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1 **Black Sesame Seeds Ethanol Extract Ameliorates Hepatic Lipid**  
2 **Accumulation, Oxidative Stress and Insulin Resistance in**  
3 **Fructose-induced Nonalcoholic Fatty Liver Disease (NAFLD)**

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22 **ABSTRACT**

23 The aim of the present study was to investigate the effect of black sesame seeds  
24 ethanol extract (BSSEE) against nonalcoholic fatty liver disease (NAFLD) in  
25 fructose-fed mice. Mice were fed diet without or with 30% fructose for 8 consecutive  
26 weeks, while mice in three BSSEE tested groups received different dose of BSSEE  
27 (0.5, 1 and 2 mL/kg) once a day from the 5th week to the 8th weeks. Administration  
28 of BSSEE dose-dependently exerted antiobesity and protective effect against  
29 metabolism disorder in fructose-fed mice. Liver histological results indicated that  
30 administration of BSSEE significantly reduced hepatic lipid accumulation. Insulin  
31 tolerance tests (ITT) and glucose tolerance tests (GTT) along with decrease of serum  
32 insulin and glucose levels by BSSEE treatment suggested the improvement of body  
33 insulin resistance, and administration of 1 and 2 mL/kg BSSEE mitigated liver insulin  
34 resistance as the evidence of down-regulated expressions of phospho-JNK1,  
35 phospho-NF- $\kappa$ B p65, phospho-IRS1 (Ser 307) and phospho-IKK, and up-regulated  
36 XBP1 expression, as well as reductions of hepatic TNF- $\alpha$  and IL-6 levels. In addition,  
37 BSSEE treatment reduced hepatic oxidative stress through increasing GSH, vitamin C  
38 and Nrf2 levels, enhancements of SOD, CAT and GSH-Px activities, and decreasing  
39 MDA and NO levels. These results demonstrated that black sesame seeds showed  
40 good effects against NAFLD-related metabolic diseases in fructose-fed mice, and may  
41 be a potent dietary supplements in the prevention of the diseases.

42 **KEYWORDS:** black sesame seeds, nonalcoholic fatty liver disease, fructose, insulin  
43 tolerance, hepatic oxidative stress.

## 44 ■ INTRODUCTION

45 Nonalcoholic fatty liver disease (NAFLD) comprises a wide pathologic spectrum  
46 of liver pathologies ranging from steatosis to steatohepatitis, fibrosis, cirrhosis and  
47 even hepatocellular carcinoma, which is characterized by the common central feature  
48 of the fatty degeneration in liver cells <sup>1</sup>. Nowadays, the disease is considered as one of  
49 the most prevalent chronic liver disease all over the world with a reported prevalence  
50 ranging 20–30% according to the studied populations <sup>2</sup>. The important reasons for this  
51 high prevalence is closely associated with the increasing prevalence of obesity,  
52 diabetes and metabolic syndrome <sup>3</sup>. Although the molecular mechanisms involved in  
53 the pathopoiesis of NAFLD are not clearly understood, it has been found that  
54 impaired insulin function is a very risk factor in the development of NAFLD. In the  
55 progression of NAFLD, pro-inflammatory cytokines such as tumor necrosis factor  
56 alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) that drive insulin resistance is closely linked  
57 with the aggravation of NAFLD through activation of specific intracellular signalling  
58 pathways, involving in not only the c-Jun NH2-terminal kinase 1 (JNK1),  $\kappa$ B kinase  
59 (IKK), nuclear factor kappa B (NF- $\kappa$ B), x-box binding protein 1 (XBP1), but also  
60 reactive oxygen species (ROS) and free fatty acids (FFA), where all these pathways  
61 could interact with insulin signalling via serine/threonine inhibitory phosphorylation  
62 of insulin receptor substrate 1 (IRS1) <sup>4</sup>. Notably, some food supplement is rich in  
63 antioxidants and have good anti-inflammatory effect, which may be more beneficial  
64 in prevention of NAFLD.

65 Fructose is one of main sugars consumption in diet. In the United States, Europe

66 and China, high-fructose corn syrup (HFSC), containing more than 42% fructose, has  
67 became a replacement of refined sugar in many foods and most sweetened beverages,  
68 such as soft drinks, juice beverages, canned fruits, jellies, jams, breakfast cereals and  
69 baked goods <sup>5</sup>. Fructose intake can cause more productions of glucose, glycogen,  
70 lactate and pyruvate, which contribute to the formation of triglycerides in liver cells <sup>5</sup>,  
71 <sup>6</sup>. Further studies indicated that high-fructose diets activates lipogenesis at least partly  
72 linked to insulin resistance <sup>5,7</sup>. In addition, dietary high fructose facilitates oxidative  
73 damage in various tissues. More production of ROS has been claimed as a persistent  
74 contributor for liver cell damage and impaired glucose tolerance in fructose-induced  
75 NAFLD <sup>8-11</sup>. Long term intake of high fructose has been repeatedly proved to be a risk  
76 factor for the pathopoiesis of NAFLD both in animals and human <sup>5,8,10</sup>.

77 Sesame seed (*sesamum indicum* L.) is one of the world's most important and  
78 oldest oil seed crops known to humanity <sup>12</sup>. Sesame seeds and its oil are popularly  
79 used in cookery to flavour foods and drinks <sup>13</sup>. Notably, sesame seed have many  
80 species (most being white sesame seeds and brown (golden) sesame seeds), one of  
81 which is black sesame seeds. The black sesame are rich in fatty oil, containing mainly  
82 oleic acid and linoleic acid, and other chemical constituents including sesamin,  
83 sesamolin, sesamol, vitamin E, phytosterols, lecithin, pedaliin, protein and minerals <sup>14</sup>.  
84 The black sesame seeds are more flavorful and have a stronger aroma than white or  
85 brown sesame seeds. Toasting black sesame seeds releases beneficial chemicals as  
86 well as enhancing the flavor. In addition, black sesame seeds are believed to have  
87 medicinal properties. In China, black sesame seeds is superior to other species as food

88 for health and often acts as a traditional Chinese medicine for promoting shiny and  
89 dark hair, invigorating liver and kidney, benefiting life essence, nourishing blood and  
90 moistening the intestines <sup>14</sup>. In addition, a comparative study found that black sesame  
91 seeds showed potent antioxidant activity than white sesame seeds <sup>15-17</sup>. It is worth  
92 mentioning that sesame oil mainly from white or brown sesames <sup>18, 19</sup>, as well as its  
93 phytochemical compounds such as sesamin <sup>20-22</sup>, sesamol <sup>23, 24</sup> and sesamol <sup>25, 26</sup>,  
94 have been reported that they show hepatoprotective effect and can alleviate steatosis  
95 in steatohepatitis by regulating lipid metabolism and enhancing anti-oxidative stress  
96 effect in animals <sup>18-21, 24, 25</sup>. However, limited studies have been performed on the  
97 investigation of the protective effect of black sesame seeds against NAFLD and its  
98 molecular biological mechanism in fructose-induced NAFLD. Since the high  
99 prevalence of NAFLD and lack of satisfactory treatments, finding the best diet to  
100 replace therapies may be a good choice for preventing and treating the disease.  
101 Therefore, the aim of the present study was to investigate the effect of black sesame  
102 seeds ethanol extract (BSSEE) against NAFLD-related metabolic diseases.

## 103 ■ MATERIALS AND METHODS

### 104 **BSSEE Extraction and Main Ingredient Analysis.**

105 Black sesame seeds (*sesamum indicum* L.) were obtained from Yurun Global  
106 Sourcing Center for Agricultural Products (Xi'an, Shaanxi, China), and pulverized  
107 using a Chinese herbal remedier (FW117, Nanchang Jiedao Scientific Instruments Co.,  
108 Ltd., Jiangxi, China). 1 kg of this powder was extracted with 10000 mL of 95%  
109 ethanol (Analytical grade, Xi'an Chemical Reagent Factory, Shaanxi, China) at 25 °C

110 and repeated three times. The ethanol extract was concentrated on a rotavapor  
111 (RE52CS-1, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China) at  
112 50 °C. Then 20.2 mL of the crude extract was obtained and stored at -20 °C for the  
113 analysis, and 1 mL of this extract weights 0.83 g. The yield of the obtained the black  
114 sesame seeds ethanol crude extract (BSSEE) from fresh black sesame seeds was  
115 1.68% (w/w), calculated by the weight of the seeds used in the extraction procedure.

116 BSSEE were analyzed using a LiChrosorb C<sub>18</sub> column (4.6 mm i.d. × 250 mm, 5  
117 μm, Merck, Darmstadt, Germany) in the analytical high performance liquid  
118 chromatography (HPLC) instrument (Waters 2695 HPLC system equipped with a  
119 PDA detector was used in addition to an Empower chromatographic workstation,  
120 Waters Corporation, Milford, Massachusetts, USA). Chromatographic separation was  
121 performed using an gradient elution at a flow rate of 1.0 mL/min with mobile phase  
122 for (A) methanol (HPLC grade, Merck, Darmstadt, Germany)/(B) distilled water:  
123 30–100 % A ( 0–60 min). The column temperature was controlled at 30 °C, and the  
124 effluents were monitored at 280 nm. 0.1 mL of the black sesame seeds extract was  
125 dissolved in 1 mL methanol (Analytical grade, Xi'an Chemical Reagent Factory,  
126 Shaanxi, China) and filtered with 0.20 μm Nylon filter (Beijing Kaiyuan Guochuang  
127 technology Co., Ltd., Beijing, China), and then 20 μl of the dilute solution was  
128 injected for analysis. Calibration curves were constructed with reference standards and  
129 used to determine the concentrations of sesaminol, sesamin and sesamolin in BSSEE.  
130 Sesaminol, sesamin and sesamolin (Purity ≥ 97%) were purchased from  
131 Sigma-Aldrich Corp. (St. Louis, MO, USA). As shown in the Figure 1, the three

132 major components of BSSEE, sesaminol (1.92%), sesamin (16.33%) and sesamol  
133 (13.06%) accounted for 31.31% of the total extract. This result is similar to previous  
134 studies <sup>26</sup>.

### 135 **Animals and Experimental Design.**

136 Fifty healthy adult male kunming mice ( $20 \pm 3$  g) were purchased from the  
137 Experimental Animal Center, Xi'an Jiaotong University (SCXK2012-003, Xi'an,  
138 Shaanxi, China). Animals were housed at standard conditions: normal light/dark  
139 (12h/12h) cyclic conditions and  $25 \pm 3$  °C, 30-70% humidity. Use of the mice was  
140 reviewed and approved by both Northwest University and the local animal Ethics  
141 Committee.

142 Mice in all groups were fed a standard diet (Jiangsu Cooperative Medical  
143 Biological Engineering Co., Ltd., Nanjing, Jiangsu, China). The composition of diet  
144 was as follows: 63% carbohydrate, 18% crude protein, 5% crude fat, 3% crude fiber,  
145 6% ash and 5% predefined minerals plus amino acids and vitamins. Representative  
146 ingredients of diet are ground wheat, ground corn, dehulled soybean meal, corn gluten  
147 meal, fish meal, chicken meal, soybean oil, methionine, lysine, cystine, calcium  
148 carbonate, brewers dried yeast, dicalcium phosphate and iodized salt. The energy  
149 content of the diet was 3.64 kcal/g. BSSEE was prepared into soybean oil (Zhejiang  
150 Tian Yushan Medicinal Oils Co., Ltd., Quzhou, Zhejiang, China) before  
151 administration to the mice. Animals were acclimated to housing conditions for one  
152 week. Subsequently mice were randomly divided into 5 groups with 10 each,  
153 including the control group, fructose group and three fructose + BSSEE (0.5, 1 and 2



154 mL/kg) tested groups. Except for the mice in the control group freely drank water  
155 without fructose, mice in all groups freely drank water containing 30% fructose  
156 (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) for 8 consecutive weeks  
157 (From the 1st week to the 8th week). However, animals in those fructose + BSSEE  
158 (0.5, 1 and 2 mL/kg) tested groups received different dose of BSSEE (0.5, 1 and 2  
159 mL/kg) by intragastric administration once a day at the end of the 4th week for 4  
160 consecutive weeks (From the 5th week to the 8th week) respectively, while mice in  
161 the control group and the fructose group received the same amount of soybean oil.  
162 During the whole experimental period, the body weight of each mouse was noted at  
163 three days intervals, and food intake and water consumption in each group was  
164 recorded every day.

165 On the last day, at the end of the drug administration, mice in all groups were  
166 fasted for 8 hours. Then all animals were anesthetized and blood of each mouse was  
167 collected through retro-orbital plexus. Subsequently all animals were euthanized, and  
168 the liver, epididymal adipose, subcutaneous adipose and perirenal adipose were  
169 collected and weighed. The liver of each mouse were collected and cut into two parts,  
170 with one part of liver lobe immersed in 4% paraformaldehyde solution for histological  
171 examinations and immunohistochemistry (IHC), while the other part of liver lobe  
172 stored at -80 °C for the biochemical analysis.

### 173 **Serum Biochemical Analysis.**

174 Serum was separated by centrifugation at 1000 g (4 °C) for 10 min. The levels of  
175 biochemical parameters, including aspartate transaminase (AST), alanine

176 transaminase (ALT), glucose, total cholesterol, and triglycerides, were measured  
177 colorimetrically and assayed by an automatic biochemistry analyzer (AU5800,  
178 Beckman Coulter, Inc., California, USA). Free fatty acids (FFA) were measured using  
179 a commercial kit (BC0590, Solarbio, Beijing, China), and insulin level was measured  
180 by a mouse insulin ELISA Kit (TWp001983, Shanghai Tongwei Industrial Co., Ltd.,  
181 Shanghai, China), in which the results were obtained by a DNM-9602 Reader (Beijing  
182 Prang New Technology Co., Ltd., Beijing, China).

### 183 **Histological Staining.**

184 Liver tissues were fixed in 4% paraformaldehyde solution (137 mM NaCl, 10  
185 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, and 2.7 mM KCl, 1000 mL deionized water, 4%  
186 paraformaldehyde, pH 7.4) for 24 hours, embedded in paraffin and cut into 5 μm  
187 sections using YD-1508R Tissue Slicer (Beijing Jiayuan Industrial Technology Co.,  
188 Ltd., Beijing, China), and then selected good tissue sections were mounted on  
189 adhesion microscope slides (Citotest Labware Manufacturing Co., Ltd., Haimen,  
190 Jiangsu, China) and stained with hematoxylin & eosin (H&E) for the examination of  
191 the morphology of liver. In order to evaluate lipid droplet accumulation, liver tissues  
192 were treated with 4% paraformaldehyde solution, embedded in optimal cutting  
193 temperature (OCT) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA), and  
194 frozen sections (10 μm) of liver tissues were washed with sterile water, infiltrated  
195 with 60% isopropanol for 2 min, and then stained with oil red O (Muto Pure  
196 Chemicals Co., Ltd., Tokyo, Japan). The severity of liver injury were evaluated by  
197 visual estimate under a LIRI-2006 microscope according to the method reported

198 previously <sup>27</sup>: Ballooning degeneration of hepatocytes: grade=0 (< 5%), grade=1 (5%  
199 -30%), grade=2 (30% - 50%), grade=3 (50% ≤); Steatosis: grade=0 (< 5%), grade=1  
200 (5%-30%), grade=2 (30% -50), grade=3 (50% ≤). Three sections and 3 fields per  
201 tissue were observed (n= 3 in each group). Photographs taken from tissue sections  
202 were digitized using CMOS camera equipped with a GMS image analysis system  
203 (Shanghai optical instrument factory, Shanghai, China).

#### 204 **Insulin Tolerance Tests (ITT) and Glucose Tolerance Tests (GTT)**

205 GTT (A) and ITT (B) were performed on weeks 7, respectively. In the ITT, mice  
206 from all five group were fasted for 8 hours and then injected intraperitoneally with 1  
207 U human insulin/kg body weight, and blood glucose values were measured at (0 min)  
208 and at 5, 10, 30, 60, and 120 min. For GTT, mice fasted 8 hours were injected  
209 intraperitoneally with d-glucose (2 g/kg body weight), and blood glucose was  
210 measured at (0 min) and at 5, 15, 30, 90, 60, and 120 min. Glucose levels both in GTT  
211 and ITT were measured using a glucometer (Sinocare Inc., Changsha, Hunan, China).  
212 The area under the curve (AUC) for GTT and ITT tests were calculated according to  
213 the trapezium rule <sup>28</sup>.

#### 214 **Analyses of TNF- $\alpha$ and IL-6 Levels in Liver and Serum.**

215 TNF- $\alpha$  and IL-6 levels in serum and liver homogenates were determined by  
216 commercial ELISA kits (KET7015 and KET7009, Abbkine Scientific Co., Ltd.,  
217 California, USA), respectively.

#### 218 **Measurement of Hepatic Triglycerides Level**

219 Liver tissue was homogenized in ice-cold PBS buffer (137 mM NaCl, 10 mM

220 Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, and 2.7 mM KCl, 1000 mL deionized water, pH 7.4).  
221 Hepatic triglycerides was extracted with the method described by Fossati <sup>29</sup>, and  
222 determined on a DNM-9602 Reader (Beijing Perlong New Technology Co., Ltd.,  
223 Beijing, China) by a assay kit (MAK266-1KT, Sigma-Aldrich Corp., St. Louis, MO,  
224 USA).

#### 225 **Hepatic Oxidative Stress Analysis.**

226 Liver tissues (100 mg) were homogenized with 1 mL of cold PBS buffer (137  
227 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, and 2.7 mM KCl, 1000 mL deionized  
228 water, pH 7.4) and then centrifuged at 10000g for 30 min at 4 °C. The homogenates  
229 were subjected to assays for nitric oxide (NO), vitamin C, glutathione (GSH),  
230 glutathione peroxidase (GSH-px), glutathione (GSH), catalase (CAT), superoxide  
231 dismutase (SOD), and malondialdehyde (MDA) using commercially available  
232 enzymatic assay kits (Wuhan ColorfulGene Biological Technology Co., Ltd., Wuhan,  
233 Hubei, China). All data acquisition was performed on an A6 semiautomatic  
234 biochemistry analyzer (Beijing Shining Sun Technology Co., Ltd., Beijing, China).

#### 235 **Western Blot Analysis.**

236 Hepatic protein expressions of phospho-JNK1, phospho-IRS1 (Ser 307),  
237 Phospho-IKK alpha/beta (Ser180/181) and XBP1 were evaluated by western blot  
238 analysis. Primary antibody against phospho-JNK1 (AF3318), phospho-IRS1  
239 (AF3272), Phospho-IKK alpha/beta (AF3013) and XBP1 (AF5110) were obtained  
240 from Affinity Biosciences (Affinity Biosciences, Cincinnati, USA) and used for the  
241 western bolt analysis, respectively. Liver tissues were homogenized in

242 cold radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 1% w/v  
243 Triton X-100, 0.1% w/v sodium dodecyl sulfate, 150 mM NaCl, 1% sodium  
244 deoxycholate, 1 mM NaF, 5 mM EDTA, protease inhibitors, and 1 mM sodium  
245 orthovanadate). The lysates were cleared by centrifugation at 15000g at 4 °C for 30  
246 min. The protein concentrations were quantified with a BCA assay kit (Huaying  
247 Biotechnology Co., Ltd., Hubei, China). Aliquots of lysates containing 25–40 µg  
248 protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis  
249 (SDS-PAGE), and electrophoretically transferred to polyvinylidene difluoride (PVDF)  
250 membranes by electrophoretic transfer (Bio-Rad Laboratories, Inc., California, UAS).  
251 After blocking with 5% BSA (0332, Amresco, USA) in TBST buffer (20 mM  
252 Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 8.0) for 2 h and rinsed three times  
253 with TBST buffer for 5 min each, the membranes were probed overnight at 4 °C with  
254 primary antibodies (Dilution 1:1000), respectively. After removal of the unbound  
255 primary antibody with TBST buffer, the membranes were washed and incubated with  
256 the secondary horseradish peroxidase-conjugated antibodies (dilution 1:50000, S0001,  
257 Affinity Biosciences, Cincinnati, USA) at room temperature for 2 h.  
258 Chemiluminescence was visualized using an ECL western blot detection kit (KF001,  
259 Affinity Biosciences, Cincinnati, USA). The signal was captured with a UVP  
260 BioSpectrum AC imaging system (UVP, Upland, CA, USA). Quantitation of protein  
261 expression was evaluated by densitometry using ImageJ software version k1.47 (NIH,  
262 Bethesda, MD, USA).  $\beta$ -actin expression with an anti- $\beta$ -actin mouse antibody  
263 (YM3028, ImmunoWay Biotechnology Company, Plano, USA) was used as an

264 internal control for equal loading of protein.

## 265 **Immunohistochemical Analysis**

266 NF-E2-related factor 2 (Nfr2) and phospho-NF-κB p65 expressions were  
267 examined by immunohistochemical analysis, respectively. The paraffin sections (5 μm)  
268 were dewaxed and hydrated followed by antigen retrieval through heat mediation in  
269 Citrate Plus solution (ab94674, Abcam Trading (Shanghai) Company Ltd., Shanghai,  
270 China.) to recover protein structure. Then sections were rinsed with 3% H<sub>2</sub>O<sub>2</sub> and  
271 TBS buffer (50 mM Tris, 100 mM NaCl, pH 7.6) to block endogenous peroxidase  
272 activity and excessive protein, and incubated with primary Nfr2 antibody or primary  
273 phospho-NF-κB antibody (AF0639 or AF2006, dilution 1:100, Affinity Biosciences,  
274 Cincinnati, USA) at 37°C for 30 minutes. After rinsing two times (5 minutes each time)  
275 with TBS buffer, sections were incubated with an horseradish peroxidase conjugated  
276 goat anti-rabbit antibody (dilution 1:100, S0001, Affinity Biosciences, Cincinnati,  
277 USA) for 30 minutes at 37°C. Then, sections were washed using TBS buffer for 3  
278 times with 5 minutes each, and incubated in diaminobenzidine (DAB) solution  
279 (ab127055, Abcam Trading (Shanghai) Company Ltd., Shanghai, China.), followed  
280 by counter-staining with 0.2% modified hematoxylin solution (Sigma-Aldrich Corp.,  
281 St. Louis, MO, USA). The stained sections were examined under a LIRI-2006  
282 microscope (Shanghai Optical Instrument Factory, Shanghai, China). The IHC  
283 analysis for Nrf2 and phospho-NF-κB p65 was evaluated according to the staining  
284 intensity of positive reaction and percentage of positive cells in a blind manner with a  
285 reported method<sup>30</sup>. The staining intensity was graded from 0 to 3 (score= 0: colorless,

286 score= 1: pale-yellow, score= 2: brown-yellow, and score= 3: saddle-brown). The  
287 score for the percentage of positive cells was graded from 0 to 4 (score= 0: negative,  
288 score= 1: fewer than 10% positive cells, score= 2: 10–50% positive cells, score= 3:  
289 51–75% positive cells, and score= 4: over 75% positive cells). Representative five  
290 animals (n = 5) were analyze.

### 291 **Statistical Analysis.**

292 The results were presented as the means  $\pm$  S.E.M (standard error of mean). The  
293 data were statistically analyzed using one-way ANOVA for multiple group  
294 comparison followed by Newman-Keuls test using SPSS 19.0 statistical software  
295 (SPSS Inc., Chicago, IL, USA).  $P \leq 0.05$  was considered statistically significant.

## 296 **■ RESULTS**

### 297 **Changes in Body Weight, Food Intake, Liver Weight and Fat Weight.**

298 In the whole experimental period, the changes in the body weight of mouse in  
299 each group were expressed as the average daily body weight of mouse in each week.  
300 The body weight of mice both in the control group and the fructose group continued  
301 to gain from beginning to end (Figure 2A and Figure 2B). Compared with mice in the  
302 fructose group, the body weight were inhibited at dose of 0.5 and 1 mL/kg BSSEE  
303 and trends to decrease at dose of 2 mL/kg of BSSEE after the BSSEE administration  
304 (Figure 2B). Before the BSSEE administration, the body weigh in mice received diet  
305 without fructose increased to 35.17 g from an initial weight of 16.7 g, whereas the  
306 body weight in fructose-fed mice increased to 39.60 (Figure 2A) and showed  
307 significant difference compared to the control group. After oral administration of the

308 BSSEE for consecutive 4 weeks, the body in fructose-fed mice slightly increased to  
309 41.83 g from 39.67 g at the dose of 0.5 mL/kg BSSEE and 40.17 g at the dose of 1  
310 mL/kg BSSEE from 39.17 g (Figure 2B), while oral administration of BSSEE at the  
311 dose of 2 mL/kg slightly decreased body weight of mice to 38.00 g from the body  
312 weight of 40.09 g at the end of the 4th week (Figure 2B). However, mice received  
313 fructose without the BSSEE administration markedly increased to 45.83 g at the end  
314 of experiment (Figure 2B). The average body weight of mice treated with BSSEE (0.5,  
315 1 and 2 mL/kg) significantly decreased compared with mice in the fructose group at  
316 the end of experiment, and significant difference was observed in mice administrated  
317 with BSSEE at 1 and 2 mL/kg compared with the control group.

318 For the changes in the food intake, fructose-fed mice showed the decrease of  
319 food intake throughout the experiment, compared with the mice in the control group.  
320 We measured the average amount of food intake per day after the BSSEE treatment,  
321 in which the average amount of food intake in those fructose-fed mice administrated  
322 with BSSEE (1 and 2 mL/kg) showed significant reduction compared with mice in the  
323 fructose group (Table 2). Although energy intake in mice administrated with BSSEE  
324 at 1 and 2 mL/kg showed less than mice in the fructose group, no significant  
325 difference were observed (Table 2).

326 At the end the of experiment, the liver weight, perinephrit fat weight,  
327 subcutaneous fat weight and epididymal fat weight in fructose group displayed  
328 significant increase compared to the control group, and the relative liver and total fat  
329 weights also significantly increased in fructose group (Table 1). Compared with mice



330 in the fructose group, BSSEE treated mice showed the decrease in the liver weight,  
331 perinephrit fat weight, subcutaneous fat weight, epididymal fat weight, and the  
332 relative liver and total fat tissue weight in a dose dependent manner (Table 1).

### 333 **Effect of BSSEE on Serum Biochemical Parameters.**

334 At the end of the experiment, fructose-fed mice showed significant high levels of  
335 serum cholesterol, triglycerides, glucose, insulin and FFA levels (Table 2), which may  
336 imply that fructose feeding cause metabolic disorders. However, mice administrated  
337 with BSSEE (0.5, 1 or 2 mL/kg) significantly decreased serum cholesterol,  
338 triglycerides, glucose, insulin and FFA levels in a dose-dependent manner (Table 2).  
339 Decreased these serum parameters by BSSEE indicated that BSSEE administration  
340 effectively improved metabolic disorders. In addition, AST and ALT levels in the  
341 fructose group are significant higher than that in the control group, whereas  
342 administration of BSSEE (0.5, 1 and 2 mL/kg) dose-dependently reversed AST and  
343 ALT levels (Table 2). Furthermore, we determined the serum TNF- $\alpha$  and IL-6 levels  
344 in mice from all group because the increase of serum TNF- $\alpha$  and IL-6 levels is closely  
345 associated with the progression of liver fat deposition<sup>31</sup>. Notably, fructose fed-mice in  
346 the fructose group showed higher levels of TNF- $\alpha$  and IL-6 than that in the control  
347 group. However, treatment with BSSEE (1 and 2 mL/kg) significantly reduced TNF- $\alpha$   
348 and IL-6 in serum.

### 349 **Effect of BSSEE on Liver Inflammation Response and Histopathological** 350 **Changes.**

351 Triglycerides are the main lipids deposited in the liver of patients with NAFLD.

352 We measured the hepatic triglycerides levels. Fructose administration significantly  
353 increased triglycerides level compared with mice in the control group, whereas mice  
354 administrated with BSSEE (1 and 2 mL/kg) significantly decreased hepatic  
355 triglycerides level (Figure.3E). Furthermore, the morphological changes of the  
356 hepatocytes of lobule were examined with H&E staining and oil red O staining, and  
357 the severity of histological changes was evaluated by the histopathological scoring  
358 system. Liver tissue stained with H&E showed some marked changes in mice from  
359 the fructose group, where the hepatocytes of lobule in some sections showed  
360 ballooning degeneration (Figure.3F), and the ballooning degeneration score  
361 significantly increased compared with mice in the control group (Figure.3C).  
362 Meanwhile, liver tissue stained with oil red O showed fatty deposition of hepatocyte  
363 and high fatty deposition score in mice from the fructose group (Figure.3D). In  
364 contrast, H&E and oil red O staining displayed that the liver sections from  
365 fructose-fed mice treated with BSSEE (1 and 2 mL/kg) alleviated these histological  
366 alterations (Figure.3F) and significantly decreased the ballooning degeneration and  
367 fatty deposition scores (Figure.3C and Figure.3D) in a dose-dependent manner.  
368 Moreover, TNF- $\alpha$  (Figure.3A) and IL-6 (Figure.3B) are two important inflammatory  
369 cytokines, in which they drive the development of hepatic steatosis and inflammation  
370 in NAFLD. IL-6 and TNF- $\alpha$  levels in fructose-fed mice without the treatment  
371 significantly increased compared with mice in the control group, and there are some  
372 inflammatory cells infiltration in liver tissue sections stained with H&E. Compared to  
373 mice in the fructose group, significant decrease in IL-6 and TNF- $\alpha$  levels with a

374 dose-dependent manner was observed in mice administrated with BSSEE (0.5, 1 and  
375 2 mL/kg), and no inflammatory cells infiltration were observed in liver tissue sections  
376 stained with H&E in BSSEE (0.5, 1 and 2 mL/kg) treated mice.

### 377 **BSSEE improved glucose homeostasis in fructose-fed mice**

378 To investigate the effect of BSSEE on body insulin sensitivity, mice were fasted  
379 and were intraperitoneally injected with either glucose (GTT) or insulin (ITT). As  
380 shown in Figure.4A, blood glucose level in fructose-fed mice without the treatment  
381 were constantly higher (statistically significant at 30 and 60 minutes) than mice in the  
382 control group mice, which was accompanied by higher levels of blood glucose level  
383 in ITT test at 60 and 90 min (Figure. 4C), suggesting that insulin sensitivity in  
384 fructose-fed mice without the treatment was relatively decreased. This notion was  
385 further strengthened by the analysis of AUC summarized in Figure.4B and Figure.4D,  
386 in which the area under the curve for the GTT and ITT in fructose group significantly  
387 increased to 29.19 and 9.24 compared with mice in the control group, respectively.  
388 However, administration with BSSEE (0.5, 1 and 2 mL/kg) lead to a significant  
389 decrease at 30 min for GTT (Figure. 4A) and at 60 and 90 min for ITT (Figure. 4C),  
390 and the total prevention of the increase in GTT area under the curve or ITT area under  
391 the curve was significantly decreased in a dose dependent manner (Figure. 4B and  
392 Figure. 4D), indicating that BSSEE treatment improved decreased insulin sensitivity  
393 in fructose-fed mice.

### 394 **Effect of BSSEE on Hepatic Oxidative Stress.**

395 SOD, CAT and GSH-Px are key antioxidant enzymes, which play important

396 roles in maintaining the intracellular redox balance. Compared with mice in the  
397 control group, hepatic SOD (Figure.5E), CAT (Figure.5F) and GSH-Px (Figure.5G)  
398 activities decreased in fructose-fed mice without the treatment, but no significant  
399 difference was found in SOD and CAT compared with the control group. However,  
400 BSSEE administration at dose of 2 mL/kg significantly increased hepatic SOD and  
401 CAT activities, and GSH-Px activities were also increased significantly in all BSSEE  
402 treated mice compared with the fructose group, indicating that BSSEE treatment  
403 enhanced hepatic antioxidant enzymes activities. In addition, vitamin C (Figure.5D)  
404 and GSH (Figure.5C) is known to be important antioxidants to scavenging free  
405 radicals in different tissues, whereas NO (Figure.5B) and MDA (Figure.5A) are  
406 indicators of oxidative stress. Mice in the fructose group showed significant low  
407 levels of hepatic vitamin C and GSH, and high levels of NO and MDA, compared to  
408 the control group. In contrast, BSSEE (0.5, 1 and 2 mL/kg) treatment increased  
409 hepatic vitamin C and GSH levels, and a significant difference showed in BSSEE 2  
410 mL/kg treated mice. Meanwhile, all BSSEE treated groups showed low level of MDA,  
411 and hepatic NO level decreased in BSSEE 1 and 2 mL/kg treated mice compared with  
412 mice in the fructose group. All of these results suggested that BSSEE administration  
413 decreased hepatic oxidative stress.

414 In order to explore whether BSSEE can improve hepatic oxidative stress,  
415 immunohistochemical staining of Nrf2 expression was carried out. Nrf2 is a  
416 transcription factor in the protection against any oxidative stress, which is translocated  
417 from sequestration in the cytoplasm to the nucleus during oxidative stress, promoting

418 several antioxidant enzymes to enhance antioxidant defense system. Nrf2 are low  
419 expressed in liver cell of nuclear in mice form the fructose group, whereas mice  
420 treatment with BSSEE at doses of 1 and 2 mL/kg elevated Nrf2 expression in cells of  
421 nuclear, suggesting that the anti-oxidant effect of BSSEE linked with increase of Nrf2  
422 expression in cells of nuclear (Figure.5H and Figure.5H).

### 423 **Effect of BSSEE on Liver Protein Expression.**

424 Insulin resistance is a risk factor for the aggravation of NAFLD, in which  
425 inflammatory mediators and lipids activate a signaling cascade that triggers  
426 inflammatory kinases such as JNK, IKK and NF- $\kappa$ B, as well as XBP1. The activation  
427 of JNK, IKK and NF- $\kappa$ B and down-regulation of XBP1 can result in the inhibition of  
428 insulin action in part through serine phosphorylation of insulin receptor substrates  
429 IRS1. In the present study, fructose-fed mice showed the high expressions of  
430 phospho-JNK1 (Figure.6C) and phospho-IKK (Figure.6B), along with low  
431 expressions of XBP1 (Figure.6A) and phospho-IRS1 (Figure.6D), compared with  
432 mice in the control group. In addition, immunohistochemical staining of  
433 phospho-NF- $\kappa$ B p65 expression obviously increased in the fructose-fed mice without  
434 the treatment (Figure.6E). However, mice treated with BSSEE decreased expressions  
435 of phospho-NF- $\kappa$ B p65, phospho-JNK1, phospho-IRS1 and phospho-IKK, and  
436 increased expression of XBP1 (Figure.6A-E), indicating that BSSEE administration  
437 markedly improved insulin resistance in liver of mice by its effect on these signalling  
438 molecules.

### 439 **■ DISCUSSION**

440 Long term intake of high fructose has been proven to be a risk factor for the  
441 development of obesity, adiposity, liver inflammation and insulin resistance, as well  
442 as NAFLD, insulin resistance and metabolic syndrome <sup>5-10</sup>. Since HFCS consumption  
443 has been found that it is closely associated with the increasing prevalence of NAFLD,  
444 obesity, insulin resistance and metabolic syndrome, we investigated the effect of  
445 BSSEE on NAFLD in fructose-fed mice. In this study, 30% of fructose intake over a  
446 period of 8 weeks caused fast body weight gain together with increase of fat tissue  
447 and liver weights in mice, which is similar to previous report <sup>10</sup>. Overweight that  
448 presents an increase of lipids in adipose tissue is considered to be one of the key  
449 events occurring in the first steps of hepatic steatosis, where adipose tissue-derived  
450 signals (e.g. classical cytokines TNF- $\alpha$ , IL-6) due to excess storage of fat has been  
451 demonstrated to play an essential role in the pathopoiesia of NAFLD <sup>3,31</sup>. However,  
452 BSSEE intake decreased body weight, adipose tissue weight and liver weight,  
453 implying that BSSEE may contribute to the improvement of NAFLD in fructose-fed  
454 mice. Importantly, in addition to the improvement of serum biochemical parameters  
455 levels after BSSEE intake in fructose-fed mice, further histopathological examinations  
456 showed that BSSEE supplementation reduced ballooning degeneration and fat  
457 deposition in liver cells compared with the fructose-fed mice without the treatment.  
458 Certainly, our experimental results indicate that BSSEE intake could alleviate hepatic  
459 steatosis in fructose-fed mice.

460 In the present study, the major active components of BSSEE were analyzed with  
461 HPLC-PDA. On the basis of the BSSEE chromatogram (Figure 1), the results

462 indicated that the major constituent of BSSEE was lignans (19.8%), which mainly  
463 contained sesaminol (1.92%), sesamin (16.33%) and sesamol (31.31%). Previously,  
464 vitamins (such as vitamins D, vitamins C and vitamins E) <sup>32,33</sup> and linoleic acids <sup>34</sup>  
465 were found to be beneficial for the improvement of NAFLD. In the present study,  
466 sugar, protein, vitamins and fatty acids in BSSEE were did not detected under that  
467 chromatographic conditions. Since soybean oil mainly contain linoleic acid  
468 (55%~67%), oleic acid (17%~26%), palmitic acid (9%~13%), stearic acid (3%~6%)  
469 as well as vitamins, mice in the fructose group received soybean oil showed no  
470 significant improvement in fructose-induced NAFLD. Therefore, lignans including  
471 sesaminol (1.92%), sesamin (16.33%) and sesamol (13.06%) might possess  
472 beneficial effect against NAFLD in this animal model.

473 In the present study, fructose consumption increased serum levels of FFA,  
474 triglyceride and total cholesterol. Disorganized these serum parameters altered change  
475 in energy metabolism in liver, and are risk factors in the development of diseases such  
476 as NAFLD, insulin resistance and metabolic syndrome. Increased free fatty acids,  
477 triglyceride and cholesterol, especially accumulation in mitochondria, are considered  
478 the “aggressive” lipids leading to TNF- $\alpha$  and IL-6-mediated liver damage and ROS  
479 formation <sup>31,35</sup>. These lipids could also shows in a nonsteatotic liver and act as early  
480 “inflammatory” hits, resulting in the whole spectrum of NAFLD pathologies <sup>36,37</sup>.  
481 However, we observed BSSEE supplementation decreased free fatty acid, triglyceride  
482 and total cholesterol, which is beneficial to the prevention in the development of  
483 NAFLD.

484 In addition, fructose consumption by mice led to insulin resistance as the results  
485 presented the increase of serum insulin and glucose levels, and ITT and GTT tests.  
486 Insulin resistance is the condition that a cell, tissue, or organism fails to respond  
487 appropriately to a given dose of insulin, in which it is an important feature of NAFLD  
488 and type 2 diabetes, and are often found in metabolic syndrome <sup>38</sup>. The improvement  
489 of NAFLD may occur independent of interference with insulin resistance <sup>39, 40</sup>.  
490 However, presence of insulin resistance may aggravate NAFLD because patients with  
491 insulin resistance are often worse in terms of liver histopathological examinations  
492 such as hepatic ballooning, steatosis and degree of fibrosis <sup>41, 42</sup>. In contrast, oral  
493 administration of BSSEE (0.5, 1 and 2 mg/kg) dose-dependently decreased serum  
494 insulin and glucose levels and improved body insulin resistance, which is important  
495 for the prevention of NAFLD complicated with type 2 diabetes and metabolic  
496 syndrome.

497 In addition to plasma insulin resistance, we found that fructose intake over a long  
498 period of time may impair hepatic insulin function as evidenced by the increase  
499 expression of phospho-IRS1 (Ser 307) in liver. Increased serine phosphorylation of  
500 IRS1 (Ser 307) may prevent insulin signal transduction, thereby resulting in  
501 impairment of insulin functions in liver <sup>4</sup>. Impaired hepatic insulin function is linked  
502 with the exacerbation of NAFLD <sup>43</sup>. Mice treated with BSSEE improved insulin  
503 function, showing decreased serine phosphorylation of IRS1 (Ser 307), which was  
504 contribute to the metabolism of glucose and lipid in liver. In order to further  
505 understand whether BSSEE could improve insulin signal transduction, we examined



506 hepatic oxidative stress and the expressions of NF- $\kappa$ B, JNK1, IKK and XBP1 in liver.  
507 One of the key aspects that disrupt insulin signaling links to major inflammatory  
508 signaling networks, including the activation of the JNK, IKK/NF- $\kappa$ B signaling  
509 pathways and production of ROS and NO <sup>44</sup>. The activation of JNK, IKK-NF- $\kappa$ B  
510 pathways can lead to the inhibition of insulin action in part through serine  
511 phosphorylation of IRS1 <sup>45, 46</sup>. However, mice by BSSEE treatment reduced  
512 phosphorylation of NF- $\kappa$ B p65, JNK1 and IKK, and consequently a decrease of serine  
513 phosphorylated IRS1 (Ser 307), suggesting that BSSEE could improve insulin signal  
514 transduction via its effect on JNK and IKK-NF- $\kappa$ B pathways. In addition, XBP1 has  
515 evolved as a critical molecule, which interacts with inflammatory cascades at various  
516 stages, including IRE1-mediated activation of JNK, activation of IKK/NF- $\kappa$ B  
517 signaling pathways and production of ROS <sup>44</sup>. XBP1 haploinsufficiency in mice  
518 exhibit markedly increased succumb to increase of body weight, hyperinsulinemia,  
519 hyperglycemia, and impaired glucose and insulin tolerance, at least in part, through  
520 activation of JNK and serine phosphorylation of IRS1 <sup>47, 48</sup>. XBP1 has proved as a  
521 critical molecule in the regulation all aspects of NAFLD <sup>49</sup>. Notably, BSSEE  
522 increased expression of XBP1 in liver compared with mice in fructose group, which  
523 may be, in part, responsible for the regulation of the lipid synthesis/accumulation,  
524 inflammation and insulin signaling/resistance in liver in fructose-fed mice.

525 As above mentioned, production of ROS and NO has been considered as strong  
526 contenders for the cause of exacerbating NAFLD and insulin resistance <sup>11</sup>. The liver is  
527 rich in antioxidant defense system, which include chemicals such as GSH, vitamins C,

528 and enzymes such as SOD, CAT, GSH-px<sup>50</sup>. Even though ROS and nitrogen species  
529 are normally produced by the metabolism of normal cells, in NAFLD the occurrence  
530 that an overproduction of free radicals which overcomes the antioxidant defenses  
531 aggravate liver injury<sup>51</sup>. The pathogenesis of NAFLD suffers multiple parallel hits,  
532 including oxidative stress<sup>31,52</sup>. Hepatic oxidative stress is also partly responsible for  
533 insulin resistance through inflammatory response<sup>53</sup>. In the present study, fructose-fed  
534 mice showed decrease of GSH and vitamins C levels, reductions of SOD, CAT and  
535 GSH-px activities, and high levels of MDA and NO in liver, suggesting decrease of  
536 antioxidant defenses function. However, BSSEE enhances antioxidant defenses  
537 function, as increase of GSH and vitamins C levels, enhancements of SOD, CAT and  
538 GSH-px activities and consequently reduction of MDA and NO level. To understand  
539 thoroughly the molecular mechanism behind the antioxidative effect of BSSEE, we  
540 further examined hepatic Nrf2 protein. Nrf2 is a transcription factor binds antioxidant  
541 response elements (AREs) in the regulatory regions of target genes, which act as a  
542 main player in the inducible expression of our cellular defense enzymes<sup>54,55</sup>. Nrf2  
543 activators have already been developed for the treatment of liver diseases involving  
544 oxidative stress and inflammatory response<sup>56,57</sup>. Similar to previous studies, the  
545 present study found that BSSEE could increased Nrf2 expression in liver cell, which  
546 might have a significant protective effect against fructose-induced oxidative stress in  
547 liver as can be seen in this study, possibly through enhancing hepatic antioxidant  
548 defense enzymes.

549 In conclusion, the present study demonstrates for the first time that BSSEE

550 showed good beneficial effects against NAFLD complicated with insulin resistance  
551 and metabolic syndrome in fructose-fed mice. These effects are most likely related to  
552 the regulation of insulin signaling molecules expressions, antioxidant defenses system  
553 and inflammatory response. Thus, black sesame seeds could be considered as a potent  
554 dietary supplements in the prevention of NAFLD-related metabolic diseases.

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##### 565 **Notes**

566 The authors declare no competing financial interest.

#### 567 ■ ABBREVIATIONS USED

568 Black sesame seeds ethanol extract (BSSEE), nonalcoholic fatty liver disease  
569 (NAFLD), free fatty acids (FFA), aspartate transaminase (AST), alanine transaminase  
570 (ALT), insulin tolerance tests (ITT), glucose tolerance tests (GTT), nitric oxide (NO),  
571 malondialdehyde (MDA), NF-E2-related factor 2 (Nrf2), superoxide dismutase (SOD),

572 catalase (CAT), glutathione peroxidase (GSH-Px), phospho-c-Jun NH2-terminal  
573 kinase 1 (phospho-JNK1), phospho-nuclear factor kappa B (NF-κB), phospho-insulin  
574 receptor substrate 1 (phospho-IRS1), phospho-IκB kinase (phospho-IKK), X-box  
575 binding protein 1 (XBP1), tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6),  
576 reactive oxygen species (ROS), high performance liquid chromatography (HPLC),  
577 high fructose corn syrup (HFCS).

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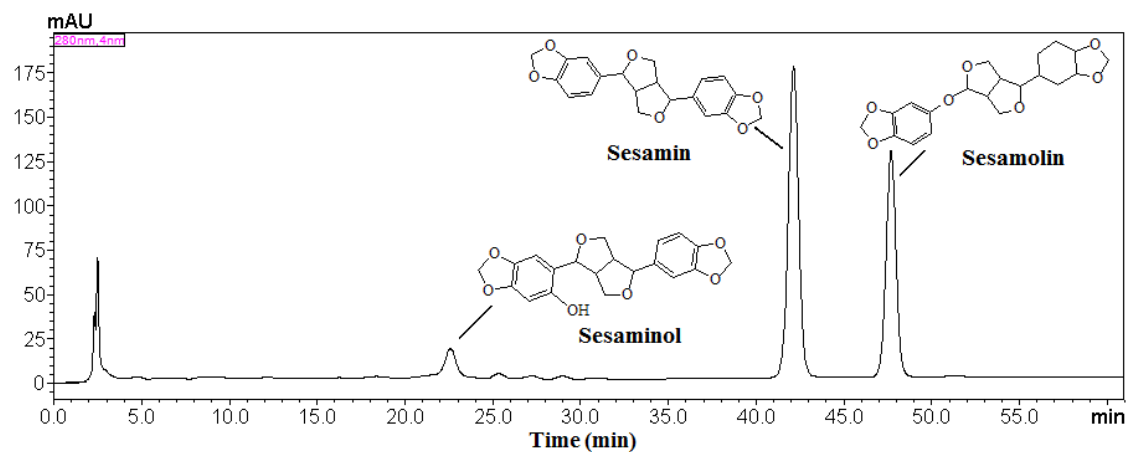
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748 Figure 1. HPLC-PDA chromatogram of black sesame seeds ethanol extract (BSSEE) at 280 nm. Retention times

749 and chemical structures of the three major components present in BSSEE are shown.

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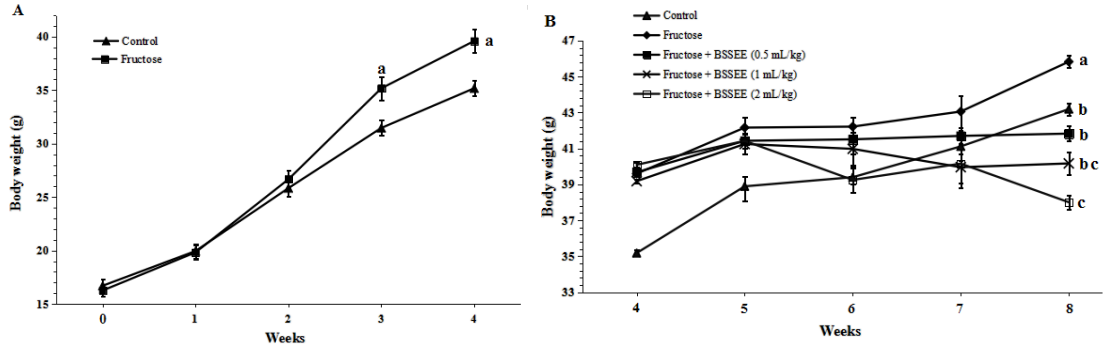
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766 Figure.2. Changes in body weight and amount of food intake. (A) Curve showing changes in average body weight

767 between the control group and the fructose-fed group before BSSEE (0.5, 1 and 2 mL/kg) treatment, in which

768 fructose feeding obviously increased body weight compared with mice in the control group. (B) Curve showing

769 changes in body weight from all group after BSSEE (0.5, 1 and 2 mL/kg) treatment, indicating that BSSEE

770 treatment significantly reduced body weight at the end of the experiment, compared with mice in the fructose

771 group. Values are expressed as means  $\pm$  SEM (n=10 per group). Results were statistically analyzed using one-way

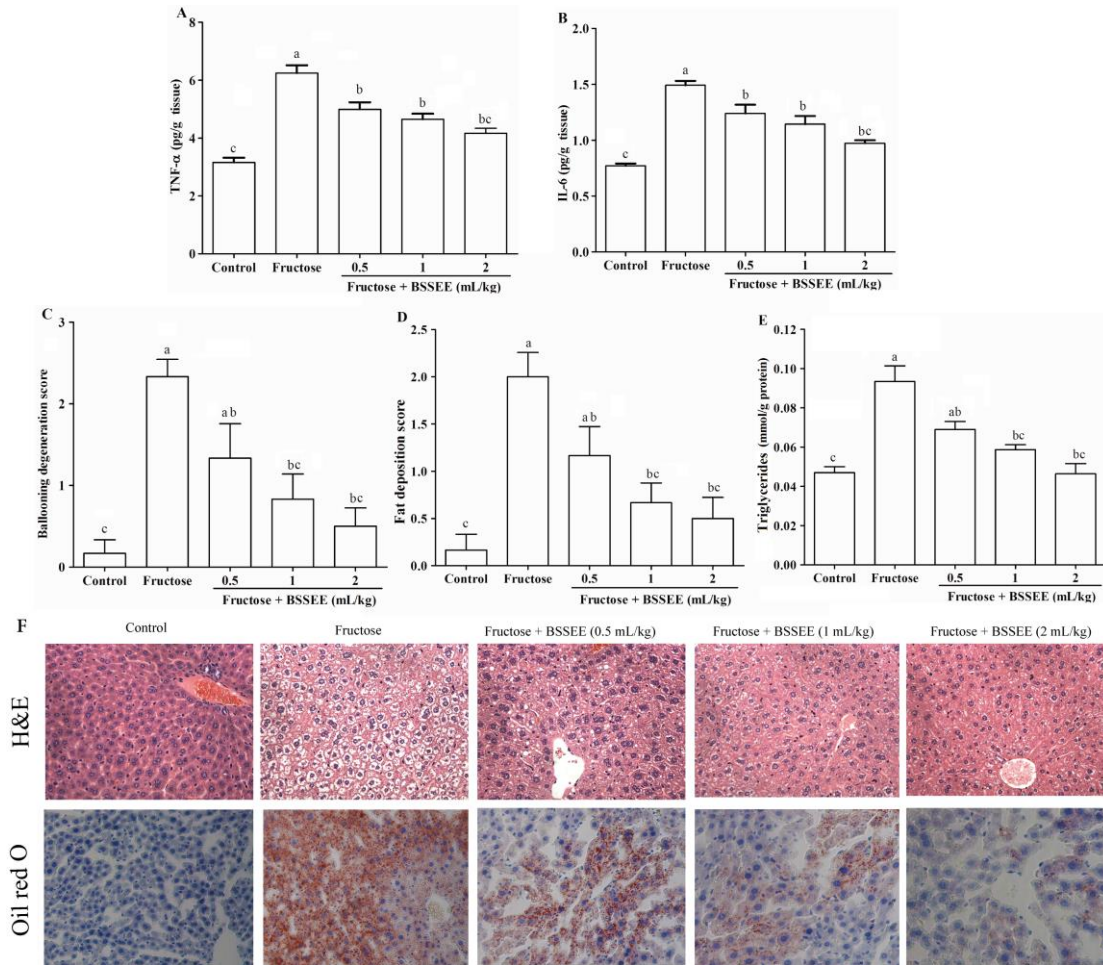
772 ANOVA coupled with Newman-Keuls's multiple-comparison test ( $p < 0.05$ ).

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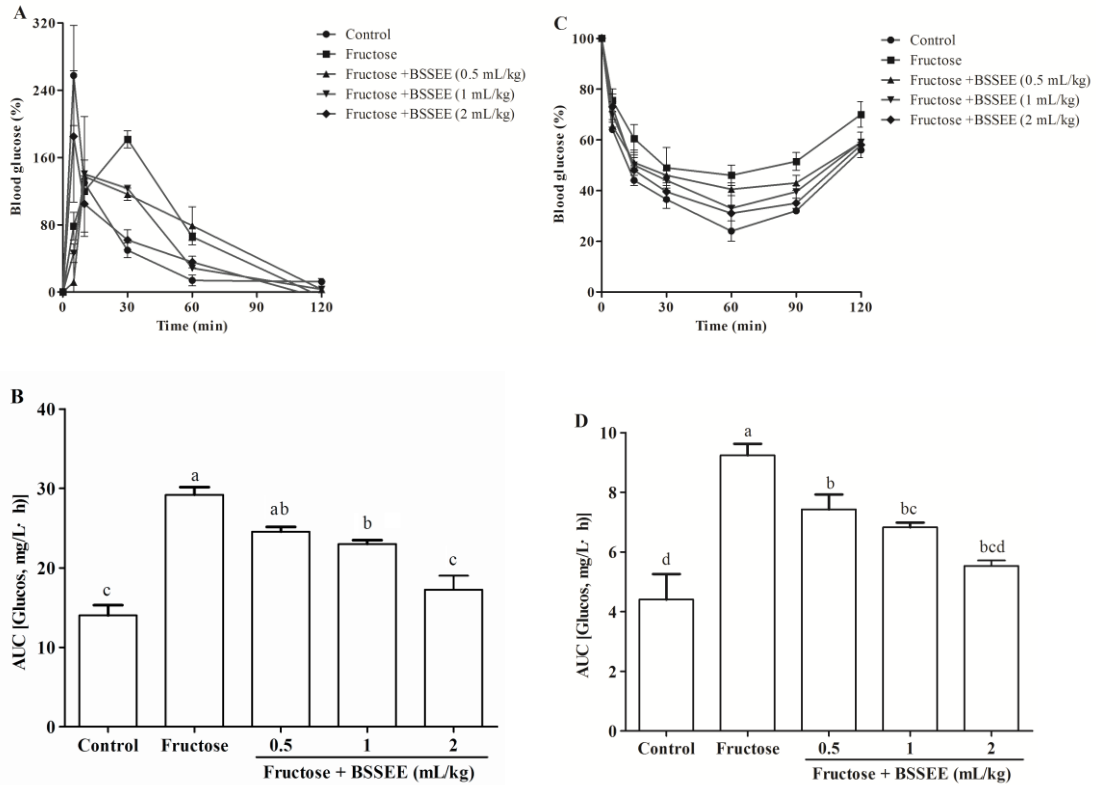


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778 Figure.3. Effects of BSSEE (0.5, 1 and 2 mL/kg) on hepatic (A) TNF- $\alpha$ , (B) IL-6 and liver histopathological  
 779 changes in fructose-fed mice: (C) ballooning degeneration score, (D) fat deposition score and (E) hepatic  
 780 triglycerides. (F) Liver tissue stained with H&E ( $\times 400$  magnification) and oil red O ( $\times 800$  magnification) show  
 781 pathomorphological changes in the fructose-fed mice. Representative analysis of each group (n =5 mice per group)  
 782 are shown. Values are expressed as means  $\pm$  SEM (n =5 mice per group). Results were statistically analyzed using  
 783 one-way ANOVA coupled with Newman-Keuls's multiple-comparison test ( $p < 0.05$ ).

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788 Fig. 4. Effects of BSSEE (0.5, 1 and 2 mL/kg) on GTT and ITT responses in fructose-fed mice. GTT (A) and ITT  
 789 (C) were performed on weeks 7, respectively. The area under the curve (AUC) for GTT (B) and ITT (D) tests were  
 790 calculated according to the trapezium rule. Values are shown as means  $\pm$  SEM and are the average of 5  
 791 animals/group. Results were statistically analyzed using one-way ANOVA coupled with Newman-Keuls's  
 792 multiple-comparison test ( $p < 0.05$ ).

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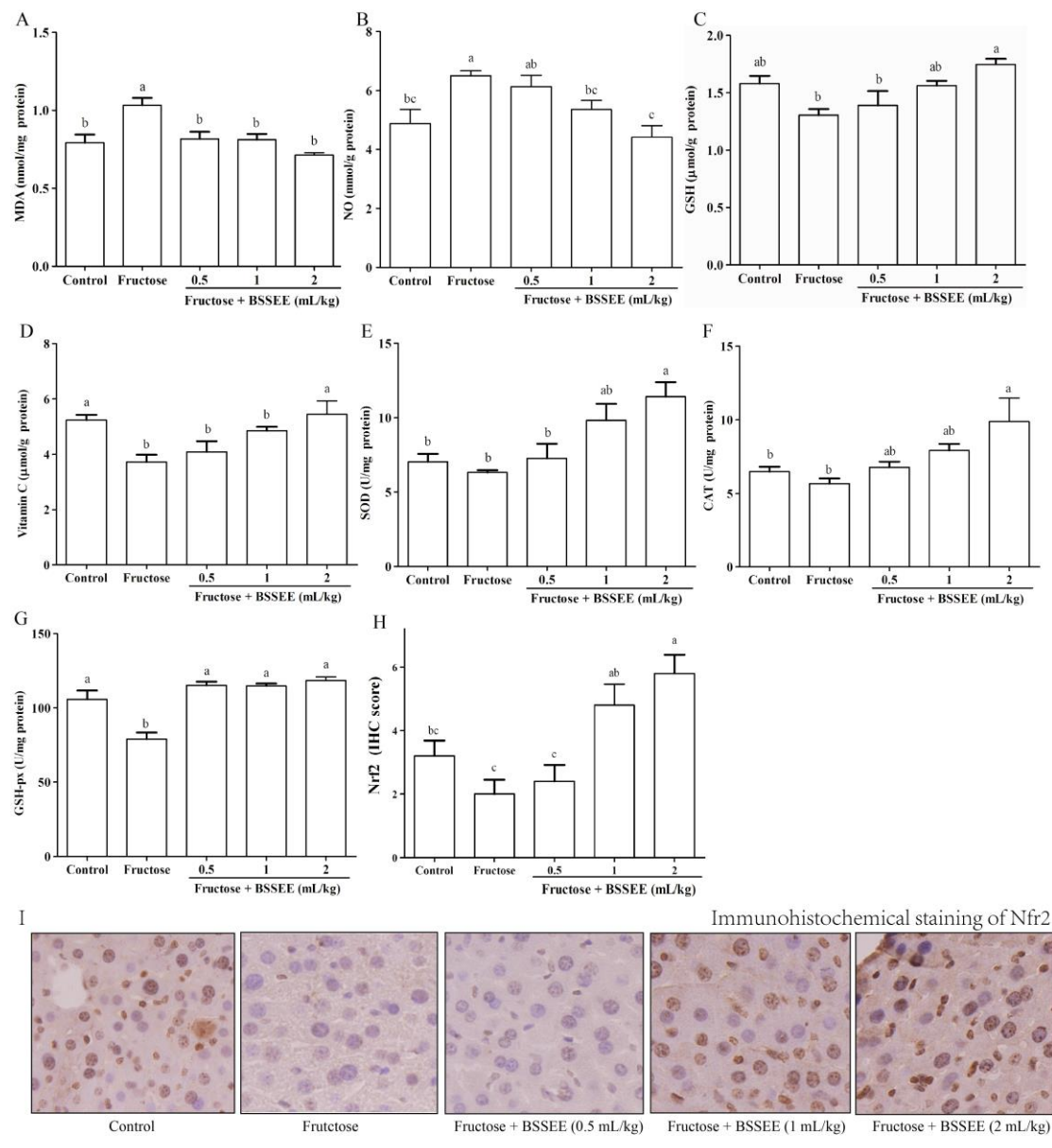
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801 Figure.5. Effects of BSSEE (0.5, 1 and 2 mL/kg) on (A)MDA, (B) NO, (C) GSH, (D) vitamin C, (E) SOD, (F)

802 CAT and (G) GSH-Px. (H and I) The expression of Nfr2 is analyzed by immunohistochemistry (IHC) and

803 semi-quantified in the fructose-fed mice, and representative images are shown here (× 400 magnification, n=5 per

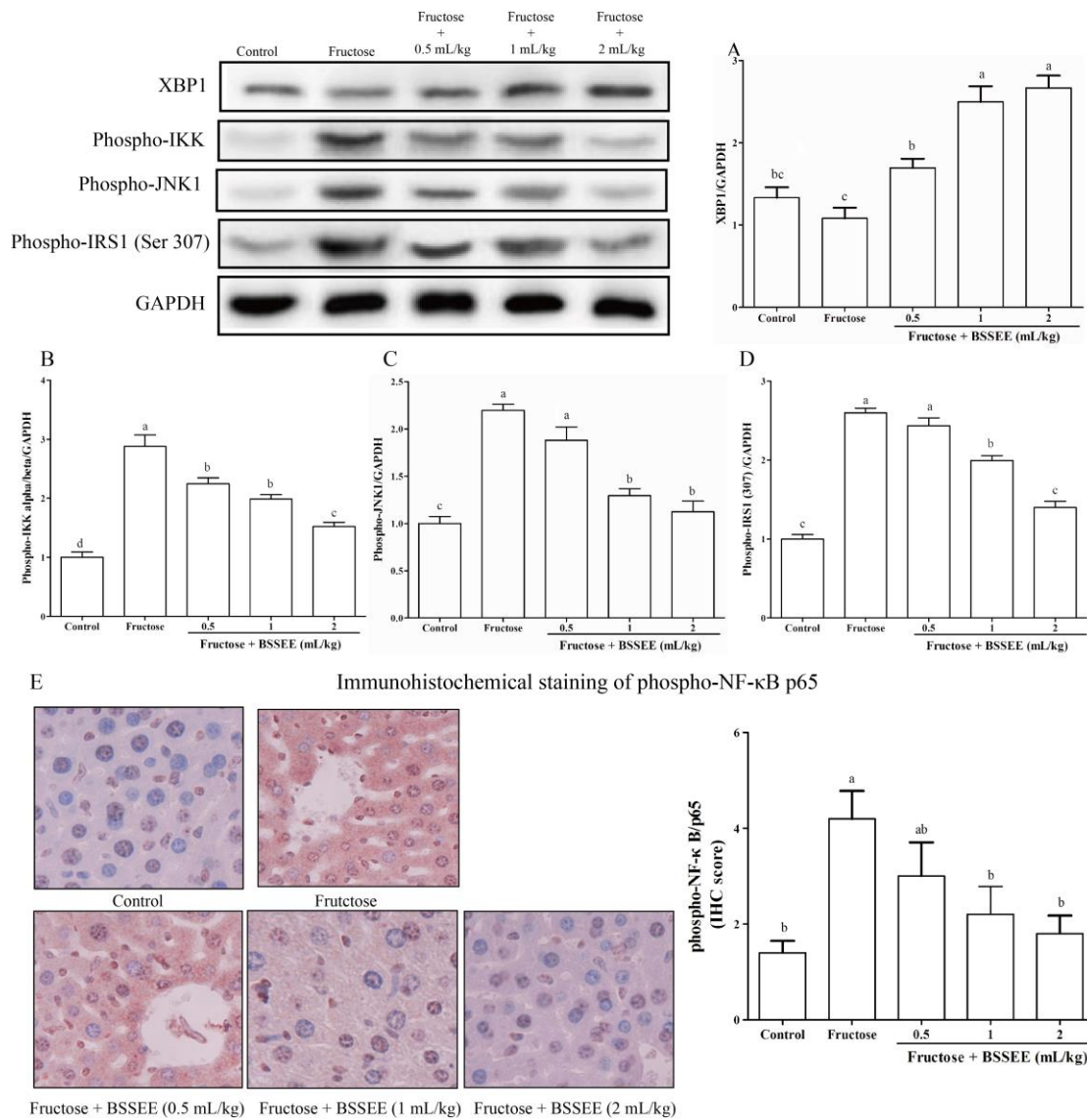
804 group). Values are shown as means ± SEM (n=10 per group). Results were statistically analyzed using one-way

805 ANOVA coupled with Newman-Keuls's multiple-comparison test ( $p < 0.05$ ).

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Fructose + BSSEE (0.5 mL/kg) Fructose + BSSEE (1 mL/kg) Fructose + BSSEE (2 mL/kg)

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Figure.6. Effects of BSSEE (0.5, 1 and 2 mL/kg) on the expressions of hepatic (A) XBP1, (B) phospho-IKK, (C)

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phospho-JNK1, (D) phospho-IRS1. (E) The expression of phospho-NF-κB p65 is analyzed by

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immunohistochemistry (IHC) and semi-quantified in the fructose-fed mice and representative images are shown

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here ( $\times 400$  magnification,  $n=5$  per group), and representative images are shown here ( $\times 400$  magnification,  $n=5$

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per group). Values are shown as means  $\pm$  SEM ( $n=5$  per group). Results were statistically analyzed using one-way

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ANOVA coupled with Newman-Keuls's multiple-comparison test ( $p < 0.05$ ).

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817 Table 1. Final tissue weights. The relative tissue weight is expressed as percentage of body weight (tissue  
818 weight/final body weight  $\times$  100). Values are shown as means  $\pm$  SEM (n=10 per group). Results were statistically  
819 analyzed using one-way ANOVA coupled with Newman-Keuls's multiple-comparison test ( $p < 0.05$ ).

Groups	Absolute weight (g)					Relative weight (/body, %)	
	Liver	Perinephrit fat	Epididymal fat	Subcutaneous fat	Total fat	Liver	Total fat
Control	1.40 $\pm$ 0.07c	0.16 $\pm$ 0.03c	0.72 $\pm$ 0.05b	0.82 $\pm$ 0.05c	1.71 $\pm$ 0.06c	3.28 $\pm$ 0.25c	4.03 $\pm$ 0.15d
Fructose	2.20 $\pm$ 0.05a	0.50 $\pm$ 0.13a	1.21 $\pm$ 0.04a	1.41 $\pm$ 0.05a	3.12 $\pm$ 0.09a	5.17 $\pm$ 0.2a	6.75 $\pm$ 0.16a
Fructose + BSSEE (0.5 mL/kg)	2.13 $\pm$ 0.08ab	0.37 $\pm$ 0.06b	0.92 $\pm$ 0.15ab	1.12 $\pm$ 0.18ab	2.42 $\pm$ 0.13ab	4.98 $\pm$ 0.15ab	5.96 $\pm$ 0.26ab
Fructose + BSSEE (1 mL/kg)	1.80 $\pm$ 0.04bc	0.33 $\pm$ 0.04b	0.80 $\pm$ 0.06b	0.92 $\pm$ 0.08bc	1.98 $\pm$ 0.08bc	4.65 $\pm$ 0.13bc	5.30 $\pm$ 0.22bc
Fructose + BSSEE (2 mL/kg)	1.50 $\pm$ 0.07c	0.25 $\pm$ 0.08bc	0.70 $\pm$ 0.05b	0.85 $\pm$ 0.07c	1.87 $\pm$ 0.09c	3.82 $\pm$ 0.19c	4.36 $\pm$ 0.24cd

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832 Table 2. Metabolic parameters in fructose-fed mice with or without treatment with BSSEE (0.5, 1 and 2 mL/kg).  
 833 Liquid intake, food intake and total energy intake show during BSSEE treatment. Cholesterol, triglycerides,  
 834 glucose, insulin, FFA, AST, ALT, TNF- $\alpha$  and IL-6 were measured in serum. Values are shown as means  $\pm$  SEM  
 835 (n=10). Results were statistically analyzed using one-way ANOVA coupled with Newman-Keuls's  
 836 multiple-comparison test ( $p < 0.05$ ).

Group	Control	Fructose	Fructose + BSSEE (0.5 mL/kg)	Fructose + BSSEE (1 mL/kg)	Fructose + BSSEE (2 mL/kg)
Water consumption (mL/day)	7.41 $\pm$ 0.44 a	7.38 $\pm$ 0.42 a	7.45 $\pm$ 0.22 a	7.92 $\pm$ 0.58 a	8.56 $\pm$ 0.56 a
Average food intake (g/day)	5.04 $\pm$ 0.02 a	3.17 $\pm$ 0.06 b	3.02 $\pm$ 0.17 bc	2.18 $\pm$ 0.11 d	2.22 $\pm$ 0.15 d
Total energy intake (kJ/day)	20.78 $\pm$ 0.07 a	22.73 $\pm$ 1.05 a	22.36 $\pm$ 1.08 a	19.86 $\pm$ 1.06 a	20.78 $\pm$ 1.16 a
Triglycerides (mmol/L)	1.05 $\pm$ 0.02b	2.17 $\pm$ 0.13a	2.07 $\pm$ 0.23a	1.42 $\pm$ 0.11b	1.04 $\pm$ 0.09b
Cholesterol (mmol/L)	2.46 $\pm$ 0.13b	3.74 $\pm$ 23a	2.24 $\pm$ 0.04b	2.12 $\pm$ 0.09b	2.04 $\pm$ 0.10b
Glucose (mmol/L)	7.63 $\pm$ 0.64b	10.62 $\pm$ 0.47a	7.70 $\pm$ 0.25b	7.33 $\pm$ 0.23b	6.00 $\pm$ 0.57b
Insulin (IU/L)	16.40 $\pm$ 1.45bc	23.09 $\pm$ 1.32a	21.16 $\pm$ 1.03ab	16.85 $\pm$ 1.68bc	12.92 $\pm$ 1.30c
HOMA-IR	4.96 $\pm$ 0.21 bcd	11.15 $\pm$ 0.65 a	7.1 $\pm$ 0.42 b	6.32 $\pm$ 0.6 bc	3.49 $\pm$ 0.51 d
FFA (mmol/L)	0.44 $\pm$ 0.03b	0.54 $\pm$ 0.01a	0.52 $\pm$ 0.01a	0.41 $\pm$ 0.03b	0.39 $\pm$ 0.02b
AST (IU/L)	81.60 $\pm$ 4.37b	124.00 $\pm$ 12.28a	84.20 $\pm$ 5.85b	79.50 $\pm$ 6.50b	77.00 $\pm$ 2.04b
ALT (IU/L)	24.00 $\pm$ 1.56b	38.80 $\pm$ 3.96 a	27.00 $\pm$ 2.81b	22.50 $\pm$ 2.18b	20.50 $\pm$ 1.32b
TNF- $\alpha$ (pg/mL)	61.85 $\pm$ 3.81b	76.32 $\pm$ 2.30a	74.24 $\pm$ 1.32a	60.94 $\pm$ 2.13b	55.28 $\pm$ 3.77b
IL-6 (pg/mL)	128.8 $\pm$ 7.99b	164.4 $\pm$ 7.84a	152.5 $\pm$ 5.68a	122.6 $\pm$ 4.80b	119.6 $\pm$ 11.0b

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