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

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Publication Details

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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.

Disciplines
Medicine and Health Sciences

Publication Details

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This journal article is available at Research Online: http://ro.uow.edu.au/ihmri/902
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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
to be continuously produced in the gut by the death of Gram-negative bacteria and its translocation into intestinal capillaries via the increased intestinal permeability in HF diet-induced obesity [15]. Endotoxinemia in turn can trigger systemic inflammation and neuroinflammation. It has been shown that an intraperitoneal (ip) injection of LPS induces neuroinflammation, cognitive impairment and memory dysfunction [16]. LPS binds to Toll-like receptor (TLR) 4 coupled with myeloid differentiation primary-response protein 88 (MyD88)-dependent pathway, and activates c-Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NFκB), two important inflammatory signaling molecules [17]. The activation of the TLR4-MyD88-JNK/NFκB signaling pathway leads to the production of pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and contributes to the development of neurodegenerative diseases [18-21].

Brain-derived neurotrophic factor (BDNF) is known to play an important role in neuronal development and synaptic plasticity in the brain regions involved in cognitive function [22]. In the perirhinal cortex, BDNF has been shown to be important for object recognition memory [23]. Studies have found that BDNF expression in the perirhinal cortex has a positive relationship with recognition memory in rats [23, 24], and that treatment with anti-BDNF serum inhibited long-term recognition memory in rats [25]. An intraperitoneal injection of IL-1β or LPS significantly decreases BDNF mRNA expression in the rat hippocampus [26]. Furthermore, the oral administration of antimicrobials in specific-pathogen-free mice transiently altered the composition of the microbiota and 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-inflammatory, 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 CO2 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°C. 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, CGCTACTTGGCTGGTTCAG; forward Lactobacillus, GAGGCAGCAGTAGGGAATCTTC; reverse Lactobacillus, GGCCCGTCTGGGTGGGTTTC; forward Bifidobacterium, CGCGTCTGGTGA AAG; reverse Bifidobacterium, CCCCATCCAGCAGCATCA; forward Desulfovibrios, CC GTGATATCTGGAGGAACATCAG; reverse Desulfovibrios, ACATCTAGGATCCCATC 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 - $\Delta Ct$ 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% H₂O₂ 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
Pty. Ltd, Sydney, NSW, Australia) for 30 min at room temperature. The sections were then washed and developed using the ImmPACT DAB peroxidase substrate kit (#4100, Vector laboratories Inc., Burlingame, CA, USA) and counterstained with haematoxylin (POCD Scientific, Artarmon, NSW, Australia). Six fields from three sections of each mouse were viewed under a Leica microscope and digital photographs were captured. Image J software was used to quantify the area of F4/80, CD11c, and CD206 immunoreactivity on each fields. The immunohistochemical staining F4/80, CD11c, and CD206 were quantified as a percentage of positive area per image. The Immunoreactivity is quantified as the % of pixels in an area of interest that have intensity greater than the background using Image J computer software, http://rsb.info.nih.gov/ij/docs/pdfs/examples.pdf.

2.5. Western blotting

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

2.6. Novel object recognition test

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 \textit{Bacteroides-Prevotella} spp, \textit{Lactobacillus} spp., \textit{Bifidobacterium} spp. and \textit{Desulfovibrios} spp. A chronic HF diet significantly altered gut microbiota in the HF group compared to the control group. The amounts of \textit{Bacteroides-Prevotella} spp. and \textit{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 \((HF: 1.02\pm0.12 \text{ EU/ml}, \ Control: 0.64\pm0.11 \text{ EU/ml}, \ p < 0.001)\) (Fig. 2A). The rhein treatment for 6 weeks prevented an increase of plasma LPS induced by the HF diet \((HF: 1.02\pm0.12 \text{ EU/ml}, \ HF+R: 0.64\pm0.13 \text{ EU/ml}, \ p < 0.001)\). There was no difference between the control and HF+R groups in the plasma LPS concentrations \((p = 1.000)\).
3.3. Rhein reduced M1 macrophage accumulation in the colon of HF diet-induced obese mice

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

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

To investigate whether endotoxemia could be related to neuroinflammation, we examined the TLR4-MyD88-NFκB/JNK signaling pathway and pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in the perirhinal cortex, an important brain region for recognition memory. Western blotting revealed that the TLR4 and MyD88 level in the perirhinal cortex was significantly higher in the HF group than the control group \( (p = 0.049, \ p < 0.001) \), while the TLR4 and MyD88 level was significantly decreased in the HF+R group (rhein treatment group) compared to the HF group \( (p = 0.038, \ p = 0.001) \) (Fig. 3). There was no statistical
difference in the TLR4 and MyD88 level between the HF+R group and the control group (both $p > 0.005$). Furthermore, the NFκB and p-JNK level was significantly higher in the HF group compared to the control group ($p = 0.047$, $p = 0.008$), while the rhein treatment prevented an increase of NFκB and p-JNK ($p = 0.036$, $p = 0.003$) (Fig. 3). The pro-inflammatory cytokine IL-1, IL-6, and TNF-α levels were significantly higher in the HF group compared to the control group ($p = 0.025$, $p = 0.011$, $p = 0.011$) (Fig. 3). The IL-1 and IL-6 levels were significantly lower in the HF+R group than the HF group ($p = 0.032$; $p = 0.006$). However, the rhein treatment did not significantly decrease the TNF-α level.

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

To assess whether rhein treatment can prevent HF diet-induced recognition memory deficits, we performed a novel object recognition test in mice fed a HF diet and given rhein treatment. During the training session of the test, the percentage of time spent exploring the identical objects in the open-field was not significantly different among the control group (18.06%), the HF group (17.05%), and the HF+R group (16.50%). One day after the training session, all mice were presented with the familiar object and a new object. The exploration time on the novel object of the HF group was significantly decreased compared to the control mice (HF: 26.43 ± 3.78 seconds, Control: 31.13±2.98 seconds, $p = 0.023$) (Fig. 4A), suggesting that the HF diet significantly impaired novel object recognition performance. However, the rhein treatment increased novel object exploration time in the HF mice (HF+R: 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: $22.82 \pm 1.73$ g, HF: $23.06 \pm 1.73$ g, $p > 0.05$) (Fig.
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 Bacteroidetes account for more than 90% of the total gut microbiota [48]. The 8-week HF diet increased Firmicutes and reduced Bacteroidetes. 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 Bacteroidetes 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.
Furthermore, rhein has been considered as an antibacterial agent against *Staphylococcus aureus* [37]. A synergistic or partial synergistic effect of rhein in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus* has also been demonstrated [51]. In the present study, we found that rhein attenuated the HF diet-induced alteration of gut microbiota. Furthermore, rhein administration prevented the elevation of HF diet-induced plasma LPS. LPS is a major component of the outer membrane in Gram-negative bacteria. The plasma LPS level was closely correlated with changes in intestinal microbiota, especially the numbers of the Gram-negative Bacteroides-like intestinal bacteria which reside within the Bacteroidetes phylum and were significantly reduced in animals fed the HF diet [14]. The elevated plasma LPS is related to the over-production of LPS in the gut by the death of Gram-negative bacteria and their translocation into the bloodstream via an increased intestinal permeability in HF diet-induced obesity [52]. In the present study, we found that a chronic HF diet decreased *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp. DNA levels, which belong to the Gram-negative bacterium Bacteroidetes and Proteobacteria respectively. A deceased Gram-negative bacterium releases LPS which leads to an increased plasma LPS level in obese mice induced by a HF diet. Furthermore, in the present study, the rhein treatment prevented the decrease of Gram-negative bacterium (including *Bacteroides-Prevotella* spp. and *Desulfovibrios* spp.) induced by a HF diet. These data strongly suggest that rhein affects the intestinal microbiota and is responsible for the attenuation of metabolic endotoxemia in HF diet-induced obesity.

LPS induce macrophage activation and accumulation [53]. M1 macrophages produce 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
the HF diet-induced decrease in the BDNF level in the perirhinal cortex in mice. Several
rodent studies have demonstrated that inflammation affects the expression of BDNF within
the brain. For example, after an intraperitoneal injection of IL-1β or LPS, the BDNF mRNA
expression was significantly decreased in the rat hippocampus [26]. A similar reduction of
BDNF at the protein level has also been observed in cortical regions [59]. The negative
impact of inflammation has important implications for pathophysiology such as the decline of
cognitive function. For example, pro-inflammatory cytokines compromise general memory
[60] and spatial memory [61], and increase apoptosis in the brain [62]. Individuals with
obesity or diabetes with low grade inflammation have an increased risk of cognitive decline
[63]. Therefore, the chronic consumption of rhein may lower the neuroinflammation and
increase BDNF in the perirhinal cortex and thus improve recognition memory in HF
diet-induced obesity.

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

Acknowledgements

The authors wish to thank the following individuals for their contributions. A/Prof. K. Russell (Department of Applied Statistics, University of Wollongong) for his suggestions regarding the statistical analysis, Ms. Linda Cohen and A/Prof. Ronald Sluyter for their editorial revision of the manuscript. This study was supported by Diabetes Australia Research Trust Research Projects to Prof XF Huang. Y.H.Y. is supported by the National Health and Medical Research Council of Australia (NHMRC 573441).

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**Figures**

![Graph showing DNA expressions](image-url)

**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)  

![Graph showing Plasma LPS levels for Con, HF, and HF+R groups.](image)

B.  

Con  

HF  

HF+R  

**F4/80**  

![Images of F4/80 staining for Con, HF, and HF+R groups.](image)

**CD11c**  

![Images of CD11c staining for Con, HF, and HF+R groups.](image)

**CD206**  

![Images of CD206 staining for Con, HF, and HF+R groups.](image)

C.  

**F4/80 area of field of view (%)**  

![Graph showing F4/80 area for Con, HF, and HF+R groups.](image)

**CD11c area of field of view (%)**  

![Graph showing CD11c area for Con, HF, and HF+R groups.](image)

**CD206 area of field of view (%)**  

![Graph showing CD206 area for Con, HF, and HF+R groups.](image)
**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)**

![Object exploration time graph](image)

**B. Discrimination index**

![Discrimination index graph](image)

**C. Relative density to Con group (%)**

![Relative density graph](image)

**D. BDNF (%Control) in PRC**

![BDNF graph](image)

**E. Plasma LPS (EU/ml)**

![Plasma LPS graph](image)

**F. Correlation**

$r = 0.622$

$r = -0.705$
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.