

Berberine Down-Regulated Myostatin Expression and Facilitated Metabolism via Smad Pathway in Insulin Resistant Mice

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Objective: To explore the effect of berberine (BBR) on skeletal muscle mass and metabolism and the possible mechanism.

Methods: Eight-week-old male C57BL/6 mice were fed with a high-fat diet (HFD) for 8 weeks to establish the insulin resistance obesity model. Then, mice were randomly divided into two groups (normal chow diet (NCD) and HFD), while NCD and HFD were further classified into two groups respectively, which were NCD+CS (Carmellose Sodium), NCD+BBR, HFD+CS and HFD+BBR. After the BBR intervention, insulin tolerance test (ITT) and glucose tolerance test (GTT) were carried out. Metabolic parameters and inflammatory biomarkers were detected. Various parts of adipose tissue and gastrocnemius were separated and measured. The gastrocnemius muscle was selected for tissue staining. The mRNA expression of myostatin (Mstn) was tested by quantitative real-time PCR (RT-PCR). Western blotting was performed to detect the expression of Mstn, phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), small ribonucleoprotein particle protein 2 (Smd2), small ribonucleoprotein particle protein 3 (Smd3), and small ribonucleoprotein particle protein 4 (Smd4).

Results: Both body weights ($P<0.01$) and various parts of fat mass ($P<0.001$) were decreased significantly, while muscle mass was increased in the HFD group after being treated with BBR. Meanwhile, the glucose and lipid metabolic disorders as well as inflammation status were improved. RT-PCR and Western blotting analysis showed that, after being fed with HFD the expression of Mstn mRNA and Mstn were significantly increased, and decreased after being treated with BBR. Western blotting analysis also showed that, compared with the NCD group, the expressions of Smad2, Smad3, and Smad4 were all increased in the HFD group, but after being treated with BBR, the expressions of Smad3 and Smad4 were decreased.

Conclusion: Our study revealed that BBR could improve metabolic disorders and inflammation status, decrease Mstn expression, and increase skeletal muscle mass, which was associated with the Smad pathway.

Keywords: skeletal muscle, myostatin, berberine, Smad pathway

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Introduction

Sarcopenia, which is a syndrome characterized by age-related progressive decline of skeletal muscle mass and function,¹ has increasing adverse clinical consequences for the development of frailty, disability, falls, even death.²⁻⁴ As skeletal muscle is an important target of insulin and responsible for the majority of the body's glucose disposal, loss of skeletal muscle may produce insulin resistance which will result in

substantial whole-body inflammation and metabolic disturbances. So far there are several studies that have demonstrated the relationship between sarcopenia and insulin resistance and metabolic disorders.^{5–7} Mstn (myostatin), which is one of the TGF- β (transforming growth factor β) superfamily members, is an important inhibitor of skeletal muscle growth and plays a critical role in the development of sarcopenia.^{8,9} A few studies have reported that Mstn can regulate body metabolism as well.^{11,12}

BBR (Berberine) is the principal bioactive ingredient of *Rhizoma coptidis*, a common traditional Chinese medicinal herb. In recent years, more and more studies have shown that BBR can improve insulin sensitivity and metabolic disorders.^{13,14} A few studies reported BBR could up-regulate Glucose transporter 4 (Glut4) expression, increase glucose uptake of skeletal muscle, improved insulin resistance and inflammatory status in skeletal muscle, and increased skeletal muscle fiber quantity,^{15,16} which was just the opposite effect of Mstn.¹² BBR regulates the metabolism in the skeletal muscle by multiple pathways. A study by Yu et al¹⁷ showed that BBR could reduce MAPK (Mitogen-activated protein kinase) activity and increase ATP (adenosine triphosphate) production in skeletal muscle cells by increasing the expression of AMPK (Adenosine 5'-monophosphate (AMP)-activated protein kinase) phosphorylation, SIRT1 (silent information regulator 1), and PGC-1 α (Peroxisome proliferator-activated receptor coactivator-1 α). Another study showed BBR improved skeletal muscle insulin resistance by down-regulating the TLR4/IKK β /NF- κ B, and PPAR α (peroxisome proliferators-activated receptor α) signaling pathways.¹⁸

Both Mstn and BBR were associated with metabolism, however, whether BBR can increase muscle mass or there is a link between Mstn and BBR is still unclear. The aim of this study was to explore the interaction between BBR and Mstn, and the possible mechanism of their influence on metabolism and skeletal muscle mass in an insulin resistant mice model.

Materials and Methods

Animals

Eight-week-old male C57BL/6 mice (20–25 g) were purchased from the laboratory animal center of Nantong University and the protocol was authorized by the IACUC (Institutional Animal Care and Use Committee) of Nantong University. In total, 24 mice were used, and there were six mice of each group. The study was conducted in accordance

with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Human or Animal Subjects of Zhongshan Hospital of Fudan University (No. 2020–058). Mice were kept in an environmentally controlled room (22–25°C, 40–70% humidity, and 12/12 circadian rhythm), with water, and diets were provided ad libitum. After 1-week acclimation, mice were fed with NCD (normal chow diet: carbohydrate 78%, fat 4%, protein 18%, Slaccas, Shanghai, China) or HFD (high-fat-diet: carbohydrate 35%, fat 45%, protein 20%, Research Diet, Mediscience Ltd, USA) for 12 weeks. Then mice were randomly divided into two groups and gavaged daily with either 0.5% CS (Carmellose Sodium) or BBR in CS suspension (Sigma, 200 mg/kg) for another 8 weeks (NCD and HFD) using flexible catheter implantation tubing attached to a cut 22-G needle tip. Then NCD and HFD were further classified into four groups, which were NCD +CS, NCD+BBR, HFD+CS, and HFD+BBR. Body weight and food intake were measured weekly. There were 24 mice in total, and six mice per each sub-group.

Measurement of Plasma Glucose, Lipid and Insulin Levels, and Inflammatory Biomarkers

Six mice in each group were fasted overnight and then were anesthetized. Mice were anesthetized (30 mg/kg of pentobarbital by intraperitoneal injection). Blood (3 mL) was collected through the abdominal aorta, and serum was centrifuged and stored at –80°C for further use. BG (Blood glucose), insulin (Ins), TG (triacylglycerol), TC (total cholesterol), LDL-C (low density lipoprotein cholesterol), and HDL-C (high density lipoprotein cholesterol), FFA (free fatty acid), were measured with an automatic biochemical analyzer. ELISA examination was carried out to detect serum TNF- α (tumor necrosis factor alpha) (MTA00B; R&D Systems, Minneapolis, MN), CRP (C-reactive protein) (MCRP00, R&D Systems, Minneapolis, MN), and Insulin (EZRMI-13K, Millipore, Billerica, MA) based on the instruction of the manufacturer.

Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

All mice were deprived of food overnight (22:00 to 8:00) before being adopted in the glucose tolerance test. After injecting intraperitoneally with D(+)-glucose (Sigma, 2 g/

kg body weight). Blood glucose concentrations were tested at 0, 15, 30, 60, 90, and 120 minutes after glucose injection. For the insulin tolerance test (ITT), all mice were deprived of food from 9:00 to 15:00 h and injected with 0.75 U/kg insulin (Novo Nordisk Pharmaceutical industries, Malov, Denmark) per 1 kg body weight.¹⁹ The blood of the mice were collected via lateral tail vein blood. The Breeze 2 Blood Glucose Meter (Bayer HealthCare LLC, Mishawaka, IN) was used for blood glucose concentration measurement.

Histology of Skeletal Muscle and Adipose Tissue

Histological analysis of skeletal muscle and adipose tissue obtained from the mice post-mortem was performed according to standard laboratory protocols. The weight of inguinal fat as subcutaneous adipose tissue (SAT) was measured. Epididymal fat, mesenteric fat, omentum fat, and perirenal fat were weighed as visceral adipose tissue (VAT). The fat in the shoulder blade area was separated as brown adipose tissue (BAT). Gastrocnemius muscle were separated as skeletal muscle mass (SMM), dried with filter paper, and weighed. A little gastrocnemius muscle tissue was fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 5 μ m were sectioned with a Leica microtome (RM2155, Wetzlar, Germany). Routine hematoxylin and eosin were carried out to stain the sections according to standard procedures. Pictures were collected by microscopy (DP71, Olympus, Japan) and processed with Image J (NIH, 64 bit).

Quantitative RT-PCR

High quality total RNA from the gastrocnemius muscle was isolated by RNAiso plus (9108, TaKaRa, Tokyo, Japan). Primary cDNA was synthesized by the HiScript II Q RT SuperMix with gDNA wiper kit (R223-01, Vazyme, Nanjing, China). Quantitative RT-PCR was performed with the AceQ qPCR SYBR Green Master Mix kit (Q141-02, Vazyme, Nanjing, China) in the sequence detector (ABI7700, Applied Biosystems, Foster City, CA, USA). The primers used for Mstn (KY441464) and 36b4 were as follows: Mstn, 5'-AGAAGATGGGCTGAATCCCT-3' (forward), 5'-GAGTGCTCATCGCAGTCAAG-3' (reverse); 36b4, 5'-GAAACTGCTGCCTCACATCCG-3' (forward), 5'-GCTGGCACAGTGACCTCACACG-3' (reverse). The data were analyzed using the $\Delta\Delta$ CT method of analysis

and expressed as arbitrary units after normalization to the levels of expression of 36b4 for each sample.

Western Blotting

Total protein extracts from the gastrocnemius muscle were homogenized in RIPA-based lysis buffer (30 mM HEPES at pH 7.6, 100 mM NaCl, 0.5% Nonidet P-40, protease inhibitor cocktail from Roche, 1 mM Sodium orthovanadate (Na_3VO_4) and 20 mM Sodium fluoride (NaF)) and incubated on ice for 10 minutes. The whole mixture was followed by centrifugation at 12,000 g for 15 minutes at 4°C, and then was denatured for 10 minutes in a boiled water bath. The concentrations of samples were detected by Enhanced BCA Protein Assay Kit (P0009, Beyotime Biotechnology, Nantong, China). Protein fractions were subsequently separated by different concentrations of SDS-PAGE according to target proteins. After the electrophoresis, proteins were blotted onto the PVDF membranes and incubated with certain antibodies which were diluted with an appropriate ratio overnight. The blots were detected by ECL development after being conjugated with the secondary antibodies. Image J software was used for quantification of labeled Western blots. The gray scale data were collected and quantified by normalizing to GAPDH (ab8245, Abcam). The other primary antibodies were all obtained from Abcam company: Anti-Smad2 (ab40855), Anti-Smad3 (ab40854), Anti-Smad4 (ab40759), Anti-Myostatin (ab71808), and Anti-pan-AKT (ab8805).

Statistical Analysis

Data were shown as means \pm SEM. Statistical differences were determined by one-way and two-way ANOVA with Tukey's multiple comparisons test using SPSS 23.0 to assess statistical differences across multiple conditions. The Student-Newman-Keuls post-hoc test was used to compare the means among specific groups. Statistical significance was set as $P<0.05$.

Results

BBR Reduced Body Weight and Fat Mass, but Increased SMM

After being fed with a HFD, significant increases were noted in the body weights ($P<0.001$), muscle mass ($P<0.01$), and fat mass, including VAT, SAT, and BAT (all $P<0.001$), when compared with their normal fed controls. After being treated with BBR for 8 weeks, both body weight ($P<0.01$) and various parts of fat mass ($P<0.001$)

were decreased significantly, while gastrocnemius muscle mass was increased ($P<0.05$) (Figure 1). HE stain showed that after being fed with a HFD, the number of skeletal muscle fibers decreased, while in those treated with BBR, the number of skeletal muscle fibers increased, which was significant in the NCD group (Figure 2).

BBR Improved Metabolic Disorders and Inflammation Status

When compared with the NCD group, there was remarkable elevation of circulating levels of glucose and lipids in the HFD group, including (FBG, TC, TG, HDL-C, LDL-C, FFA, and insulin as well as inflammatory biomarkers

CRP and TNF- α) (all $P<0.001$). After being treated with BBR, both the glucose and lipid metabolism as well as inflammation status were improved. But all of the above values were still higher than the NCD group, which indicated BBR could only partially improve metabolic disorders and inflammation (Figure 3). Moreover, GTT and ITT indicated that BBR treated mice had an increased glucose clearance rate and improved insulin sensitivity in the HFD group (Figure 4).

BBR Down-Regulated Mstn Expression

When compared with the NCD group, the results showed that both the expression of Mstn mRNA and Mstn protein

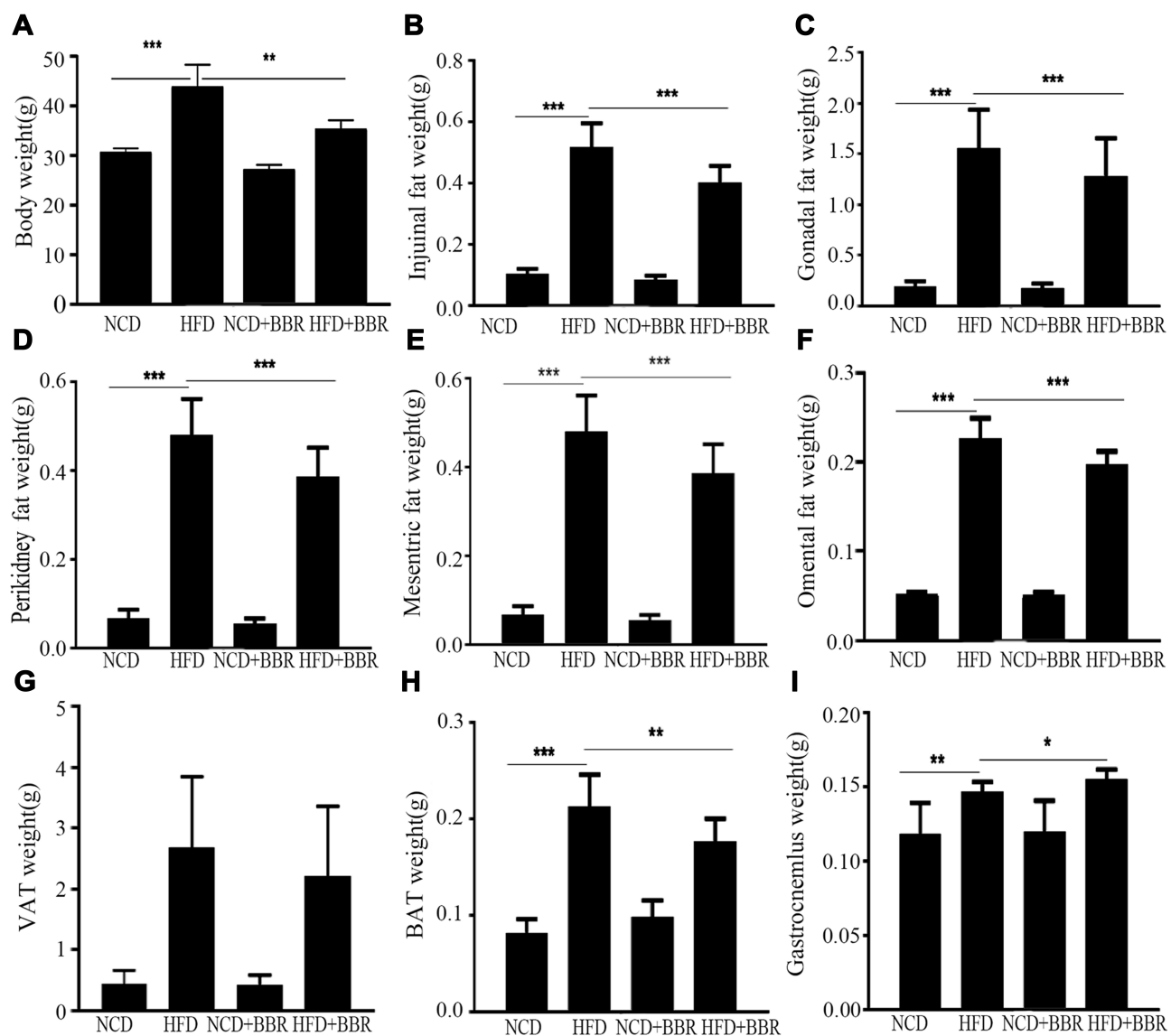


Figure 1 The changes of body weight (A), inguinal fat weight (B), gonadal fat weight (C), peri-kidney fat weight (D), mesenteric fat weight (E), omental fat weight (F), VAT weight (G), SAT weight (H), and gastrocnemius weight (I) among the NCD, HFD, NCD+BBR, and HFD+BBR groups. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

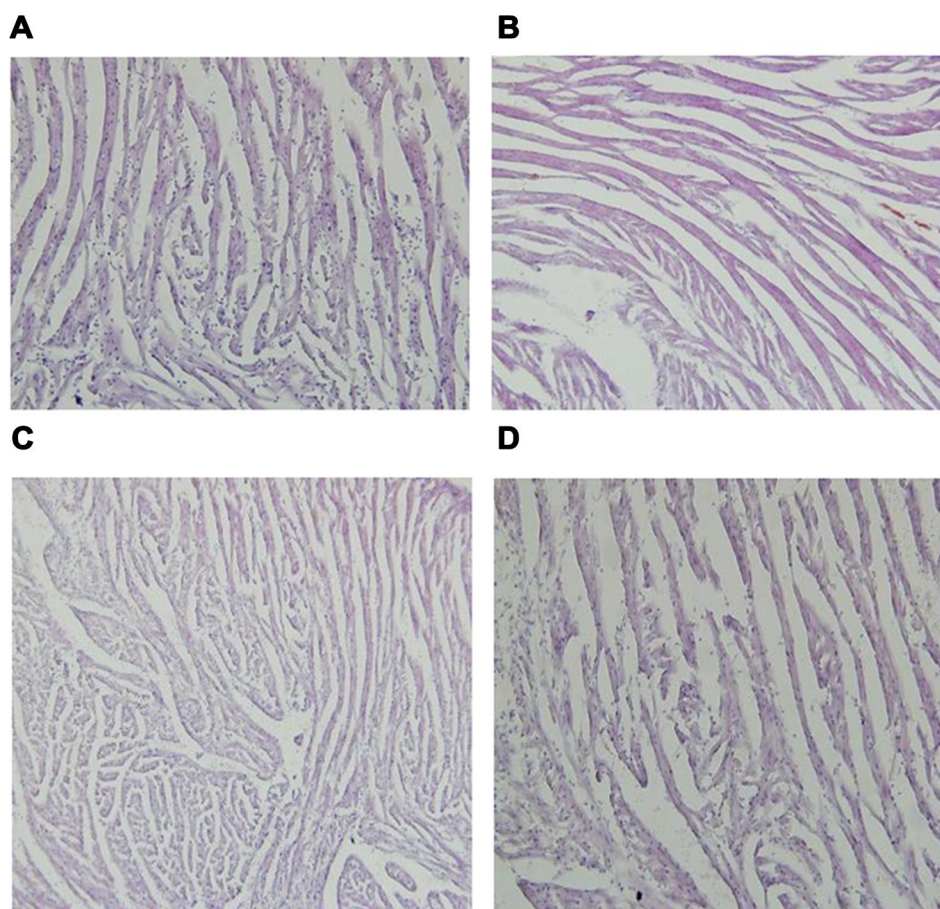


Figure 2 The changes of HE stain of gastrocnemius muscle in NCD (A), HFD (B), NCD+BBR (C), and HFD+BBR (D) groups.

were increased dramatically ($P<0.01$) after being fed with a HFD. After being treated with BBR, the Mstn expression was decreased dramatically, which was presented as Mstn mRNA expression decreased by 48% ($P<0.01$) and Mstn protein decreased by 32% by fold change ($P<0.01$). However, no significant changes were noted in NCD mice (Figure 5).

Smad Pathway Was Inhibited During the Effects of BBR

When fed with HFD, the expression of Smad2, Smad3, and Smad4 were all increased when compared to the NCD group, but no changes of PI3K or Akt were observed. After being treated with BBR, the expression of Smad3 and Smad4 were decreased in the HFD group, and still showed no significant difference of PI3K and Akt, which prompted that the Smad pathway was activated by HFD, and BBR could inhibit it. These results indicated that the Smad pathway was related with BBR, Mstn, and metabolism in skeletal muscle.

Discussion

This study presented that HFD-induced insulin resistance was associated with metabolism homeostasis dysfunction and increased Mstn expression. BBR could down-regulate the expression of Mstn, increase SMM, and facilitate metabolic disorders and inflammation status, which was associated with the Smad pathway. Mstn is a critical inhibitor of skeletal muscle growth and development. Beyond the confines of its traditional role, Mstn has recently been shown to play an important role in the regulation of metabolism.^{11,12} Several studies showed elevated levels of Mstn in muscle have been associated with obesity and insulin resistance.^{11,12,20} Mstn (-/-) mice and treatment of Mstn antagonist exhibited reduced fat accumulation, peripheral insulin resistance, and enhanced glucose disposal,^{21,22} and these were consistent with our results that Mstn increased with adiposity as well as glucose and lipids metabolic disorders in HFD mice, while BBR induced reduction of Mstn was associated with improvement of metabolic disorders and reduced fat accumulation in adipose tissue. A study by Zhu et al²³ showed ectopic fat

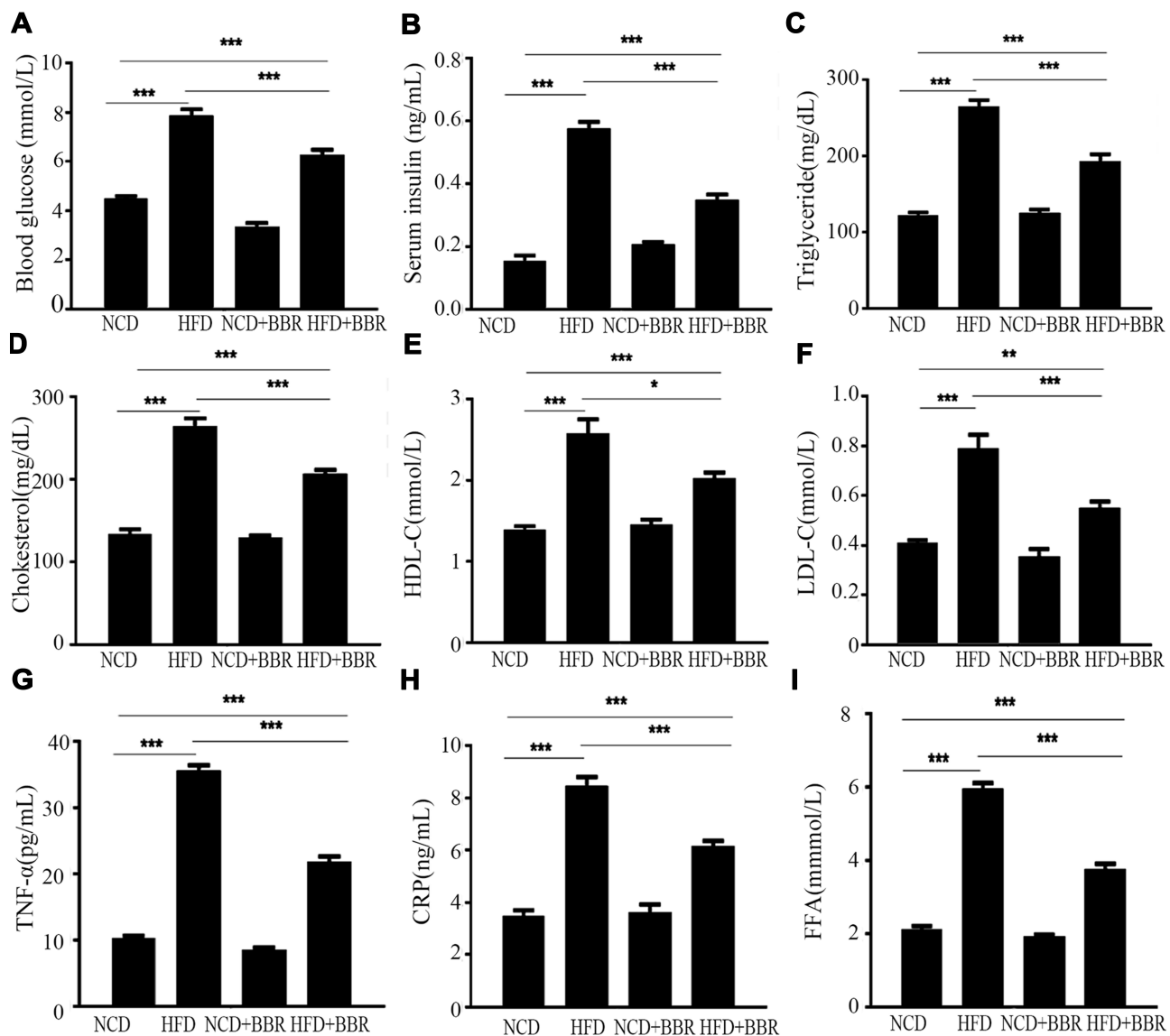


Figure 3 The changes of blood glucose (A), serum insulin (B), triglyceride (C), cholesterol (D), HDL-C (E), LDL-C (F), TNF- α (G), CRP (H), and FFA (I) among the NCD, HFD, NCD+BBR, and HFD+BBR groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

deposition including accumulation of peri-muscular adipose tissue could accelerate age- and obesity-induced muscle atrophy. The gastrocnemius muscle weight increase with ascending Mstn in the HFD group might be due to the ectopic fat deposition in muscle, which can be examined in our further research. Prolonged high caloric intake leads to metabolic overload and insulin resistance, resulting in macrophage accumulation and leading to an increased inflammatory response.^{24,25} The overproduced pro-inflammatory cytokines, such as TNF- α and other inflammatory molecules including FFA, and CRP, resulted in further requirement of macrophages and impaired fat oxidation and synthesis of glycogen and protein.^{25,26} The above is also one of the

pathogenesis related with sarcopenia.²⁷ Therefore, the treatment of BBR could improve inflammation which partly contributed to facilitating metabolic dysfunction and increased muscle mass. When compared with the NCD group, there was remarkable elevation of circulating levels of glucose and lipids in the HFD group, including FBG, TC, TG, HDL-C, LDL-C, and FFA. However, the results of HDL-C were controversial, as it should be inversely proportional to other lipid profile biomarkers. It should be that TG, TC, and LDL-C were increased, but HDL-C was decreased. This needed to be further evaluated.

Mstn, one of the TGF β superfamily members, can activate several signal pathways, but the Smad pathway is

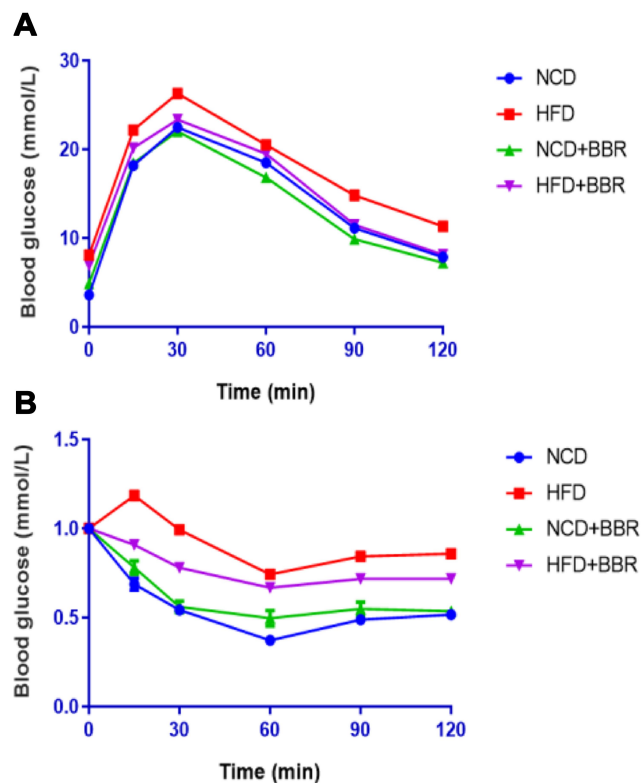


Figure 4 GTT (A) and ITT (B) results of NCD, HFD, NCD+BBR, and HFD+BBR groups at different times.

considered to be the most significant. Mstn binds ActRIIB (activin type IIB receptor) to activate downstream signals, ultimately resulting in Smad2/3 phosphorylation and translocation to the nucleus to modulate the transcription of numerous genes together with the co-mediator Smad4.^{27,28} The present study showed that the expression of Smad3 and Smad4 increased with Mstn when fed with HFD, while treatment with BBR down-regulated the expression of Smad3 and Smad4 with Mstn expression decreasing, which was similar to the studies by Watts et al²⁸, and Fuentes et al.²⁹, and Fuentes et al. FuentesFuentes As the PI3K/AKT pathway is importantly involved in insulin-stimulated glucose uptake, we further tested whether Mstn modulated metabolism interacted with the insulin pathway. The result presented no significant difference of the expression of PI3K and AKT between groups, which was inconsistent with previous studies.³⁰ However, the study by Liu et al¹² used C2C12 cells to explore perturbations in insulin induced intracellular signaling of insulin action, and the study by Retamales et al³⁰ investigated crosstalk between the IGF-1 and MSTN signaling pathways were different. Both these two studies applied interventions directly involved in insulin pathway,

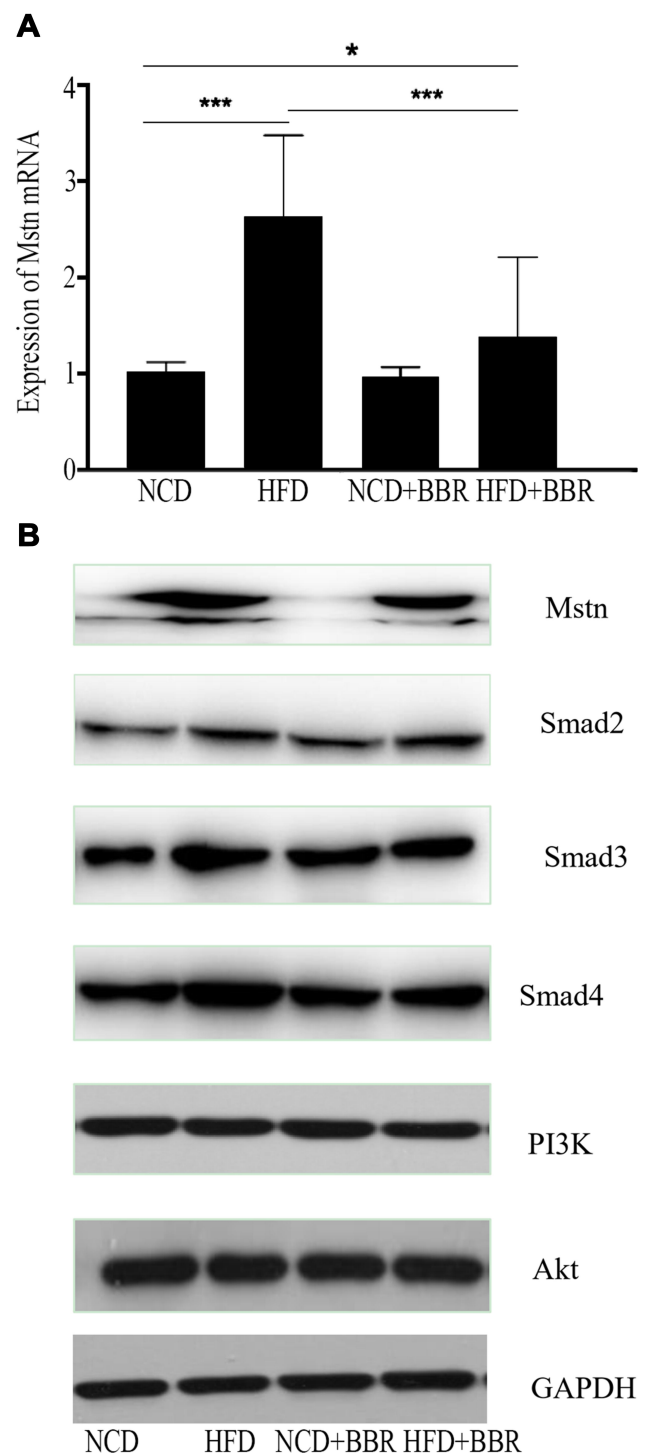


Figure 5 The changes of expression of Mstn mRNA among NCD, HFD, NCD+BBR, and HFD+BBR groups (A). The Western blot results of Mstn, Smad pathway, and PI3K/Akt pathway NCD, HFD, NCD+BBR, and HFD+BBR groups (B). * $P < 0.05$; *** $P < 0.001$.

and did not use BBR for intervention, so there is no comparability between them. The results indicated that up-regulated Smad signaling accounted for at least a proportion of adiposity and Mstn related metabolic

disorders, and BBR can facilitate muscle metabolism and increased muscle mass at least partly by the Smad pathway, which was insulin independent.

Our study first discovered the relationship between BBR and Mstn, put forward the possible mechanism, and provided a new direction for the treatment of sarcopenia and metabolic disorders. Further study should be carried out to examine the role of Smad pathway in the relationship between BBR and metabolism as well as Mstn. Whilst it is unlikely that Mstn alone are implicated in the atrophic effects on skeletal muscle, subsequent myogenic factors such as MyoD and myogenin attributed to myogenesis should also be considered. Since many other molecules such as AMPK are critical in insulin action and metabolism, further experiments need to be performed to examine whether BBR and Mstn may regulate the metabolism by other pathways involved in the insulin effect.

Conclusion

Our findings indicated that high caloric intake induced metabolic disorders was associated with Mstn over-expression, and the treatment with BBR could improve metabolic disorders, down-regulate Mstn expression, and increase skeletal muscle mass, which was associated with inhibiting the Smad signaling pathway.

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Disclosure

The authors report no conflicts of interest in this work.

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