**Raspberry seed flour attenuates high sucrose diet-mediated hepatic stress
and adipose tissue inflammation**

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Running title: Ellagic acid and sugar toxicity

Key words: ellagic acid; raspberry seed flour; obesity; hepatic stress; adipose inflammation.

1. Abstract

Chronic intake of high sucrose (HS) diet exacerbates high fat (HF) diet-induced obesity and its associated metabolic complications. Previously, we have demonstrated that ellagic acid (EA), an abundant polyphenol found in some fruits and nuts, exerts distinct lipid-lowering characteristics in hepatocytes and adipocytes. In this study, we hypothesized that EA supplementation inhibits HS diet-mediated hepatic toxicity and its accompanied metabolic dysregulation. To test this hypothesis, C57BL/6 male mice were randomly assigned to three isocaloric HF diets (41 % calories from fat) containing either no-sucrose (HF), high-sucrose (HFHS), or high-sucrose plus EA (HFHS-R) from raspberry seed flour (RSF, equivalent to 0.03 % of EA), and fed for 12 weeks. The inclusion of EA from RSF significantly improved HFHS diet-mediated dyslipidemia and restored glucose homeostasis levels similar to the HF diet-fed mice. Despite marginal difference in hepatic triglyceride content, the addition of EA substantially reversed the activation of endoplasmic reticulum (ER) stress and oxidative damage triggered by HFHS diet in the liver. These effects of EA were further confirmed in human hepatoma cells by reducing ER stress and reactive oxygen species (ROS) production. Moreover, HFHS-R diet significantly decreased visceral adipocyte hypertrophy and adipose tissue inflammation evidenced by reduced pro-inflammatory gene expression and macrophage infiltration. In summary, EA supplementation from RSF was effective in reducing HFHS diet-mediated metabolic complication by attenuating hepatic ER and oxidative stresses as well as adipocyte inflammation. Our results suggest that the inclusion of EA in diets may normalize metabolic insults triggered by HS consumption.

2. Introduction

Obesity is defined as a chronic low-grade inflammatory condition, which causes major health problems in the United States and many parts of the world [1]. The obesity epidemic appears to have emerged largely from the imbalance between energy intake and energy expenditure. The critical role of HF diets on the pathogenesis of obesity has been well-established over the past fifteen years [2, 3]. In addition to the positive correlation between obesity and HF diets, accumulating evidence pinpoints that consumption of high contents of sucrose (or fructose) is an independent metabolic risk factor to exacerbate obesity and its accompanied health complications [4, 5].

Although it is controversial, the overall consumption of total sugar and sweeteners has increased in the United States over the past few decades [6, 7]. The surge of fructose into portal vein perturbs hepatic glucose and lipid metabolisms. High sugar intake is associated with reduced glucose uptake, elevated gluconeogenesis and hepatic glucose output, enhanced *de novo* TG synthesis, and promoted ER stress and hepatic inflammation [8]. These metabolic modifications appear to underlie the induction of insulin resistance commonly observed with HS feeding in both humans and rodent models of obesity. Moreover, epidemiological studies have revealed that sugar consumption is positively correlated with weight gain [7]. Given the deleterious contribution of HS intake to metabolic syndrome, there is an immense need for developing new strategies to counteract the HS diet-mediated metabolic insults.

EA is a dietary polyphenol abundantly found in many fruits such as pomegranate, berries, muscadine grapes and mangos, as well as nuts [9, 10]. EA exhibits anti-proliferative, chemopreventive, and anti-atherogenic properties in various cell types [11-14]. The major mechanism

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4 of action of EA includes 1) inhibition of redox stress responses [15] and 2) attenuating
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6 inflammatory damage through the regulation of NF- κ B [16]. In addition, our group has identified
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8 that EA exerts lipid-lowering characteristics by inhibiting hyperplastic and hypertrophic
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10 expansion of adipocytes [17, 18], and by attenuating hepatic lipid accumulation [17]. Based on
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12 these unique properties, EA may downregulate the signaling modification triggered by HS diets,
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14 attenuating the attendant metabolic dysregulation. However, this possibility has not been
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16 investigated yet.
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22 The objective of this study is to explore the role of EA-containing RSF on HS diet-
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24 mediated hepatic toxicity and its metabolic consequences. To achieve this goal, we prepared
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26 three isocaloric HF diets containing no sucrose (HF), high sucrose (HFHS) and HS plus EA
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28 (HFHS-R) from RSF as a source of EA. Here, we report that inclusion of EA in HFHS diets was
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30 effective in reversing HS-mediated 1) ER and oxidative stress responses in liver and 2) adipocyte
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32 hypertrophy and pro-inflammatory responses in adipose tissue.
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3. Materials and Methods

3.1. Chemical reagents

All cell culture dishes were purchased from Fisher Scientific. Fetal bovine serum (FBS) was purchased from GIBCO. Pure EA (from tree bark) was purchased from Sigma Chemical Co. (#E2250). EA stock at 10 mM was prepared in dimethyl sulfoxide (DMSO) as described previously [17, 18]. Small aliquots of EA stocks were kept at -20 °C and freshly diluted to 10 µM with DMSO at the time of treatment in Huh7 cells. All other chemicals and reagents were purchased from Sigma Chemical Co., unless otherwise stated.

3.2. Analysis of phenolic compounds in RSF

Raspberry seed flour was purchased from Millennium©Health (Meeker Red Raspberry seed flour). For the analysis of phenolic compounds of RSF, 10 mg of RSF was extracted with 1 mL of methanol:DMSO:water (40:40:20) acidified with 0.1% HCl. The mixture was vortexed, subjected to ultrasonic bath for 10 minutes, and centrifuged at 14,000g for 5 min in a Sigma 1-13 microcentrifuge (Braun Biotech. International, Germany). The supernatant was filtered through a 0.45 µm polyvinylidene difluoride (PVDF) filter. The sample was analyzed in an Agilent 1200 high-performance liquid chromatography (HPLC) system equipped with a ultraviolet-visible diode array detector (UV-Vis-DAD) (Agilent Technologies, Waldbronn, Germany) and an Esquire 1100 ion trap mass spectrometer (IT) with an electrospray interface (ESI) (Agilent). Chromatographic separation was carried out on a reverse phase Pursuit XRs C18 column (Agilent) (250x4 mm, 5 µm particle size) using water:formic acid (99:1, v/v) (A) and acetonitrile (B) as mobile phases. The gradient profile was: 0–20 min, 5–30% B; 20–30 min, 30–55% B; 30–38 min, 55–90% B. This percentage was maintained for 2 min and then the column was

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4 equilibrated with the initial conditions for 8 min. A volume of 8 μ L of sample was injected onto
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6 the column operating at room temperature and a flow rate of 0.8 mL/min. The separated
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8 compounds were monitored in sequence with DAD (280 and 360 nm) and with a mass
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10 spectrometry (MS) detector. Nitrogen was used as drying gas and nebulizing gas in the mass
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12 spectrometry detector with the following conditions: nebulizer pressure 65 psi, dry gas flow 11
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14 L/min, and dry gas temperature 350 °C. Mass scan (MS) and daughter (MS-MS) spectra were
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16 measured in the m/z range of 100-1800 in the negative ionization mode. Phenolics were
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18 identified according to their UV and MS spectra, as well as MS/MS fragments. The extracts were
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20 chemically hydrolyzed as previously reported [19].
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28 3.3. *Animal study*

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31 Male C57BL/6 mice at six weeks of age were purchased from Jackson Laboratory (Bar
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33 Harbor, ME) and housed in a specific-pathogen free facility at the University of Nebraska-
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35 Lincoln. All animal experimental procedures were approved by the Institutional Animal Care
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37 and Use Committee at the University of Nebraska-Lincoln. At eight weeks of age, mice were
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39 randomly assigned to one of three experimental groups fed with different diets: isocaloric HF
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41 diets (41% calories from fat) containing either no-sucrose (HF group), HS (HFHS group, 37%
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43 calories from sucrose), or HS plus EA (HFHS-R group) from RSF (equivalent to 0.03% of EA
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45 based on RSF analysis) and fed for 12 weeks. Diet preparation was adapted from the AIN-93G
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47 diets. For HFHS-R diet, cellulose was substituted for RSF (mainly dietary fibers), and thus the
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49 total dietary fibers among the diets were not different (Supplemental Table 1). Mice were given
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51 fresh ration every other day and fed ad libitum. The daily food consumption per mouse was
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measured for 3 days at the last week of feeding before sacrifice. Changes in body weight (BW) of all mice were monitored weekly throughout the study (Supplemental Fig. 1).

3.4. Measurement of blood biochemical parameters

Enzymatic colorimetric assay kits were used to determine plasma levels of TG (Wako Diagnostics), total cholesterol (Roche Applied Sciences), and high-density lipoprotein (HDL) cholesterol (BioAssay Systems). Plasma low-density lipoprotein (LDL) cholesterol (mg/dL) was calculated from the Friedwald Equation [20]. Fasting blood glucose levels (mg/dL) were measured using a glucometer (Bayer, Contour). Commercial ELISA kit was used to determine plasma levels of insulin (Crystal Chem).

3.5. Glucose and insulin tolerance tests

A glucose tolerance test (GTT) was performed on overnight fasted C57BL/6 mice by intraperitoneal (i.p.) injection of 10% D-glucose solution (0.5 g/kg BW). Blood glucose levels (mg/dL) were measured at 0, 15, 30, 60 and 120 min after injection using a glucometer (Bayer, Contour). For insulin tolerance test (ITT), overnight fasted C57BL/6 mice were administered 0.75 U/kg BW of insulin (Novolin R). Blood glucose levels were measured at 0, 10, 20, 30, 60 and 120 min after injection. The HOMA-IR (homeostasis model assessment of insulin resistance) index was calculated as $[\text{fasting plasma glucose} \times \text{fasting plasma insulin} / 22.5]$ to assess insulin resistance.

3.6. H&E staining, adipocyte size measurement and F4/80 staining.

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4 Upon necropsy, liver and adipose tissues were dissected from the mice and immediately
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6 fixed in 10% buffered formalin. Tissues were embedded in paraffin, cut to 5-7 micrometer
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8 sections, and processed for hematoxylin and eosin (H&E) staining as described previously [17].
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10 H&E stained-sections of epididymal adipose tissue were used for size determination by
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12 following the published protocol by Chen et al. [21]. Briefly, adipocyte size was examined by
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14 analyzing digital images of H&E stained paraffin sections by using Image J software. For
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16 fluorescent immunohistochemistry (IHC)-F4/80 staining, paraffin embedded adipose tissue
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18 sections were stained with a primary F4/80 antibody (dilution 1:50, Abcam), followed by
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20 incubation with Alexa Fluor® 488 (Cell Signaling). Images were taken by using an EVOS
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22 microscope (AMG Inc.)
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31 *3.7. High fructose and palmitate stimulation of Huh7 cells*

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33 Huh7 cells were maintained in low-glucose Dulbecco's modification of Eagle's medium
34 (DMEM) medium containing 1 mM glucose, 1% L-glutamine, 10% fetal bovine serum, 100
35 units/mL penicillin, and 100 g/mL streptomycin for 2 weeks before experiments. For experiment
36 in Figure 3F and G, Huh7 cells were seeded into 6-well plates (0.25×10^6) and pre-incubated with
37 given dose of EA (10-40 μ M) for 48 hours. To mimic the postprandial conditions of HFHS diet,
38 Huh7 cells were challenged with mixtures of glucose (25 mM), fructose (50 mM) and palmitate-
39 BSA complex (800 μ M) (Glc/ Fr/ PA) for 12 hr. Huh 7 cells were harvested for either western
40 blot analysis (Figure 3F) or stimulated with 100 nM of insulin for detecting insulin-stimulated
41 Akt phosphorylation (Figure 3G) as we previously described [22].
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58 *3.8. Hepatic lipid accumulation*

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The colorimetric triglyceride quantification kit (BioVision, K622-100) was used to quantify the hepatic TG contents according to the manufacturer's protocol.

3.9. Quantitative Real-Time PCR

Gene expression analysis was performed as described previously [17]. Relative gene expression was determined based on the $2^{-\Delta\Delta CT}$ method with normalization of the raw data to 36B4 (primer sequences in Supplemental Table 1).

3.10. Western blotting analysis

Snap frozen adipose and liver samples were homogenized with a polytron homogenizer (Elkhart, IN). Huh7 cell cultures were scraped in ice-cold radioimmune precipitation assay (RIPA) buffer (Thermo Scientific) with protease inhibitors and phosphatase inhibitor as described previously [22]. Proteins were separated using 8 or 10% SDS-PAGE gels, transferred to PVDF membranes, and incubated with the relevant antibodies. Chemiluminescence was detected with ECL solution (PerkinElmer) using a FluorChem E system (Protein Simple). Polyclonal or monoclonal antibodies targeting phospho-JNK (4668), phospho-p38 (4511), phospho-eukaryotic translation initiation factor 2 α (eIF2 α) (9721), phospho-Akt (Ser473, 4060), total Akt (9272) and β -actin (4967) were purchased from Cell Signaling Technology (Danvers, MA). Phospho-insulin receptor substrate 2 (IRS2) (Ser388, 07-15171) or total-IRS2 (MABS15) were purchased from Invitrogen.

3.11. Measurement of hepatic oxidative stress

For the determination of hepatic oxidative stress, liver tissue (50-100 mg) was homogenized. Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) were used to determine hepatic H₂O₂ content according to the provider's instruction. To measure the intracellular accumulation of ROS in Huh7 cells, commercial kit of 2,7-dichloro-dihydro-fluorescein diacetate (DCFDA) cellular ROS detection assay was used (Abcam). Briefly, Huh7 cells were pre-incubated with either DMSO or EA (0-40 µM) in a dose-dependent manner for 2 days. Then, Huh7 cells were treated with Glc (25 mM), Fr (50 mM), and PA (800 µM) with or without EA. After 12 hours later, Huh7 cells were washed with hanks' balanced salt solution (HBSS), and loaded with 20 µM DCFDA for 1 hour at 37 °C. After 1 hour incubation, unincorporated dye was removed by washing with HBSS. Then 250 µM of H₂O₂ (*t*-butyl hydroperoxide (Sigma Aldrich)) was spiked to Huh7 cell with experimental conditioned media in the presence or absence of EA. Fluorescence intensity was measured by Synergy H1 (Biotech). Oxidation of DCFDA to the highly fluorescent 2,7-dichloro-fluorescein (DCF) was proportionate to ROS generation.

3.12. *Inflammatory cytokine assay*

Homogenized epididymal adipose tissue and plasma were prepared for testing the levels of multiple inflammatory cytokines by using the Mouse Inflammation Array C1 (Ray Biotech, Norcross, GA) according to the manufacturer's protocol. The complete blots were imaged by a FluorChem E System (Proteinsimple, Santa Clara, CA).

3.13. *Statistical analysis*

The data were statistically analyzed using one-way ANOVA analysis of variance with Tukey's multiple comparison tests or Student's *t*-test. All analyses were performed with GraphPad Prism 5 (Version 5.04). Results are presented as mean $\pm$ SEM.

4. Results

4.1. Raspberry seed flour (RSF) is a natural source of free EA and its precursors

To investigate the potential role of dietary EA in normalizing the HS intake-mediated metabolic complications, we prepared HFHS diet with or without EA from natural sources. We selected RSF as the source of EA as it has been reported to contain high levels of EA, ellagitannins (ETs), and its derivatives [23]. Our analysis shows that there were a negligible amount of anthocyanins or flavonols in RSF (Table 1). RSF mainly contained different proanthocyanidins (condensed tannins) and ETs (hydrolysable tannins), confirming that RSF was prepared from achenes of raspberries. Free EA content in the RSF was 1.1 mg/g whereas gallic acid was found only in trace amounts. To release the EA from tannins, RSF was hydrolyzed and neutralized for further analysis. After hydrolysis, the amount of free EA increased up to 7 mg/g and gallic acid up to 0.39 mg/g (Fig. 1). In addition, valoneic acid dilactone (2 mg/g), another hydrolysis product of ETs, was also found upon hydrolysis. Based on this analysis, the maximally attainable EA contents by the ingestion of RSF (sum of parental compounds, EA and potential other metabolites) would be approximately 10 mg/g (1% in RSF). As we added 3 % of RSF to HFHS diet (Supplementary Table 1), we assume that total EA and its responsible derivatives in HFHS-R preparation would be equivalent to 0.03 % of EA. Furthermore, as the complete hydrolysis of ETs may not be achievable *in vivo*, we regarded HSHS-R as a source of EA no more than 0.03 % for the rest of the study.

4.2. RSF supplementation attenuated HFHS diet-induced metabolic parameters and dyslipidemia in C57BL/6 mice

We first investigated whether RSF supplementation alters HFHS diet-induced obesity. As seen in Table 2, there was no difference in food intake between the groups. After 12 weeks of the diet, mice fed with HFHS diet significantly promoted BW gain compared to mice fed with HF alone, which was partially normalized in mice fed with HFHS-R. Similarly, the extent to which HFHS diet promoted liver and visceral fat (epididymal and mesenteric) mass gain compared to HF diet, was partly attenuated by HFHS-R diet. However, liver/BW ratio did not reach statistical significance among groups despite the trend of stepwise decline in mice fed with HFHS>HFHS-R>HF. Next, we investigated whether the inclusion of RSF in HFHS diet improves plasma lipid profiles. As we expected both plasma TG and total cholesterol levels were higher in HFHS group than HF control. The elevated levels of TG and total cholesterol by HFHS diet were almost completely dampened in HFHS-R group comparable to HF control. Consistently, decreased HDL cholesterol and elevated LDL cholesterol levels were restored in HFHS-R group close to the HF-fed control mice (Table 2). These data demonstrated that EA-containing RSF supplementation confers a resistance to HS-mediated exacerbation of obesity and dyslipidemia.

4.3. RSF supplementation normalized HS diet-mediated abnormal glucose metabolism

Next, we asked whether RSF modulates HS-mediated abnormal glucose metabolism. As expected, HFHS fed mice exhibited higher fasting glucose and insulin levels than HF fed mice. Consistent with the improved plasma lipid profile by RSF (Table 2), inclusion of RSF decreased HS-mediated abnormal increase of blood glucose and insulin concentration to the levels of HF only group (Fig. 2A, B). The HOMA-IR index, an indicator of insulin resistance, revealed the HFHS-R fed group was approximately 3-fold more sensitive to insulin than HFHS group; sensitivities between HFHS-R fed group and HF fed group were similar (Fig. 2C). To confirm

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4 this, glucose (GTT) and insulin tolerance tests (ITT) were conducted. During GTT, glucose
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6 disposal was significantly slower in HFHS fed mice than HF fed mice. HFHS diet-mediated
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8 glucose tolerance was improved by RSF supplementation, which was confirmed by
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10 quantification of GTT area under curve (AUC) (Fig. 2D). In parallel, HFHS fed mice maintained
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12 higher glucose levels than HF fed mice during ITT. In contrast, glucose disposal rate between
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14 mice fed HFHS-R and HF alone was almost identical, showing no difference in AUC (Fig. 2E).
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16 These data suggested that EA-containing RSF supplementation normalized the HS-induced
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18 glucose and insulin resistance in mice.
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26 **4.4. RSF supplementation attenuated HS-mediated hepatic ER stress.**

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28 HS intake has known to alter hepatic lipid metabolism and cause hepatic ER stress [24].
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30 Given the significant improvement in plasma lipid profile by RSF (Table. 2), we hypothesized
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32 that RSF supplementation attenuates HS diet-induced hepatic steatosis and ER stress. The gross
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34 images of liver fed HFHS-R showed darker brownish color than HFHS fed liver, implicating that
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36 TG accumulation might be decreased. H&E staining of liver section revealed that TG
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38 accumulation appeared to be lower in HFHS-R fed group than HFHS fed mice (Fig. 3A). There
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40 was a decrease in TG content in HFHS-R fed group from HFHS, but it was not significant (Fig.
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42 3B). It may reflect that HFHS-R diet showed a tendency to decrease liver mass compared to
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44 HFHS, but it was not significant (Table 2). Despite the marginal difference in liver mass,
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46 lipogenic related gene expression of stearoyl CoA desaturase-1 (SCD-1), lipoprotein lipase
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48 (LPL) and diacylglycerol acyltransferases 2 (DGAT2) were markedly decreased in mice fed with
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50 HFHS-R compared to HFHS. However, hepatic gene expression levels of carbohydrate response
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52 element-binding protein (ChREBP) and sterol regulatory element-binding protein 1c (SREBP1c)
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4 did not differ between HFHS and HSHS-R fed mice (Fig. 3C). Intriguingly, the two genes
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6 promoting gluconeogenesis, glucose 6-phosphase (G6Pase) and phosphoenolpyruvate carboxyl
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8 kinase (PEPCK) were also significantly down-regulated, suggesting that HS-mediated hepatic
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10 glucose output may be decreased by EA-containing RSF supplementation (Fig. 3D).

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14 HS-mediated hepatic ER stress is associated with hepatic steatosis and upregulation of
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16 gluconeogenesis [25]. Thus, we determined the expression of ER stress markers in mice fed with
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18 HFHS vs. HFHS-R diet. RSF supplementation attenuated phosphorylation of JNK (p-JNK), p38
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20 mitogen-activated protein kinase (p-p38 MAPK), and eukaryotic translation initiation factor 2A
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22 (p-eIF2 α) compared to HFHS feeding (Fig. 3E). To validate these results *in vitro* system, we
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24 simulated ER stress condition in human hepatoma Huh7 cells by insulting cells with the
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26 combination glucose (Glc), fructose (Fr) and palmitate (PA). Pre-incubation of EA abolished the
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28 HFHS-triggered activation of p-JNK and p-p38 in a dose-dependent manner (Fig. 3F). To
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30 determine whether EA attenuates HFHS-mediated inhibition of hepatic insulin signaling, basal
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32 and insulin-stimulated phosphorylation of Akt were measured in huh 7 cells (Fig. 3G). Huh 7
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34 cells pre-treated with or without EA were simulated for ER stress by adding a high concentration
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36 of Glc, Fr and PA. Even in basal conditions, phosphorylation levels of Akt appear to be
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38 increased slightly in the presence of 10 μ M EA (Fig 3G, lane 1 vs. 2). Insulin-stimulated p-Akt
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40 were markedly higher in EA treated Huh7 cells (Fig 3G, lane 3 vs. 4), indicating that EA protects
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42 insulin signaling pathways against HSHS-mediated ER stress.
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53 **4.5. RSF supplementation attenuated hepatic oxidative stress.**

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55 Prolonged ER stress leads to ROS production and causes oxidative stress [26]. We
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57 hypothesized that RSF supplementation reduces HFHS-mediated hepatic oxidative stress. To
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4 address this, we first determined the hepatic H₂O₂ levels (μmol/ mg protein), an indicator of ROS
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7 production in liver, was significantly higher in mice fed with HFHS diet compared to mice fed
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10 with HSHF-R and mice fed with HF diet alone, which showed similar values (Fig. 4A). To
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12 confirm the effects of EA on ROS production *in vitro*, we measured ROS production in the
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14 presence or absence of EA by measuring DCF fluorescence levels in Huh7 cells. The ROS
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16 production rate (relative DCF florescence) was remarkably increased upon challenging with
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18 high concentration of Glc, Fr and PA cocktail, which was significantly lower in EA-pretreated
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20 Huh7 cells (Fig. 4B also see Supplemental Fig. 2 for kinetic production of ROS). Collectively,
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22 these data suggest that EA in RSF may contribute to a reduction in HSHS-mediated hepatic ROS
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26 production.
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31 **4.6. RSF supplementation attenuated the HS-mediated adipose tissue inflammation**

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33 To determine whether inclusion of RSF alters HFHS diet-induced adipose tissue
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35 remodeling, we first examined morphological changes of epididymal fat. H&E staining of
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37 adipose tissue clearly revealed that HFHS diet promotes adipocyte hypertrophy and macrophage
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39 infiltration compared to isocaloric HF diet. Despite the marginal difference in visceral fat mass
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41 (Table 2), HFHS-R diet significantly decreased adipocyte size and immune cell infiltration
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43 compared to HFHS diet (Fig. 5A). The analysis of adipocyte size and distribution confirmed
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45 these morphological changes. HFHS-R diet normalized HFHS-mediated adipocyte size
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47 expansion (111.53 ± 1.09 vs. 95.37 ± 0.86 , HFHS vs. HFHS-R) (Fig. 5B) nearer to the HF
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49 control. Similarly, adipocyte size distribution from HFHS-R fed mice was shifted toward smaller
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51 sizes similar to HF diet feeding (Fig. 5C). Reflecting the smaller and more insulin-sensitized
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53 adipocytes, plasma levels of adiponectin levels (Fig. 5D) as well as adiponectin gene expression
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(Fig. 5E) were significantly higher with HFHS-R diet than HFHS diet. The proinflammatory gene expressions including IL-6, IL-8, F4/80 (a marker for monocytic cells), tumor necrosis factor α (TNF α) and monocyte chemoattractant protein 1 (MCP-1) were significantly reduced in HFHS-R-fed adipose tissue compared to HFHS-fed adipose tissue (Fig. 5E). The decrease of macrophage infiltration by HFHS-R was also confirmed by immunostaining of F4/80 (Fig. 5F). Inflammatory protein profiles were also determined using the membrane-based inflammatory cytokine array in adipose tissue and plasma. HFHS diet decreased proinflammatory adipokine production from epididymal adipose tissue including leptin, C-X-C motif chemokine 5 (CXCL5), chemokine (C motif) ligand (XCL1), MCP-1, macrophage colony-stimulating factor (M-CSF), soluble tumour necrosis factor receptor 1 (sTNFR1), and sTNFR2 (Fig. 5G, upper) and decreased plasma levels of proinflammatory cytokines XCL1, MCP-1, M-CSF, sTNFR1, and sTNFR2 (Fig. 5G, below). Collectively, these data demonstrated that EA-containing RSF supplementation attenuated HS diet-mediated adipose inflammation and as well as systemic levels of inflammation.

5. Discussion

Western diet, high in saturated fat and sugar, but low in fresh fruits and vegetables, is the primary culprit to increase the risk of obesity and its associated metabolic dysfunction. This study was specifically designed to test the hypothesis whether the inclusion of EA in HFHS diet reverses the HS-induced metabolic complications. Here, we demonstrated that addition of EA-containing RSF significantly normalizes HFHS-induced dyslipidemia (Table 2), enhances hepatic and systemic insulin sensitivity (Fig. 2, 3), reduces hepatic ER and oxidative stress (Fig. 3, 4), and inhibits proinflammatory adipose tissue remodeling and adipose dysfunction (Fig. 5) in C57BL/6 mice. To our knowledge, this is the first study to report the previously unrecognized function of EA in attenuating high sugar intake-inducible metabolic dysfunction by alleviating hepatic stress and adipose tissue inflammation.

Despite the numerous health beneficial effects of EA, low bioavailability is the biggest caveat for the practical use of EA [27]. However, a recent study has suggested that plasma concentrations of free EA in peripheral human plasma could be higher than previously expected (80 nM vs. 10 nM), and the concentrations of EA in liver tissues could also be relevant [28]. Furthermore, EA is extensively metabolized by gut microbes producing urolithins. Although there are several studies showing that urolithins may resemble metabolic characteristics of EA [29, 30], it is difficult to estimate the exact physiological contribution of urolithins to metabolism. Moreover, urolithin production is highly compounding with the individual variability of microbiome [31]. Prior to this study, we had conducted a pilot study by feeding HF diet with 0.1 % of pure EA from tree bark (unpublished data). To our disappointment, we were not able to observe physiological benefits that we proposed in our cellular studies [17, 18]. We speculated that free EA consumption may dilute the EA's health benefits due to its insolubility or

prompt microbial conversion into urolithins. In this study, our criteria for selecting EA source were, 1) to use natural dietary source (ET form) rather than synthetic EA or isolated EA from tree bark, 2) to avoid complete conversion into urolithins before absorption, and 3) to minimize the other bioactive polyphenolic contamination to isolate the role of EA. RSF appeared to fit our experimental requirements because 1) raspberry is an affordable fruit with easy access, 2) RSF contains very few other polyphenolic compounds (Table 1), and 3) the ratio of free EA to EA precursors (mostly ETs) was roughly 1:6 (based on before and after hydrolysis of RSF), which may allow sufficient time to be released from tannin and absorbed in the gut, delaying microbial action. Supporting our rationales, Kosmala et al. has recently analyzed the chemical composition of raspberry vs. strawberry seeds; the main component of raspberry seed was fiber and the major polyphenols were polymerized ETs (dimer or trimer), while strawberry seed mainly composed of monomers of ET [23]. Proanthocyanidins (condensed tannin), the other polyphenolic compounds found in RSF, were relatively low compared to ET-containing compounds [23]. Furthermore, the concentrations to exert bioactivity (lipid lowering and anti-inflammation) of depolymerized proanthocyanidins were in range of 50-100 µg/mL *in vitro* (our unpublished data), whereas EA is in 10 uM (2.7 µg/mL) [17, 18]. Therefore, RSF effects are likely due to combinatory of EA, ET, and its metabolites rather than other phytochemicals. In accordance with our results, raspberry ETs was more effective in lowering plasma TG levels than that of strawberry seed, suggesting that degree of conjugation with tannin (dimer/trimer>monomer> free EA) might regulate bioavailability of EA [23].

A growing body of evidence suggests that there is a substantial link between HS consumption and metabolic dysfunction including non-alcoholic fatty liver diseases (NAFLD), obesity, dyslipidemia, and insulin resistance (sugar toxicity) [8, 32]. To test the central

hypothesis that EA inhibits sugar toxicity, we prepared HFHS diets with and without RSF and compared their metabolic markers with isocaloric HF diet with no sucrose. The inclusion of RSF in HFHS diet was associated with at least three metabolic outcomes attenuating 1) obesity and dyslipidemia, 2) hepatic ER/oxidative stress and 3) adipose inflammation.

The first systemic metabolic benefit that we immediately noticed after RSF supplementation was the improvement of dyslipidemia and insulin sensitivity against HFHS diet (Table 1 and Fig. 1). Numerous studies in experimental animals and humans (both epidemiological and clinical intervention studies) have established that consumption of high-fructose promotes obesity, dyslipidemia (increased in plasma TG, LDL cholesterol, but decreased in HDL cholesterol) and insulin resistance [8, 33]. In agreement with this notion, switching carbohydrate source from dextrin (HF) to sugar (HFHS) without altering total calories, effectively promoted weight gain, dyslipidemia, and insulin resistance. These sugar toxicity-mediated metabolic abnormalities were ameliorated in the presence of RSF (Table 1). As RSF contains few other bioactive phytochemicals, the improvement of lipid profile and insulin sensitivity must be originated from EA and its derivatives in RSF. It is supported by growing evidence that consumption of EA-containing fruits protects high fructose-mediated metabolic abnormal modifications [34, 35]. The most noticeable benefits by RSF was a normalization of HS-mediated elevation of total and LDL cholesterol (Table 2). It implicates that RSF may promote LDL catabolism. In fact, HFHS diet reduced hepatic LDL receptor (LDLr) resulting in an accumulation of LDL particles while inclusion of EA in HFHS diet upregulated LDLr and apoA-I (Supplemental Fig. 3). In agreement with our results, Yoshimura et al. showed that EA effectively upregulates LDLr gene expression in diabetic KK-Ay mice [36]. Several papers reported that either free EA or EA (or ET)-containing berries improve glucose and insulin

1 tolerance against diet-induced obesity [35, 37]. As an underlying mechanism, it has been
2
3 suggested EA and gallic acid selectively inhibit α -glycosidase, suppressing entry of
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5 monosaccharides from the lumen to intestinal epithelium [38]. If the inhibition of α -glycosidase
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7 enzyme activity by RSF was the key mechanism to reduce plasma glucose level, HFHS-R-fed
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9 mice may exhibit lower blood glucose than HF-fed mice, because HF diet contains the same
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11 amount of α -glycosidase bondages with HFHS-R. However, it does seem to be the case since
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13 RSF supplementation improved the only HS-mediated metabolic abnormality (comparable with
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15 HF only group). However, at this point, we do not entirely exclude the possibility that inhibition
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17 of α -glycosidase by RSF contributes to the improvement of insulin tolerance against sugar
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19 toxicity. Unexpectedly, the apparent improvement in dyslipidemia and insulin sensitivity with
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21 0.03% of EA supplementation was not directly correlated with the decrease in BW or adipose
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23 tissue weight; HS-mediated weight gain was significantly ($p<0.05$) reversed by RSF but to a
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25 lesser magnitude. We assume that modulation of adiposity may require a higher concentration of
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27 EA than controlling insulin sensitivity or plasma lipids. Taken together, the inclusion of no more
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29 than 0.03 % of EA in HFHS diet (roughly 30 mg EA /kg BW/day or ~1.8 g/60 kg BW)
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31 substantially reversed sugar-mediated insulin resistance and dyslipidemia. We believe that RSF-
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33 mediated normalization of glucose tolerance would be the consequences of alleviated metabolic
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35 stress primarily from liver.

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37 Subsequently, we examined the potential benefit of RSF against sugar toxicity in the
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39 liver, the primary metabolic target organ for sugar toxicity. There is convincing literature
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41 demonstrating that HS intake promotes hepatic lipogenesis. It is due to unique properties of
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43 fructose metabolism that 1) fructose is mostly metabolized in liver (about 80% in contrast to only
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45 20% for glucose) [39], 2) entry of fructose into hepatic glycolysis bypasses the regulatory control
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of phosphofructokinase (PFK) producing unregulated lipogenic and proinflammatory precursors [39], and thus 3) fructose activates lipogenic transcription factors of SREBP1c, and ChREBP [25] leading to ER stress and insulin resistance. Furthermore, high fructose intake also impedes fatty acid oxidation and augments very-low-density lipoprotein (VLDL) secretion expediting hepatic lipid accumulation and systemic obesity [40]. Complying with metabolic modification by HS intake, our results showed that HFHS diet was associated with a 2-fold increase of hepatic TG deposition and significant upregulation of both lipogenic transcription factors (SREBP1c and ChREBP) and their target genes compared to isocaloric HF diet. Unexpectedly, hepatic TG contents were not apparently different between mice fed with HFHS and HFHS-R. These results are conflicting with our previous *in vitro* study showing that EA attenuated hepatic TG accumulation by decreasing both *de novo* synthesis FA and its esterification [17]. As our experimental design did not include the animal group of HF-R (HF plus RSF supplementation/no sugar), it is difficult for us to tease out the role of RSF on fatty acid metabolism from fructose metabolism. The marginal impact of RSF on hepatic TG accumulation upon HFHS consumption, may attribute to the synergistic activation of ChREBP and SREBP1c (reviewed in [41]) via simultaneous influx of fatty acids (FA) and fructose to liver. We speculate that metabolic modification by concurrent stimulation of FA and fructose will facilitate TG accumulation overriding TG-lowering role of EA. We are currently under investigation to address this possibility. However, it is notable that hepatic lipogenic gene expressions of SCD-1, LPL and DGAT2 were downregulated in mice fed with HFHS-R compared to HFHS (Fig. 3C), despite insignificant differences in transcription factor levels of SREBP1c and ChREBP. More importantly, two critical regulators of gluconeogenesis and hepatic glucose output, G6Pase and PEPCK, were significantly lower in HFHS-R mice (Fig. 3D). This supports our finding that

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4 HSHF-R-fed mice were more glucose tolerant than HFHS-fed mice (Fig. 2). Augmented insulin
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6 sensitivity may be attributed to the decreased hepatic ER stress and ROS production (Fig. 3, 4).
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8 In agreement with our results, a few studies supported the notion that EA is capable of
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10 scavenging free radicals and decreasing ROS production [42]. It is unclear why EA is more
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12 sensitive in repressing oxidative stress signaling than the transcriptional repression of lipogenic
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14 genes. It could be attributed to limited accessibility of EA to the nucleus or higher concentration
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16 of EA may be required for optimal regulation of gene regulation. Further studies should be
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18 conducted to validate these possibilities. Taken together, herein we report that EA
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20 supplementation by RSF could effectively suppress HS-induced hepatic ER stress, ROS
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22 production, and hepatic glucose output in the absence of an obvious reduction in hepatic TG
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24 content.
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32 Lastly, we assessed the impact of RSF on obesity and adipose tissue inflammation. The
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34 rank order of visceral adiposity among the mice fed with 12 weeks of isocaloric diet was,
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36 HFHS>HFHS-R>>HF, paralleling changes in body and liver weight. More importantly, there
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38 was a distinct improvement of adipose tissue remodeling by decreasing adipocyte size, immune
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40 cell infiltration and pro-inflammatory gene and protein expression (Fig. 5). Supporting our
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42 finding, the recent study by Winand et al. demonstrated that EA decreased TNF α , IL-6 and
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44 chemokine (C-C motif) ligand-2 (CCL-2) secretion in lipopolysaccharides (LPS) induced
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46 macrophage and adipocyte [43]. Notably, minor changes in adiposity by HFHS-R, promoted
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48 almost complete reversal of adipose tissue inflammation. At this point, it is unknown whether the
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50 reversal of HS-mediated adipose tissue inflammation reflects role of EA in adipose tissue *in situ*,
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52 or indirect influence primarily from metabolic adaptation in liver (e.g., reduced VLDL
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54 secretion). We have previously reported that EA may impose direct lipid-lowering effects in
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human adipocytes. Several other studies have demonstrated that EA can impede adipocyte differentiation *in vitro* [44, 45]. Also, our preliminary work in human adipocytes showed that urolithins are able to regulate adipogenesis or lipogenesis *in vitro* (unpublished). Future studies that analyze tissue levels of EA and its urolithin metabolites are necessary to identify whether EA or its metabolites urolithins directly target adipose tissue metabolism.

In summary, here we tested an innovative idea whether inclusion of EA in HF diet could inhibit high sugar intake-mediated metabolic dysfunction. We identified that RSF, as a source of EA, effectively antagonized the effects of sugar toxicity including dyslipidemia, hepatic ER stress and ROS, and pro-inflammatory adipose tissue remodeling. Whether these initial findings in rodents will be confirmed in humans must await future studies. Nevertheless, we believe that our study shed new insight into EA-containing foods consumption for the prevention of sugar toxicity.

References

- [1] Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr* 2006;83:461s-5s.
- [2] Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, et al. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* 1999;4:597-609.
- [3] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008;57:1470-81.
- [4] Malik VS, Popkin BM, Bray GA, Despres JP, Hu FB. Sugar-sweetened beverages, obesity, type 2 diabetes mellitus, and cardiovascular disease risk. *Circulation* 2010;121:1356-64.
- [5] Parks EJ, Hellerstein MK. Carbohydrate-induced hypertriacylglycerolemia: historical perspective and review of biological mechanisms. *Am J Clin Nutr* 2000;71:412-33.
- [6] Carden TJ, Carr TP. Food availability of glucose and fat, but not fructose, increased in the U.S. between 1970 and 2009: analysis of the USDA food availability data system. *Nutr J* 2013;12:130.
- [7] Malik VS, Schulze MB, Hu FB. Intake of sugar-sweetened beverages and weight gain: a systematic review. *Am J Clin Nutr* 2006;84:274-88.

- [8] Stanhope KL, Schwarz JM, Keim NL, Griffen SC, Bremer AA, Graham JL, et al. Consuming fructose-sweetened, not glucose-sweetened, beverages increases visceral adiposity and lipids and decreases insulin sensitivity in overweight/obese humans. *J Clin Invest* 2009;119:1322-34.
- [9] Lee JH, Johnson JV, Talcott ST. Identification of ellagic acid conjugates and other polyphenolics in muscadine grapes by HPLC-ESI-MS. *J Agric Food Chem* 2005;53:6003-10.
- [10] Vasudev SS, Ahmad FJ, Khar RK, Bhatnagar A, Kamal YT, Talegaonkar S, et al. Validated HPLC method for the simultaneous determination of taxol and ellagic acid in a *Punica granatum* fruit extract containing combination formulation. *Pharmazie* 2012;67:834-8.
- [11] Adams LS, Zhang Y, Seeram NP, Heber D, Chen S. Pomegranate ellagitannin-derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. *Cancer Prev Res(Phila)*. 2010;3:108-13.
- [12] Losso JN, Bansode RR, Trappey A, Bawadi HA, Truax R. In vitro anti-proliferative activities of ellagic acid. *J Nutr Biochem* 2004;15:672-8.
- [13] Aiyer HS, Gupta RC. Berries and ellagic acid prevent estrogen-induced mammary tumorigenesis by modulating enzymes of estrogen metabolism. *Cancer Prev Res (Phila)* 2010;3:727-37.
- [14] Park SH, Kim JL, Lee ES, Han SY, Gong JH, Kang MK, et al. Dietary ellagic acid attenuates oxidized LDL uptake and stimulates cholesterol efflux in murine macrophages. *J Nutr* 2011;141:1931-7.
- [15] Hseu YC, Chou CW, Senthil Kumar KJ, Fu KT, Wang HM, Hsu LS, et al. Ellagic acid protects human keratinocyte (HaCaT) cells against UVA-induced oxidative stress and apoptosis through the upregulation of the HO-1 and Nrf-2 antioxidant genes. *Food Chem Toxicol* 2012;50:1245-55.
- [16] Umesalma S, Sudhandiran G. Differential inhibitory effects of the polyphenol ellagic acid on inflammatory mediators NF-kappaB, iNOS, COX-2, TNF-alpha, and IL-6 in 1,2-dimethylhydrazine-induced rat colon carcinogenesis. *Basic Clin Pharmacol Toxicol* 2010;107:650-5.
- [17] Okla M, Kang I, Kim dM, Gourineni V, Shay N, Gu L, et al. Ellagic acid modulates lipid accumulation in primary human adipocytes and human hepatoma Huh7 cells via discrete mechanisms. *J Nutr Biochem* 2015;26:82-90.
- [18] Kang I, Okla M, Chung S. Ellagic acid inhibits adipocyte differentiation through coactivator-associated arginine methyltransferase 1-mediated chromatin modification. *J Nutr Biochem* 2014;25:946-53.
- [19] Garcia-Villalba R, Espin JC, Aaby K, Alasalvar C, Heinonen M, Jacobs G, et al. Validated Method for the Characterization and Quantification of Extractable and Nonextractable Ellagitannins after Acid Hydrolysis in Pomegranate Fruits, Juices, and Extracts. *J Agric Food Chem*. 2015;63:6555-66.
- [20] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-502.
- [21] Chen HC, Farese RV, Jr. Determination of adipocyte size by computer image analysis. *J Lipid Res* 2002;43:986-9.

- [22] Zhao L, Kang I, Fang X, Wang W, Lee MA, Hollins RR, et al. Gamma-tocotrienol attenuates high-fat diet-induced obesity and insulin resistance by inhibiting adipose inflammation and M1 macrophage recruitment. *Int J Obes (Lond)* 2015;39:438-46.
- [23] Kosmala M, Zdunczyk Z, Juskiewicz J, Jurgonski A, Karlinska E, Macierzynski J, et al. Chemical composition of defatted strawberry and raspberry seeds and the effect of these dietary ingredients on polyphenol metabolites, intestinal function, and selected serum parameters in rats. *J Agric Food Chem* 2015;63:2989-96.
- [24] Kelley GL, Allan G, Azhar S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. *Endocrinol* 2004;145:548-55.
- [25] Zhang C, Chen X, Zhu RM, Zhang Y, Yu T, Wang H, et al. Endoplasmic reticulum stress is involved in hepatic SREBP-1c activation and lipid accumulation in fructose-fed mice. *Toxicol Lett* 2012;212:229-40.
- [26] Bhandary B, Marahatta A, Kim HR, Chae HJ. An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *Int J Mol Sci.* 2012;14:434-56.
- [27] Landete J. Ellagitannins, ellagic acid and their derived metabolites: a review about source, metabolism, functions and health. *Food Res Int* 2011;44:1150-60.
- [28] González-Sarriás A, García-Villalba R, Núñez-Sánchez MA, Tomé-Carneiro J, Zafrilla P, Mulero J, et al. Identifying the limits for ellagic acid bioavailability: A Crossover pharmacokinetic study in healthy volunteers after consumption of pomegranate extracts. *J Funct Foods* 2015. 19:225-235.
- [29] Larrosa M, Gonzalez-Sarrias A, Yanez-Gascon MJ, Selma MV, Azorin-Ortuno M, Toti S, et al. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. *J Nutr Biochem* 2010;21:717-25.
- [30] Ishimoto H, Shibata M, Myojin Y, Ito H, Sugimoto Y, Tai A, et al. In vivo anti-inflammatory and antioxidant properties of ellagitannin metabolite urolithin A. *Bioorg Med Chem Lett* 2011;21:5901-4.
- [31] Tomas-Barberan FA, Garcia-Villalba R, Gonzalez-Sarrias A, Selma MV, Espin JC. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. *J Agric Food Chem* 2014;62:6535-8.
- [32] Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, et al. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol* 2008;48:993-9.
- [33] Caton PW, Nayuni NK, Khan NQ, Wood EG, Corder R. Fructose induces gluconeogenesis and lipogenesis through a SIRT1-dependent mechanism. *J Endocrinol* 2011;208:273-83.
- [34] Anhe FF, Roy D, Pilon G, Dudonne S, Matamoros S, Varin TV, et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. *Gut* 2015;64:872-83.
- [35] Jaroslawska J, Juskiewicz J, Wroblewska M, Jurgonski A, Krol B, Zdunczyk Z. Polyphenol-rich strawberry pomace reduces serum and liver lipids and alters gastrointestinal metabolite formation in fructose-fed rats. *J Nutr.* 2011;141:1777-83.

- [36] Yoshimura Y, Nishii S, Zaima N, Moriyama T, Kawamura Y. Ellagic acid improves hepatic steatosis and serum lipid composition through reduction of serum resistin levels and transcriptional activation of hepatic ppara in obese, diabetic KK-A(y) mice. *Biochem Biophys Res Commun* 2013;434:486-91.
- [37] Torronen R, Sarkkinen E, Tapola N, Hautaniemi E, Kilpi K, Niskanen L. Berries modify the postprandial plasma glucose response to sucrose in healthy subjects. *Br J Nutr* 2010;103:1094-7.
- [38] Kam A, Li KM, Razmovski-Naumovski V, Nammi S, Shi J, Chan K, et al. A comparative study on the inhibitory effects of different parts and chemical constituents of pomegranate on alpha-amylase and alpha-glucosidase. *Phytother Res* 2013;27:1614-20.
- [39] Havel PJ. Dietary fructose: implications for dysregulation of energy homeostasis and lipid/carbohydrate metabolism. *Nutr Rev* 2005;63:133-57.
- [40] Mayes PA. Intermediary metabolism of fructose. *Am J Clin Nutr* 1993;58:754s-65s.
- [41] Wang Y, Viscarra J, Kim SJ, Sul HS. Transcriptional regulation of hepatic lipogenesis. *Nat Rev Mol Cell Biol* 2015;16:678-89.
- [42] Galano A, Francisco Marquez M, Perez-Gonzalez A. Ellagic acid: an unusually versatile protector against oxidative stress. *Chem Res Toxicol* 2014;27:904-18.
- [43] Winand J, Schneider YJ. The anti-inflammatory effect of a pomegranate husk extract on inflamed adipocytes and macrophages cultivated independently, but not on the inflammatory vicious cycle between adipocytes and macrophages. *Food Funct* 2014;5:310-8.
- [44] Jeong MY, Kim HL, Park J, An HJ, Kim SH, Kim SJ, et al. Rubi Fructus (*Rubus coreanus*) Inhibits Differentiation to Adipocytes in 3T3-L1 Cells. *Evid Based Complement Alternat Med* 2013;2013:475386.
- [45] Wang L, Li L, Ran X, Long M, Zhang M, Tao Y, et al. Ellagic Acid Reduces Adipogenesis through Inhibition of Differentiation-Prevention of the Induction of Rb Phosphorylation in 3T3-L1 Adipocytes. *Evid Based Complement Alternat Med* 2013;2013:287534.

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Table 1. Main phenolic compounds identified in RSF.

Compound	Retention time	m/z	MS/MS
Flavan-3-ol and proanthocyanidins			
Proanthocyanidin dimers (epi)catechin-(epi)catechin	10.82; 12.33;14.82	577	451, 425, 407, 289
(Epi)catechin (monomer)	11.76	289	271, 245, 161, 125
Proanthocyanidin trimer 3 (epi)catechin	12.90	865	289
Proanthocyanidin dimers (epi)afzelechin-(epi)catechin	13.19; 14.26; 15.25;17.06	561	543, 435, 407, 289
proanthocyanidin trimer 2 (epi)catechin-(epi)afzelechin	15.04	849	
Proanthocyanidin dimer (epi)afzelechin-(epi)afzelechin	16.05	545	549,419,273,164
Proanthocyanidin trimer 2 (epi)afzelechin-(epi)catechin	16.41	833	
Ellagic acid derivatives and ellagitannins			
*HHDP-hexoside	3.28	481	301
bis-HHDP-hexoside (pedunculagin isomers)	7.47; 9.52	783	481,301
Castalagin/vescalagin isomers	8.52; 15.69, 16.70	466 (double charged)	865, 781, 631, 481, 451, 301
Galloyl-HHDP-glucose (corilagin isomers)	8.91;9.69;11.42	633	481, 463, 301
Valoneic acid dilactone isomers	8.98;11.54;14.20	469	425
Digalloyl hexoside	10.60	483	331, 313, 271, 169
Ellagitannin (unidentified)	13.50	631 (double charged)	938, 783, 695, 451, 301
Galloyl bis HHDP-glucoside isomers	14.97;15.69;16.70, 17.35	467(double charged)	751, 633, 451, 391, 301
Di-galloyl HHDP glucoside	15.25	785	633, 483, 301
Ellagitannin (unidentified)	15.54	551(double charged)	765, 631, 448, 301
Ellagic acid pentoside isomers	16.84;17.35	433	301
Ellagic acid	18.28	301	257
Phenolic acid derivatives			
Caffeoyl hexoside	2.51	341	179, 161

*HHDP, hexahydroxydiphenic acid.

Table 2. Food intake, metabolic parameters and blood lipid profiles *

Group	HF	HFHS	HFHS-R	<i>p</i> -value
Food intake (g/mouse/day)	3.43 ± 0.13	3.45 ± 0.13	3.27 ± 0.04	0.840
Phenotypes				
BW (g)	36.67 ± 0.74 ^b	45.00 ± 0.59 ^a	40.50 ± 0.73 ^{ab}	0.011
Δ BW (g)	10.00 ± 0.46 ^b	18.5 ± 0.27 ^a	14.50 ± 0.77 ^{ab}	0.002
Liver (g)	1.57 ± 0.04 ^b	2.97 ± 0.18 ^a	1.97 ± 0.07 ^{ab}	0.046
Liver/BW (%)	4.27 ± 0.10	5.93 ± 0.49	4.86 ± 0.18	0.223
Epididymal fat (g)	1.82 ± 0.07 ^b	2.51 ± 0.048 ^a	2.14 ± 0.08 ^{ab}	0.040
Mesenteric fat (g)	0.60 ± 0.07 ^b	1.08 ± 0.07 ^a	0.88 ± 0.07 ^{ab}	0.015
Blood Chemistry				
Triglyceride (mg/dL)	25.59 ± 0.77 ^{ab}	38.93 ± 2.41 ^a	21.67 ± 2.07 ^b	0.056
Total Cholesterol (mg/dL)	110.33 ± 4.12 ^b	184.50 ± 2.36 ^a	122.83 ± 1.83 ^b	0.001
HDL (mg/dL)	71.28 ± 0.48 ^a	60.82 ± 0.22 ^b	72.13 ± 0.40 ^a	< 0.0001
LDL (mg/dL)	33.94 ± 0.48 ^c	115.80 ± 0.22 ^a	45.89 ± 0.40 ^b	<0.0001

* Values are mean ± SEM. All groups, n = 6. Column not sharing a common letter are significantly different (p < 0.05)

Figure legends

Figure 1. Identification of ellagic acid and its derivatives after hydrolysis of RSF.

Figure 2. RSF supplementation ameliorated glucose and insulin tolerance in C57BL/6 mice against HS diet. Eight-week old male C57BL/6 mice were fed with isocaloric HF (grey), HFHS (white), or HFHS-R (black) diet for 12 weeks (n = 6 per group). (A) Fasting plasma glucose levels (mg/dL), (B) Fasting plasma insulin levels (ng/mL) quantified by ELISA, (C) HOMA-IR, (D) Glucose tolerance tests (GTT), (E) Insulin tolerance tests (ITT). Data are means expressed as mean $\pm$ SEM (n = 6). Bars with different letters are significantly different by one-way ANOVA. * $P < 0.05$ (HF vs. HFHS), # $P < 0.01$, and ### $P < 0.0001$ (HFHS vs. HFHS-R) by Student's *t*-test.

Figure 3. RSF supplementation attenuated hepatic ER stress and insulin sensitivity against HS diet. Eight-week old male C57BL/6 mice were fed with isocaloric HF (grey), HFHS (white), or HFHS-R (black) diet for 12 weeks (n=6 per group) (A-E). (A). Gross images and representative microscopic images of liver revealed by H&E staining after 12 weeks of feeding with HFHS or HFHS-R, (B) Hepatic TG contents (mg/g liver), (C) Hepatic mRNA expression of SCD-1, LPL, DGAT2, ChREBP and SREBP1c by qPCR, (D). Hepatic mRNA expressions of gluconeogenic genes of G6Pase and PEPCK by qPCR, (E). Western blot analysis of phosphorylation of JNK, p38, and eIF2 α in liver fed with either HFHS or HFHS-R. Huh7 cells were pre-incubated with either DMSO or EA before stimulation with the mixture of glucose (Glc), fructose (Fr) and palmitate (PA) (F, G). (F) Phosphorylation of JNK, and p38 expression by western blot. (G) Phosphorylation of Akt by western blot with or without insulin (100 nM) treatment. β -actin was used for loading control. Data are expressed as mean $\pm$ SEM (n = 6). Bars with different letters are significantly different by one-way ANOVA.

Figure 4. RSF supplementation reduced hepatic oxidative stress against HS diet. Eight week-old male C57BL/6 mice were fed with HF (grey), HFHS (white), or HFHS-R (black) diet for 12 weeks (n=6 per group). (A) H₂O₂ level (μmol/ mg protein) in liver sample. (B) Relative ROS production in huh 7 cells with or without EA using DCF florescence as molecular probe for detecting ROS production (Left). DCF fluorescence was visualized by fluorescence microscopy (Right). Data are expressed as mean ± SEMs. Bars with different letters are significantly different by one-way ANOVA. DCF: dichlorofluorescein

Figure 5. RSF supplementation attenuated visceral adipocyte hypertrophy and adipose inflammation against HS diet. Eight week-old male C57BL/6 mice were fed with HF, HFHS, or HFHS-R diet for 12 weeks (n=6 per group). (A) Representative microscopic images of epididymal adipose tissue by H&E staining. Arrows indicate macrophage infiltration, (B) Average size (diameter) of epididymal adipocytes (n=6 mice per group), (C) Adipocyte size distribution, The line of best fit is shown (Gaussian curve fitting), (D) Plasma adiponectin levels (ng/mL) quantified by ELISA, (E) mRNA expression levels of adiponectin, IL-6, IL-8, F4/80, TNFα and MCP1 in epididymal adipose tissue quantified by qPCR. (F) Immunofluorescent staining of F4/80 (Green). The nuclei counterstained by DAPI (Blue) and phase contrast images were overlapped. (G) Homogenized epididymal adipose tissue and plasma (pooled sample n=6 per group) from HFHS or HFHS-R fed mice were used for simultaneous detection of multiple inflammatory cytokines using mouse inflammatory array C1. Data are expressed as mean ± SEMs. Bars with different letters are significantly different by one-way ANOVA. ***P* < 0.01, ****P* < 0.001 (HFHS vs. HFHS-R) by Student's *t*-test.

Figure 1

Figure 2

Figure 3

Figure 3

Figure 4

A **B**

Figure 5