Flos Chrysanthemi Indici extract improves a high-sucrose diet-induced metabolic disorder in Drosophila

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Abstract. Flos Chrysanthemi Indici (CI) is a traditional medicinal plant used in the treatment of inflammatory diseases. However, the pharmacological role of CI in metabolic diseases, especially in diseases induced by insulin metabolism disorders, remains poorly understood. In the present study, Drosophila melanogaster (Drosophila) were fed with high-sugar diet (HSD) to induce a model similar to Type 2 diabetes (T2D) in order to determine whether CI extracts improve the metabolic disorder. It was demonstrated that the CI extracts could improve growth rate, body size, lifespan, reproductive capacity and fat storage, and CI especially improved the fat metabolism and cell size in S6k and Akt1 mutant flies. In conclusion, the present study provides novel evidence that CI may be an effective drug for the treatment of T2D.

Introduction

A high-sugar diet (HSD) includes more and more types of foods, such as baked goods, convenience foods and a variety of sugary drinks. Dietary sugar is the main source of sugars in the body; sugar is ingested and absorbed, converted into monosaccharides, and then transported by the blood to cells and tissues for metabolism (1). However, a long-term, excessively high-sugar or high-calorie diet damages the homeostasis of glucose metabolism in the body and causes obesity, which further leads to metabolic disorders and other problems such as hypertension, fatty liver, cancer, and especially the onset of Type 2 diabetes (T2D) (2). T2D, a complex metabolic disease characterized by insulin resistance, is related to metabolic abnormalities, such as high blood glucose levels and weight loss, and can cause serious blindness, amputation and disability, renal failure, uremia, and so on (3). Currently, the prevalence of this type of chronic disease is increasing; while though T2D has traditionally only developed among adults, the disease has begun to appear in children (4). The current treatment of T2D consists mainly oral hypoglycemic agents and insulin injections, and the side effects and safety of these agents need to be further studied (5).

Traditional medicinal plants, which are low-cost, easy to obtain, and have the advantages of small side effects, have long been widely used around the world (6). Among them is Flos Chrysanthemi Indici (CI), the capitulum of the perennial herb Chrysanthemum indicum L. of Compositae. The chemical composition of CI includes sesquiterpenes, flavonoids, and phenolic compounds. CI extracts, which have anti-inflammatory, anti-oxidative and anti-microbial activities, exhibit inhibitory activity against rat lens aldose reductase, a mediator of pathogens involved in diabetic complications (7,8). However, the regulatory effect of CI on abnormal metabolic functions induced by a high sugar diet is poorly understood.

Drosophila melanogaster (Drosophila) has become an excellent model for investigating T2D because approximately 74% of human disease-causing genes are conserved in this species, and more importantly, the mechanisms of glucose homeostasis are highly conserved between mammals and Drosophila (4,9). In a number of previous studies, Drosophila were fed with an HSD to establish models of T2D that exhibit features of T2D patients, including hyperglycemia and insulin resistance (10). Therefore, we analyzed the effects of aqueous CI extracts on improving T2D-like features in an HSD-induced Drosophila model. The results indicated that CI improved HSD-induced metabolic abnormalities as well as growth rate, body size, lifespan, productive capacity and fat storage. In addition, CI improved fat metabolism and cell size in S6k and Akt1 mutant flies. These results provide a valuable reference for preclinical drug discoveries that take the CI of this medicinal plant into account.
Materials and methods

Fly stocks and culture conditions. Wild-type w+; and Da-Gal4 flies were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). S6k+/TM6B flies were obtained from Tian Xu, and Akt1 RNAi flies were obtained from the Tsinghua Fly Center (Beijing, China). Fly stocks were maintained on standard cornmeal-yeast medium at 25±1˚C and 60±5% humidity under a 12-h light/12-h dark cycle.

Preparation of CI aqueous extract and Drosophila growth medium. CI was purchased from the Renmin Tongtai Pharmacy (Harbin, China). Aqueous CI extract was obtained as previously described (11). Chopped capitula (20 g) were soaked overnight in deionized water (200 ml; yield, ~5-14%) at room temperature and then heated until boiling for 3 h. The extraction process was repeated twice and the filtrate was collected and concentrated to 100 ml. The LSD (low-sugar diet) and HSD contained 0.15 and 1 M of sucrose, respectively. Aside from sucrose, no additional sugar was added to any of the growth media. Flies fed the LSD or HSD media containing the CI extracts comprised the experimental groups, and the final concentrations of the CI extracts were 5 or 10% in weight/volume. The choice of extract concentration was based in previous tests performed in flies which showed that CI aqueous extract did not affect the size and growth rate of Drosophila (data not shown).

Lifespan. To test the lifespan, after mating for 24 h, males and females were separated into vials containing experimental media. The flies were transferred to vials with fresh food once every 2 days. The number of dead flies were recorded at the time of transfer until all flies were dead. Each vial contained 30 flies, and each lifespan assay was repeated 4 times independently.

Body weight, pupal and larval volume. Newly enclosed adult flies (less than 8 h old) of each group were collected and maintained on the fresh respective medium for 24 h. Then, males and females from each group were separated under CO2 anesthesia and weighed on a balance. Five experiments per group were performed and the mean body mass was calculated. To determine the pupal or larval volume, the pupae and larvae were photographed and the volumes were calculated with the formula 4/3π(L/2)(l/2)^2 (L, length; l, width) using ImageJ software (V1.47; National Institutes of Health, Bethesda, MD, USA) (12).

Fecundity and hatching rate. Five-day-old adult flies were placed on apple juice agar plates containing yeast as the only food source. The apple juice agar plates were replaced every 2-3 h and the numbers of eggs on each plate were counted. The egg production was calculated by dividing the total egg production by the total number of h in each cage. After 22 h, the number of 1st instar larvae (L1) on each plate was counted again. The hatching rate was calculated by dividing the total number of larvae by the total number of fertilized eggs on each plate.

BODIPY and Phalloidin staining assay. Phalloidin staining was performed as previously described (13). The fat body was dissected and fixed for 30 min with 4% paraformaldehyde in PBS at room temperature. Then, the dissected tissue was stained with Phalloidin and BODIPY (Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 30 min each in a humidified chamber and washed three times for 5 min in PBST. The tissues stained with DAPI for 10 min and mounted using SlowFade Diamond Antifade Mountant (Thermo Fisher Scientific, Inc.). Fluorescence was analyzed using a Zeiss Axioplan 2 microscope (Zeiss AG, Oberkochen, Germany). The cell and lipid droplet areas were measured using ImageJ software.

Wing and cell area assay. To determine the wing and cell sizes, 19 wings from males were analyzed. Cell size was estimated by counting the number of trichomes in a defined area of the wing blade. The wing area was measured using ImageJ software (V1.47; National Institutes of Health).

Statistical analysis. The data are representative of at least three independent experiments, and images were analyzed using ImageJ (v.1.47; National Institutes of Health). The Kaplan-Meier method was used to analysis survival and performed using SPSS Statistics v.19.0 software (IBM Corporation, Armonk, NY, USA), and survival significances were used by log-rank test and performed using GraphPad Prism v.6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) (P-values were calculated for HSD and LSD <0.0167 was considered statistically significant). The remaining statistical analyses were performed through one-way ANOVA with post hoc Dunnett test using GraphPad Prism 6.0 software (P-values >0.05 indicated no significance; *P<0.05; **P<0.01; ***P<0.001). Error bars indicate the means ± standard error of the means.

Results

CI extracts increase the lifespan of flies fed an HSD. Consumption of an HSD is often associated with a decreased survival rate in flies (14). To assess whether CI could increase the lifespan of flies fed an HSD, the flies were fed an HSD either alone or supplemented with CI. We showed that the mean lifespan, 50% survival rate and maximum lifespan of the adult flies were obviously decreased in the HSD group compared with those of the LSD group (Table I, Fig. 1). However, supplementation with 5 or 10% CI significantly extended the HSD-induced lifespan in female and male flies (Table I). The mean lifespan was 33.1 and 29.7 days in HSD-fed female and male flies, respectively; however, the lifespan was significantly increased by more than 10 days in both females and males when supplemented with 5 or 10% CI, respectively. Furthermore, the 50% survival rates increased by more than 8 or 10 days in the 5 and 10% CI groups compared with those of the HSD group, and the maximum lifespan increased by more than 20 and 24 days in females and males, respectively. These results suggest that CI can limit the adverse effects of the HSD and can increase the lifespan of flies. The above results indicated that the effect of supplementation with 10% CI was better than that of 5%. Thus, the subsequent experiments were performed using 10% CI.
CI increases the body weight and pupal volume of flies fed an HSD. An HSD decreases the size of both larvae and adults due to insulin resistance (4). To determine whether supplementation with CI in an HSD changes the size of individual flies, we calculated the pupal volume and adult body weight. The results showed that the pupal volume and adult weight of flies fed an HSD were significantly decreased compared with those fed LSD (Fig. 2A). However, this small size was improved markedly when CI was added to the HSD. The pupal volume increased by 52.4% and the weights of both male and female flies increased by 81.0 and 66.1%, respectively, in flies fed diets supplemented with CI as compared with those fed an HSD (Fig. 2B and C). These results suggest that CI can reverse the phenomenon in which individuals decrease due to feeding with an HSD.

CI promotes larval development and increases the fecundity of female flies fed an HSD. LSD can severely decrease the speed of larval development (15). To analyze the effects of CI on the speed of larval development, we recorded the developmental time and the size of larvae that hatched from fertilized eggs for 96 h starting after egg laying (AEL) until the larvae reached the 3rd instar stage at different time points. There was no significant difference in size or developmental speed between the HSD and LSD groups before 96 h (data not shown). The HSD significantly retarded larval development by delaying the time to become pupa, and the individual sizes of HSD-fed larvae were smaller than those of the LSD-fed larvae. However, larvae volume was significantly increased when CI was added to the HSD, and CI accelerated larval growth and increased individual size (Fig. 3A and B). Fecundity and development are inseparable; therefore, we determined the fecundity of 5-day-old female flies in different experimental groups by measuring the egg production over 1 h. Compared with the LSD group, egg production was noticeably decreased by 73.8% in flies fed an HSD. However, the egg production was significantly increased when the HSD was supplemented with CI, and the numbers of eggs were similar to those of the LSD group (Fig. 3C).

### Table I. Lifespan of flies fed diets supplemented with and without CI extract.

| Strain | Sex | Diet<sup>a</sup> | Mean lifespan<sup>b</sup> (days ± SE) | Change of mean lifespan<sup>c</sup> (%) | P-value<sup>d</sup> | 50% Survival lifespan<sup>e</sup> (days) | Maximum lifespan<sup>e</sup> (days ± SE) | Change of maximum lifespan<sup>f</sup> (%) |
|--------|-----|------------------|-----------------------------|---------------------------------|------------------|-----------------------------|-----------------------------|-------------------------------|
|        |     |                  |                             |                                 |                  |                             |                             |                               |
| w^1118 | Female | LSD             | 57.5±1.4                     |                                 |                  |                             |                             |                               |
|        |     | HSD             | 33.1±1.1                     | -42.4                          | <0.0001<sup>e</sup> | 34.0±1.7                     | 48.3±0.6                     | -37.0                         |
|        |     | HSD+5% CI       | 43.9±1.8                     | -23.7                          | <0.0001<sup>f</sup> | 48.0±2.6                     | 68.5±0.4                     | -10.7                         |
|        |     | HSD+10% CI      | 44.9±1.9                     | -21.9                          | <0.0001<sup>f</sup> | 42.0±2.1                     | 73.5±0.6                     | -4.2                          |
|        | Male | LSD             | 56.3±1.5                     |                                 |                  |                             |                             |                               |
|        |     | HSD             | 29.7±1.0                     | -47.2                          | <0.0001<sup>e</sup> | 30.0±1.2                     | 46.2±0.9                     | -39.1                         |
|        |     | HSD+5% CI       | 40.9±1.8                     | -27.4                          | <0.0001<sup>f</sup> | 42.0±4.1                     | 70.8±0.7                     | -7.0                          |
|        |     | HSD+10% CI      | 41.6±1.8                     | -26.1                          | <0.0001<sup>f</sup> | 40.0±2.3                     | 72.0±0.8                     | -5.0                          |

<sup>a</sup>LSD refers to the standard diet containing 0.15M sugar, and the percentage value indicates the final concentration CI in the food. <sup>b</sup>Lifespan values are expressed as days ± standard error. <sup>c</sup>P-values <0.0001 are italicized, P-values were calculated by log-rank analysis for lifespan of all flies. <sup>d</sup>Maximum lifespan was based on lifespan of the top 10% longest lived flies. <sup>e</sup>P-values were calculated for HSD and LSD. P-values <0.0167 was considered statistically significant. P-values were calculated for HSD and HSD + CI, P-values <0.00833 was considered statistically significant. LSD, low-sugar diet; HSD, high-sugar diet; HSD+5% CI, high-sugar diet+ 5% *Flos Chrysanthemi Indici*; HSD+10% CI, high-sugar diet+ 10% *Flos Chrysanthemi Indici*; SE, standard error.

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by 17.8% compared with LSD, feeding with CI significantly increased the hatching rate by 17.9% (Fig. 3D). These results suggest that CI can improve the HSD-induced larval developmental time, female fecundity and egg hatching rate.

**CI reduces lipid accumulation in the fat bodies of HSD-fed larvae.** During the initial stage, T2D is often accompanied by obesity (16). The fat body of Drosophila is a functional homolog of the liver and white fat in vertebrates and is used to store fat (17). To analyze whether CI controls the accumulation of lipids, we stained the lipid droplets of the larval fat body with BODIPY. The results showed that after feeding with an HSD, the lipid droplets of fat bodies were significantly larger than those in the LSD group (Fig. 4A). Moreover, analysis using ImageJ showed a large distribution of lipid droplets in the HSD group, which demonstrated the uneven size of the average relative area of lipid droplets in this group (Fig. 4B). However, Phalloidin staining showed that though the size of the fat cells did not increase, the numbers of lipid droplets decreased (Fig. 4C and D). We also showed that after the addition of CI to the HSD group, the size of the lipid droplets was significantly reduced, the distribution of lipid droplets was smooth, and the number of lipid droplets was increased (Fig. 4A). These results suggest that the CI extract can reduce HSD-induced lipid accumulation without changing the size of the fat cells.

**CI increases the body weight, pupal volume and wing area in S6k mutants.** Insulin and insulin-growth-factor-like signaling (IIS) play vital roles during development by increasing the levels of phosphatidylinositol 3,4,5-triphosphate through the activation of 40S ribosomal protein S6 kinase (S6k, or dS6k in the case of Drosophila S6k) and protein kinase B (PKB, or dAkt in case of Drosophila protein kinase B) (18). S6k is involved in metabolic processes, cell growth and reproduction (19), and though dS6K^{+/−} mutants are viable, they have smaller body sizes due to a decrease in cell size (20). We found that CI significantly improved the HSD-induced disorders especially by increasing the individual size. These results indicate that CI may affect the insulin metabolic pathway to improve the state of insulin resistance in Drosophila. To further investigate whether CI regulates insulin metabolism in Drosophila, we fed the S6k mutant diet containing CI. Compared with the control flies, the S6k mutants showed 14.8 and 23.5% decreases in wing size and pupal volume, respectively, and the body weight of males decreased by 22.7% (Fig. 5). The S6k mutants after feeding with CI, the wing size and pupal volume increased by 13.5 and 22.1%, respectively, and the body weight of males increased by 17.0% (Fig. 5).

Next, we analyzed the sizes of both fat and wing cells. Phalloidin was used to stain the membrane of fat cells, and the relative average cell area was calculated using ImageJ. In addition, we measured the number of trichomes, a type of single bristle that accessorizes each cell of the wing blade, in a defined area of the wing blade. Though the loss of S6k can significantly decrease both fat cell and wing cell size by 28.7 and 20.7%, respectively, the cell sizes of S6k mutants were significantly increased by 20.6 and 24.2%, respectively, after the addition of 10% CI to the LSD (Fig. 6). These results suggest that the CI extract can restore the developmental defects observed in S6k mutants and can increase body weight and the cell size of peripheral tissue (thereby increasing the wing and pupa sizes).
Figure 3. CI promotes larval development and increases the fecundity of females fed an HSD. (A) Developmental stage of third instar larvae 96 h after egg laying; (B) pictures were taken every 24 h until the third instar larval stage. Relative larval volume at different developmental timing points; the larval volume is normalized to the volume of 96 h larvae fed an LSD. (C) Eggs laid by 100 female flies in each cage were counted every 2-3 h and (D) the fertilized egg hatching ability was analyzed. Experimental results of C and D were normalized to the values obtained in flies fed the LSD. All percentages in the graph vs. the LSD group **P<0.01, ***P<0.001. CI, Flos Chrysanthemi Indici; HSD, high-sugar diet; AEL, after egg laying.

Figure 4. CI decreases the size of lipid droplets but increases the number of lipid droplets in flies fed an HSD. Lipid droplets and cell membranes of fat bodies were stained with BODIPY (green) and Phalloidin (red), respectively; (A) DAPI (blue) was used to stain nuclei. (B) Quantification of relative lipid droplet area, (C) fat cell area and lipid droplet number in (D) (n=100). All experimental results were normalized to the values obtained in flies fed the LSD. The boxed areas show a highly magnified view, indicated by the white borders point. All percentages in the graph vs. the LSD group **P<0.001, ***P<0.001. Scale bars: 50 µm. CI, Flos Chrysanthemi Indici; HSD, high-sugar diet.
CI increases the fat cell area and lipid accumulation in Akt1 larvae. Akt1, a downstream effector of PI3K that regulates cell growth and organ size, can phosphorylate and antagonize the transcription factor FOXO (19). As previous experiments showed that CI can improve the knockdown of S6k-induced defects in development, we next knocked down Akt1 levels under the control of a UAS driver to further confirm the role of CI in the insulin signaling pathway. Phalloidin and BODIPY were used to stain the fat cell membranes and lipid droplets, respectively. We showed that the cell area and the size of lipid droplets were significantly decreased in fat bodies of Akt1 knockdown larvae. However, when CI was added, the cell area and lipid droplets were obviously increased by 12.6 and 78.1%, respectively, and they were similar to normal size (Fig. 7). Altogether, these results suggest that CI can increase the cell size and fat storage resulting from Akt1 knockdown.

Discussion

CI has been widely used in the treatment of various diseases due to its anti-inflammatory and antioxidative properties (21). CI is also usually used in Chrysanthemum tea, Chrysanthemum pillow, as a food additive, and in medicated baths in folk medicine (22). Previous studies have demonstrated that Chrysanthemi Flos, the same genus as CI, might have therapeutic potential in diabetic complications (8). However, the effects of aqueous CI extracts on T2D have not been previously characterized. Previous studies have demonstrated that an HSD induces insulin-resistant phenotypes in Drosophila; these phenotypes involve decreases in the individual size, growth rate, fecundity and lifespan while increasing fat deposition (10). Due to the high level of conservation between Drosophila and mammalian insulin metabolism (23), our results provide a theoretical basis for exploring the potential use of CI for the clinical treatment of diabetes.

In the present study, we observed that CI significantly improved the HSD-induced disorders by increasing the lifespan, individual size, growth rate, fecundity and hatching rate. In addition, CI decreased the fat content, and the developmental state of CI-supplemented flies was similar to those of LSD group. These results indicate that CI may affect the insulin metabolic pathway to improve the state of insulin resistance in Drosophila. However, it is not clear whether this improvement simply strengthens the absorption of excess carbohydrates by the peripheral tissue in Drosophila. To further confirm the pharmacological effects of CI, we used transgenic flies in which important components of insulin signaling pathway were knocked down. Our findings indicated that CI can increase the individual size of HSD-fed flies. In addition, previous studies have indicated that S6k and Akt knockdown can decrease cell size (24). Therefore, we fed
Figure 6. CI increases cell size in S6k mutants. (A) Fat cell membranes were stained with Phalloidin (red), and wing trichome was captured with natural light (A) in S6k mutants after feeding with an LSD or LSD contain with 10% CI. (B) Relative fat cell (n=200) and (C) wing cell (n=19) areas were calculated from (A). All experimental results were normalized to the values obtained in w1118/TM6B. The boxed areas show a highly magnified view, indicated by the black borders point. All percentages in the graph vs. the w1118/TM6B group. ***P<0.001. Scale bars: 50 µm.

Figure 7. CI increases the fat cell area and lipid accumulation of the Akt1 larvae. (A) Representative images of lipid droplets and cell membranes were stained with BODIPY (green) and Phalloidin (red); DAPI (blue) was used to stain nuclei. (B) Relative droplet (n=100) and (C) fat cell (n=200) areas were calculated from (A). All experimental results were normalized to the values obtained in Da-Gal4>w1118. The boxed areas show a highly magnified view, indicated by the white borders point. All percentages in the graph vs. the Da-Gal4>w1118 group ***P<0.001. Scale bars: 50 µm.
S6k and Akt mutants with 10% CI, and the results indicated that CI could significantly increase the sizes of fat cells in S6k and Akt mutants and lipid droplets of Akt mutants.

The two signaling pathways that control energy metabolism in Drosophila are the insulin signaling and the AKH signaling, respectively (25,26). The way of lipid metabolism of Drosophila is similar to that of mammals. Excess lipids are stored as fat droplets in the fat body cells (4). Previous studies indicate that the activation of insulin signaling pathways in non-fat tissues leads to an increase in fat storage, and that fat bodies regulate secretion of DILPs in the brain by sensing changes in carbohydrate content in the diet (17,27). Therefore, the storage of fat and insulin metabolism are inextricably linked. Most T2D patients have long-term obesity accompanied by the gradual onset of abnormal fat metabolism (28). We observed similar symptoms in HSD-fed flies, in which a large amount of fat accumulated in the fat body; however, after the addition of CI, the excessive storage of fat was significantly improved. Perhaps CI improves the metabolic homeostasis by improving the fat storage of Drosophila to further influence growth and development, but the specific mechanism requires further exploration. Therefore, we predict that aqueous CI extracts may have potential as antiobesity agents, and further experimentation is required to fully understand the pharmacological functions of CI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LHJ made substantial contributions to conception and design, and revised the manuscript critically for important intellectual content. YB performed the experiments, and analyzed the growth rate, lifespan, fat storage, cell size and wing size, and was a major contributor in writing the manuscript. KL performed and analyzed the data regarding the body weight. JYS performed the experiments and analyzed the data regarding the reproductive capacity. QXL performed statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.
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