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

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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, hypoendotoxinemia, 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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Abstract

High-fat (HF) diet modulates gut microbiota and increases plasma concentration of lipopolysaccharide (LPS), metabolic endotoxemia, 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 a HF diet for 8 weeks to induce obesity, and then treated with oral rhein (120 mg/kg body weight per day in HF diet) for a further 6 weeks. Chronic rhein treatment prevented the HF diet-induced recognition memory impairment assessed by novel object recognition test, neuroinflammation and BDNF deficits in the perirhinal cortex. Furthermore, rhein inhibited the HF diet-induced increased plasma LPS level and the pro-inflammatory 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, hypoendotoxinemia, 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.

Keywords: rhein; gut microbiota; recognition memory; lipopolysaccharide; inflammation; perirhinal cortex

1. Introduction

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

Nowadays, it is widely accepted that obesity and its associated cognitive decline is associated with low-grade systemic and central inflammation, despite the fact that the molecular origin of the inflammation is poorly understood [9, 10]. Increased fat intake has been found to be strongly correlated with increased plasma lipopolysaccharide (LPS), endotoxemia [10]. LPS is a major component of the outer membrane in Gram-negative bacteria. Emerging evidence from animal studies suggests a link between the alteration of gut microbiota, increased intestinal permeability, and endotoxemia in HF diet-induced obesity [11]. An imbalance of Bacteroidetes and Firmicutes, the primary bacterial phyla comprising the gastrointestinal microbiota, has been reported in rodents fed a HF diet and obese individuals [12, 13]. The plasma LPS level was closely correlated with altered intestinal microbiota, in which the number or diversity of the Gram-negative, Bacteroidetes phylum, 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]. Endotoxinemia 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 (NFκB), two important inflammatory signaling molecules [17]. The activation
56 of the TLR4-MyD88-JNK/NFκB signaling pathway leads to the production of
57 pro-inflammatory cytokines, such as interleukin-1β (IL-1β), 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-1β 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

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

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

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

2. Methods

2.1. Animals and treatments

Twenty-four C57Bl/6J male mice (8 weeks old) were obtained from the Australian Bio-Resource Centre (Moss Vale, NSW), and housed in environmentally controlled conditions (temperature 22°C, 12 hour light/dark cycle). Eight mice were fed a lab chow diet as a control (Con group). Sixteen mice were fed a HF diet (HF group) containing 60% fat by calories (SF13-092; Specialty Feeds, Glen Forrest, WA). After 8 weeks, the 16 mice fed a HF diet were divided into two groups: 8 mice continued to receive the HF diet, and the other 8 mice received the rhein treatment (HF+R group) for 6 weeks. Rhein was mixed in the HF diet (dosage: 120 mg/kg body weight per day) [41]. Rhein (98%, C15H8O6, MW = 284.21) was purchased from Sangon Biotech Co. Ltd, China. Body weight was measured on the last day in every week. Food intake was recorded on the first day in every week. A weighed amount of fresh diet was given at the beginning of the dark cycle. The remaining food in the cage plus spillage were collected and weighed 24 hours later. After 6 weeks of treatment, the novel object recognition test and the intraperitoneal glucose tolerance test (IPGTT) were carried out. The mice were asphyxiated in chambers prefilled with CO_2 4 days after the tests were carried out. Plasma, cecal contents and brain tissue were collected, snap frozen and stored at

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

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

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

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

2.3. Lipopolysaccharide (LPS) determination

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

2.4. Immunohistochemistry

The immunohistochemical staining has been described in our previous work (Dinh et al., 2015). Fixed colon tissues were embedded in paraffin and sectioned at 5 μm. The sections were rehydrated in xylene and then in graded ethanol solutions. The sections were then washed in 0.3% H_2O_2 in methanol for 10 min, blocked with 5% normal rabbit serum or goat serum, and incubated overnight at 4 °C with primary antibodies. Primary antibodies were anti-F4/80 (1: 500 dilution; ab6640), anti-CD11c (1: 1000 dilution; ab6640), and anti-CD206 (1: 1000 dilution; ab6640) (all from Abcam Inc, Cambridge, MA). Sections were then washed 3 times with TBST and incubated consecutively with the appropriate biotinylated secondary antibodies: rabbit anti-rat IgG (1: 500 dilution; ab6733), goat anti-Armenian hamster IgG H&L (1: 500 dilution; ab5744) and goat anti-rabbit IgG H&L (1: 500 dilution; ab6720) (all from Abcam Inc, Cambridge, MA) for 30 minutes at room temperature. The sections were 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), NFκB (sc-7178), p-JNK (sc-81502), IL-1β
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

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

2.7. Intraperitoneal glucose tolerance test

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

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

2.8. Statistical analysis

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

3. Results

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

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

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

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

To further determine whether changes in the gut microbiota could be associated with systemic inflammation, we measured the plasma concentration of LPS, a trigger of inflammation. The concentration of plasma LPS was 59% higher in the HF group than the control group (HF: 1.02±0.12 EU/ml, Control: 0.64±0.11 EU/ml, $p < 0.001$) (Fig. 2A). The rhein treatment for 6 weeks prevented an increase of plasma LPS induced by the HF diet (HF: 1.02±0.12 EU/ml, HF+R: 0.64±0.13 EU/ml, $p < 0.001$). There was no difference between the 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-NFκB/JNK signaling pathway and pro-inflammatory cytokines (TNF-α,
263 IL-6 and IL-1β) 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 NFκB 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 NFκB 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

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

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

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

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

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

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

4. Discussion

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

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

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

Rhein is the main ingredient of the rhubarb plant. An increased bacterial diversity was observed in the ileum of rhubarb-exposed rats [33]. The 380 bp product (region of the 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-1β, while M2 macrophages

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

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

BDNF has been shown to be important for object recognition memory in the perirhinal cortex [23]. Previous studies found that BDNF expression in the perirhinal cortex has a positive relationship with recognition memory in rats [23, 24]. An intracerebroventricular injection of anti-BDNF serum inhibited recognition memory and altered Trk receptor and 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-1β 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

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

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

Abbreviations

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

Competing interests

The authors declare no conflict of interest.

Authors' contributions

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

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Figures

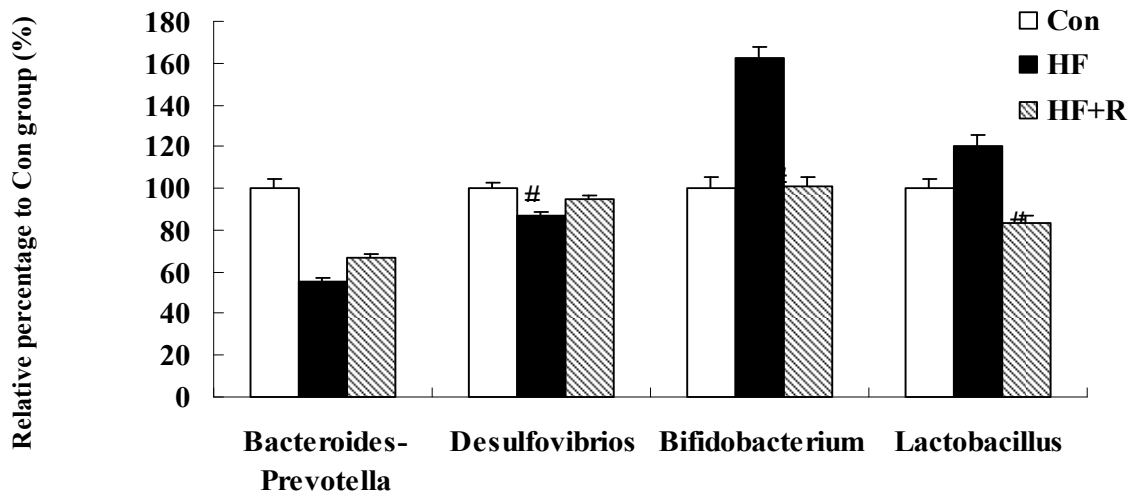


Figure 1. Bacteroides-Prevotella, Desulfovibrios, Bifidobacterium and Lactobacillus DNA expressions in gut microbiota of the control group (Con), high-fat diet group (HF), and HF with rhein treatment group (HF+R) (n = 8 per group). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the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

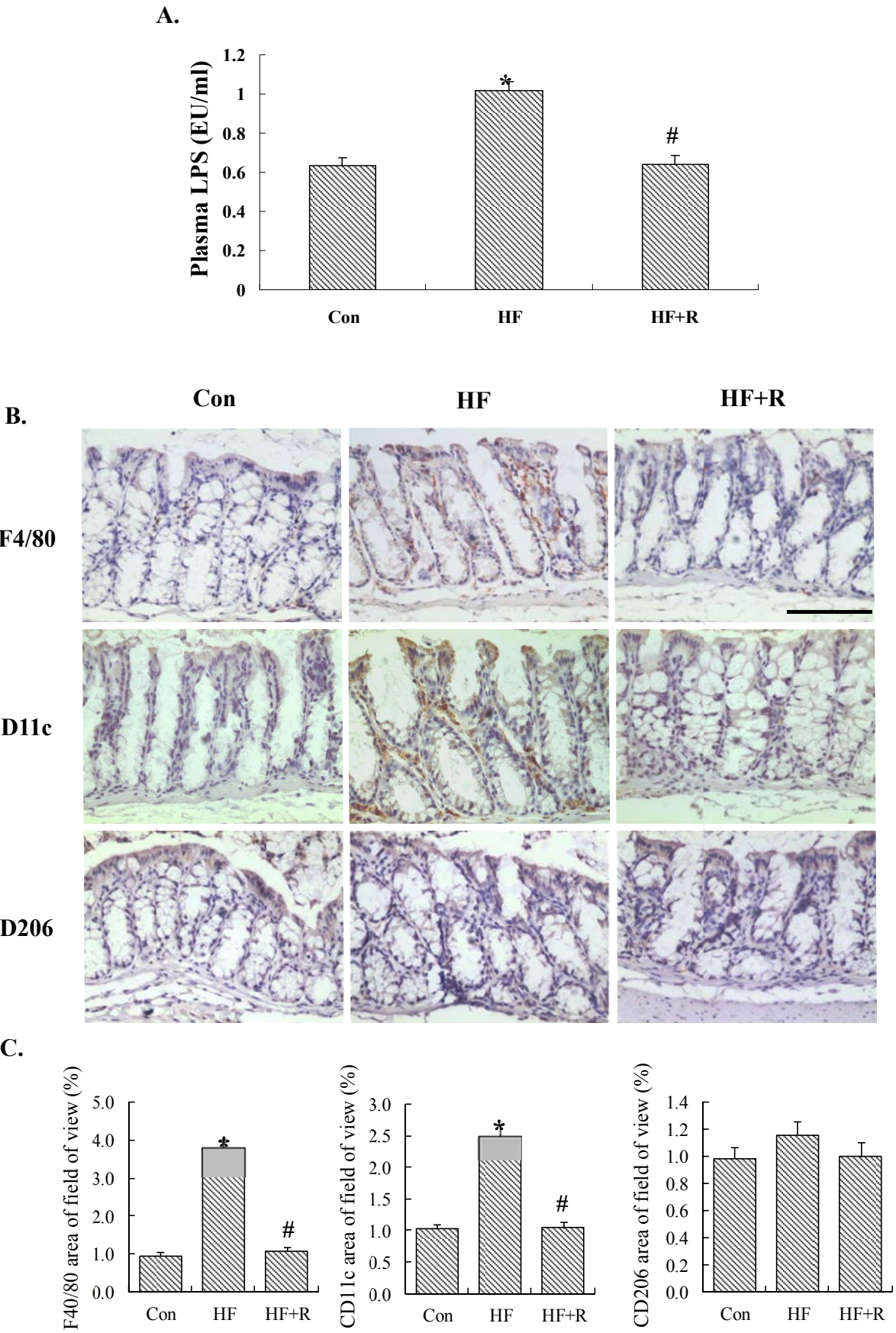

A.
Plasma LPS (EU/ml)
1.2
1
0.8
0.6
0.4
0.2
0
*
#
Con
HF
HF+R
B.
Con
HF
HF+R
F4/80
CD11c
CD206
C.
F40/80 area of field of view (%)
5.0
4.0
3.0
2.0
1.0
0.0
CD11c area of field of view (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CD206 area of field of view (%)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Con
HF
HF+R

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 μM. 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 ± SEM.

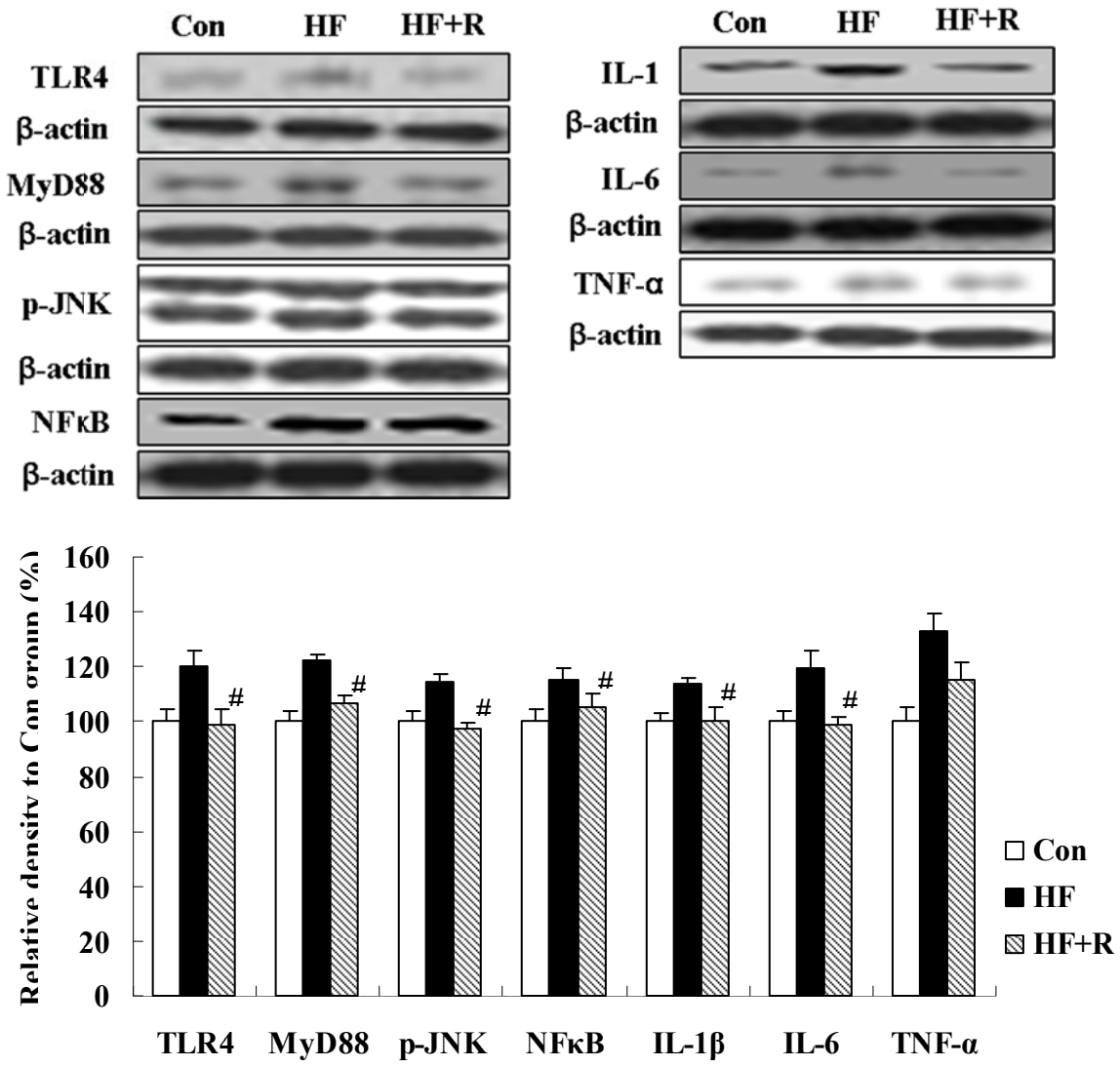
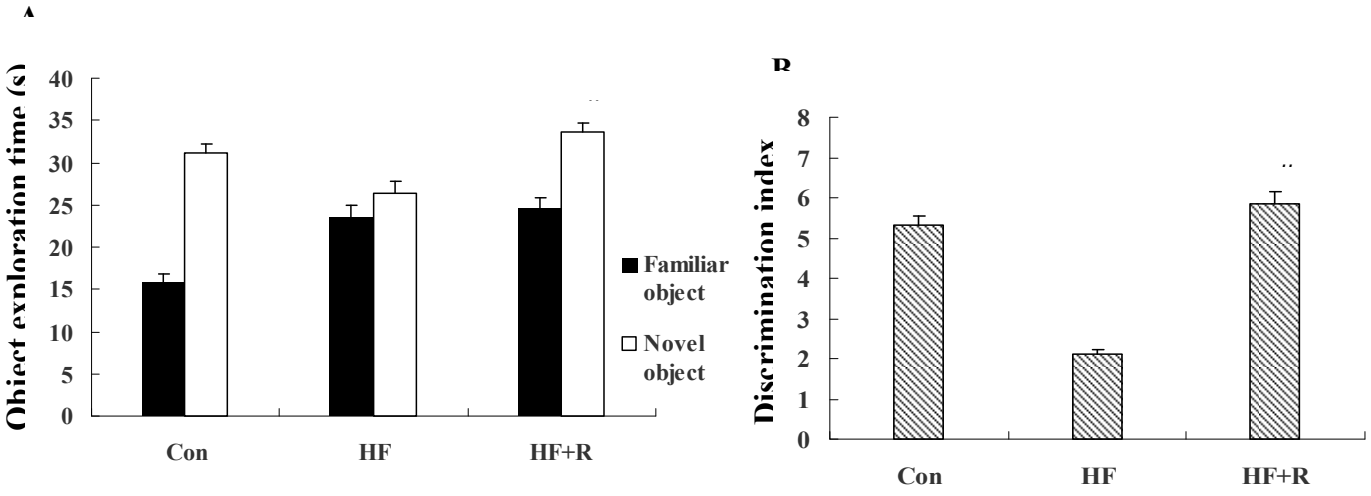
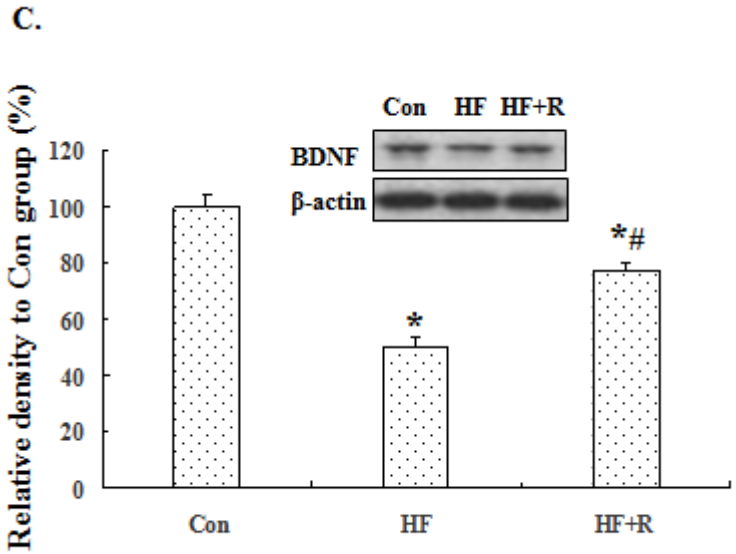
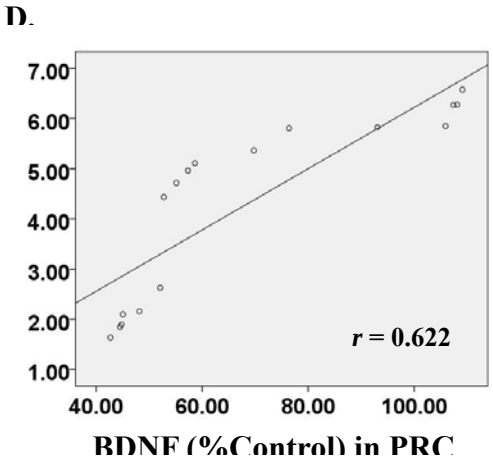


Figure 3. Protein expression levels of TLR4, MyD88, p-JNK, NFκB, IL-1β, IL-6, and TNF-α in the perirhinal cortex of the control group (Con), high-fat diet group (HF), and HF with rhein treatment group (HF+R) (n = 8 per group). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group,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


A
Object exploration time (s)
40
35
30
25
20
15
10
5
0
Con
HF
HF+R
Familiar object
Novel object
B
Discrimination index
8
7
6
5
4
3
2
1
0
Con
HF
HF+R


C.
Relative density to Con group (%)
120
100
80
60
40
20
0
Con
HF
HF+R
BDNF
β-actin
*
*#


D.
7.00
6.00
5.00
4.00
3.00
2.00
1.00
40.00
60.00
80.00
100.00
r = 0.622
BDNF (%Control) in PRC

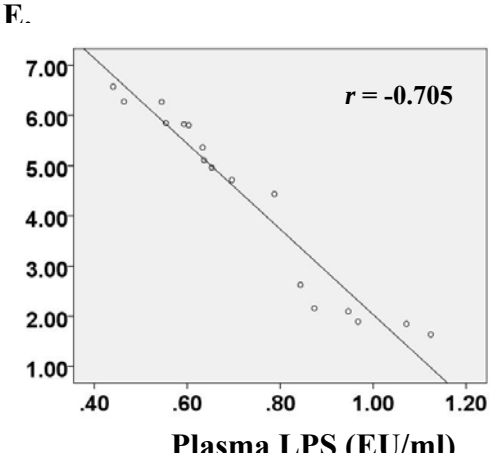
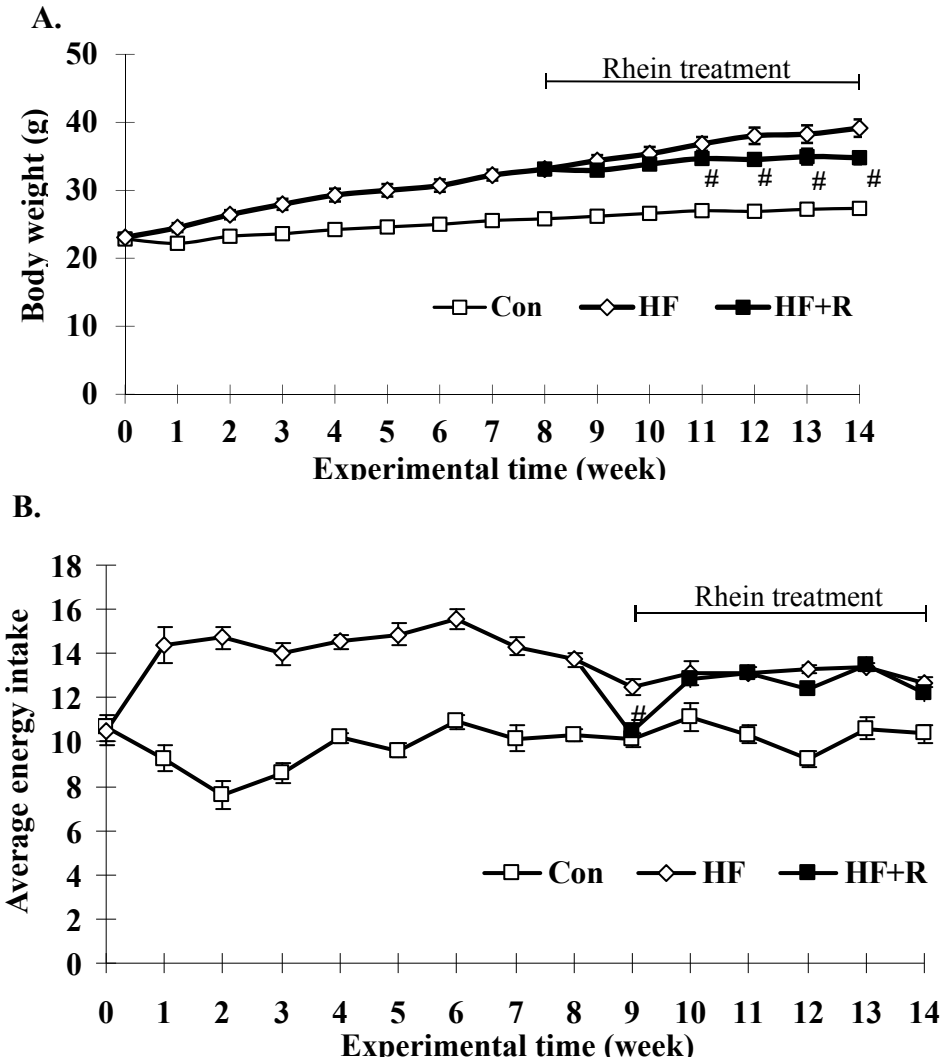

E.
7.00
6.00
5.00
4.00
3.00
2.00
1.00
.40
.60
.80
1.00
1.20
r = -0.705
Plasma LPS (EU/ml)

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 ± SEM.



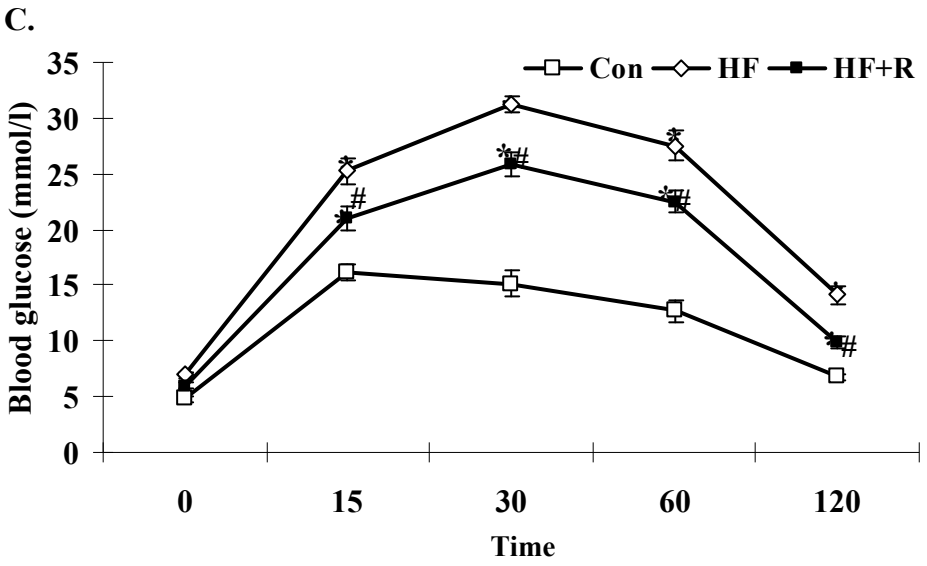


Figure 5. Body weight (A), energy intake (B) and glucose tolerance (C) of the control group (Con), high-fat diet group (HF) and HF with rhein treatment group (HF+R). * $p < 0.05$ compared to the Con group, # $p < 0.05$ compared to the HF group, values are means ± SEM.