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Chronic rhein treatment improves recognition memory in high-fat diet-induced obese male mice

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Chronic rhein treatment improves recognition memory in high-fat diet-induced obese male mice

Abstract

High-fat (HF) diet modulates gut microbiota and increases plasma concentration of lipopolysaccharide (LPS) which is associated with obesity and its related low-grade inflammation and cognitive decline. Rhein is the main ingredient of the rhubarb plant which has been used as an anti-inflammatory agent for several millennia. However, the potential effects of rhein against HF diet-induced obesity and its associated alteration of gut microbiota, inflammation and cognitive decline have not been studied. In this study, C57BL/6J male mice were fed an HF diet for 8 weeks to induce obesity, and then treated with oral rhein (120 mg/kg body weight/day in HF diet) for a further 6 weeks. Chronic rhein treatment prevented the HF diet-induced recognition memory impairment assessed by the novel object recognition test, neuroinflammation and brain-derived neurotrophic factor (BDNF) deficits in the perirhinal cortex. Furthermore, rhein inhibited the HF diet-induced increased plasma LPS level and the proinflammatory macrophage accumulation in the colon and alteration of microbiota, including decreasing *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA and increasing *Bifidobacterium* spp. and *Lactobacillus* spp. DNA. Moreover, rhein also reduced body weight and improved glucose tolerance in HF diet-induced obese mice. In conclusion, rhein improved recognition memory and prevented obesity in mice on a chronic HF diet. These beneficial effects occur via the modulation of microbiota, hypoendotoxemia, inhibition of macrophage accumulation, anti-neuroinflammation and the improvement of BDNF expression. Therefore, supplementation with rhein-enriched food or herbal medicine could be beneficial as a preventive strategy for chronic HF diet-induced cognitive decline, microbiota alteration and neuroinflammation.

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Chronic rhein treatment improves recognition memory in high-fat diet-induced obese mice

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1 **Abstract**

2 High-fat (HF) diet modulates gut microbiota and increases plasma concentration of
3 lipopolysaccharide (LPS), metabolic endotoxemia, which is associated with obesity and its
4 related low-grade inflammation and cognitive decline. Rhein is the main ingredient of the
5 rhubarb plant which has been used as an anti-inflammatory agent for several millennia.
6 However, the potential effects of rhein against HF diet-induced obesity and its associated
7 alteration of gut microbiota, inflammation and cognitive decline have not been studied. In
8 this study, C57BL/6J male mice were fed a HF diet for 8 weeks to induce obesity, and then
9 treated with oral rhein (120 mg/kg body weight per day in HF diet) for a further 6 weeks.
10 Chronic rhein treatment prevented the HF diet-induced recognition memory impairment
11 assessed by novel object recognition test, neuroinflammation and BDNF deficits in the
12 perirhinal cortex. Furthermore, rhein inhibited the HF diet-induced increased plasma LPS
13 level and the pro-inflammatory macrophage accumulation in the colon and alteration of
14 microbiota, including decreasing *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA
15 and increasing *Bifidobacterium* spp. and *Lactobacillus* spp. DNA. Moreover, rhein also
16 reduced body weight and improved glucose tolerance in HF diet-induced obese mice. In
17 conclusion, rhein improved recognition memory and prevented obesity in mice on a chronic
18 HF diet. These beneficial effects occur via the modulation of microbiota, hypoendotoxemia,
19 inhibition of macrophage accumulation, anti-neuroinflammation and the improvement of
20 BDNF expression. Therefore, supplementation with rhein-enriched food or herbal medicine
21 could be beneficial as a preventive strategy for chronic HF diet-induced cognitive decline,
22 microbiota alteration and neuroinflammation.

23 **Keywords:** rhein; gut microbiota; recognition memory; lipopolysaccharide; inflammation;

24 perirhinal cortex

25

26 **1. Introduction**

27 Obesity is a major risk factor for the development of insulin resistance, type 2 diabetes, and
28 cognitive decline in neurodegenerative diseases such as Alzheimer's disease (AD) and
29 vascular dementia [1, 2]. Patients with AD have been characterized by deficits in recognition
30 memory [3]. The perirhinal cortex plays an important role in higher object recognition
31 memory [4]. Lesions in the perirhinal cortex severely disrupt object recognition [5],
32 object-in-place memory, and temporal order recognition memory [6, 7] in rodent studies.
33 Empirical evidence has linked high-fat (HF) diet-induced obesity with impairments in
34 learning and memory, including a decline in recognition memory [8] as assessed with the
35 novel object recognition test.

36 Nowadays, it is widely accepted that obesity and its associated cognitive decline is
37 associated with low-grade systemic and central inflammation, despite the fact that the
38 molecular origin of the inflammation is poorly understood [9, 10]. Increased fat intake has
39 been found to be strongly correlated with increased plasma lipopolysaccharide (LPS),
40 endotoxemia [10]. LPS is a major component of the outer membrane in Gram-negative
41 bacteria. Emerging evidence from animal studies suggests a link between the alteration of gut
42 microbiota, increased intestinal permeability, and endotoxemia in HF diet-induced obesity
43 [11]. An imbalance of Bacteroidetes and Firmicutes, the primary bacterial phyla comprising
44 the gastrointestinal microbiota, has been reported in rodents fed a HF diet and obese
45 individuals [12, 13]. The plasma LPS level was closely correlated with altered intestinal
46 microbiota, in which the number or diversity of the Gram-negative, Bacteroidetes phylum,
47 were significantly reduced in animals fed a HF diet [14]. The endogenous LPS is considered

48 to be continuously produced in the gut by the death of Gram-negative bacteria and its
49 translocation into intestinal capillaries via the increased intestinal permeability in HF
50 diet-induced obesity [15]. Endotoxemia in turn can trigger systemic inflammation and
51 neuroinflammation. It has been shown that an intraperitoneal (ip) injection of LPS induces
52 neuroinflammation, cognitive impairment and memory dysfunction [16]. LPS binds to
53 Toll-like receptor (TLR) 4 coupled with myeloid differentiation primary-response protein 88
54 (MyD88)-dependent pathway, and activates c-Jun N-terminal kinase (JNK) and nuclear
55 factor-kappa B (NF κ B), two important inflammatory signaling molecules [17]. The activation
56 of the TLR4-MyD88-JNK/NF κ B signaling pathway leads to the production of
57 pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor
58 necrosis factor- α (TNF- α), and contributes to the development of neurodegenerative diseases
59 [18-21].

60 Brain-derived neurotrophic factor (BDNF) is known to play an important role in neuronal
61 development and synaptic plasticity in the brain regions involved in cognitive function [22].
62 In the perirhinal cortex, BDNF has been shown to be important for object recognition
63 memory [23]. Studies have found that BDNF expression in the perirhinal cortex has a
64 positive relationship with recognition memory in rats [23, 24], and that treatment with
65 anti-BDNF serum inhibited long-term recognition memory in rats [25]. An intraperitoneal
66 injection of IL-1 β or LPS significantly decreases BDNF mRNA expression in the rat
67 hippocampus [26]. Furthermore, the oral administration of antimicrobials in
68 specific-pathogen-free mice transiently altered the composition of the microbiota and
69 increased exploratory behavior and the level of BDNF in the brain [27]. Moderate colonic

70 inflammation induced anxiety-like behavior and decreased BDNF mRNA expression in the
71 brain [28]. Our previous study found that a HF diet impaired recognition memory, decreased
72 BDNF, and increased inflammation in the prefrontal cortex of mice [29]. Notably, numerous
73 studies have shown that the beneficial effects of prebiotics and probiotics in obesity occur via
74 the modulation of gut microbial homeostasis [30]. Accordingly, the maintenance of a healthy
75 gut microbial environment is important for the treatment of obesity and its related BDNF and
76 cognitive decline.

77 Rhubarb is usually considered to be a vegetable in western countries. The dried rhubarb
78 rhizome is an important herbal medicine and has been used for thousands of years. Rhubarb
79 or extract of rhubarb has been reported to possess antibacterial, anti-inflammation,
80 antioxidative, antidiabetic, and neuroprotective properties [31, 32]. Rhein (4, 5'-
81 dihydroxy-anthraquinone-2-carboxylic acid) is the main ingredient of rhubarb. It has been
82 shown that rhubarb-exposed rats have increased bacterial diversity in the ileum [33]. Several
83 reports have shown that rhein prevents activation of NF- κ B and the ERK1/ERK2 pathway
84 [34], and inhibits the synthesis and activity of proinflammatory cytokines [35, 36]. It has
85 been shown that rhubarb-exposed rats have increased bacterial diversity in the ileum [33].
86 Rhein has also been reported to be an antibacterial agent which inhibits *Staphylococcus*
87 *aureus* [37]. Previously rhein has been reported to decrease body weight gain and fat
88 accumulation in HF diet-induced obese mice [38-40]. However, the potential effects of rhein
89 against alteration of gut microbiota and cognition in HF diet-induced obesity have not been
90 studied. The present study used a chronic HF diet-induced obese mouse model to investigate
91 whether rhein supplementation prevents endotoxemia, alteration of gut microbiota,

92 recognition memory decline, body weight gain, and glucose intolerance in these mice.
93 Furthermore, the neuroinflammatory TLR4-MyD88-JNK/NF κ B signaling pathway,
94 pro-inflammatory cytokines (TNF- α , IL-6 and IL-1 β), and neurotrophin BDNF were assessed
95 in the perirhinal cortex.

96

97 **2. Methods**

98 *2.1. Animals and treatments*

99 Twenty-four C57Bl/6J male mice (8 weeks old) were obtained from the Australian
100 Bio-Resource Centre (Moss Vale, NSW), and housed in environmentally controlled
101 conditions (temperature 22°C, 12 hour light/dark cycle). Eight mice were fed a lab chow diet
102 as a control (Con group). Sixteen mice were fed a HF diet (HF group) containing 60% fat by
103 calories (SF13-092; Specialty Feeds, Glen Forrest, WA). After 8 weeks, the 16 mice fed a HF
104 diet were divided into two groups: 8 mice continued to receive the HF diet, and the other 8
105 mice received the rhein treatment (HF+R group) for 6 weeks. Rhein was mixed in the HF diet
106 (dosage: 120 mg/kg body weight per day) [41]. Rhein (98%, C₁₅H₈O₆, MW = 284.21) was
107 purchased from Sangon Biotech Co. Ltd, China. Body weight was measured on the last day
108 in every week. Food intake was recorded on the first day in every week. A weighed amount
109 of fresh diet was given at the beginning of the dark cycle. The remaining food in the cage
110 plus spillage were collected and weighed 24 hours later. After 6 weeks of treatment, the novel
111 object recognition test and the intraperitoneal glucose tolerance test (IPGTT) were carried
112 out. The mice were asphyxiated in chambers prefilled with CO₂ 4 days after the tests were
113 carried out. Plasma, cecal contents and brain tissue were collected, snap frozen and stored at

114 -80 °C for further analyses as detailed below. The colon tissue was fixed in 10% buffered
115 formalin for immunohistochemistry. The study was approved by the University of
116 Wollongong Animal Ethics Committee (AE13/11) and all animal experiments were
117 conducted in compliance with the National Health and Medical Research Council Australian,
118 Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).

119

120 2.2. Real-time PCR (qRT-PCR) to quantify microbial strains from cecal content

121 The cecal contents of mice were collected immediately after the mice were sacrificed and
122 stored at -80°C. The QIAamp DNA Stool Minikit (QIAGEN, Germany) was used to extract
123 DNA from cecal contents according to the manufacturer's instructions. Group-specific
124 primers based on 16S rDNA sequences PCR assay were forward *Bacteroides*-Prevotella,
125 GAGAGGAAGGTCCCCAC; reverse *Bacteroides*-Prevotella, CGCTACTTGGCTGGTTC
126 AG; forward *Lactobacillus*, GAGGCAGCAGTAGGGAATCTTC; reverse *Lactobacillus*, GG
127 CCAGTTACTACCTCTATCCTTCTTC; forward *Bifidobacterium*, CGCGTCTGGTGTGA
128 AAG; reverse *Bifidobacterium*, CCCACATCCAGCATCCA; forward *Desulfovibrios*, CC
129 GTAGATATCTGGAGGAACATCAG; reverse *Desulfovibrios*, ACATCTAGCATCCATC
130 GTTTACAGC. Quantitative real-time PCR was performed in a 20-µL final reaction volume
131 using a SYBR green I master in a Lightcycler 480 (F. Hoffmann-La Roche Ltd, Switzerland).
132 Amplification was carried out with 40 cycles of 95°C for 5 seconds, 60°C for 10 seconds, and
133 72°C for 10 seconds. Each assay was performed in duplicate in the same run. The level of
134 expression for each gene was calculated using the comparative threshold cycle value (Ct)
135 method, using the formula $2^{-\Delta\Delta Ct}$ (where $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ reference}$). The final

136 results were expressed as normalized fold values relative to the normal group as described
137 previously [42].

138

139 *2.3. Lipopolysaccharide (LPS) determination*

140 The concentration of plasma LPS was measured by enzyme-linked immunosorbent assay
141 (LAL assay kit, Hycult Biotech, The Netherlands). The absorbance at 405 nm was measured
142 with a spectrophotometer. A measurable concentration ranges from 0.04 to 10 EU/ml. All
143 samples for LPS measurements were performed in duplicate.

144

145 *2.4. Immunohistochemistry*

146 The immunohistochemical staining has been described in our previous work (Dinh et al.,
147 2015). Fixed colon tissues were embedded in paraffin and sectioned at 5 μ m. The sections
148 were rehydrated in xylene and then in graded ethanol solutions. The sections were then
149 washed in 0.3% H₂O₂ in methanol for 10 min, blocked with 5% normal rabbit serum or goat
150 serum, and incubated overnight at 4 °C with primary antibodies. Primary antibodies were
151 anti-F4/80 (1: 500 dilution; ab6640), anti-CD11c (1: 1000 dilution; ab6640), and anti-CD206
152 (1: 1000 dilution; ab6640) (all from Abcam Inc, Cambridge, MA). Sections were then washed
153 3 times with TBST and incubated consecutively with the appropriate biotinylated secondary
154 antibodies: rabbit anti-rat IgG (1: 500 dilution; ab6733), goat anti-Armenian hamster IgG
155 H&L (1: 500 dilution; ab5744) and goat anti-rabbit IgG H&L (1: 500 dilution; ab6720) (all
156 from Abcam Inc, Cambridge, MA) for 30 minutes at room temperature. The sections were
157 then washed and incubated with streptavidin-HRP polymer conjugate (#2438, Sigma-Aldrich

158 Pty. Ltd, Sydney, NSW, Australia) for 30 min at room temperature. The sections were then
159 washed and developed using the ImmPACT DAB peroxidase substrate kit (#4100, Vector
160 laboratories Inc., Burlingame, CA, USA) and counterstained with haematoxylin (POCD
161 Scientific, Artarmon, NSW, Australia). Six fields from three sections of each mouse were
162 viewed under a Leica microscope and digital photographs were captured. Image J software
163 was used to quantify the area of F4/80, CD11c, and CD206 immunoreactivity on each fields.
164 The immunohistochemical staining F4/80, CD11c, and CD206 were quantified as a
165 percentage of positive area per image. The Immunoreactivity is quantified as the % of pixels
166 in an area of interest that have intensity greater than the background using Image J computer
167 software, <http://rsb.info.nih.gov/ij/docs/pdfs/examples.pdf>.

168

169 *2.5. Western blotting*

170 Perirhinal cortexes were dissected and homogenized in a NP-40 lysis buffer. The following
171 antibodies were used: MyD88 (sc-74532), NF κ B (sc-7178), p-JNK (sc-81502), IL-1 β
172 (sc-7884), BDNF (sc-20981), and IL-6 (sc-7920) from Santa Cruz Biotechnology (Santa
173 Cruz, CA); TNF- α (#11948) and TLR4 (#2219) from Cell Signaling Technology (Beverly,
174 MA). The bands corresponding to the proteins of interest were scanned and band densities
175 were analyzed using the automatic imaging analysis system, Quantity One (Bio-Rad
176 Laboratories, Hercules, CA). All quantitative analyses were compared to the control group.

177

178 *2.6. Novel object recognition test*

179 Recognition memory was assessed by performing a novel object recognition test based on

180 our group's previous studies [29]. Briefly, a white open-field square box measuring 55 cm in
181 length, 55 cm in width, and 35 cm in height was used. The open-field box was located in a
182 sound proof room, and lit at approximately 14 lux. The experimental procedure consisted of
183 habituation, training, and retention sessions, which were recorded using a video camera
184 placed above the open-field box. All objects and the open-field box were cleaned with 70%
185 ethanol between each mouse. For habituation, mice were individually placed in the box for 5
186 minutes to explore the environment in the absence of objects. During the training session, two
187 identical objects (A) were placed at opposing corners of the box, 5 cm from the adjacent wall.
188 Each mouse was then placed in the middle of the open-field box and left to explore the
189 objects for 5 minutes. A mouse was considered to be exploring the object if it was sniffing,
190 touching, or facing the object within 2 cm or less, and measurements were recorded in
191 seconds. For the retention session, one familiar object (A) was replaced with one novel object
192 (B) and measurements were taken according to how much time each mouse spent at each
193 object as per the training session. The retention session commenced upon placing each mouse
194 in the middle of the open-field box 90 minutes after its training session, and leaving it to
195 explore for another 5 minutes. Novel object exploration time and the discrimination index (DI
196 = [(Novel Object Exploration Time/Total Exploration Time) – (Familiar Object Exploration
197 Time/Total Exploration Time)] × 100) were used to evaluate the recognition memory of
198 the mice [43].

199

200 *2.7. Intraperitoneal glucose tolerance test*

201 Mice were fasted overnight before a glucose tolerance test was performed to assess glucose

202 clearance, following an intraperitoneal injection of glucose (0.5 g/kg; Sigma-Aldrich, St
203 Louis, MO, USA). Blood samples were taken from the tail vein at 0, 15, 30, 60 and 120
204 minutes following the injection of glucose. Blood glucose was measured using an Accu-Chek
205 glucometer (Roche Diagnostics GmbH Mannheim, Germany).

206

207 *2.8. Statistical analysis*

208 Data were analyzed using the statistical package SPSS 20 (SPSS, Chicago, IL). Data was
209 first tested for normality before differences among the Con, HF, and HF+R groups were
210 determined using one-way analysis of variance (ANOVA). This was followed by the post hoc
211 Tukey-Kramer honestly significant difference (HSD) test for multiple comparisons among the
212 groups. A *p* value of <0.05 was considered to be statistically significant. Values are expressed
213 as mean ± SEM. Pearson's correlations were used to examine the relationship between the
214 discrimination index in the novel object recognition test and plasma LPS and BDNF levels in
215 the perirhinal cortex.

216

217 **3. Results**

218 *3.1. Rhein reversed the alteration of gut microbiota induced by a HF diet*

219 To investigate the effect of rhein on intestinal microbiota, we used qRT-PCR to evaluate
220 the abundance of several vital strains of gut flora in the cecal content, including
221 *Bacteroides-Prevotella* spp, *Lactobacillus* spp., *Bifidobacterium* spp. and *Desulfovibrios* spp.
222 A chronic HF diet significantly altered gut microbiota in the HF group compared to the
223 control group. The amounts of *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA

224 were significantly decreased ($p < 0.001$, $p < 0.001$), while *Bifidobacterium* spp. and
225 *Lactobacillus* spp. DNA were significantly increased ($p < 0.001$, $p = 0.003$) (Fig 1). A
226 6-week rhein oral treatment reversed the altered gut microbiota induced by the HF diet. The
227 amount of *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA were significantly
228 increased in the HF+R group compared to the HF group ($p = 0.044$, $p = 0.044$), although the
229 amount of *Bacteroides-Prevotella* spp. DNA in the HF+R group was still lower than the
230 control mice ($p < 0.001$) (Fig. 1). Meanwhile, the rhein treatment prevented the HF
231 diet-induced alteration of the amount of *Bifidobacterium* spp. DNA in the HF+R group
232 compared to the HF group ($p < 0.001$). There was no significant difference in
233 *Bifidobacterium* spp. DNA between the HF+R group and the control group ($p = 0.997$) (Fig.
234 1). The amount of *Lactobacillus* spp. DNA of the HF+R group was lower than the HF group
235 ($p = 0.003$), but there was no significant difference between the HF+R group and the control
236 group ($p = 0.159$) (Fig. 1).

237

238 3.2. Rhein decreased plasma LPS concentration in obese mice induced by a HF diet

239 To further determine whether changes in the gut microbiota could be associated with
240 systemic inflammation, we measured the plasma concentration of LPS, a trigger of
241 inflammation. The concentration of plasma LPS was 59% higher in the HF group than the
242 control group (HF: 1.02 ± 0.12 EU/ml, Control: 0.64 ± 0.11 EU/ml, $p < 0.001$) (Fig. 2A). The
243 rhein treatment for 6 weeks prevented an increase of plasma LPS induced by the HF diet (HF:
244 1.02 ± 0.12 EU/ml, HF+R: 0.64 ± 0.13 EU/ml, $p < 0.001$). There was no difference between the
245 control and HF+R groups in the plasma LPS concentrations ($p = 1.000$).

246

247 *3.3. Rhein reduced M1 macrophage accumulation in the colon of HF diet-induced obese mice*

248 To investigate the effect of rhein on macrophage accumulation in the colon of HF diet mice,
249 we stained macrophages with F4/80 antibody (Fig 2B and C). The positive immunoreactivity
250 of F4/80 was significantly increased in the colon of obese mice, however this was reduced by
251 the rhein treatment (Fig. 2B). Furthermore, we characterized the type of macrophages. CD11c
252 was used to detect M1 macrophages which produce pro-inflammatory cytokines, and CD206
253 was used to detect M2 macrophages which produce anti-inflammatory cytokines [44, 45].
254 Rhein significantly reduced the CD11c positive staining in the colon of obese mice compared
255 to the obese mice without rhein treatment (Fig. 2B and C). There were no significant
256 differences in the CD206 staining in the colon of obese mice compared to the obese mice
257 without rhein treatment (Fig. 2B and C).

258

259 *3.4. Rhein suppressed the inflammation in the perirhinal cortex in HF diet-induced obese* 260 *mice*

261 To investigate whether endotoxemia could be related to neuroinflammation, we examined
262 the TLR4-My88-NF κ B/JNK signaling pathway and pro-inflammatory cytokines (TNF- α ,
263 IL-6 and IL-1 β) in the perirhinal cortex, an important brain region for recognition memory.
264 Western blotting revealed that the TLR4 and MyD88 level in the perirhinal cortex was
265 significantly higher in the HF group than the control group ($p = 0.049$, $p < 0.001$), while the
266 TLR4 and MyD88 level was significantly decreased in the HF+R group (rhein treatment
267 group) compared to the HF group ($p = 0.038$, $p = 0.001$) (Fig. 3). There was no statistical

268 difference in the TLR4 and MyD88 level between the HF+R group and the control group
269 (both $p > 0.005$). Furthermore, the NF κ B and p-JNK level was significantly higher in the HF
270 group compared to the control group ($p = 0.047$, $p = 0.008$), while the rhein treatment
271 prevented an increase of NF κ B and p-JNK ($p = 0.036$, $p = 0.003$) (Fig. 3). The
272 pro-inflammatory cytokine IL-1, IL-6, and TNF- α levels were significantly higher in the HF
273 group compared to the control group ($p = 0.025$, $p = 0.011$, $p = 0.011$) (Fig. 3). The IL-1 and
274 IL-6 levels were significantly lower in the HF+R group than the HF group ($p = 0.032$; $p =$
275 0.006). However, the rhein treatment did not significantly decrease the TNF- α level.

276

277 *3.5. Rhein improved recognition memory and increased BDNF levels in the perirhinal cortex* 278 *of HF diet-induced obese mice*

279 To assess whether rhein treatment can prevent HF diet-induced recognition memory
280 deficits, we performed a novel object recognition test in mice fed a HF diet and given rhein
281 treatment. During the training session of the test, the percentage of time spent exploring the
282 identical objects in the open-field was not significantly different among the control group
283 (18.06%), the HF group (17.05%), and the HF+R group (16.50%). One day after the training
284 session, all mice were presented with the familiar object and a new object. The exploration
285 time on the novel object of the HF group was significantly decreased compared to the control
286 mice (HF: 26.43 ± 3.78 seconds, Control: 31.13 ± 2.98 seconds, $p = 0.023$) (Fig. 4A),
287 suggesting that the HF diet significantly impaired novel object recognition performance.
288 However, the rhein treatment increased novel object exploration time in the HF mice (HF+R:
289 33.57 ± 3.29 seconds, HF: 26.43 ± 3.78 seconds, $p = 0.002$) (Fig. 4A). Consistent with the

290 result of novel object exploration time, the rhein treatment significantly improved recognition
291 memory as assessed by the discrimination index. The HF diet decreased the discrimination
292 index by 60.60% compared to the control group ($p = 0.009$), while the rhein treatment
293 increased the discrimination index by 178.57% compared with the HF group ($p = 0.013$) (Fig.
294 4B). There was no difference between the HF+R group and the control group in novel object
295 exploration time and the discrimination index ($p = 0.230$, $p = 0.785$). These results show that
296 recognition memory deficits caused by a HF diet may be prevented by rhein treatment.

297 We evaluated the effect of rhein on the level of BDNF in the perirhinal cortex of HF diet
298 fed mice using western blotting analysis. The BDNF level was significantly lower in the HF
299 group than the control group ($p < 0.001$) (Fig. 4C), while the rhein treatment significantly
300 increased the BDNF level in the HF+R group compared to the HF group ($p < 0.001$).
301 However, the BDNF level was still lower in the HF+R group than the control group ($p =$
302 0.036) (Fig. 4C). Pearson's correlation analysis revealed a significantly positive correlation
303 between the discrimination index of the novel object recognition test and the BDNF level in
304 the perirhinal cortex ($r = 0.622$, $p = 0.008$) (Fig. 4D). Furthermore, there was a negative
305 correlation between the discrimination index value and the plasma LPS level ($r = -0.705$, $p =$
306 0.002) (Fig. 4E).

307

308 *3.6. Rhein reduced body weight and food intake, and improved glucose tolerance in HF* 309 *diet-induced obese mice*

310 Before the HF diet feeding, there was no significant difference in body weight between the
311 control group and the HF group (Control: 22.82 ± 1.73 g, HF: 23.06 ± 1.73 g, $p > 0.05$) (Fig.

312 5A). After 8 weeks on the HF diet, the HF group had significant higher body weight than the
313 control group (HF: 33.11±2.41 g, Control: 25.80±1.56 g, $p < 0.05$). The rhein treatment
314 prevented the body weight gain from week 9. After the rhein treatment for 6 weeks, the body
315 weight was significantly lower in the HF+R group than the HF group (HF+R: 33.37±2.85 g,
316 HF: 38.22±3.56 g, $p < 0.05$), although it was still higher than the control group (HF+R:
317 33.37±2.85 g, Control: 27.47±1.93 g, $p < 0.05$) (Fig. 5A). Furthermore, the energy intake was
318 significantly decreased on the first day following rhein treatment compared to the HF diet (p
319 < 0.001) (Fig. 5B). However, there was no significantly difference in the energy intake for
320 the remaining 5 weeks of treatment between HF+R and HF group (all $p > 0.05$).

321 Glucose tolerance tests were performed to assess glucose homeostasis. The highest blood
322 glucose level in the HF group and the HF+R group occurred at 30 minutes, while the highest
323 blood glucose level in the control group occurred at 15 minutes (Fig. 5C). The blood glucose
324 level of the rhein treatment group significantly decreased at 15, 30, 60, and 120 minutes
325 compared with the HF group (all $p < 0.05$), but they were still higher than those of the control
326 group (all $p < 0.05$).

327

328 **4. Discussion**

329 In this study, we found that a chronic HF diet altered gut microbiota, increased plasma LPS,
330 increased macrophage accumulation in colon, increased the neuroinflammation response, and
331 decreased the BDNF level in the perirhinal cortex, and impaired recognition memory in obese
332 mice. Rhein oral treatment for 6 weeks significantly ameliorated the altered gut microbiota,
333 lowered plasma LPS, reduced macrophage accumulation, decreased neuroinflammation, and

334 increased BDNF in the perirhinal cortex and improved recognition memory in diet-induced
335 obese mice.

336 Previous studies have provided compelling evidence to suggest an association between the
337 gut microbiota, HF diet and body weight regulation [46, 47]. Firmicutes and Bacteroidetes
338 account for more than 90% of the total gut microbiota [48]. The 8-week HF diet increased
339 Firmicutes and reduced Bacteroidetes. It has also been reported that the HF diet for 8 weeks
340 post-weaning changes the microbiota, decreases the overall bacterial abundance, and
341 increases the ratio of Firmicutes to Bacteroidetes in mice [47]. Ley *et al.* observed that a
342 reduced abundance of Bacteroidetes and an increased abundance of Firmicutes were observed
343 in leptin-deficient obese mice compared with their lean littermates [13]. Consistent with
344 animal models, a similar difference of an increased ratio of Firmicutes/Bacteroidetes in the
345 gut microbiota has also been reported in obese humans [49]. In the present study, we found
346 that a chronic HF diet decreased the DNA level of *Bacteroides-Prevotella* spp. and increased
347 the DNA level of *Lactobacillus* spp. belonging to Bacteroidetes and Firmicutes respectively
348 at the species level [50]. Therefore, our study at the species level supports the finding from
349 previous studies at the phylum level that a HF diet alters the microbiota in Bacteroidetes and
350 Firmicutes [13, 47]. Importantly, we also found that the rhein treatment prevented the
351 alteration of gut microbiota induced by a HF diet in obese mice, which may contribute to
352 rhein's ability to prevent HF diet-induced obesity.

353 Rhein is the main ingredient of the rhubarb plant. An increased bacterial diversity was
354 observed in the ileum of rhubarb-exposed rats [33]. The 380 bp product (region of the
355 *Bacteroides* genome) was increased in the feces and bowel mucosa of rhubarb-exposed rats

356 [33]. Furthermore, rhein has been considered as an antibacterial agent against *Staphylococcus*
357 *aureus* [37]. A synergistic or partial synergistic effect of rhein in combination with ampicillin
358 or oxacillin against methicillin-resistant *Staphylococcus aureus* has also been demonstrated
359 [51]. In the present study, we found that rhein attenuated the HF diet-induced alteration of gut
360 microbiota. Furthermore, rhein administration prevented the elevation of HF diet-induced
361 plasma LPS. LPS is a major component of the outer membrane in Gram-negative bacteria.
362 The plasma LPS level was closely correlated with changes in intestinal microbiota, especially
363 the numbers of the Gram-negative Bacteroides-like intestinal bacteria which reside within the
364 Bacteroidetes phylum and were significantly reduced in animals fed the HF diet [14]. The
365 elevated plasma LPS is related to the over-production of LPS in the gut by the death of
366 Gram-negative bacteria and their translocation into the bloodstream via an increased
367 intestinal permeability in HF diet-induced obesity [52]. In the present study, we found that a
368 chronic HF diet decreased *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA levels,
369 which belong to the Gram-negative bacterium Bacteroidetes and Proteobacteria respectively.
370 A deceased Gram-negative bacterium releases LPS which leads to an increased plasma LPS
371 level in obese mice induced by a HF diet. Furthermore, in the present study, the rhein
372 treatment prevented the decrease of Gram-negative bacterium (including
373 *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp.) induced by a HF diet. These data
374 strongly suggest that rhein affects the intestinal microbiota and is responsible for the
375 attenuation of metabolic endotoxemia in HF diet-induced obesity.

376 LPS induce macrophage activation and accumulation [53]. M1 macrophages produce
377 pro-inflammatory cytokines, such as TNF- α , IL-6, and IL-1 β , while M2 macrophages

378 produce anti-inflammatory cytokines, such as IL-10 [44, 45]. This study showed that there is
379 an increased level of plasma LPS concentration in obese mice on a chronic HF diet. This is
380 accompanied by an increased M1 macrophage accumulation in the colon. These results
381 support previous findings which show that TNF- α expression is significantly increased in the
382 colon of obese mice [54, 55]. Importantly, our present study shows that rhein treatment
383 significantly reduced colon inflammation in obese mice on a chronic HF diet.

384 TLR4 is the receptor for LPS and it plays a critical role in innate immunity [56]. The
385 stimulation of TLR4 activates the MyD88-dependent pathway to induce NF κ B and JNK
386 activation, which in turn leads to the production of pro-inflammatory cytokines such as
387 TNF- α , IL-6, and IL-1 β [57]. In this study, we detected the activation of the
388 TLR4-My88-JNK/NF κ B inflammatory signaling pathway. We also found an over-expression
389 of pro-inflammatory cytokines in the perirhinal cortex of mice on a chronic HF diet. It has
390 previously been shown that peripheral inflammation acutely impairs object location memory
391 in humans with perirhinal cortex lesions [58]. Importantly, in the present study, rhein
392 treatment prevented the inflammatory response in the perirhinal cortex of HF diet-induced
393 obese mice. This suggests that the anti-inflammatory effect of rhein may contribute to its
394 prevention of recognition memory decline.

395 BDNF has been shown to be important for object recognition memory in the perirhinal
396 cortex [23]. Previous studies found that BDNF expression in the perirhinal cortex has a
397 positive relationship with recognition memory in rats [23, 24]. An intracerebroventricular
398 injection of anti-BDNF serum inhibited recognition memory and altered Trk receptor and
399 BDNF levels in the perirhinal cortex in rats [25]. In our study, the rhein treatment prevented

400 the HF diet-induced decrease in the BDNF level in the perirhinal cortex in mice. Several
401 rodent studies have demonstrated that inflammation affects the expression of BDNF within
402 the brain. For example, after an intraperitoneal injection of IL-1 β or LPS, the BDNF mRNA
403 expression was significantly decreased in the rat hippocampus [26]. A similar reduction of
404 BDNF at the protein level has also been observed in cortical regions [59]. The negative
405 impact of inflammation has important implications for pathophysiology such as the decline of
406 cognitive function. For example, pro-inflammatory cytokines compromise general memory
407 [60] and spatial memory [61], and increase apoptosis in the brain [62]. Individuals with
408 obesity or diabetes with low grade inflammation have an increased risk of cognitive decline
409 [63]. Therefore, the chronic consumption of rhein may lower the neuroinflammation and
410 increase BDNF in the perirhinal cortex and thus improve recognition memory in HF
411 diet-induced obesity.

412 Previous studies have reported that rhein treatment did not significantly influence energy
413 intake in either HF mice or db/db mice. For example, rhein delivered by gavage, did not
414 significantly decrease energy intake of HF mice over 6 weeks [64]. While others showed that
415 food intake was not significantly decreased by the rhein (oral gavage) over 2 weeks in *db/db*
416 mice [65]. In the present study, the energy intake of the rhein group only decreased on the
417 first day following rhein treatment but not thereafter. This initial drop in energy intake may
418 be due to an adaptation period of transition from the HF diet to HF diet mixed with rhein.
419 Consistent with rhein's hypoglycemic effect in streptozotocin-induced diabetic mice [66], our
420 study showed that rhein has an anti-obesity effect by reducing body weight and improving
421 glucose intolerance in HF diet-induced obese mice. Cognitive deficits have been observed in

422 older people with glucose intolerance or diabetes but these deficits appear to be attenuated by
423 improving glycemic control [67]. A previous study found that rhein oral treatment decreased
424 body weight gain with increased oxygen consumption suggesting that rhein increased energy
425 expenditure [64]. Furthermore, weight loss in obese older people can significantly improve
426 cognition [68]. Therefore, rhein-induced weight loss and improved glucose metabolism can
427 contribute to improved recognition memory.

428 In conclusion, this study demonstrated that rhein can improve recognition memory and
429 glucose intolerance and prevent weight gain in mice fed a chronic HF diet. The rhein
430 treatment also prevented the HF diet-induced alterations of gut microbiota,
431 hyperendotoxemia, macrophage accumulation in colon, neuroinflammation, and increased
432 BDNF in the perirhinal cortex in mice. The behavioral and neurochemical improvements
433 suggest that supplementation with rhein-enriched food could be a promising strategy to
434 improve HF diet-induced obesity and cognitive decline.

435

436 **Abbreviations**

437 HF: high fat; LPS: lipopolysaccharide; IPGTT: intraperitoneal glucose tolerance test; BDNF: brain-derived
438 neurotrophic factor; TLR: Toll-like receptor; MyD88: myeloid differentiation primary-response protein 88;
439 JNK: c-Jun N-terminal kinase; NF κ B: nuclear factor-kappa B; IL-1 β : interleukin-1 β ; IL-6: interleukin-6;
440 TNF- α : tumor necrosis factor- α .

441

442 **Competing interests**

443 The authors declare no conflict of interest.

444

445 **Authors' contributions**

446 Y.Y. and W.S. contributed to the experimental design, researched data, and wrote the manuscript. Z.P.,
447 W.H., and Z.Q., researched data and contributed to discussions. Y.S. and X.F.H contributed to data
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449

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Figures

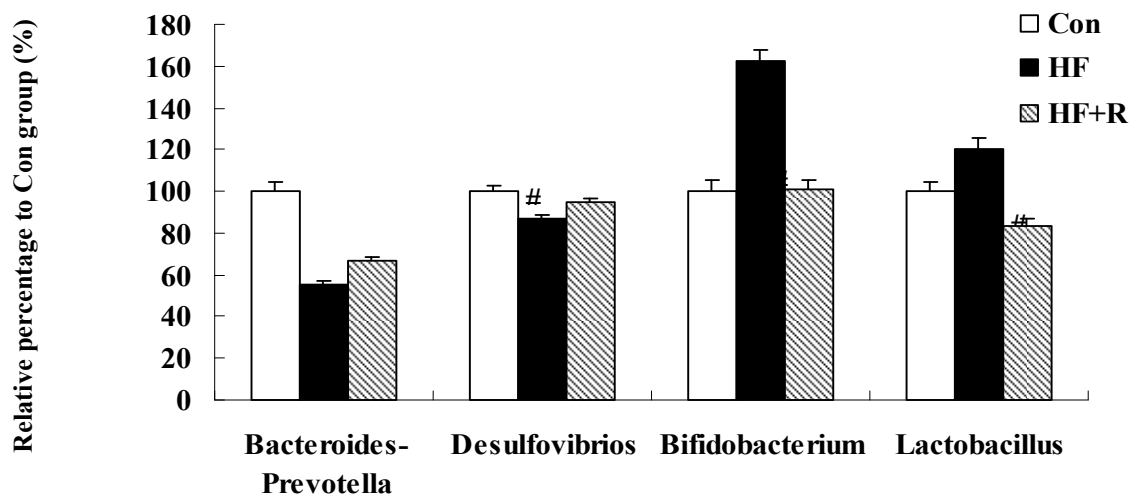
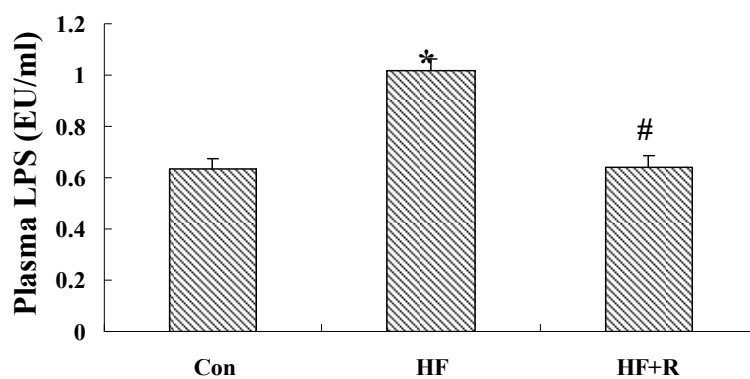
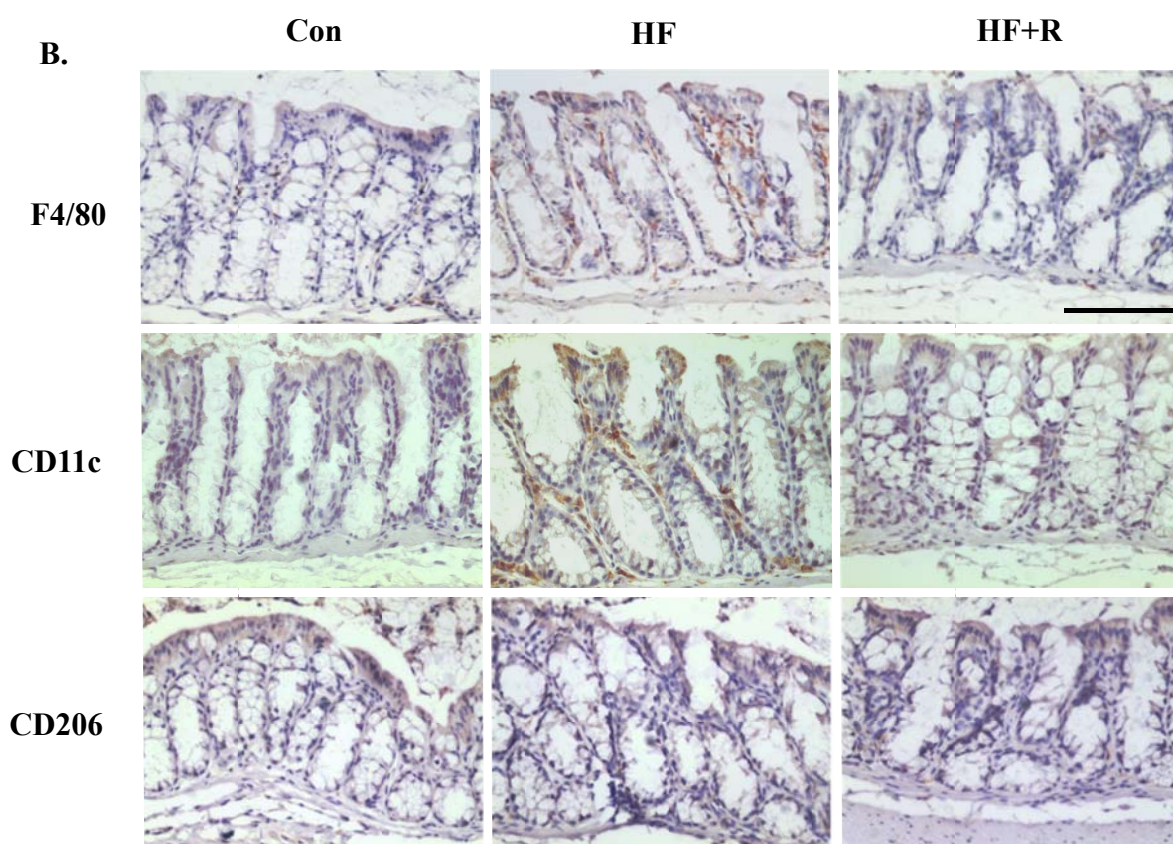


Figure 1. Bacteroides-Prevotella, Desulfovibrios, Bifidobacterium and Lactobacillus DNA expressions in gut microbiota of the control group (Con), high-fat diet group (HF), and HF with rhein treatment group (HF+R) (n = 8 per group). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means \pm SEM.

A.



B.



C.

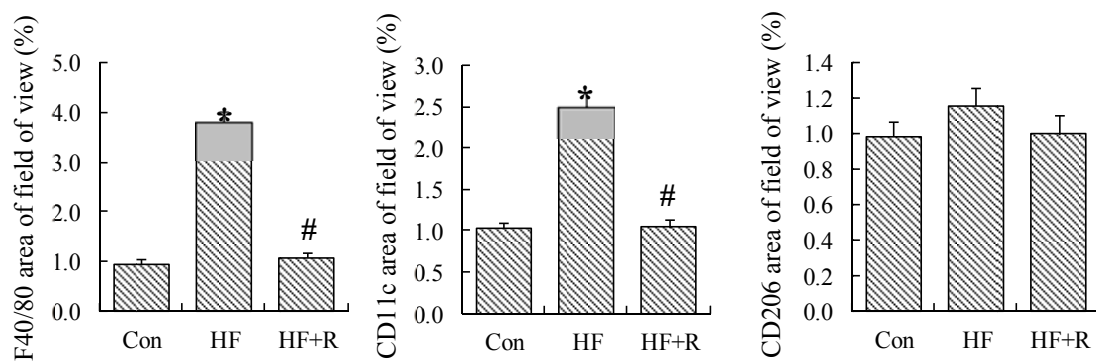


Figure 2. The plasma LPS level of control group (Con), high-fat diet group (HF), and HF with rhein treatment group (HF+R) ($n = 8$ per group) (A). (B and C) The expression of F4/80, CD11c, and CD206 macrophages in the colon. B: Immunohistochemical staining, Bar = 100 μ M. C: Quantification of the F4/80, CD11c, and CD206-positive areas (%). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means \pm SEM.

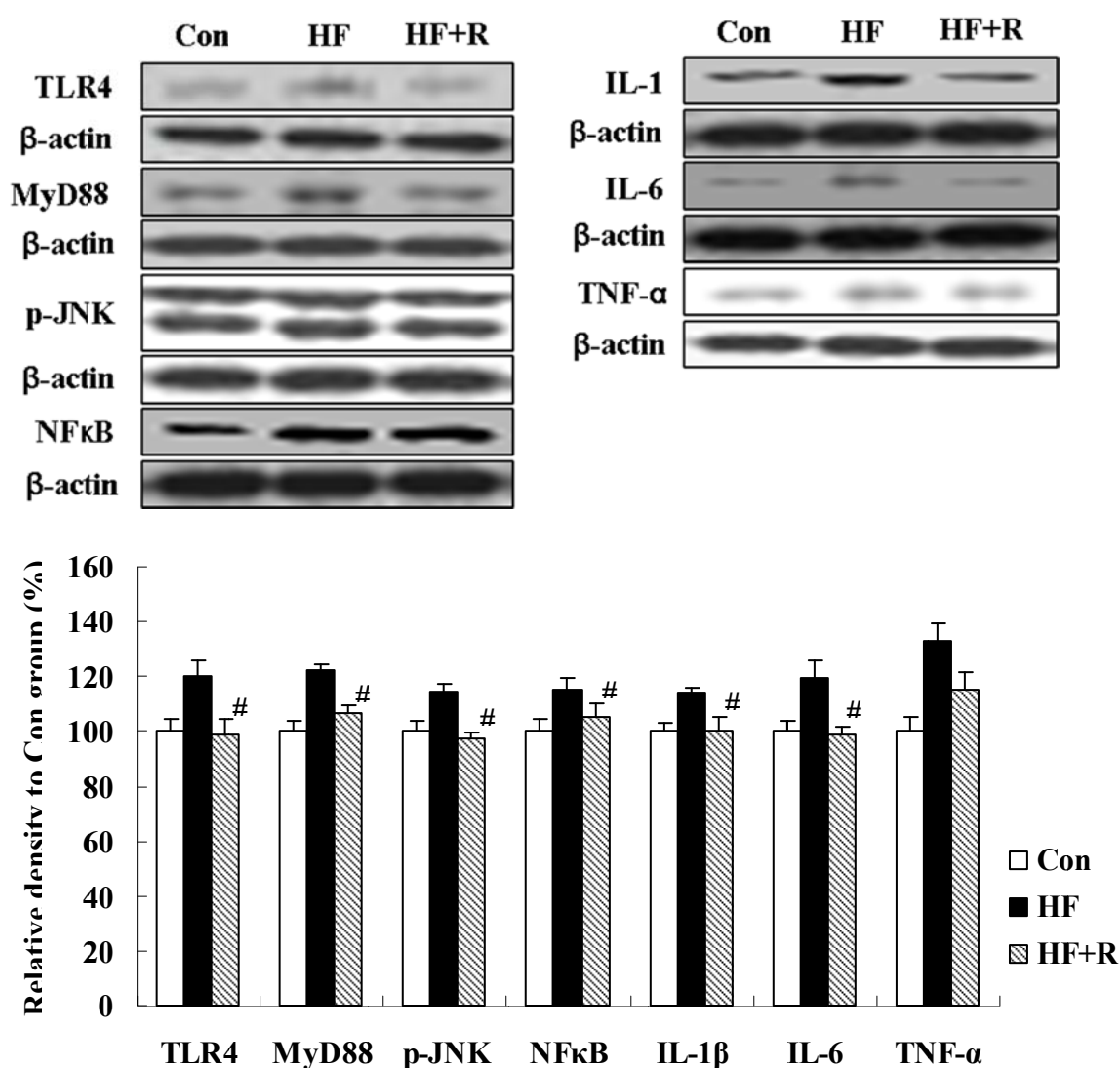


Figure 3. Protein expression levels of TLR4, MyD88, p-JNK, NFκB, IL-1β, IL-6, and TNF-α in the perirhinal cortex of the control group (Con), high-fat diet group (HF), and HF with rhein treatment group (HF+R) ($n = 8$ per group). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means \pm SEM.

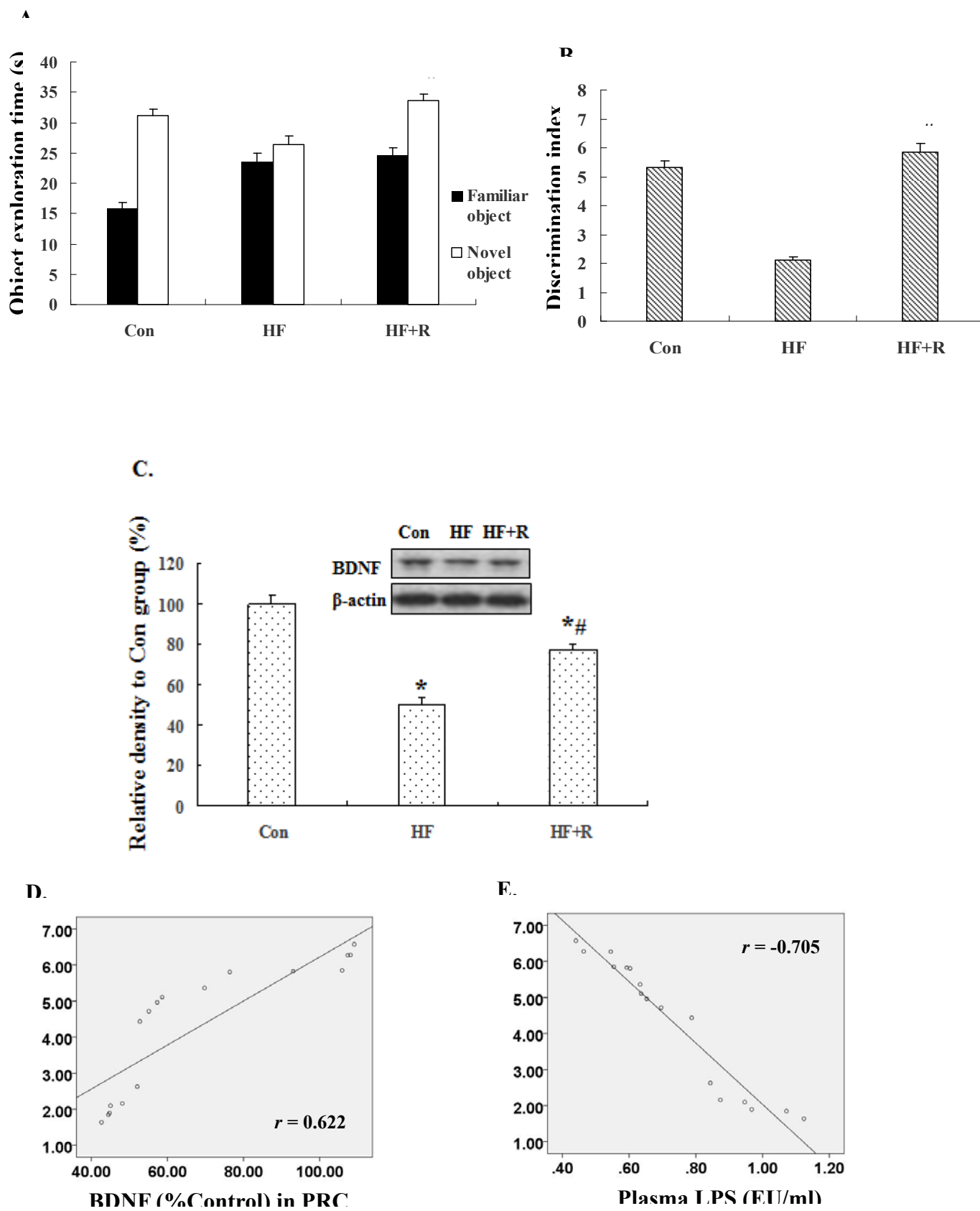
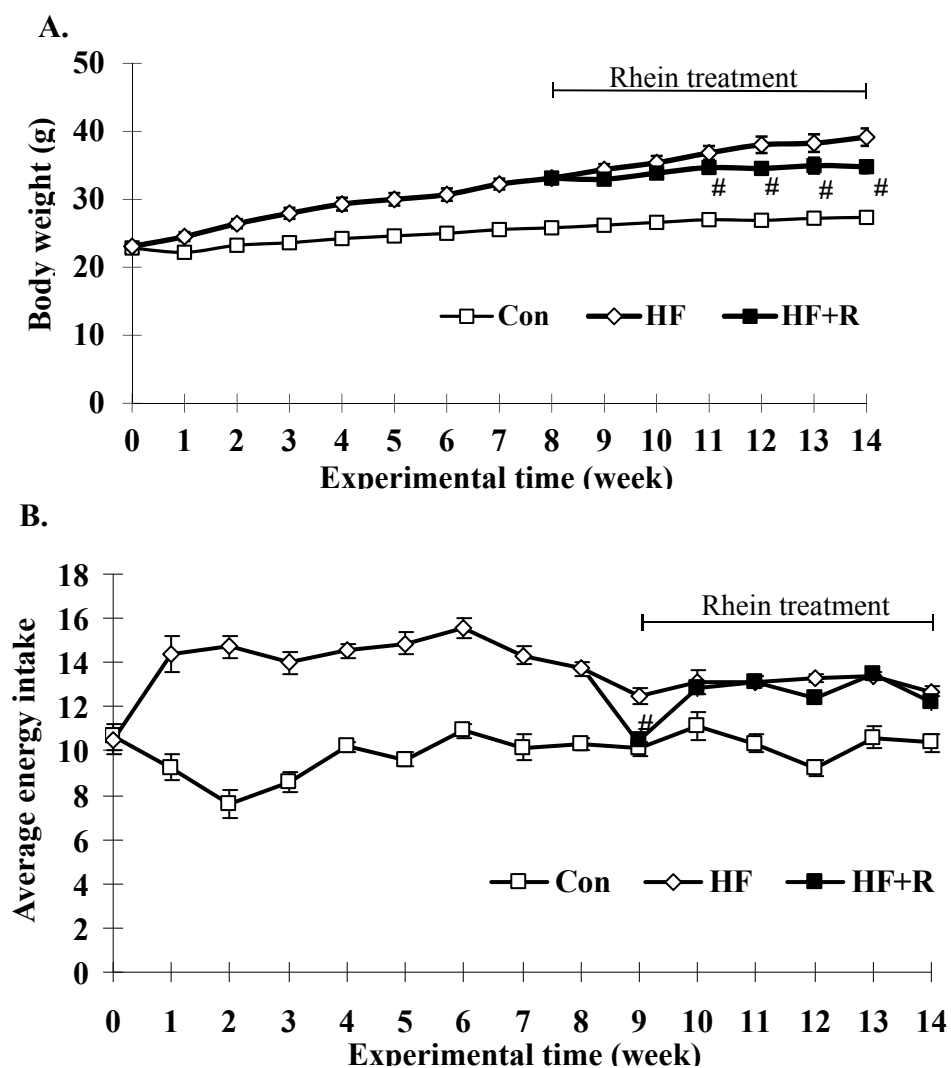


Figure 4. The object exploration time (A) and the discrimination index (B) in the novel object recognition test of the control group (Con), high-fat diet group (HF) and HF with rhein treatment group (HF+R). (C) The level of BDNF expression in the perirhinal cortex of mice (n = 8 per group). The discrimination index in novel object recognition was positively correlated with BDNF in the perirhinal cortex (D), and negatively correlated with plasma LPS (E). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means \pm SEM.



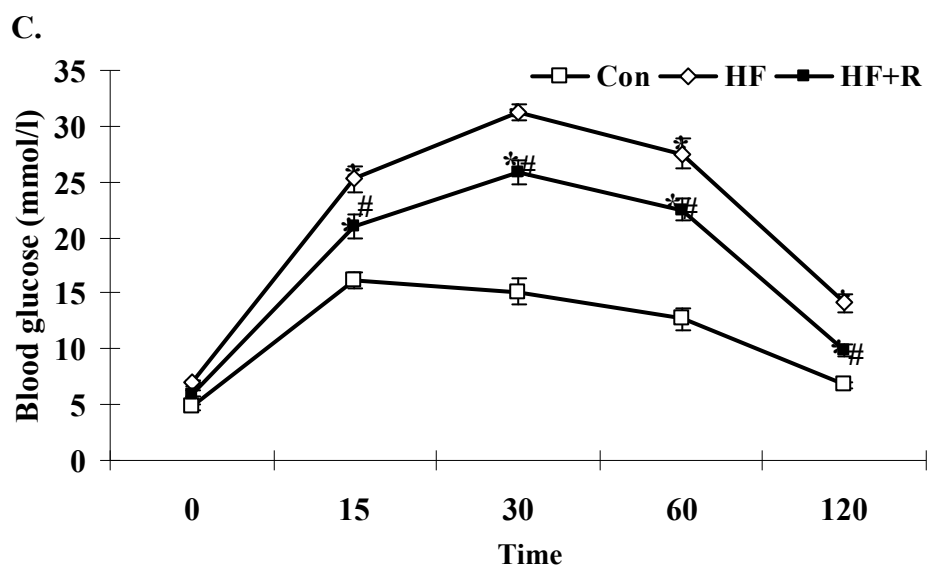


Figure 5. Body weight (A), energy intake (B) and glucose tolerance (C) of the control group (Con), high-fat diet group (HF) and HF with rhein treatment group (HF+R). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means \pm SEM.