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Berberine treatment increases *Akkermansia* in the gut and improves high-fat diet-induced atherosclerosis in *Apoe*$^{-/-}$ mice

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ABSTRACT

Background and aims: Gut microbiota plays a major role in metabolic disorders. Berberine is used to treat obesity, diabetes and atherosclerosis. The mechanism underlying the role of berberine in modulating metabolic disorders is not fully clear because berberine has poor oral bioavailability. Thus, we evaluated whether the antiatherosclerotic effect of berberine is related to alterations in gut microbial structure and if so, whether specific bacterial taxa contribute to the beneficial effects of berberine.
Methods: *Apoe*$^{-/-}$ mice were fed either a normal-chow diet or a high-fat diet (HFD). Berberine was administered to mice in drinking water (0.5 g/L) for 14 weeks. Gut microbiota profiles were established by high throughput sequencing of the V3–V4 region of the bacterial 16S ribosomal RNA gene. The effects of berberine on metabolic endotoxemia, tissue inflammation and gut barrier integrity were also investigated.
Results: Berberine treatment significantly reduced atherosclerosis in HFD-fed mice. *Akkermansia* spp. abundance was markedly increased in HFD-fed mice treated with berberine. Moreover, berberine decreased HFD-induced metabolic endotoxemia and lowered arterial and intestinal expression of proinflammatory cytokines and chemokines. Berberine treatment increased intestinal expression of tight junction proteins and the thickness of the colonic mucus layer, which are related to restoration of gut barrier integrity in HFD-fed mice.
Conclusions: Modulation of gut microbiota, specifically an increase in the abundance of *Akkermansia*, may contribute to the antiatherosclerotic and metabolic protective effects of berberine, which is poorly absorbed orally. Our findings therefore support the therapeutic value of gut microbiota manipulation in treating atherosclerosis.

1. Introduction

Atherosclerosis causes coronary heart disease (CHD), which is the leading cause of death worldwide. Despite the widespread use of statin-based lipid-lowering therapies, percutaneous coronary intervention, and risk factor modification, which has decreased CHD mortality rates, the global burden of this disease remains high [1]. Identifying other environmental risk factors and novel therapeutic strategies in CHD, therefore, remains a topic of interest.

Gut microbiota is an important environmental factor that interacts and co-evolves with its host [2,3]. Emerging evidence highlights the role of gut microbiota (microbiome) in obesity [2,4,5], type 2 diabetes [6,7], hypercholesterolemia [8] and metabolic syndrome [9–11]. These disorders are all related to CHD and recently trimethylamine-N-oxide (TMAO), an intestinal microbiota-derived metabolite (derived from choline, phosphatidylcholine and L-carnitine), has been identified to induce atherosclerosis [12,13]. In addition, elevated plasma TMAO levels are associated with increased risk of adverse cardiovascular events [13,14].

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Moreover, atherosclerotic heart disease is not only a lipid disorder but also a chronic inflammatory disease [15]. The formation and rupture of atherosclerotic plaque is related to higher levels of gut microbiota-derived lipopolysaccharide (LPS) and inflammatory cytokines in the circulation [15,16]. Studies indicate that high fat diet (HFD) elevates plasma LPS levels by altering gut microbiota composition, inducing intestinal barrier dysfunction and increasing gut permeability [17–19]. LPS embedded in gut enterocyte-derived chylomicrons pass through the intestinal barrier into the bloodstream where they bind toll-like receptors. LPS initiates the proinflammatory response of the host innate immune system, which results in endothelial dysfunction, atherosclerotic plaque formation and rupture [20,21]. Furthermore, systemic chronic low grade LPS-derived inflammation promotes insulin resistance, impaired glucose tolerance and obesity, which is defined as metabolic endotoxemia [17,22]. These findings suggest that HFD-induced atherosclerosis could be treated by targeting gut microbiota-induced chronic inflammation and intestinal barrier dysfunction.

Berberine is extracted from *Coptis chinensis*, which in Chinese medicine is traditionally used to treat bacterial diarrhea. Berberine is also used to treat obesity, diabetes, nonalcoholic fatty liver disease and atherosclerosis [23–27]. The beneficial metabolic and anti-atherosclerotic effects of berberine are mainly mediated by AMP-activated protein kinase (AMPK) activation [23,26,28]. Moreover, unlike statins, berberine reduces cholesterol levels by upregulating hepatic low-density lipoprotein receptor expression [29]. Nevertheless, it is still difficult to fully explain the clinical metabolic effects of berberine given its poor oral bioavailability [30,31]. Accordingly, modulation of gut microbiota by berberine has been proposed as one of the mechanisms during its treatment in metabolic disorders [30,32].

However, whether or not gut microbiota modifies the anti-atherosclerotic efficacy of berberine has not been studied to date. We therefore assessed the preventive effect of berberine on HFD-induced atherosclerosis in *Apoe*$^{-/-}$ mice and the contribution of the gut microbiota. Our results indicate that berberine reduces the development of atherosclerosis by manipulating the gut microbiota.

2. Materials and methods

2.1. Animal model

All animal experimental procedures were performed in accordance with the institutional ethics committee regulations and guidelines on animal welfare and were approved by the Institutional Animal Care and Use Committee (IACUC) office of Fudan University (Approval Number: 20161041C005). Five-week-old female *Apoe*$^{-/-}$ mice on a C57BL background (SPF grade) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. [Beijing, China, certified No. SCXK (Jing) 2011-0011]. Animals were housed in a controlled environment (temperature of 22 ± 2 °C and a 12 h day/night cycle) with free access to food and water. All mice were fed a normal-chow diet (#TP26338, TROPHIC Animal Feed High-Tech Co., Ltd. Nantong, China) during their one-week acclimatization period and then divided into 4 groups as follows: (1) normal-chow diet (NCD) without berberine treatment (NC group), (2) normal-chow diet with berberine treatment (NC-BBR group), (3) HFD [containing 0.2% (wt/wt) cholesterol, 42% fat (kcal/100 g), #TP26303, TROPHIC Animal Feed High-Tech Co., Ltd. Nantong, China] without berberine treatment (HF group) or (4) HFD with berberine treatment (HF-BBR group). Berberine (Berberine chloride hydrate), obtained from Alfa Aesar (China) chemical Co., Ltd., was added to drinking water (0.5 g/L) during the 14-week intervention period. Food intake was recorded and calculated as described previously [11]. Mean values for the weekly assessment were reported. Mouse body weight was monitored every two weeks. Mice were fasted for 8 h before anesthesia by intraperitoneal injection of 5% chloral hydrate (0.1 ml/10 g). Blood was obtained by retro-orbital bleeding into microfuge tubes and centrifuged at 4000 rpm for 15 min at 4 °C, and serum was collected and frozen at −80 °C until biochemical analysis.

2.2. Analysis of atherosclerotic lesions

After collection of blood samples, the circulatory system was rinsed with phosphate-buffered saline (PBS) and then fixed with PBS containing 4% paraformaldehyde. The heart and aortic tissues were then removed from the aortic root to the iliac bifurcation and placed in 4% paraformaldehyde for 6 h. To evaluate atherosclerotic lesion areas, the aorta was cut longitudinally to expose the intimal surface, and the plaque-containing area was visualized by fat staining (Oil Red O). Lesions in the aortic area were quantified from the aortic arch to the iliac bifurcation (excluding the brachiocephalic trunk, the left common carotid artery, the left subclavian artery and other branch vessels). The aortic arch was defined as ranging from the beginning of the ascending arch to 3 mm distally of the left subclavian artery. The heart with 2 mm of proximal aorta was embedded in paraffin and cut to 4 μm sections for hematoxylin and eosin staining for analysis of atherosclerotic lesion size in the aortic sinus. The lesion area and size were quantified using Image J software by a blinded observer.

2.3. Measurements of serum lipid profiles and inflammation levels

Serum levels of cholesterol, triglycerides and high density lipoprotein-cholesterol (HDL-C) were determined using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Serum LPS concentration was determined using a homogeneous fluorescence end-point assay kit (Hyglos GmbH, Bernried, Germany) based on an improved limulus recombinant Factor C. Inflammatory molecules, including tumor necrosis factor (TNF)-α, were measured by immunoassays from Anogen (Ontario, Canada). Interleukin (IL)-1β levels were measured by immunoassay from R&D Systems (Minneapolis, MN, USA). All experimental procedures were carried out according to the manufacturer's instructions.

2.4. Quantitative real-time PCR

Individual ileal, colonic, or carotid tissue samples (10–20 mg) were homogenized with grinding pestles and buffer RL (TIANGEN BIOTECH CO., LTD, Beijing, China) using the TGrinder Electric Tissue Grinder (TIANGEN, Beijing, China). Total RNA was isolated according to the manufacturer's instructions (RNAprep Pure Tissue Kit, TIANGEN). Complementary DNA (cDNA) was prepared using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) from 1 μg of total RNA. RT-qPCR conditions were: 30 s of predenaturation at 95 °C followed by 40 cycles of two-step PCR denaturation at 95 °C for 5 s and annealing extension at 60 °C for 34 s; PCR reactions were performed on an ABI 7500 Real-Time PCR System. The PCR mixture contained 50–100 ng cDNA, 10 μL of SYBR Premix Ex Taq II (TaKaRa), 0.8 μL of forward and reverse primers (10 μM) respectively, and 0.4 μL of ROX II (TaKaRa) in a final volume of 20 μL. The same reaction was performed in triplicate, and the data were analyzed using the $2^{-\Delta\Delta Ct}$ method with β-actin serving as an endogenous control gene. The PCR primers are listed in Supplementary Table 1.