice that had been continuously fed with a HFD for 27 weeks. The results may help develop a new type of food intervention for the treatment of prediabetes or T2DM.

Materials and methods

Instruments and chemicals

Blood glucose meter was obtained from Roche, France. Total cholesterol (TC) (cat. no. A111-2-1), triglyceride (TG) (cat. no. A110-2), high density lipoprotein cholesterol (HDL-C) (cat. no. A112-2) and low density lipoprotein cholesterol (LDL-C) (cat. no. A113-2) kits were from Bioengineering Institute, Nanjing, China. Malonaldehyde (MDA) (cat. no. S0131), nitric oxide (NO) (cat. no. S0023), total superoxide dismutase (SOD) (cat. no. S0101) and catalase (CAT) (cat. no. S0051) were determined using kits purchased from Beyotime Biotech, Shanghai, China. Peroxisome proliferator-activated receptor gamma (PPAR-γ) and sterol regulatory element-binding protein 1c (SREBP-1c) were determined using kits from Abcam (Cambridge, UK). All assays were performed according to the manufacturer's instructions and in triplicate.

Animals

Male mice (C57BL/6J, 4-weeks old, weighting 10-16 g) were purchased from the Medical Laboratory Animal Center, Guangdong, China. The animal care and study protocols were approved by the Institutional Ethics Committee of Southern Medical University (Approved No: K2018014). Before the experiments, the animals were individually housed at 22 ± 2°C with 55 ± 5% relative humidity in a room with a 12 h light (06:00-18:00)/12-h dark (18:00-06:00) cycle, and fed with a normal chow diet and water *ad libitum*. The compositions of experimental diets (HFD or HFD containing UC extract (UC-HFD) or green tea (GT) powder (GT-HFD) are shown in **Table 1**.

UC and extract preparation

UC (batch number: 20180815) was brought from a Chinese herbal medicine store in Qingping, Guangzhou and was identified as *U. crinita* Desv. ex DC. The roots were washed, dried, and ground into powder. A total of 500 g UC powder was added with 50% ethanol at a ratio of 1:10 (g:mL) and extracted three times for 2 h each. The extracts were pooled, filtered, concentrated under vacuum, dissolved in water and filtered. The filtrates were further extracted three times with 500 mL diethyl ether. The solutions were then extracted three times with 500 mL ethyl acetate and concentrated to obtain an extract that contains total flavonoids. The extract was stored at 4°C for subsequent use as diet supplement.

HFD-induced prediabetic models

One week after acclimatization, the animals were randomly divided into five groups, each consisting of six mice. The control group was fed with normal diet (ND), the models were fed HFD, 2% or 4% UC-HFD. Four% GT-HFD was used as positive control. The animals were fed for 27 weeks to imitate the process of prediabetes development. The consumption of diets was recorded and efforts were taken to ensure that equal amount foods were consumed in each group.

Oral glucose tolerance assay

One day before sacrifice by carbon dioxide inhalation, the animals were fasted for 12 h. Blood samples were collected from the tail veins to determine FBG. Then, 20% glucose at 2 g/kg b.w. was administered, and the blood glucose was determined 30, 60 and 120 min later to determine oral glucose tolerance as described previously [15].

Sample collection and biochemical measurements

On the last day of the experiment, the animals were fasted for 12 h and anesthetized, and the blood was collected, maintained at room temperature for 1 h and centrifuged at 3000 g for 15 min to obtain serum. The serum was then stored at -20°C, and the serum TC, TG, HDL-C, LDL-C and SOD and CAT activities as well as MDA contents in the liver tissues were determined.

Mice were then sacrificed, and the epididymal white adipose tissues (WAT) and liver tissues were collected and weighed. A portion of the liver tissues was fixed in a 10% formalin solution for pathological examination.

Western blot analysis

Total protein was prepared from the liver tissues using RIPA lysis buffer. The protein level was determined using a protein assay kit (Pierce, USA). Fifty μg of protein was separated on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) for 2 h and then transferred to polyvinylidene difluoride membranes at 300 mA for 80 min. The membranes were blocked with 5% nonfat milk and then reacted overnight at 4°C with primary antibodies against PPAR-γ and SREBP-1c (1:1000, Abcam) and GAPDH (1:7000, Santa Cruz Biotechnology) in 5% nonfat milk with gentle shaking. The membranes were then washed with TBST, incubated at room temperature for 1 h with the HRP-conjugated secondary antibody in 5% nonfat milk, washed again and treated with a super ECL kit for visualization.

Histopathological analysis

The hepatic tissues embedded in paraffin were cut into 5 μm slices and stained with hematoxylin and eosin (H&E). The histopathological changes in the liver tissues were observed under an Imager microscope at a magnification of ×200.

Statistical analysis

All the data were expressed as mean ± SEM of at least three independent experiments. The statistical analyses were carried out with GraphPad Prism 5.0 software. One-way ANOVA and the Dunnett's test were used to evaluate the differences among the groups, and differences between groups were examined using the Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

UC extract reduced weight, FBG and glucose level

As shown in **Figure 1**, the weight and FBG were significantly greater after mice were fed with HFD than with ND at the end of the 27-week experimental period. UC extract significantly reduced the gains in a dose-dependent manner. The same effect was observed for GT powder as well, which was used as positive control in the experiments. In addition, the oral glucose tolerance test (OGTT) indicated that blood glucose levels increased sharply during the first 30 min in all groups. However, the glucose levels were significantly lower in the mice fed with UC-HFD than those fed with HFD, and were similar to those in the GT group (**Figure 1D**). As shown in **Figure 1E**, 2% UC, 4% UC and 4% GT significantly decreased the areas under the OGTT curves (**Figure 1E**).

UC extract reduced serum TC, TG, and LDL-C

As shown in **Figure 2**, the serum levels of TC, TG, and LDL-C were significantly higher in the model group than in the control group, althou-

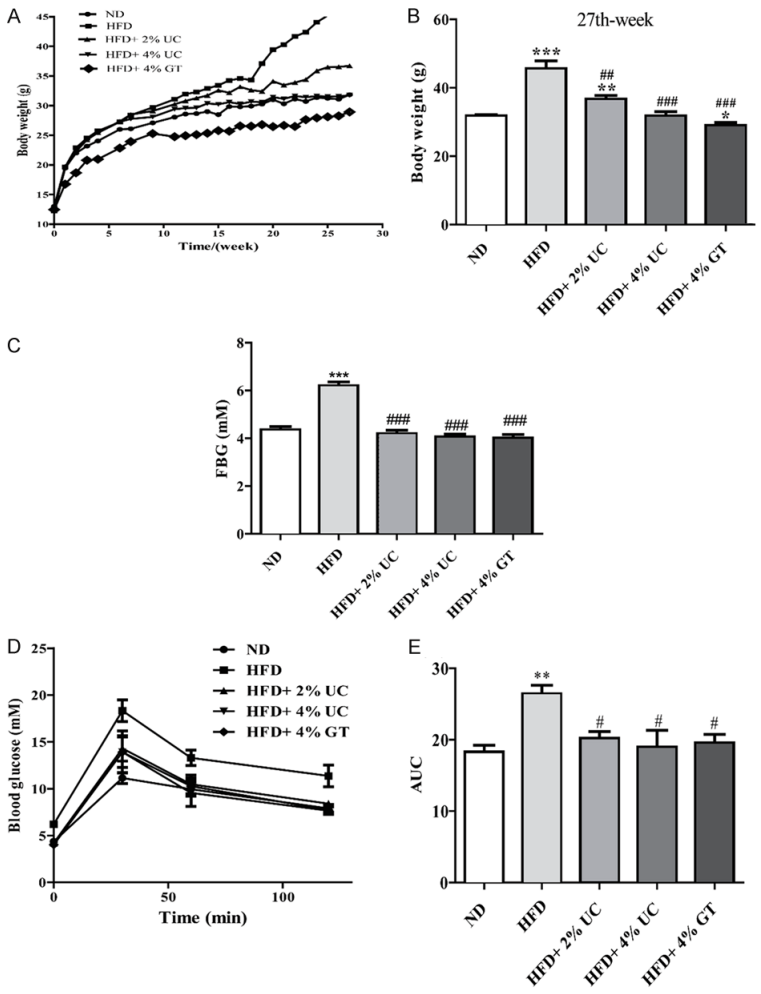

Figure 1. Effect of UC extract on weight, fasting blood glucose and glucose resistance of C57BL/6 mice fed with UC-HFD during 27 week feeding period. (A) Weight over the entire study period, (B) final weight on the last day of the study, (C) FBG levels on the last day of the study, (D) blood glucose concentration after oral glucose administration, and (E) areas under the OGTT curves shown in (D). ** and *** denote $P<0.01$ and $P<0.001$ vs the control group, respectively; #, ## and ### denote $P<0.05$, <0.01, and <0.001 vs the model group, respectively.

gh the HDL-C level was lower but the reduction was not statistically significant. Compared with the model, animals fed with 2% and 4% UC extract had significantly lower levels of TC, TG and LDL-C levels, but reduction was not significantly different between the 2% and 4% UC groups. Additionally, the impact of 4% GT was similar to UC as previously observed in diabetic mice [16].

UC extract increased antioxidant activity

Additionally, the total SOD and CAT activities were significantly lower in HFD group than in the control group (**Figure 3A**, **3B**), but similar between UC extract and GT powder groups. Meanwhile, MDA content was significantly higher in the livers of HFD-fed than ND-fed mice (**Figure 3C**), while it was significantly lower in the livers of mice fed with UC-HFD or TG-HFD than those fed with HFD, suggesting that UC has antioxidant activity.

UC extract reduced the weight gain of liver and WAT tissue

As shown in **Figure 4A**, UC extract and GT powder reduced the liver weights of mice compared with those of the models, suggesting that these ingredients may have protective activity against the weight gain in the liver due to HFD. On the other hand, the WAT weights in the bilateral epididymis significantly increased in the mice fed with HFD; and UC extract significantly reduced the increase in a dose-dependent manner (**Figure 4**). In addition, similar effect was observed for GT powder on the WAT weight as well as the 4% UC extract.

UC extract down-regulated the expression of adipogenesis-related proteins in the liver

To study the mechanism underlying the improved lipid profiles achieved in the prediabetic mice, PPAR-γ and SREBP-1c expressions were detected to find the proteins crucial roles in lipid metabolism in the liver [17, 18]. The results are presented in **Figure 5**. The protein levels of PPAR-γ and SREBP-1c in the livers of the model group were significantly increased. On the other hand, both UC extract and GT powder significantly down-regulated the expression of these two proteins, which is consistent with the results presented in **Figure 4A**.

UC extract reduced the damage to liver tissue

As shown in **Figure 6B**, clear lesions were observed in the livers of the mice in the model group, exhibited as increased number of cyto-

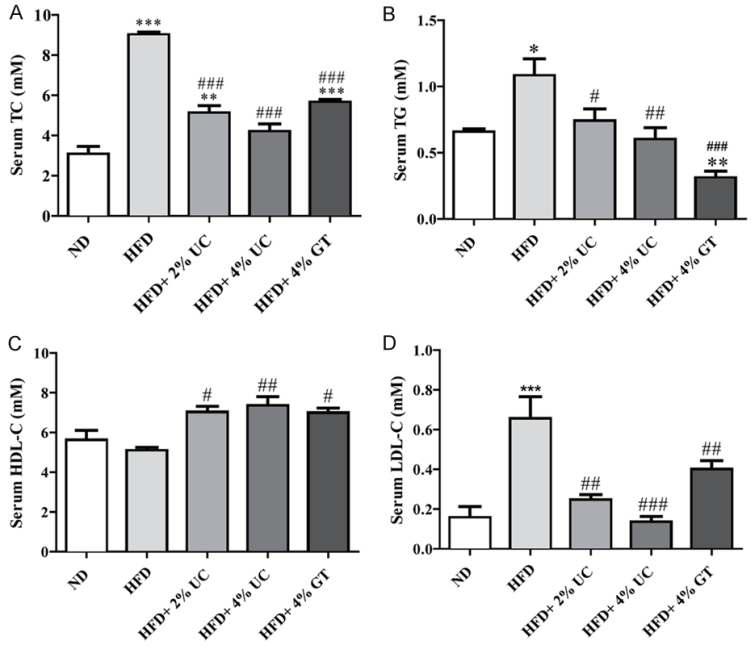

Figure 2. Effects of UC extract on the serum levels of TC (A), TG (B), HDL-C (C) and LDL-C (D) in mice fed with UC-HFD. ** and *** denote $P<0.01$ and $P<0.001$ vs the control group, respectively; #, ## and ### denote $P<0.05$, <0.01, and <0.001 vs the model group, respectively.

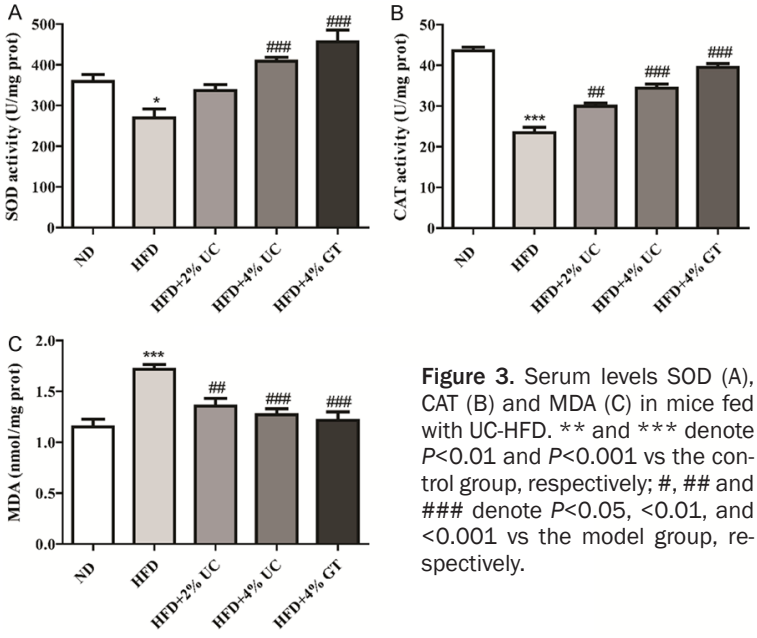

Figure 3. Serum levels SOD (A), CAT (B) and MDA (C) in mice fed with UC-HFD. ** and *** denote $P<0.01$ and $P<0.001$ vs the control group, respectively; #, ## and ### denote $P<0.05$, <0.01, and <0.001 vs the model group, respectively.

plasmic vesicles with greater amounts of inflammatory cells and more severe necrosis than control group. The application of UC and GT powder significantly improved the liver pathology. As clearly shown in **Figure 6C-E**, 4% UC powder exerted a stronger protective effect, more than 2% UC or 4% GT powder did.

Discussion

Our experiments showed that UC extract in HFD reduces weight gain, FBG, and improves IFG, IGT, and the serum lipid profile. At the protein level, the expression of PPAR-γ and SREBP-1c associated with lipid metabolism are down-regulated in the liver tissues after feeding with UC-HFD. Based on the H&E analysis, UC-HFD can reduce the liver damage induced by HFD diet. To our knowledge, this is the first study showing that UC extract has hypoglycemic and hypolipidemic effects in mice.

The identification and treatment of individuals with prediabetes (IGT/IFG) have become increasingly important. In fact, patients with prediabetes are prone to suffer from diabetes complications [19], and in the absence of any interventions, 37 to 70% of patients might progress from prediabetes to diabetes within 4 years [20-22]. Lifestyle interventions could successfully prevent the progression to diabetes and are always cost-effective [23, 24]. Recent studies have demonstrated the positive outcomes of dietary interventions, such as lower glycemic excursions [25]. In this study, we first established mouse models of prediabetes through the administration of a HFD alone. In parallel, UC extract was administered from the early stage of diabetes (i.e., prediabetes). At the end of the experiment, although the mice in the model group did not show any clear symptoms of polydipsia, polyphagia, or polyuria, the animals exhibited obesity, IFG, and IGT, which are symptoms of prediabetes. In contrast, UC extract sig-

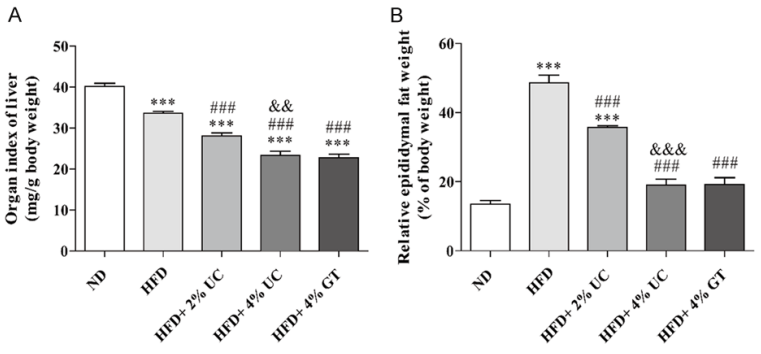

Figure 4. Weights of liver (A) and WAT (B) in mice fed with UC-HFD. ** and *** denote $P<0.01$ and $P<0.001$ vs the control group, respectively; #, ## and ### denote $P<0.05$, <0.01, and <0.001 vs the model group, respectively; && and &&& denote <0.01 and <0.001 vs 2% UC group, respectively.

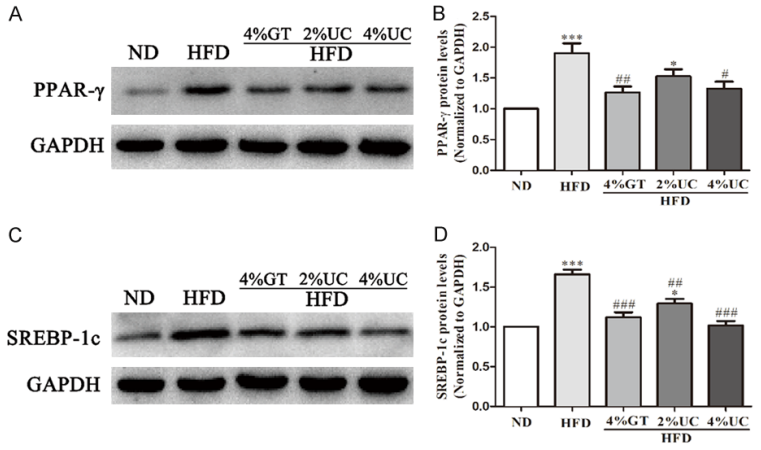

Figure 5. Expression of the adipogenesis-related proteins PPAR-γ (A, B) and SREBP-1c (C, D) in liver tissues of mice fed with UC-HFD. Left panels, representative Western blots; right panel, relative protein content. **and *** denote $P<0.01$ and $P<0.001$ vs the control group, respectively; #, ## and ### denote $P<0.05$, <0.01, and <0.001 vs the model group, respectively.

nificantly reduced the body weight and improved IGT and IFG as compared with the model group. Patients with IGT/IFG have high risk of developing diabetes, and only 1.5% to 10% of the UC-treated individuals in this group exhibit progression to diabetes every year, directly suggesting the effectiveness of UC powder on controlling prediabetes [25, 26]. Additionally, H&E analysis showed that the liver of the model group fed HFD showed clear macrovesicular steatosis and infiltration of inflammatory cells. Liver steatosis is the first step towards nonalcoholic fatty liver. As shown in **Figure 6**, no fat globules were observed in the ND group; both UC and GT supplementation reduced hepatic steatosis and the infiltration of inflammatory cells in a dose-dependent manner, suggesting that UC can effectively improve IGT/IFG and protect liver tissues. At the molecular level, UC extract decreased PPAR-γ and SREBP-1c levels in liver tissue, both of which are important in lipid metabolism indicators in diabetic or obese mice [27-29]. These findings indicate that the potential effects of UC extract on prediabetes might result from the down-regulation of PPAR-γ and SREBP-1c.

Numerous studies have shown that elevated blood glucose is usually accompanied with dyslipidemia in diabetes [30, 31]. If not well controlled, dyslipidemia symptoms will be aggravated [32]. High TC and TG levels increase the risk of insulin resistance [33] and are major contributors to the occurrence of coronary artery diseases and related complications [34, 35]. In our study, we found that UC extract could reduce the TG, TC, and LDL-C levels, increase the HDL-C level, and significantly reduce the weights of epididymal white adipose and liver tissues; indicating that UC extract has potential effects on lipogenesis. Studies have shown that in patients with T2DM, HDL-C exerts a protective effect on early vascular complications [36]. Decreased HDL-C can easily cause diabetic cardiovascular diseases. During the course of diabetes, insufficient insulin secretion and insulin resistance enhance the oxidation and glycosylation of LDL, promoting the production of oxygen free radicals and thereby aggravating the damage of islet β cells. This would result in reduced activity of LDL receptors, decreased clearance of LDL-C and elevated serum levels of LDL-C [37]. Therefore, the effects of UC extract on LDL-C and HDL-C might help improve dyslipidemia and prevent certain diabetic complications.

In 2005, Brownlee et al proposed that T2DM and its vascular complications have a common pathogenesis, namely, oxidative stress [38]. As

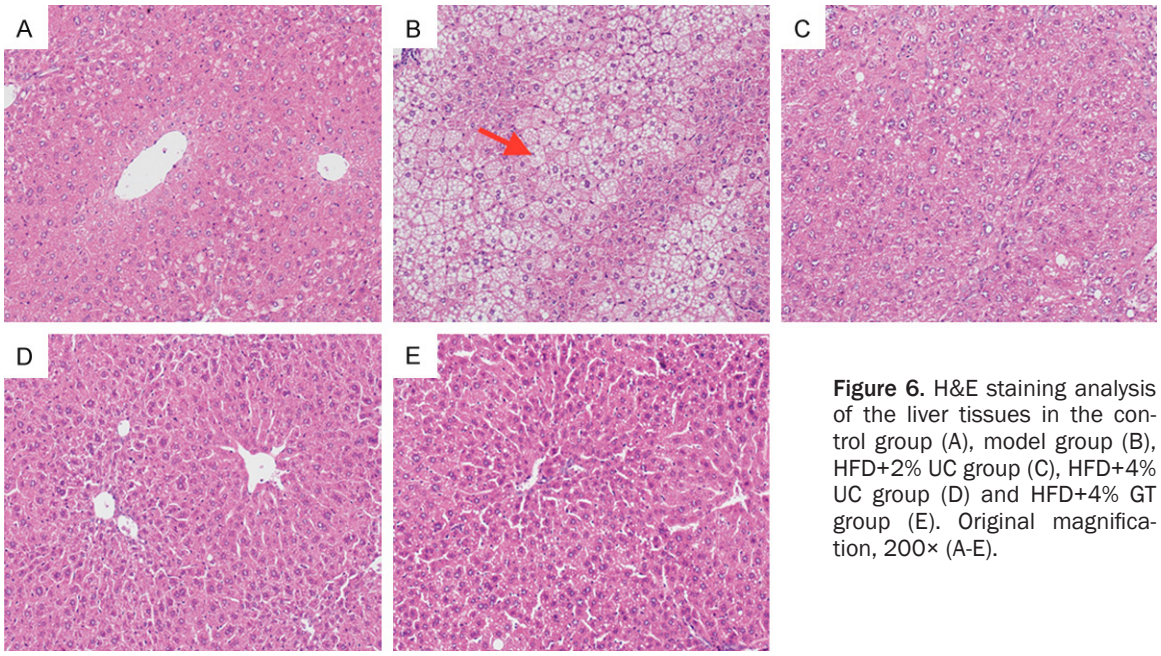

Figure 6. H&E staining analysis of the liver tissues in the control group (A), model group (B), HFD+2% UC group (C), HFD+4% UC group (D) and HFD+4% GT group (E). Original magnification, 200× (A-E).

the chief culprit of diabetes and its chronic complications, oxidative stress has been detected throughout the process. The exposure of islet β cells to oxidative stress aggravates prediabetes [39]. Our previous study showed that the total flavonoids in UC have significant anti-oxidative effects *in vitro* [40]. Here, we first found that UC extract could have anti-oxidative activity *in vivo*. We hypothesize that the anti-oxidative activity is likely due to the rich flavonoids in the UC extract because it has been widely accepted that flavonoids are able to exert effects on diabetes [41, 42]. Beside, Wang et al have identified fourteen compounds in the roots of UC that might be related to the anti-oxidative activity such as β-sitosterol, genistein, octadecanoic acid, palmitic acid and β-daucosterol [43]. Among them, genistein [44-46], β-sitosterol [47-49], silybin [50, 51] and botulin [52, 53] have been reported to have significant hypoglycemic effects and can therefore be used for T2DM treatment, further suggesting that the hypoglycemic effect of UC might be due to the presence of these ingredients.

There are limitations in our study. The active ingredients in UC have not been fully elucidated and their contents may be affected by the source of materials. Therefore, further study is needed to use isolated and purified active ingredients for therapeutic treatment. Furthermore, prediabetes at different stages needs to be treated to identify optimal treatment windows and finally human subjects should be used to validate the hypoglycemic and hypolipidemic effects.

Taken together, we have demonstrated, for the first time, that UC extract has hypoglycemic and hypolipidemic effects on mice with prediabetes. It reduces liver damage and down-regulates the expression of PPAR-γ and SREBP-1c with improved liquid profiles.

Disclosure of conflict of interest

None.

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