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3M-Brazzein as a natural sugar substitute rescued obesity in maternal and offspring mice

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ABSTRACT

Weight loss is among the greatest challenges facing public health in the globesity epidemic. Reducing or eliminating sugar from the diet is the main dietary factor for weight loss. Here, 3M-brazzein, a natural sweet protein, was investigated as a possible sugar substitute for weight loss. High-fat diet-induced female mice were fed normal chow and exposed to 3M-brazzein or 10 % sucrose of equivalent sweetness in drinking water before mating through pregnancy and lactation. Consumption of 3M-brazzein reduced body weight and adiposity and induced a partial recovery of the metabolic disorder before mating, unlike sucrose consumption. Additionally, 3M-brazzein consumption by obese mice before pregnancy mitigated offspring obesity risk, whereas offspring of sucrose-consuming obese mice displayed adiposity, hypertrophy, and metabolic disorder. By virtue of its naturally low-calorie content, 3M-brazzein could be an effective sugar substitute to support weight loss in obesity, even during pregnancy and lactation, reducing adiposity and attenuating metabolic disorders.

1. Introduction

The escalating rates of overweight and obesity among adults and children worldwide are a growing concern. More than 1.9 billion adults are overweight, including 650 million suffering from obesity, as reported by the World Health Organization (Organization, 2022). This accounts for 39 % of the adult population globally, with the prevalence of obesity having nearly tripled since 1975. Such a surge in obesity significantly elevates the risk of various noncommunicable diseases, including cardiovascular diseases, diabetes mellitus, musculoskeletal disorders, and some cancers (Kulkarni, Karssiens, Kumar, & Pandit, 2016; Singh & Lin, 2013). Childhood obesity is especially associated with a wide range of serious health complications and an increased risk of premature onset of related illnesses (Sahoo et al., 2015). In addition, strengthening evidence links maternal obesity with increased offspring risks of obesity, coronary heart disease, stroke, type 2 diabetes, and asthma. Maternal obesity may also lead to poorer cognitive performance in the offspring and an increased risk of cerebral palsy and other neurodevelopmental disorders (Godfrey et al., 2017; Kankowski et al., 2022).

Recent epidemiological studies have shown a rising trend in obesity among mothers and children. According to a reported study, about 30 % of pregnant women now fall into the obese category, a notable increase with serious health implications (Lane, Wilcox, Wingard, McLean, & Liu, 2023). Similarly, WHO (Organization, 2022) observes a surge in child obesity, increasing from 4 % to over 18 % since 1975, influenced by dietary, physical, and socioeconomic factors. Furthermore, a significant link between maternal and childhood obesity was highlighted, emphasizing the need for comprehensive public health strategies (Shipp et al., 2024). These findings reinforce the relevance of our study's focus on obesity interventions for mothers and children, aiming to provide actionable insights for tackling this growing public health issue.

Optimal diets for weight management have been rigorously debated among researchers, nutrition experts, healthcare professionals, and the general public (Makris & Foster, 2011). Weight management depends upon complex factors, such as the amount of food eaten, the type of food eaten, and the timing of meals. Evidence shows that calorie restriction is the most important factor for weight loss, and a low-calorie diet with a

Abbreviations: AUC, area under the curve; 3M-brazzein, brazzein with triple mutations (H31R/E36D/E41A); EpiWAT, epididymal white adipose tissue; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter type 4; GTT, glucose tolerance test; H&E, hematoxylin, and eosin; HFD, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; IngWAT, inguinal white adipose tissue; i.p., intraperitoneal; IRS2, insulin receptor substrate 2; ITT, insulin tolerance test; SSB, sugar-sweetened beverage. WAT, white adipose tissue.

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low-fat or low-carbohydrate content has been recommended to reduce the daily calorie intake. However, metabolic adaptations to decreased energy intake can lead to reduced energy expenditure (J. Y. Kim, 2021). The main cause of obesity is an energy imbalance between calories consumed and calories expended due to an increased intake of energy-dense foods high in fat and sugars (Hall, 2018). Evidence from human studies demonstrates that increasing the overall amount of food available for consumption leads to increased *ad libitum* energy intake. The increased energy availability in the food supply is likely an important driver of the obesity epidemic (Hall, 2018). Especially, sugar-sweetened beverages (SSBs) have emerged as an important risk factor for weight gain and obesity-related diseases (Malik, Popkin, Bray, Despres, & Hu, 2010). SSBs are the largest source of added sugar in the diet. Many health authorities have called for reductions in SSB consumption and implemented public policies to limit SSB intake to improve health (Malik & Hu, 2022).

Brazzein is a water-soluble and heat-stable sweet-tasting natural protein with a sweetness that is 500 to 2000 times greater than sucrose (Ming & Hellekant, 1994). One of the recombinant brazzein mutant proteins, the brazzein protein with triple mutations (H31R/E36D/E41A; 3M-brazzein), was produced in our laboratory using yeast *Kluyveromyces lactis (K. lactis)*, which is generally recognized as safe (GRAS), and showed 22,500 times stronger sweetness than sucrose (J. W. Lee, Cha, Jo, & Kong, 2013). It has been reported that 3M-brazzein did not induce obesity and does not appear to disrupt glucose homeostasis and insulin sensitivity (H. Kim et al., 2020). Therefore, brazzein is an attractive candidate sugar substitute for improving public health by reducing sugar overconsumption.

Our study is aims to investigate the effects of 3M-brazzein, a natural sweet protein, as a potential sugar substitute in mitigating obesity and its associated concerns, particularly focusing on maternal and offspring obesity. We hypothesized that the introduction of 3M-brazzein in the diet of high-fat diet-induced obese female mice will not only lead to a decrease in their body weight and fat accumulation but also have a beneficial effect on the obese and metabolic health of their offspring. Hence, this investigation primarily seeks to explore the efficacy of 3M-brazzein as a sugar alternative in diminishing obesity and ameliorating metabolic outcomes in both the maternal subjects and their offspring, presenting a potentially groundbreaking strategy in the global fight against obesity.

2. Materials and methods

2.1. Animals and diet

All the animal care and experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee at Chung-Ang University (Seoul, Korea). Six-week-old female C57BL/6J mice ($n = 40$) were purchased from DBL (Chungcheongbuk-do, Korea). The animals were maintained in an animal facility controlled on a 12 h light–dark cycle at room temperature. After 1 week of habituation, mice were randomly divided into two groups and fed either an HFD ($n = 30$; Seongnam-si, Korea) or an NC diet ($n = 10$; Cargill, Wayzata, MN, USA) for 6 weeks. On a caloric basis, the HFD consisted of 58 % fat from lard, 25.6 % carbohydrate, and 16.4 % protein (total 23.4 kJ/g), whereas the NC diet contained 11.4 % fat, 62.8 % carbohydrate, and 25.8 % protein (total 12.6 kJ/g).

Six weeks later, the HFD group of mice was uniformly divided into three subgroups and switched to the NC diet supplemented with water ($n = 10$, HFD/NC-water group), 10 % sucrose (Sigma-Aldrich Co., St. Louis, MO. USA) solution ($n = 10$, HFD-NC/10 % sucrose group), or 3M-brazzein solution ($n = 10$, HFD-NC/3M-brazzein group) before mating through pregnancy and lactation. 3M-brazzein was purified and diluted in water to have an equivalent sweetness to 10 % sucrose on a weight basis, as described previously.(H. Kim et al., 2020). The control group of mice received continuous feeding of the NC diet with water. The amount of food and solution intake was monitored daily by measuring the weight and volume, and the body weights of the mice were measured weekly by precision electronic scales during the study.

After 5–6 weeks on the NC diet with water, 3M-brazzein, or 10 % sucrose, when the HFD/NC-water group of mice lost weight and returned from obesity back to normal, all female mice were mated with the mice fed with NC-water. All pups were monitored and weighed daily from 4 days of age. On day 21 of birth, all dams and pups were euthanized. The blood was collected, and their white adipose tissue (WAT) and liver were isolated, weighed, instantly frozen in liquid nitrogen, and stored at −80 °C for further experiments.

2.2. Blood biochemical analysis

Mice fasted for 12 h before the glucose tolerance test (GTT) and insulin tolerance test (ITT). Glucose was intraperitoneally (i.p.) injected at 1.5 g/kg of body weight for GTT. Insulin (Humlin® R, Eli-Lilly and Co., Indianapolis, IN, USA) was i.p. injected at 0.75 U/kg of body weight for ITT. Blood glucose level was measured by a glucometer (CareSens® Pro, Seoul, Korea) at 0, 15, 30, 60, and 120 min after glucose or insulin injection through the tail vein. Blood glucose levels were plotted against time, and the area under the curve (AUC) was calculated. The serum was separated from the fresh blood samples by centrifugation at 500 × *g*, 4 °C, for 15 min. Plasma C-peptide and insulin levels were determined using commercial ELISA kits (Crystal Chem, Elk Grove Village, IL, USA) according to the manufacturer's protocols. Briefly, blood was drawn from the mice's tail vein and immediately serum was separated by centrifugation. From this, 5 μL of serum was used for insulin measurement. The homeostatic model assessment of insulin resistance (HOMA-IR) was determined by [fasting glucose level (mg/dL) × fasting insulin level (mU/L)] / 405.(Wallace, Levy, & Matthews, 2004).

2.3. Histological analysis of WAT and liver tissue

The fresh inguinal white adipose tissue (IngWAT) and liver were immediately implanted in O.C.T. compound (Tissue Tek, Torrance, CA, USA) and frozen in dry ice-chilled isopentane. Cross-sections with a thickness of 15 ~ 20 μm for WAT and 10 ~ 15 μm for the liver were prepared and stained with hematoxylin and eosin (H&E), and liver samples were additionally stained with Oil-Red-O. Images of the stained sections were captured using a microscope (Eclipse Ti2, Nikon, Japan).

2.4. Total RNA extraction from tissue and qRT-PCR

Total RNA was isolated from the liver samples using the TRIzol™ reagent (Thermo Fisher Scientific, MA, USA). First-strand cDNA was synthesized using MultiScribe™ reverse transcriptase (Thermo Fisher Scientific) and random primers. Quantitative polymerase chain reaction (qPCR) was conducted using Power SYBR™ Green Master Mix (Thermo Fisher Scientific). Primer sequences used in qPCR are listed in Table S1. Target gene expression was normalized to the internal control, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the relative mRNA level was determined by fold change based on the comparative cycle threshold method $2^{-\Delta\Delta Ct}$, with C_t values obtained using the QuantStudio™ 1 Real-Time PCR System (Thermo Fisher Scientific).

2.5. Statistical analysis

Data were analyzed and expressed as mean ± standard error of the mean (SEM) using the GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). For determining the significance of all experiments, including GTT, ITT, and biochemical parameter tests, Student's *t*-test and two-way ANOVA were conducted. Tukey's multiple comparisons test was performed by ANOVA interactions for multiple comparisons. Significance was defined as $p < 0.05$.

3. Results

3.1. 3M-Brazzein mitigated HFD-Induced obesity in mice

An HFD is commonly used to develop diet-induced obesity models (Gupta et al., 2017; Y. S. Lee et al., 2011). In this study, C57BL/6J female mice were fed an HFD *ad libitum* over 6 weeks to induce obesity. Initially, the mean body weight and glucose level of the mice in the HFD-fed group ($n = 30$) and NC-fed control group ($n = 10$) showed no difference (17.90 *vs.* 18.00 g). After 6 weeks, HFD-exposed mice showed significantly increased obesity and insulin resistance-related phenotypes, including elevated body weight (21.80 *vs.* 27.26 g, $p < 0.0001$), accumulated fat mass, high blood glucose level, and impaired tolerance to both glucose and insulin relative to NC-fed control mice (Fig. 1B–E, Fig. S1A–C). HFD-exposed mice consumed lower amounts of diet than NC diet-exposed mice; however, total calorie intake was significantly higher in HFD-fed mice (Fig. S2A, B).

Once obese, the HFD-female mice were divided into three subgroups: HFD-NC/water group, HFD-NC/10 % sucrose group, and HFD-NC/3M-brazzein group, before mating through pregnancy and lactation for 12 weeks (Fig. 1A). NC-fed mice were continuously fed with NC and water as a control group throughout the study (NC-water or control group). The body weight of the HFD-NC/water group started to decrease when switched to the NC diet until it reached that of the NC-water group. Similarly, the 3M-brazzein−exposed mice exhibited a decrease in body weight. However, the 10 % sucrose-drinking mice showed a continuous increase in body weight despite the change to the NC diet (Fig. 1F). At the end of the study, the average body weight of the four groups reached 25.10 ± 0.547 g (HFD-NC/3M-brazzein group), 25.63 ± 0.575 g (NC-water group), 26.60 ± 0.542 g (HFD-NC/water group), and 30.00 ± 0.730 g (HFD-NC/10 % sucrose group). The 3M-brazzein supplement neither affected the amount of solid food and fluid intake nor altered the total calorie intake, unlike 10 % sucrose consumption (Fig. 1G,H). 3M-brazzein did not cause overconsumption but contributed to weight loss when treated at a similar sweetness level to 10 % sucrose.

3.2. 3M-Brazzein consumption diminished HFD-Induced body fat accumulation

Excess body fat accumulation is usually observed in obesity. To check the body fat, the IngWAT of all female mice was removed, photographed, and weighed at the end of the study. The size and weight of IngWAT were similar between the HFD-NC/3M-brazzein group, control group, and HFD-NC/water group, whereas they were significantly increased in the HFD-NC/10 % sucrose group ($p < 0.05$; Fig. 2A,B). Furthermore, the IngWAT weight-to-body weight ratio showed no difference between the HFD-NC/water and HFD-NC/3M-brazzein groups but was dramatically increased in the HFD-NC/10 % sucrose group compared to that in the control group (Fig. 2C). 3M-brazzein consumption in liquid form did not affect fat increase but decreased body fat accumulated by HFD, suggesting that 3M-brazzein may substitute sugar to alleviate obesity caused by overconsumption.

Histological analysis of IngWAT showed that the adipocyte size was similar between the HFD-NC/3M-brazzein group and the control group and smaller than that in the HFD-NC/10 % sucrose group (Fig. 2D). The average size of adipocytes in IngWAT was similar between the control and 3M-brazzein groups, which had smaller adipocytes than the HFD-NC/10 % sucrose group (Fig. 2E). Moreover, most of the adipocytes in the IngWAT of the control group, HFD-NC/water group, and HFD-NC/3M-brazzein group (94.6 %, 90.0 %, and 93.6 % of IngWAT, respectively) had diameters of 30—–60 μm, unlike the adipocytes in the IngWAT from the HFD-NC/10 % sucrose group, which had a broader diameter range of 40–100 μm, and most of them measured > 50 μm in diameter (98.0 % of the IngWAT) (Fig. 2F).

3.3. 3M-Brazzein affected the recovery of HFD-Altered glucose homeostasis

A previous study found that the chronic consumption of 3M-brazzein in liquid form did not disturb glucose homeostasis (H. Kim et al., 2020). Therefore, we investigated if chronic 3M-brazzein drinking could recover the HFD-altered metabolic homeostasis using *in vivo* GTT assay by i.p. glucose injection after 12 h fasting. The three randomly assigned HFD groups exhibited a high level of blood glucose and impaired tolerance to both glucose and insulin after starting the treatment with different solutions (Fig. 1D,E). As expected, the diet change from HFD to NC induced weight loss and showed a recovery of the disrupted glucose homeostasis in the HFD-NC/water group. Blood glucose levels of the HFD-NC/3M-brazzein group also returned to similar levels of the control group. However, despite the diet change to NC, the HFD-NC/10 % sucrose group exhibited continuous impairment in glucose homeostasis (Fig. 3A). This was confirmed by AUC analysis. Mice exposed to water or 3M-brazzein had similar GTT-AUC, whereas the AUC of the 10 % sucrose group was significantly higher than that of the control group (Fig. 3B). Furthermore, insulin response indicated that the diet change from HFD to NC recovered the impaired metabolic homeostasis, and 3M-brazzein consumption aided the recovery of impaired glucose levels. However, the blood glucose level was still impaired in mice treated with 10 % sucrose despite the diet change from HFD to NC (Fig. 3C). Again, this was confirmed by AUC calculation (Fig. 3D).

Serum insulin and C-peptide levels were measured in blood samples at the end of the study. Insulin and C-peptide levels were similar among the HFD-NC/3M-brazzein group, control group, and HFD-NC/water group. However, the serum insulin and C-peptide levels of mice treated with 10 % sucrose were significantly elevated compared to those in the control group (Fig. 3E,F). Moreover, the HOMA-IR index used as an index of insulin resistance was significantly increased in the HFD-NC/10 % sucrose group compared with those in the other groups ($p < 0.0005$) (Fig. 3G).

3.4. 3M-Brazzein did not affect insulin signaling and hepatic fat accumulation

We have previously demonstrated that chronic 3M-brazzein consumption does not affect insulin signaling and that it does not cause fat accumulation in the liver of wild-type mice.(H. Kim et al., 2020) In the present study, we investigated whether 3M-brazzein drinking would have similar outcomes in HFD-induced mice. Low insulin response is generally associated with a decrease in the expression of hepatic insulin receptors and insulin-regulated glucose transporters involved in insulin signaling, resulting in high blood glucose. Hepatic mRNA levels of glucose transporter type 4 (*GLUT4*) and insulin receptor substrate 2 (*IRS2*) genes were measured. The *IRS2* and *GLUT4* expression levels were similar among the 3M-brazzein, NC-water, and HFD-NC/water groups and significantly decreased in the HFD-NC/10 % sucrose group (Fig. 4A,B). It indicated that the disturbed glucose metabolism was linked to impaired insulin signaling in the liver.

Furthermore, only the HFD-NC/10 % sucrose group had significantly different wet liver weight and size from the control group ($p < 0.05$; Fig. 4C,D), showing that chronic 3M-brazzein drinking could aid in reducing accumulated fat in the liver. A previous report showed that wet liver weight was not significantly affected in mice treated with 10 % sucrose (H. Kim et al., 2020). The HFD may have caused the wet liver weight difference. The switch from HFD to NC reduced hepatic fat accumulation in the water and 3M-brazzein groups, whereas the HFD-NC/10 % sucrose group did not lose hepatic fat accumulated by the HFD. It demonstrated that 3M-brazzein consumption did not affect hepatic fat accumulation, whereas it did aid in reducing the accumulated fat. H&E staining and Oil-Red-O staining confirmed that 3M-brazzein consumption did not lead to hepatic fat accumulation, while 10 % sucrose consumption resulted in the accumulation of hepatic fat in HFD-induced

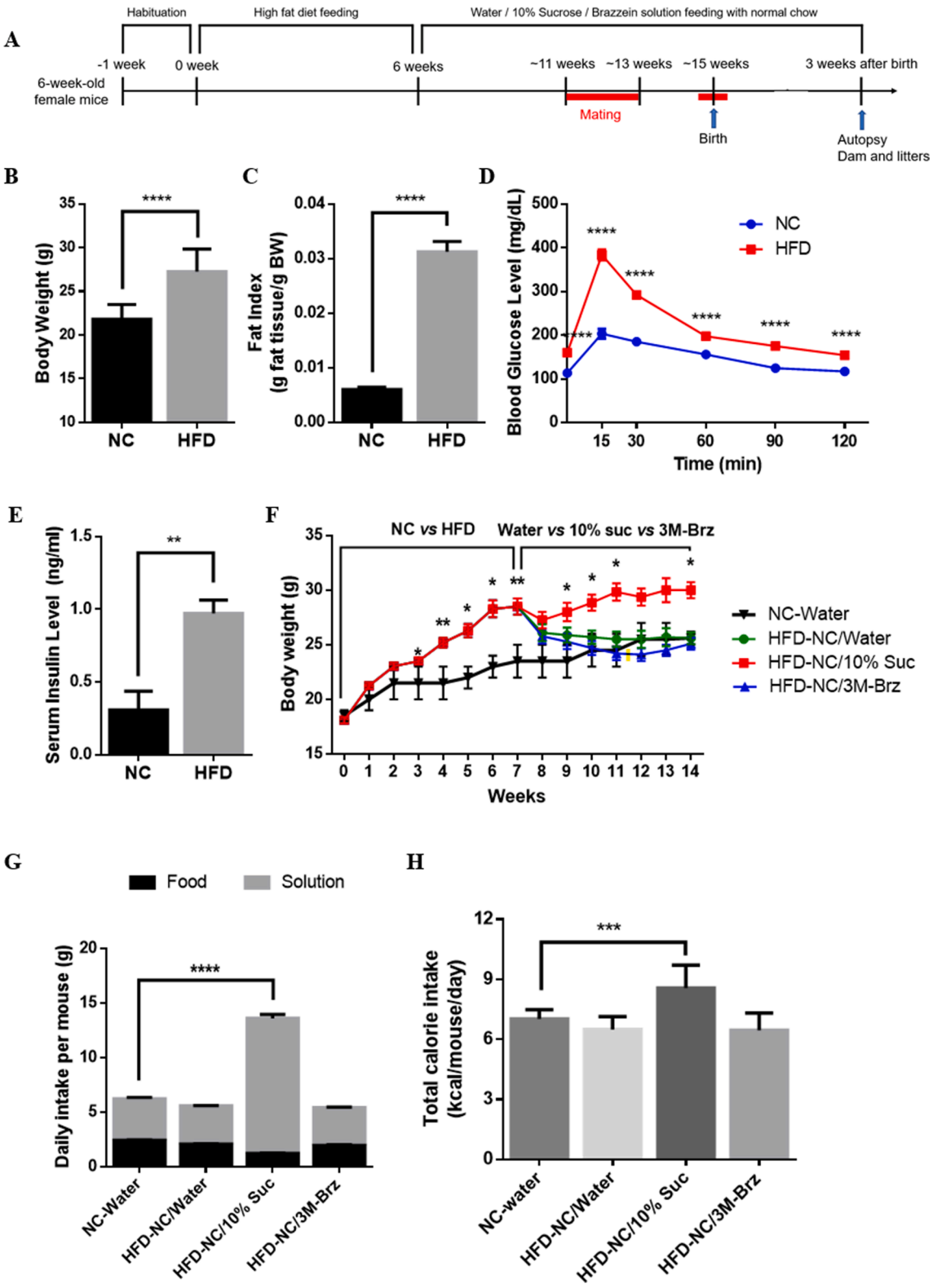

Fig. 1. 3M-**Brazzein mitigated HFD-induced obesity in mice.** (**A**) Experimental design of the study. (**B**) Body weight. (**C**) WAT fat index. (**D**) GTT performed after 12 h of fasting. Blood glucose levels are shown at baseline and following i.p. administration of glucose (2 mg/kg). (**E**) Serum insulin level. HFD, mice supplied with high-fat diet ($n = 30$); NC, mice supplied with normal-chow ($n = 10$). (**F**) Weekly body weight. (**G**) Average daily food intake for a mouse. (**H**) Average daily calorie intake for a mouse. NC-water, mice supplied with NC and water ($n = 10$); HFD-NC/Water, mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc, mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz, mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

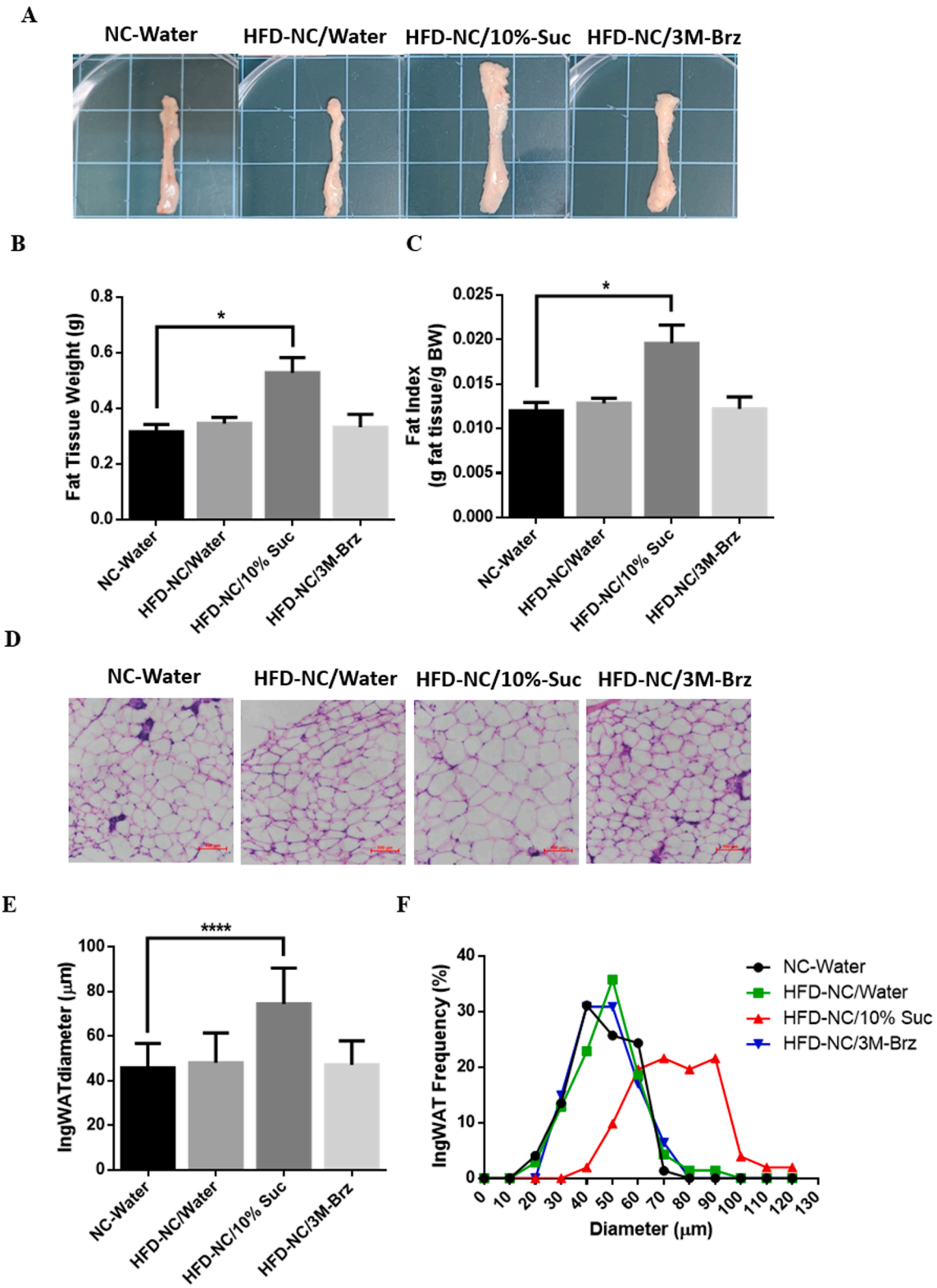

Fig. 2. 3M-**brazzein consumption diminished HFD-induced body fat accumulation.** (**A**) Representative pictures of IngWAT. (**B**) The amount of IngWAT. (**C**) IngWAT fat index. (**D**) Representative images of H&E staining of IngWAT for each group. (**E**) Quantitative analysis of adipocyte size for IngWAT. (**F**) The adipocyte size distribution of IngWAT. NC-water, mice supplied with NC and water ($n = 10$); HFD-NC/Water, mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc, mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz, mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-test, $^{*}p < 0.05$, $^{****}p < 0.0001$.

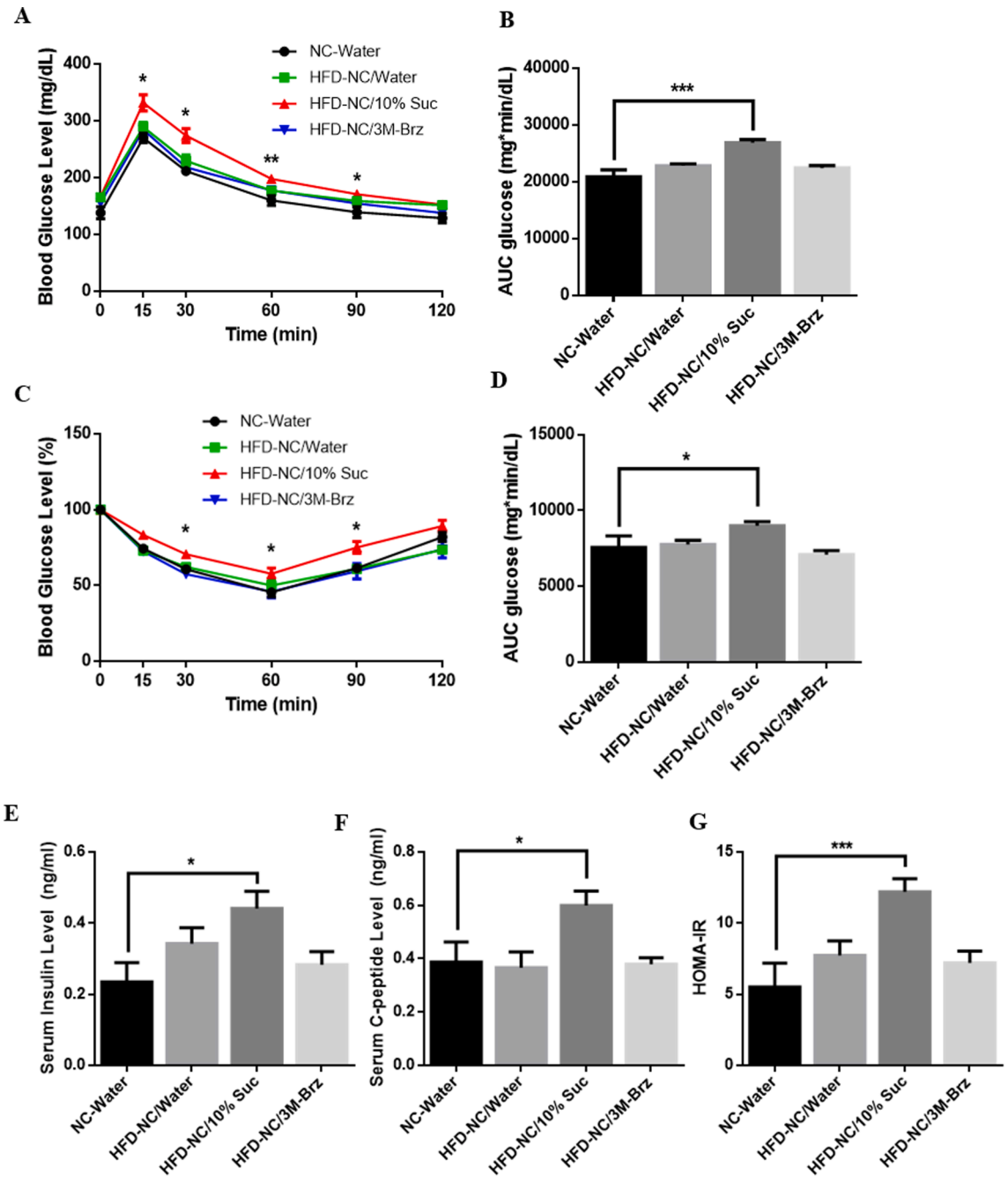

Fig. 3. 3M-**brazzein affected the recovery of HFD-altered glucose homeostasis.** (**A**) GTT performed after 12 h of fasting. Blood glucose levels are shown at baseline and following i.p. administration of glucose (2 mg/kg). (**B**) AUC representation of GTT data. (**C**) ITT performed after 12 h of fasting. Blood glucose levels are shown at baseline and following i.p. administration of insulin (0.75 U/kg). (**D**) AUC representation of ITT data. (**E**) Serum insulin level. (**F**) Serum C-peptide level. (**G**) HOMA-IR presented as a median. NC-water, mice supplied with NC and water ($n = 10$); HFD-NC/Water, mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc, mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz, mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-tests for fasting blood glucose, insulin, and c-peptide and two-way ANOVA for GTT and ITT, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

obese mice (Fig. 4E,F).

3.5. 3M-Brazzein did not cause pediatric obesity

Several studies have suggested maternal obesity can lead to obesity in their baby and child (Heslehurst et al., 2019; Leddy, Power, & Schulkin, 2008). In this study, we investigated whether the chronic drinking of 3M-brazzein during pregnancy and lactation would cause obesity in offspring. The body weight and body length were measured in all pups at birth and 3 weeks of age.

Compared to pups born from NC-water control mice (NC-water F1), the body weight of male and female pups born from HFD-NC/3M-brazzein−exposed mice (HFD-NC/3M-brazzein F1) showed no significant differences at ages 4 days and 3 weeks, while the pups born from 10 % HFD-sucrose exposed mice (HFD-NC/10 % sucrose F1) had a higher body weight at age 4 days ($p < 0.0001$) and 3 weeks ($p < 0.0005$) (Fig. 5A,B). However, there was no significant difference in the number of pups, gender, and body length of pups among all four groups (Fig. 5C).

At 3 weeks of age, both the IngWAT and epididymal WAT (EpiWAT)

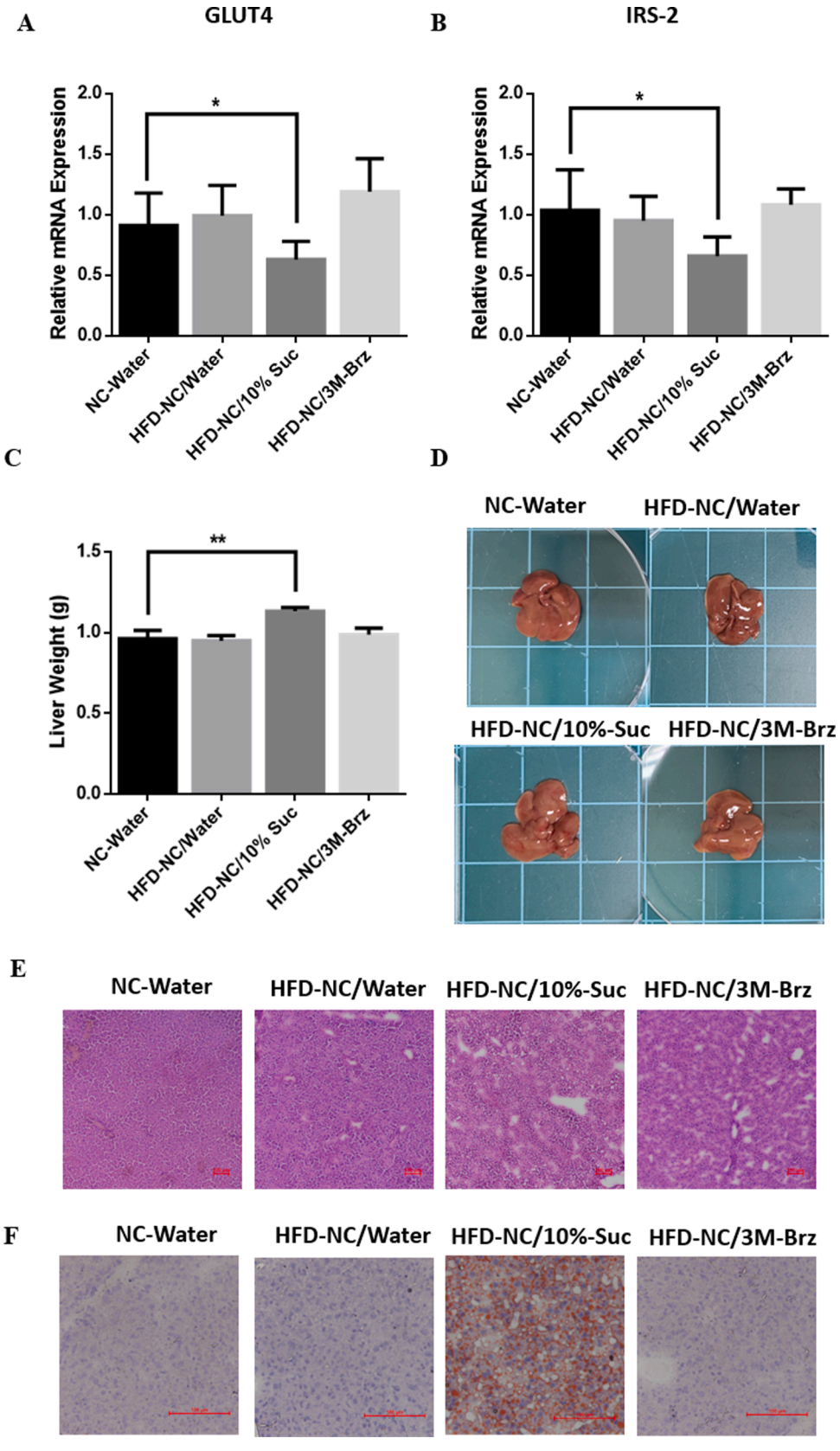

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Fig. 4. 3M-**brazzein did not affect insulin signaling and hepatic fat accumulation.** Hepatic mRNA expression of (**A**) IRS2, (**B**) GLUT4. (**C**) Liver wet weight in each group. (**D**) Representative images of livers in each group. (**E**) Representative images of H&E staining of livers in each group. (**F**) Representative images of Oil-Red-O staining of the liver in each group. NC-water, mice supplied with NC and water ($n = 10$); HFD-NC/Water, mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc, mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz, mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from all pups were removed, photographed, and weighed. The IngWAT and EpiWAT removed from HFD-NC/3M-brazzein F1 group were of similar size and weight to those of the NC-water F1 group, while the HFD-NC/10 % sucrose F1 group showed significantly increased weight and size of IngWAT and EpiWAT (Fig. 5D–F, Fig. S3), indicating that chronic 3M-brazzein drinking in female mice before pregnancy and throughout pregnancy and lactation did not cause fat accumulation in the offspring. Moreover, histological analysis of IngWAT indicated that adipocyte size was similar between the HFD-NC/3M-brazzein F1 group and control group, whereas the adipocyte size was increased in the HFD-NC/10 % sucrose F1 group (Fig. 5G). The average size of adipocytes in IngWAT was similar between the HFD-NC/3M-brazzein F1 group and the NC-water F1 group, which had smaller adipocytes than the HFD-NC/10 % sucrose F1 group (Fig. 5H). In addition, most of the adipocytes in the NC-water F1, HFD-NC/water F1, and HFD-NC/3M-brazzein F1 groups had diameters of 20–40 μm (97 %, 93 %, and 93 % of IngWAT, respectively). By contrast, adipocytes from the HFD-NC/10 % sucrose F1 group showed a broader diameter range of 20–70 μm, and most of them (65 %) measured > 40 μm in diameter (Fig. 5I).

3.6. 3M-Brazzein did not alter the glucose homeostasis and hepatic fat accumulation in offspring

HFD-induced obesity and impaired glucose metabolism were recovered by diet change from HFD to NC with water or 3M-brazzein in drinking water, whereas consumption of 10 % sucrose in drinking water increased maternal obesity and impaired glucose metabolism in HFD-induced obese mice (Figs. 2 and 3). We further investigated the relationship between maternal 3M-brazzein drinking and the metabolism of the descendants. The *in vivo* GTT test by i.p. glucose injection after 12 h fasting revealed no significant difference among the NC-water F1, HFD-NC/water F1, and HFD-NC/3M-brazzein F1 groups, whereas the HFD-NC/10 % sucrose F1 mice already showed impaired glucose homeostasis at 3 weeks of age (Fig. 6A). This was confirmed by AUC analysis. Mice born from mothers who drank water or 3M-brazzein in drinking water had similar GTT-AUC, whereas the HFD-NC/10 % sucrose F1 mice showed significantly higher AUC compared to the NC-water F1 group (Fig. 6B). To measure insulin sensitivity, an ITT assay was performed by i.p. insulin injection, and the glucose levels were measured and presented as the percentage of basal glucose. Insulin sensitivity was not changed in all mice groups at 3 weeks of age, unlike GTT sensitivity (Fig. 6C). AUC analysis also showed that ITT-AUC was similar in all four groups (Fig. 6D). Serum insulin and C-peptide levels were measured in blood samples at the end of the study. The insulin and C-peptide levels were similar among the HFD-NC/3M-brazzein F1, NC-water F1, and HFD-NC/water F1 groups. However, the serum insulin and C-peptide levels of 10 %-sucrose-drinking mice pups were significantly elevated compared to those of the control group mice pups (Fig. 6E,F). Moreover, the HOMA-IR index used as an index of insulin resistance was significantly increased in the HFD-NC/10 % sucrose F1 group compared with those in the other groups ($p < 0.0005$) (Fig. 6G).

Liver insulin signaling pathway gene expression and hepatic fat accumulation were not affected by chronic 3M-brazzein consumption in HFD-induced obese mice, unlike HFD-NC/10 % sucrose consumption (Fig. 4). We further investigated whether the insulin signaling pathway and hepatic fat accumulation were altered in descendants born from maternal chronic 3M-brazzein−consuming mice.

Hepatic mRNA levels of *GLUT4* and *IRS-2* genes in offspring from the 3M-brazzein group were similar to those in offspring from the NC-water and HFD-NC/water group, whereas the *IRS2* and *GLUT4* expression levels were significantly decreased in the HFD-NC/10 % sucrose F1 group (Fig. 7A,B). Furthermore, only the HFD-NC/10 % sucrose F1 group had significantly different wet liver weight and size from the control group ($p < 0.0001$; Fig. 7C,D), showing that chronic 3M-brazzein drinking during pregnancy and lactation did not affect fat accumulation in the liver. H&E staining and Oil-Red-O staining confirmed that 3M-brazzein consumption during pregnancy and lactation did not lead to hepatic fat accumulation in mice pups, while 10 % sucrose consumption resulted in accumulation of hepatic fat in offspring of HFD-induced obese mice (Fig. 7E,F). Chronic consumption of 3M-brazzein in liquid form reduced body weight, adiposity, and hypertrophy and attenuated metabolic disorder in HFD-induced obese mice, unlike sucrose consumption. Furthermore, throughout pregnancy and lactation, 3M-brazzein drinking exhibited no significant effects on offspring obesity and metabolic pathway compared to sucrose drinking. Our study suggests that 3M-brazzein may be an effective sugar substitute for weight loss, even during pregnancy and lactation.

4. Discussion

Combating obesity, recognized worldwide as a major public health challenge, requires groundbreaking and enduring dietary strategies. This study contributes to the expanding knowledge base by examining the role of 3M-brazzein as a natural sweetener, particularly for controlling and preventing obesity. The excessive consumption of added sugars is a widely acknowledged factor in the rise of obesity (Makris & Foster, 2011). Our study's focus on decreasing sugar consumption is aligns with recommendations for low-calorie diets, which are key in addressing the obesity crisis. The ongoing debate about artificial and natural sweeteners centers on their role as substitutes for high-calorie sugars. While artificial sweeteners offer a low-calorie alternative to sugar, concerns about their long-term effects on health, particularly gut microbiota, warrant careful consideration (Suez et al., 2014). In contrast, natural sweeteners like brazzein provide a promising alternative, offering sweetness without the caloric burden and additional health benefits, including antimicrobial and anti-inflammatory effects (Perez Espitia et al., 2012). Future research should continue to explore these alternatives, focusing on long-term health impacts and potential therapeutic applications.

Brazzein, in particular, has emerged as a promising natural sweetener due to its high sweetness potency and favorable sensory qualities, devoid of the undesirable aftertastes often associated with many artificial sweeteners (Assadi-Porter, Aceti, & Markley, 2000; Izawa, Ota, Kohmura, & Ariyoshi, 1996). In contrast to high sugar consumption, which is often linked to weight gain and metabolic disorders, 3M-brazzein does not contribute to these health issues. The study by H. Kim et al. (2020) provides evidence supporting these beneficial health effects (H. Kim et al., 2020). Future research could explore the long-term health impacts and potential uses of brazzein. Its applications in the food industry, especially in sugar-reduced products, are promising.

This study extends previous research to explore the effects of 3M-brazzein on weight management in obese mice and assess its safety during pregnancy and lactation, with a special focus on the implications of sugar cravings and excessive sugar intake during pregnancy. It is estimated that 50–90 % of pregnant women have one or more food cravings during gestation. There is substantial evidence that sugar

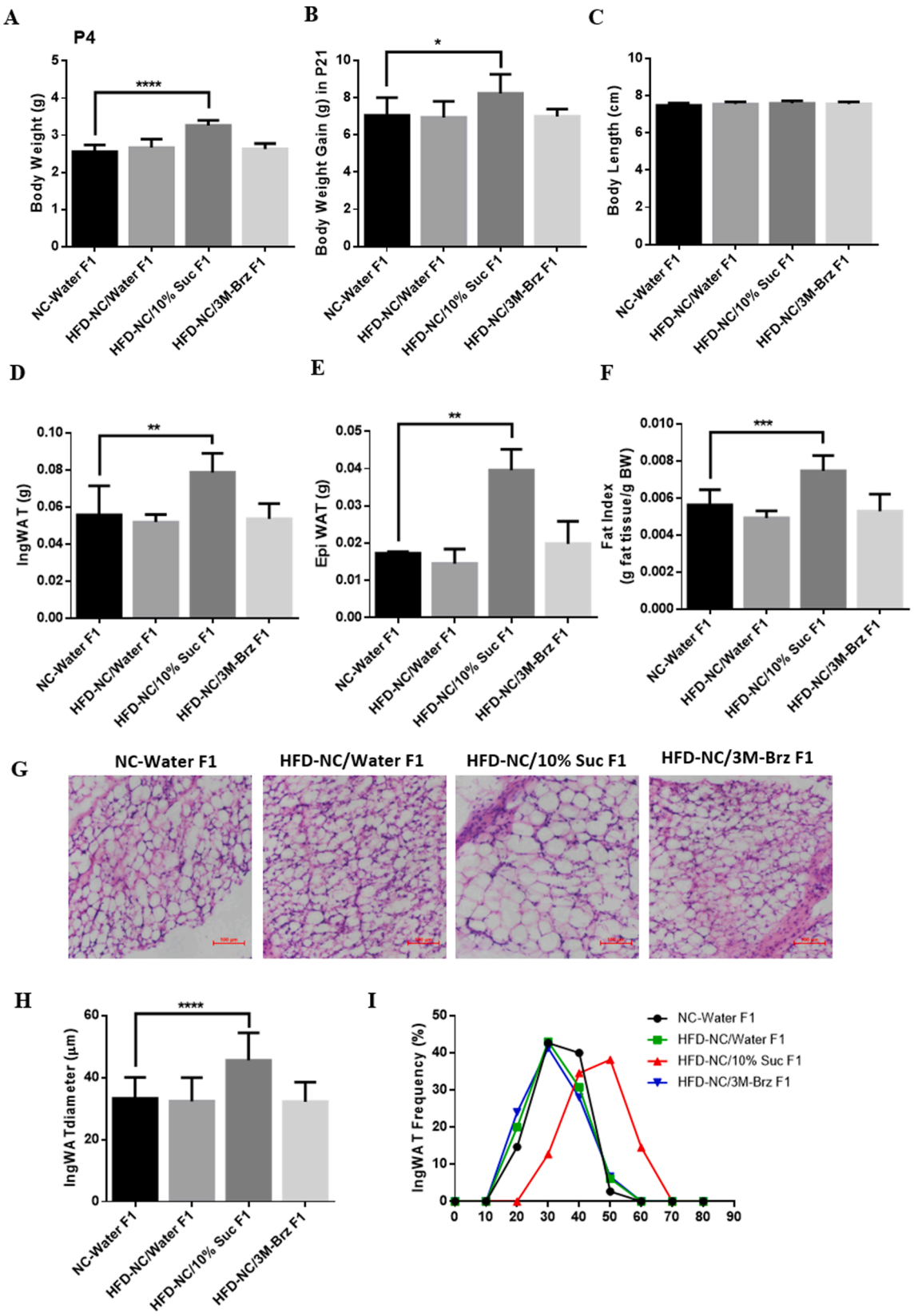

Fig. 5. 3M-**brazzein did not cause fat accumulation in offspring.** (A) Body weight of F1 mice aged 4 days. (**B**) body weight gain of F1 mice from 4 to 21 days of age. (**C**) Body length of F1 mice on day 21 after birth. The amount of (**D**) IngWAT, (**E**) EpiWAT. (**F**) WAT fat index. (**G**) Representative images of H&E staining of IngWAT for each group. (**H**) Quantitative analysis of adipocyte size for IngWAT. (**I**) Adipocyte size distribution of IngWAT. NC-water F1, pups born from mice supplied with NC and water ($n = 10$); HFD-NC/Water F1, pups born from mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc F1, pups born from mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz F1, pups born from mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

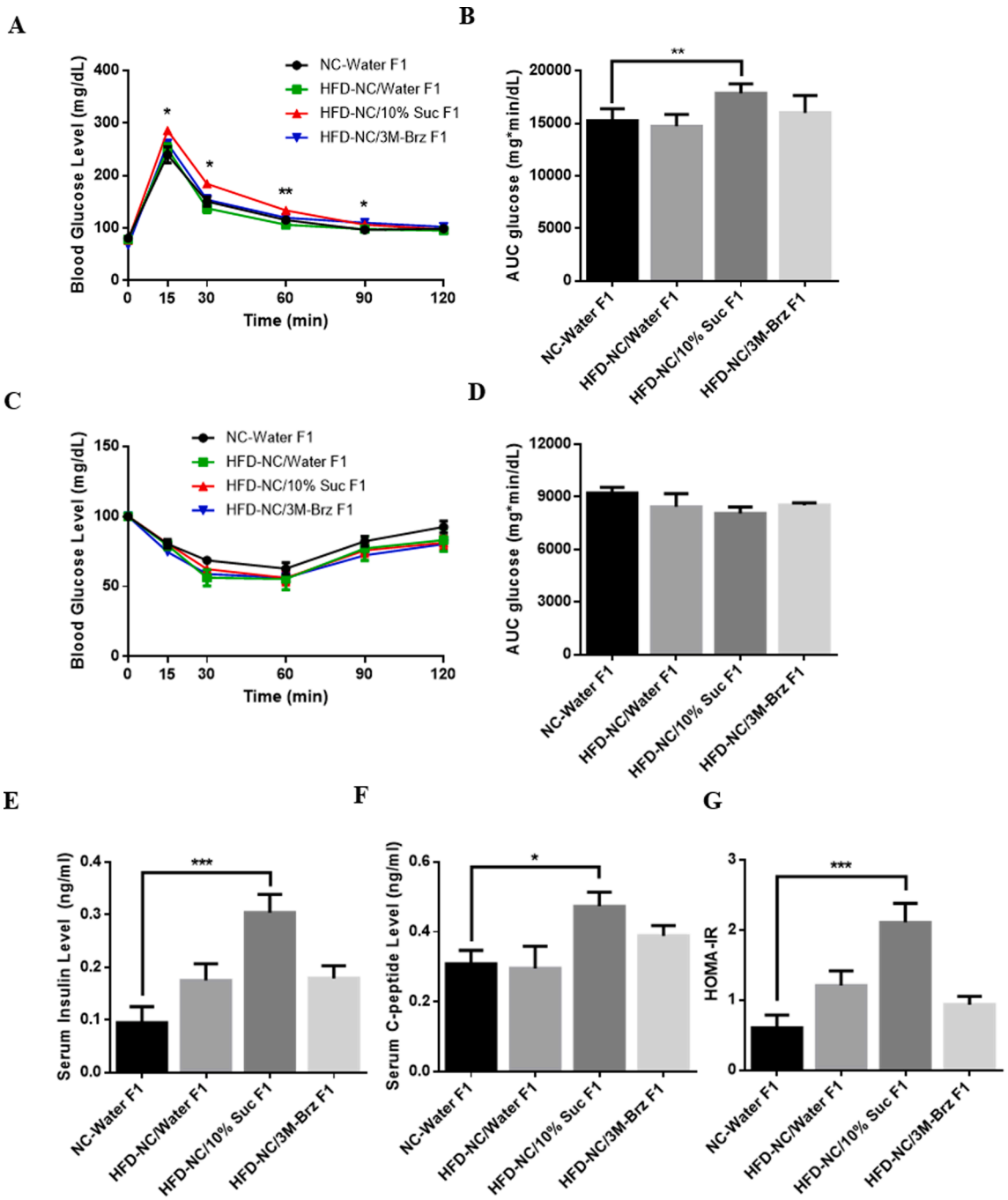

Fig. 6. 3M-**brazzein did not alter the glucose homeostasis in offspring** (**A**) GTT performed after 12 h of fasting. Blood glucose levels are shown at baseline and following i.p. administration of glucose (2 mg/kg). (**B**) AUC representation of GTT data. (**C**) ITT performed after 12 h of fasting. Blood glucose levels are shown at baseline and following i.p. administration of insulin (0.75 U/kg). (**D**) AUC representation of ITT data. (**E**) Serum insulin level. (**F**) Serum C-peptide level. (**G**) HOMA-IR presented as a median. NC-water F1, pups born from mice supplied with NC and water ($n = 10$); HFD-NC/Water F1, pups born from mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc F1, pups born from mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz F1, pups born from mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-tests for fasting blood glucose, insulin, and C-peptide and two-way ANOVA for GTT and ITT, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

intake during pregnancy is directly associated with gestational weight gain and the development of several pregnancy complications, such as gestational diabetes mellitus, preeclampsia, and preterm birth (Casas, Castro Barquero, & Estruch, 2020; Orloff & Hormes, 2014). Our findings suggest that 3M-brazzein emerges as a potentially viable alternative to high-calorie sweeteners, with particular relevance for obesity management. While the preliminary findings are promising, the long-term implications of brazzein consumption require extensive research, especially during sensitive life stages such as pregnancy. Questions about potential hormonal interactions, developmental impacts on the fetus, and the long-term metabolic effects of brazzein consumption need to be systematically investigated to ensure safety and efficacy. It is imperative for upcoming research endeavors to focus extensively on the effects of 3M-brazzein among different demographic categories, particularly in vulnerable groups such as pregnant women and children. This investigation is key to understanding the wider consequences of

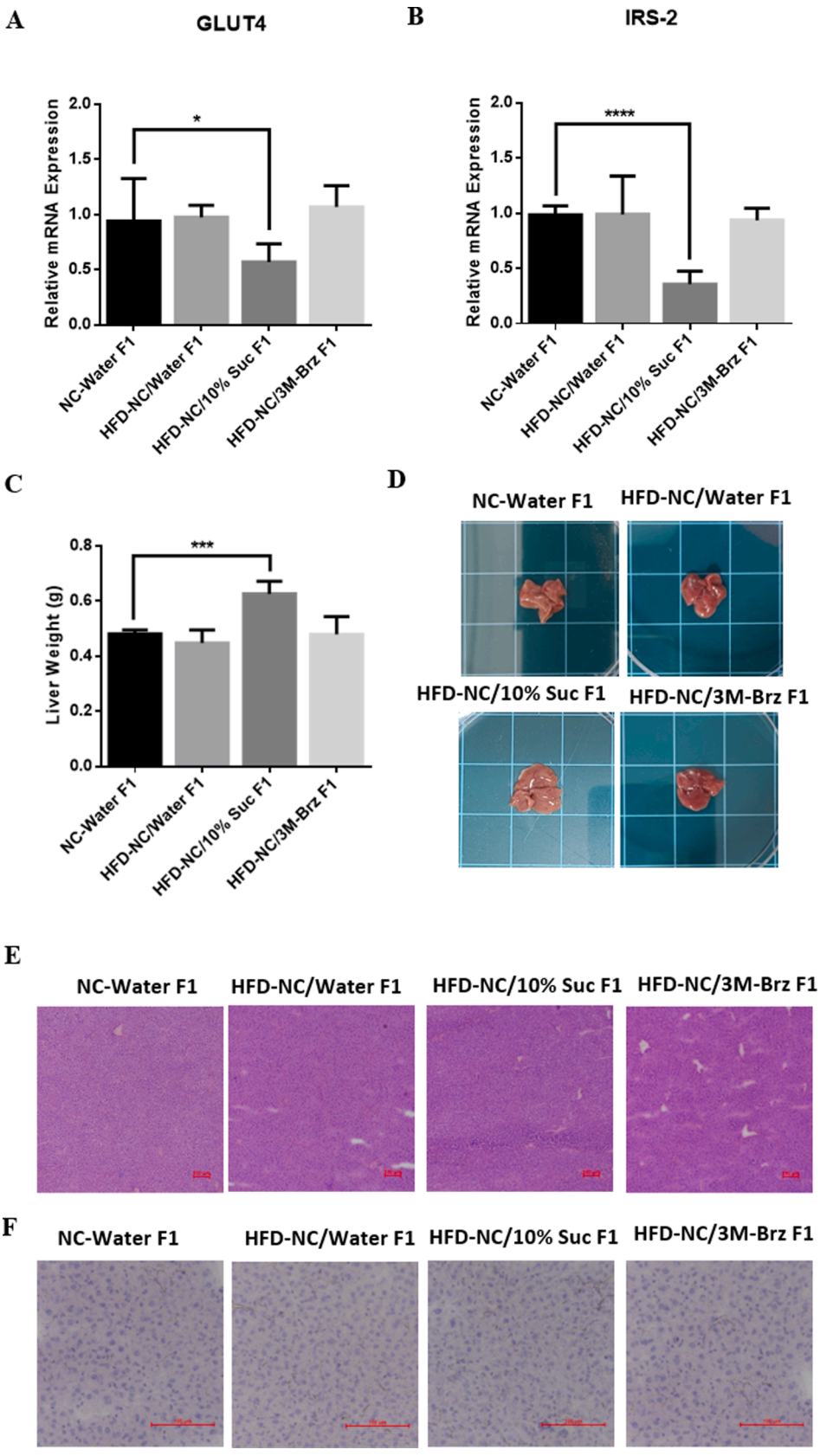

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Fig. 7. 3M-**brazzein did not affect hepatic fat accumulation in offspring** (**A**) Hepatic mRNA expression of (**A**) IRS2, (**B**) GLUT4. (**C**) Liver wet weight in each group. (**D**) Representative images of livers in each group. (**E**) Representative images of H&E staining of livers in each group. (**F**) Representative images of Oil-Red-O staining of the liver in each group. NC-water F1, pups born from mice supplied with NC and water ($n = 10$); HFD-NC/Water F1, pups born from mice supplied with HFD and followed with NC and water ($n = 10$); HFD-NC/10 % Suc F1, pups born from mice supplied with HFD and followed with NC and 10 % sucrose in drinking water ($n = 10$); HFD-NC/3M-Brz F1, pups born from mice supplied with HFD and followed with NC and 3M-brazzein in drinking water with an equivalent sweetness to 10 % sucrose ($n = 10$). Data are expressed as the mean ± SEM. Analyses were performed using the Student's *t*-test, $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

integrating 3M-brazzein in dietary habits, which could significantly influence nutrition guidelines and health policies. These studies are essential to gain a holistic view of the role of natural sweeteners in public health and nutrition.

5. Conclusions

Chronic 3M-brazzein consumption in a liquid form affects weight loss in obese mice and that maternal chronic 3M-brazzein consumption throughout pregnancy and lactation does not induce offspring obesity in mice. Moreover, the drinking of 3M-brazzein could reduce an associated risk of sugar consumption during pregnancy and lactation. Ultimately, our research points to 3M-brazzein as a promising natural, low-calorie sweetener that may play a crucial role in combating obesity worldwide. Its favorable characteristics, supported by preliminary data on safety and effectiveness, warrant its further investigation within functional foods and the expansive discipline of nutrition science. Although further study is needed to guarantee its safety, brazzein is a potential alternative to sugar, and a natural sweetener with beneficial biological characteristics.

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7. Ethics statement

All animal experiments in this study were approved by Chuang-Ang University (approval number A2022015). All the animal care and experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee at Chung-Ang University (Seoul, Korea).

CRediT authorship contribution statement

Seungwoo Hong: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Hansaem Kim:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Kwang-Hoon Kong:** Writing – review & editing, Supervision, Project administration. **Sungguan Hong:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2024.106104.

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