Capsaicin Improves The Hypoglycemic Effect of Metformin Benefitted From Gut Microbiota

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Research

Keywords: Capsaicin, Gut microbiota, Metformin, Hypoglycemic effect, Akkermansia

Posted Date: September 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-882559/v1>

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Abstract

Background: Capsaicin(CAP) possesses a hypoglycemic activity through remodulating gut microbiota as well as the first-line oral hypoglycemic drug, metformin(MET). However, CAP on the hypoglycemic effect of MET pertinent to gut microbiota remained poorly known.

Methods: The glucose and insulin tolerance of diabetic rats were monitored. The glycolipid metabolism were analyzed by detecting blood biochemical parameters. Liver pathological changes were observed by HE staining. The inflammatory cytokines and intestinal tight junction proteins were detected by RT-PCR and Western blot. 16S rRNA sequencing was employed to analyze gut microbiota.

Results: Compared with MET monotherapy, CAP and MET co-treatment could significantly reduce fasting blood glucose, improve glucose tolerance, lessen the degree of liver injury and inflammatory infiltration, reduce the expression of inflammatory cytokines and increase the expression of intestinal tight junction proteins in diabetic rats. Moreover, CAP and MET co-treatment altered gut microbiota profiles by regulating microbial abundances such as *Akkermansia*.

Conclusion: CAP showed significant hypoglycemic effect of MET benefitted from remodulating gut microbiota in diabetic rats.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic and complex metabolic disease, which has become one of the greatest threats to global health[1]. T2DM mainly involves in hyperglycemia, dyslipidemia, islet β -cell dysfunction and insulin resistance[1]. Metformin (MET), as a first-line oral hypoglycemic agent, is able to inhibit hepatic glucose production and intestinal glucose absorption in the proximal small intestine. Importantly, MET could restore gut microbiota dysbiosis associated with T2DM[15, 16]. Moreover, the interplay between MET and gut microbiota could lead to individual hypoglycemic discrimination of MET[17]. Therefore, it may be useful to improve hypoglycemic effect of MET by remodulating gut microbiota.

Diet is the most important factor in modulating gut microbiota. It indicates that spicy food preferred regional distribution is negatively correlated with diabetes prevalence in China [9]. As the main irritant component in pepper, dietary capsaicin (CAP) intake will result in diversity variations of gut microbiota, which has various beneficial metabolic properties, such as antioxidant, anti-inflammatory, anti-obesity and cardiovascular protection[10, 11]. CAP protects against high-fat diet (HFD)-induced metabolism disorder and low-grade inflammation through reshaping gut microbiota, such as increasing the abundance of *Akkermansia muciniphila*[13, 14]. Excitedly, it is recently reported that MET exerts hypoglycemic effect via increasing the abundance of *A. muciniphila*. Therefore, we hypothesized that long-term intake of CAP had better hypoglycemic effect of MET through remodulating gut microbiota.

In this study, CAP and MET co-treatment could significantly reduce fasting blood glucose and improve impaired glucose tolerance in diabetic rats, which showed stronger hypoglycemic effects than MET monotherapy. Combination therapy could lessen the degree of liver injury and inflammatory infiltration, reduce the expression of inflammatory cytokines and increase the expression of intestinal tight junction proteins in diabetic rats. The relative abundances of *Akkermansia*, as well as other gut beneficial bacterial, were significantly increased in diabetic rats, which was negatively correlated with fasting insulin (FINS), plasma lipopolysaccharide (LPS) and interleukin-1 β (IL-1 β). CAP showed significantly hypoglycemic effect of MET by remodulating gut microbiota in diabetic rats.

Methods

Animals and T2DM model

Forty-five male SD rats (140 $\pm$ 20 g, SPF grade) were purchased from Hunan SLYK Jingda experimental animal Co., Ltd. All animal experiments were approved by the laboratory animal management and ethics committee of Central South University. The rats were housed under the condition at constant temperature (24 $\pm$ 1 °C) and humidity (55 $\pm$ 5%). After 7 days for adaptive feeding, SD rats were randomly divided into 5 groups: high-fat CAP group (CAP(H), n=12), high-fat control group (V(H), n=11), normal CAP group (CAP(N), n=10), normal vehicle group (V(N), n=12). CAP was administered intragastrically in soybean oil. CAP(H) and V(H) groups were fed with 34.5% high-fat diet with/without CAP (12.5 mg/kg/day), respectively, while CAP(N) and V(N) groups were fed with normal chow diet with CAP and soybean oil, respectively (Fig. 1). T2DM model was established by intraperitoneal injection of STZ (35 mg/kg) in high-fat fed rats (CAP(H) and V(H) group). Fasting blood glucose $\geq$ 11.1 mmol/L and random blood glucose $\geq$ 16.7 mmol/L were considered as a diabetic state.

Experiment design

The experimental animals were randomly divided into 8 groups: CAP combined with MET group (CAP-MET(H), n=6), CAP monotherapy group (CAP(H), n=6), MET monotherapy group (MET(H), n=6), model control group (MODEL, n=5), CAP combined with MET normal group (CAP-MET(N), n=5), CAP monotherapy normal group (CAP(N), n=5), CAP monotherapy normal group (MET(N), n=6), normal control group (CON, n=6), respectively. MET was administered in 0.9% normal saline by gavage (200 mg/kg/day). The rats were fed for 8 weeks. Body weight (BW) and fasting body weight (FBW) were measured weekly.

Oral glucose tolerance test and Intraperitoneal insulin tolerance test

After fasting for 16 hours, SD rats were administered with glucose solution (0.9% normal saline) by gavage at the dose of 2 g/kg. Blood glucose was detected at 0, 15, 30, 60, 120 and 180 minutes after glucose loading. In 8 weeks, the rats were fasted for 4 hours and injected with intraperitoneal insulin solution at the dose of 0.6 IU/kg. Blood samples were collected from the tail vein to detect blood glucose by Roche dynamic blood glucose analyzer.

Blood glucose and insulin resistance index

Random blood glucose (RBG) and fasting blood glucose (FBG) were measured once a week. The insulin resistance index was calculated according to the formula $\text{HOMA-IR} = \text{fasting blood glucose (FBG, mmol/L)} \times \text{fasting insulin (fins, } \mu\text{IU/ml)} / 22.5$.

Samples collection

Fresh fecal samples were collected and stored in liquid nitrogen tank. Rats were sacrificed under anesthesia with a combination of Zoletil and Rompun (1:1, v/v). The blood samples were placed at room temperature for 30 minutes, then centrifuged at 3000 r/min, 4 °C for 20 min. The serum and plasma samples were collected and stored in -80 °C refrigerator. The liver, epididymal fat, jejunum and ileum tissue were quickly excised and weighed, and then stored in liquid nitrogen tank.

Hematoxylin eosin staining

A piece of liver tissue was placed in 4% paraformaldehyde fixative and stored at room temperature. Hematoxylin eosin staining of liver tissues were completed by Wuhan Seville Biotechnology Co., Ltd.

Detection of biochemical indexes

Blood biochemical indexes, including low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG) and alanine aminotransferase (ALT), were detected by automatic biochemical analyzer in Wuhan Seville Biotechnology Co., Ltd. Serum insulin (FINS), plasma lipopolysaccharide (LPS) and inflammatory cytokines (TNF- α , IL-1 β and IL-6) levels were detected by ELISA kits.

RNA extraction and real-time fluorescence quantitative analysis

Total RNA was extracted using Trizol Reagent. 500 ng of total RNA was reverse-transcribed using the PrimeScript RT Master Mix to produce cDNA. SYBR Green qPCR SuperMix was used to perform qPCR. Real-time PCR was carried out in LightCycler 480 using the forward and reverse primers shown in Table S1. PCR programming was set as pre-denaturation at 95 °C for 30s, denaturation at 95 °C for 5s, and annealing at 60 °C for 30s in 45 cycles. Data were calculated by $2^{-\Delta\Delta CT}$ method with β -actin as internal reference.

Western blotting

Tissue samples were crushed and incubated in RIPA lysis buffer to produce lysate. Protein concentrations were determined using a BCA kit (Beyotime Institute of Biotechnology, Jiangsu, China). Total protein (20 ng per sample) was separated using SDS-PAGE on a 10% polyacrylamide gel and then electrophoretically transferred onto a polyvinylidene difluoride membrane. The membranes were cut and incubated with the following specific primary antibodies, rabbit anti-mouse Occludin (1:1000, Abcam, USA) and rabbit anti-

mouse β -actin (1:10000, Proteintech, USA). Bands were detected using an ECL chemiluminescence system after treated with secondary antibodies.

16S rRNA sequencing

The 16S rRNA sequencing was completed by Beijing Boao Jingdian Biotechnology Co., Ltd. The effective tags were selected from the original data, which were analyzed by OTU cluster analysis. The α -diversity was evaluated by calculating the ACE, Chao1, Shannon and Simpson indexes. According to the distance matrix, the β -diversity was evaluated and compared among different groups by principal component analysis (PCA) and principal coordinate analysis (PCoA: binary jaccard distance algorithm). The marker species were selected by LefSe analysis, while the linear discriminant analysis (LDA) value of significant difference was set as 4.0. KEGG database and PICRUSt software were used to predict the function of fecal microbiota.

Statistical analysis

Graphpad prism 7.0 software was used for analysis and mapping. The experimental data were expressed as mean $\pm$ SEM. T test and one-way ANOVA or two-way ANOVA combined with LSD multiple test were used for statistically analysis. Spearman correlation analysis was performed on R software. Welch's t test and Benjamin Hochberg multiple test were used for comparison between two groups. $p < 0.05$ was considered statistically significant.

Results

CAP and MET co-treatment reduced blood glucose levels

FBW showed significant differences between diabetic and normal groups (Fig. 2A), but not among diabetic groups. Combination therapy ($p < 0.0001$) and MET ($p < 0.01$) could control FBW (Fig. 2C). FBG in the treated groups was lower than model group (Fig. 2B), while FBG of CAP-MET(H) group ($p < 0.0001$) decreased by comparing with model group, as well as CAP(H) ($p < 0.05$) and MET(H) groups ($p < 0.01$) (Fig. 2D).

CAP and MET co-treatment improved glucose tolerance and insulin resistance

0w-AUC for OGTT test increased in model group (Figure S1). As shown in Fig. 3A, 8w-AUC ($p < 0.001$) of CAP-MET(H) group decreased by comparing with model group, as well as 8w-AUC ($p = 0.052$) of MET(H) group. 8w-AUC value of the combination therapy group was lower than CAP ($p < 0.01$) and MET monotherapy group ($p < 0.05$), respectively.

As shown in Fig. 3B, IPITT- AUC of each group (CAP-MET(H) ($p < 0.01$), CAP(H) ($p < 0.05$) and MET(H) ($p < 0.05$)) was lower than model group. The HOMA-IR values decreased in CAP-MET(H) ($p = 0.001$), CAP(H) ($p < 0.05$) and MET(H) groups ($p = 0.004$) (Fig. 3C). However, the FINS values showed no significant difference among each group (Fig. 3D).

CAP and MET co-treatment alleviated hyperlipidemic and liver injury

The contents of serum TG, TC and LDL-C in treatment groups showed a downward trend (Fig. 4A). CAP and MET co-treatment reduced the levels of serum TC ($p = 0.063$) and LDL-C ($p < 0.05$) in diabetic rats. The HE staining of liver tissue were presented in Fig. 4B. The pathological damage of liver was observed in model group with serious expansion of hepatic sinusoid and obvious increase of inflammatory infiltrating cells. The liver damage in treatment groups was alleviated, while the effects of combination therapy were much better than monotherapy.

CAP and MET co-treatment attenuated inflammation and improved intestinal integrity

The levels of plasma LPS, IL-1 β , IL-6 and TNF- α in treatment groups were lower than model group. CAP and MET co-treatment could significantly decrease the contents of LPS ($p < 0.001$), IL-1 β ($p < 0.001$) and TNF- α ($p < 0.05$). LPS level in CAP-MET(H) group were also much lower than CAP(H) group ($p < 0.001$) and MET(H) group ($p < 0.05$), respectively. Additionally, the gene expression of inflammation-related molecules in liver (Fig. 5B), fat (Fig. 5C) and intestinal tissues (Fig. 5D-E) was determined. In liver, the mRNA expression of TLR4 ($p < 0.001$), NF- κ B ($p < 0.05$) and IL-1 β ($p < 0.01$) in CAP-MET(H) group was lower than model group, while the combination therapy could decrease the expression of TLR4 ($p < 0.05$) and IL-1 β ($p < 0.05$) by comparing with MET monotherapy. In epididymal fat, the mRNA expression of NF- κ B treated with combination therapy ($p < 0.01$) and MET monotherapy ($p < 0.05$) was decreased. The mRNA expression of TNF- α was different between CAP-MET(H) group and MET(H) group ($p < 0.05$). In jejunum and ileum, compared with model group, the mRNA expression of TLR4 and NF- κ B in CAP-MET(H) and MET(H) groups, TNF- α in CAP-MET(H) group were statistically significant, as well as the mRNA expression of tight junction proteins, Occludin (Fig. 6A and 6B, $p < 0.01$). The protein expression of Occludin in ileum of CAP-MET(H) group was higher than CAP(H) group (Fig. 6C, $p < 0.05$).

CAP and MET co-treatment modulated gut microbiota

The OUT amounts (Table 1) and microbial α -diversity (Table 2) in treatment groups were obviously increased, which were much higher in CAP-MET(H) group than MET(H) group ($p = 0.090$). According to PCA and PCoA analysis, β -diversity varied between diabetic and normal rats (Figure S2 A), while CAP-MET(H) group and model group could be distinctly clustered (Fig. 7A, Anosim: $p = 0.002$; Adonis: $p = 0.003$). As shown in Fig. 7B and 7C, β -diversity were significantly different between CAP-MET(H) group and MET(H) group (Anosim: $p = 0.020$; Adonis: $p = 0.003$) or CAP(H) group (Anosim: $p = 0.003$; Adonis: $p = 0.003$), which was not found in normal group (Figure S2 C, D).

Table 1
16S rRNA sequencing data

Group	OTU number
CAP-MET(H)	238.30 ± 7.44 ^{##}
CAP(H)	228.20 ± 6.58 [#]
MET(H)	222.20 ± 5.26 ^{&,+}
MODEL	202.60 ± 6.67 ^{****}
CAP-MET(N)	322.80 ± 4.03
CAP(N)	325.80 ± 7.74
MET(N)	300.50 ± 6.30 ^{**}
CON	323.80 ± 3.78
<p>CAP-MET(H): capsaicin combined with metformin model group (n = 6), CAP(H): capsaicin monotherapy model group (n = 6), MET(H): metformin monotherapy model group (n = 6), MODEL: model control group (n = 5), CAP-MET(N): capsaicin combined with metformin normal group (n = 5), CAP(N): capsaicin monotherapy normal group (n = 5), MET(N): metformin monotherapy normal group (n = 6), CON: normal control group (n = 6). OTU: operational taxonomy unit. ^{**}<i>p</i> < 0.01 vs. CON group, ^{****}<i>p</i> < 0.0001 vs. CON group, [#]<i>p</i> < 0.01 vs. MODEL group, ^{##}<i>p</i> < 0.01 vs. MODEL group, ^{&}<i>p</i> = 0.053 vs. MODEL group, ⁺<i>p</i> = 0.090 vs. CAP-MET(H) group.</p>	

Table 2
α diversity index

Group	ACE	Chao1	Simpson	Shannon
CAP-MET(H)	289.30 ± 4.45 ^{####}	286.80 ± 5.86 [#]	0.16 ± 0.01 ^{###}	2.62 ± 0.03 ^{##}
CAP(H)	265.70 ± 7.56 ⁺⁺	269.10 ± 8.43	0.16 ± 0.02 ^{###}	2.60 ± 0.11 ^{##}
MET(H)	256.40 ± 4.09 ⁺⁺⁺	259.70 ± 6.24 ⁺	0.18 ± 0.04 ^{##}	2.57 ± 0.19 ^{##}
MODEL	250.30 ± 4.75 ^{****}	252.10 ± 10.80 ^{****}	0.37 ± 0.06 ^{**}	1.82 ± 0.24 ^{****}
CAP-MET(N)	339.20 ± 3.95	341.90 ± 3.97	0.09 ± 0.01	3.54 ± 0.11
CAP(N)	341.10 ± 5.27	341.80 ± 6.31	0.05 ± 0.01 [*]	3.89 ± 0.18
MET(N)	328.60 ± 5.18	329.90 ± 5.55	0.10 ± 0.03	3.31 ± 0.20
CON	340.60 ± 2.52	341.80 ± 3.59	0.08 ± 0.01	3.55 ± 0.10
CAP-MET(H): capsaicin combined with metformin model group (n = 6), CAP(H): capsaicin monotherapy model group (n = 6), MET(H): metformin monotherapy model group (n = 6), MODEL: model control group (n = 5), CAP-MET(N): capsaicin combined with metformin normal group (n = 5), CAP(N): capsaicin monotherapy normal group (n = 5), MET(N): metformin monotherapy normal group (n = 6), CON: normal control group (n = 6). * <i>p</i> < 0.05 vs. CON group, ** <i>p</i> < 0.01 vs. CON group, **** <i>p</i> < 0.0001 vs. CON group, # <i>p</i> < 0.05 vs. MODEL group, ## <i>p</i> < 0.01 vs. MODEL group, ### <i>p</i> < 0.001 vs. MODEL group, #### <i>p</i> < 0.0001 vs. MODEL group, + <i>p</i> < 0.05 vs. CAP-MET(H) group, ++ <i>p</i> < 0.01 vs. CAP-MET(H) group, +++ <i>p</i> < 0.001 vs. CAP-MET(H) group.				

CAP and MET co-treatment could increase microbial abundances such as *Allobaculum*, *Akkermansia*, *Bacteroides*, *Lactobacillus*, *Faecalibaculum*, *Parabacteroides*, *Ruminiclostridium_9*, *Lachnospiraceae-NK4A136*, *Lachnospiraceae-UCG-006*, *Roseburia*, *Oscillibacter* and *Phascolarctobacterium*, which were much higher than model group (*p* < 0.01), CAP(H) group (*p* < 0.01) and MET(H) group (*p* < 0.05) (Fig. 8), and increased the relative abundance of Cyanobacteria (*p* < 0.01), Verrucomicrobia (*p* < 0.001) (Figure S3), as well as Akkermansiaceae (*p* < 0.001) (Fig. 9A, Figure S4). LEfSe analysis indicated that *Akkermansia* could be the predominant biomarker of CAP-MET(H) group (Fig. 9B, C).

Actinobacteria, *Clostridium_sensu_stricto_1*, ratio of Firmicutes to Bacteroidetes, *Clostridiaceae_1*, *Bifidobacteria* and *Agathobacter* were positively correlated with T2DM indicators (*p* < 0.05), while *Akkermansia*, *Parabacteroides*, *Parasutterella*, *Phascolarctobacterium*, *Oscillibacter*, *Ruminiclostridium_9*, *Lachnospiraceae-UCG-006*, *Bacteroides* and *Muribaculaceae* were negatively associated with T2DM indicators (*p* < 0.05) (Fig. 10). The relative abundance of *Akkermansia* was negatively correlated with FINS, LPS, IL-1β and TNF-α (*p* < 0.05, *Q* < 0.05).

Functional microbial pathways

According to the prediction analysis of microbial function by PICRUST, biosynthesis of amino acids, 2-oxocarboxylic acid metabolism, biosynthesis of antibiotics, biosynthesis of secondary metabolites, quorum sensing, C5-Branched dibasic acid metabolism, valine, leucine and isoleucine biosynthesis and nitrogen metabolism were enriched in model group ($p < 0.01$, $Q < 0.01$), while butanoate metabolism, pyruvate metabolism, citrate cycle (TCA cycle), flagellar assembly, glycolysis/gluconeogenesis, glycerolipid metabolism, amino sugar and nucleotide sugar metabolism were enriched in CON group ($p < 0.01$, $Q < 0.01$) (Fig. 11A). CAP and MET co-treatment enhanced the activity of fatty acid metabolism, tryptophan metabolism, lysine degradation, biosynthesis of unsaturated fatty acids, butanoate metabolism and citrate cycle and inhibited the activity of biosynthesis of amino acids, valine, leucine and isoleucine biosynthesis, nicotinate and nicotinamide metabolism and peptidoglycan biosynthesis in diabetic rats (Fig. 11B). Compared with CAP(H) and MET(H) groups, the enrichment in histidine metabolism pathway was significantly reduced in CAP-MET(H) group ($p < 0.01$) (Fig. 11C and D).

Discussion

Gut microbiota play an important role in metabolic syndrome. The composition and function of gut microbiota in patients with T2DM are significantly different from healthy individuals [18, 19]. CAP can regulate gut microbiota, which has anti-obesity, anti-inflammatory, hypoglycemic and lipid-lowering effects. In the study, we found that CAP and/or MET can both improve the impaired glucose tolerance. The fasting blood glucose of rats treated with CAP and MET co-treatment reached the lowest level at the third week. In the mid-term (3–5 weeks), the combination therapy could significantly reduce the fasting blood glucose and improve the impaired glucose tolerance and insulin resistance. After 8 weeks, the combination therapy showed better control of blood glucose and improvement of impaired glucose tolerance than MET monotherapy.

Chronic inflammation is an important mechanism of diabetes and other chronic diseases [20]. T2DM is often accompanied by peripheral insulin resistance and low-grade systemic inflammation. High fat diet can increase the expression of inflammatory cytokines IL-6, IL-1 β and TNF- α , which can reduce the insulin sensitivity and accelerate the occurrence of insulin resistance. CAP and MET co-treatment could improve the hyperlipidemia of diabetic rats, reduce the levels of serum TC and LDL-C and alleviate liver injury and inflammatory infiltration. The plasma IL-1 β , IL-6 and TNF- α and the mRNA expressions of IL-1 β and TNF- α in liver, epididymal fat and intestinal tissues were relatively increased. Compared with MET monotherapy, the results suggested that the combination therapy may have a better anti-inflammatory effects.

Gut microbiota in low-level inflammatory response involve bacterial LPS, SCFA and branched chain amino acid (BCAA) metabolism, which have effects on the pathogenesis and treatment of T2DM [21]. LPS, as a ligand of TLR4, can activate related inflammatory signaling pathways and induce tissue inflammatory response. The results showed that the CAP and MET co-treatment could significantly reduce the content of LPS in plasma and the mRNA expression of TLR4 and NF- κ B in liver and intestine. Meanwhile, the combination treatment could relatively increase the expression of intestinal tight junction

molecule, occludin, which was able to maintain the intestinal barrier homeostasis and reduce gut microbiota-derived LPS entering the systemic circulation.

The imbalance of gut microbiota can induce inflammatory reactions, change intestinal permeability and cause metabolic endotoxemia. In diabetic rats, the ratio of Firmicutes to Bacteroidetes and the relative abundances of *Actinobacteria* and *Bifidobacteria* were significantly increased [22]. CAP and MET co-treatment could significantly regulate the composition and function of gut microbiota, which could increase the α -diversity and alter β -diversity of gut microbiota. Evidences have proved that the abundances of pro-inflammatory microbials in T2DM patients are significantly increased [23–25]. CAP and MET co-treatment could increase the relative abundances of a variety of beneficial bacteria, such as *Akkermansia*, *Allobaculum*, *Bacteroides*, *Faecalibaculum*, *Lactobacillus*, *Lachnospiraceae-UCG-006*, *Ruminiclostridium_9*, *Lachnospiraceae-NK4A136*, *Roseburia*, *Oscillibacter*, *Parabacteroides* and *Phascolarctobacterium*, all of which were SCFA producing microbials. SCFA are a subset of fatty acids produced by gut microbiota during polysaccharide fermentation, which play an important role in glucose and lipid metabolism, intestinal barrier homeostasis and anti-inflammatory response [26]. In the correlation analysis, metabolic pathway analysis indicated the co-treatment could increase the enrichment of lysine degradation pathway and butanoate metabolism pathway, which were reported to be related to butyric acid synthesis [27]. Compared with MET monotherapy, combination therapy could reduce the proportion of some pathogenic bacteria, such as *Blautia*, *Escherichia*, *Shigella* and *Enterococcus*, which are correlated to the adverse gastrointestinal reactions of MET [28].

Alternation in gut microbial composition can influence the type and quantity of microbial metabolites. Amino acids are the key substrates in many metabolic pathways, while clinical data have shown that the serum amino acid level is higher in diabetic patients by comparing with healthy individuals [29]. CAP combined with MET could inhibit the biosynthesis of amino acids pathway and reduce the amino acid synthesis of gut microbiota. BCAA (such as valine, leucine and isoleucine) can increase the risk of diabetes [30, 31], which can promote liver lipid storage and lead to insulin resistance [32], while gut microbiota are involved in BCAA synthesis. CAP combined with MET can reduce the synthesis of valine, leucine and isoleucine. Moreover, the co-treatment could enhance the tryptophan metabolism function, which could be directly metabolized into indole derivatives by gut microbiota. These metabolites have important effects on regulating immune homeostasis and protecting the intestinal barriers [33]. The synthesis of unsaturated fatty acids may be related to the improvement of blood lipid, blood glucose and antioxidant levels in T2DM [34]. The metabolism of nicotinic acid and nicotinamide was also related to the occurrence of T2DM and inflammatory response [35, 36]. Our results showed that the above-mentioned related pathways could be regulated by CAP and MET co-treatment [37]. Compared with MET monotherapy, the combination therapy could significantly reduce the microbial metabolism of histidine, which was associated with T2DM. The gut microbiota of T2DM patients have a stronger histidine metabolism ability than healthy subjects. The histidine metabolites lead to decreased glucose tolerance and impaired insulin signal [38]. These results suggested gut-derived microbial metabolites played important role in therapeutical efficacy.

Conclusions

In the study, CAP and MET co-treatment showed better effects than MET monotherapy on blood glucose control, enhancing insulin sensitivity, improving impaired glucose tolerance and reducing inflammatory reactions. CAP and MET co-treatment could remodulate the profiles and metabolic function of gut microbiota, which may play an important role in therapeutic effects of T2DM. Our study provided experimental evidence for the treatment of T2MD by combination drugs with diet intervention.

Abbreviations

AUC, area under the receiver operating characteristic curve; **ALT**, alanine aminotransferase; **BCAA**, branched-chain amino acid; **CAP**, capsaicin; **ddH₂O**, double distilled water; **FBG**, fasting blood glucose; **FINS**, fasting insulin; **GCG**, proglucagon; **HE**, hematoxylin-eosin; **HFD**, high-fat diet; **HOMA**, homeostasis model assessment; **IPITT**, intraperitoneal insulin tolerance test; **IR**, insulin resistance; **LDL-C**, low-density lipoprotein cholesterol; **LPS**, lipopolysaccharide; **MET**, metformin; **OGTT**, oral glucose tolerance test; **PCR**, polymerase chain reaction; **RNA**, ribonucleic acid; **SCFA**, short chain fatty acids; **STZ**, streptozotocin; **T2DM**, type 2 diabetes mellitus; **TC**, total cholesterol; **TG**, triglyceride.

Declarations

Authors' contributions

W. Z. and W.-H. H. conceived and designed the study. Z.-Q. K. and J.-L. H. performed the experiments, analyzed the data and wrote the manuscript. M.-Y. C. and N. Y. provided technical help and revised the manuscript.

Acknowledgements

None.

Competing interests

None of the authors has any conflict of interest regarding this study.

Availability of data and materials

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Fund

This work was supported by the National Natural Scientific Foundation of China (82074000, 81903784), the Hunan Provincial Natural Science Foundation of China (2020JJ4878), Science and Technology Benefiting People Plan of Zhengzhou (2020KJHM0028), Medical Science and Technology Research Plan of Henan Province (2018020762), the Scientific Research Project of Department of Education of Hunan Province (20K136), Sanming Project of Medicine in Shenzhen(SZSM201811057) and NHC Key Laboratory of Birth Defect for Research and Prevention (Hunan Provincial Maternal and Child Health Care Hospital, No. KF2020002).

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Figures

Figure 1

Animal experimental protocol.

Figure 2

CAP combined with MET reduced blood glucose levels. (A) Fasting body weight level of rats in 8 weeks medication. (B) Fasting blood glucose level of rats in 8 weeks administration. (C) Fasting body weight of normal rats at 0, 4th and 8th week. (D) Fasting blood glucose of diabetic rats at 0, 4th and 8th week. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. CON group, # $p < 0.05$, ## $p < 0.01$, #### $p < 0.0001$ vs. MODEL group, + $p < 0.05$, ++ $p < 0.01$ vs. CAP-MET(H), ▲▲ $p < 0.01$ vs. CAP-MET(N), n.s. no significance.

Figure 3

CAP combined with MET improved glucose tolerance and insulin resistance. (A) Oral glucose tolerance test (OGTT) of rats at 8th week of administration. (B) The intraperitoneal insulin tolerance test of rats at 8th week of administration. (C) The fasting insulin level of rats at 8th week of administration. (D) Insulin resistance index of rats at 8th week of administration. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6), MET(H): metformin monotherapy

model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). * $p < 0.05$ vs. CON group, *** $p < 0.001$ vs. CON group, **** $p < 0.0001$ vs. CON group, # $p < 0.05$ vs. MODEL group, ## $p < 0.01$ vs. MODEL group, ### $p < 0.001$ vs. MODEL group, #### $p < 0.0001$ vs. MODEL group. + $p < 0.05$ vs. CAP-MET(H) group, ++ $p < 0.01$ vs. CAP-MET(H) group, +++ $p < 0.001$ vs. CAP-MET(H) group, n.s. no significance.

Figure 4

CAP combined with MET alleviated hyperlipidemic and liver injury. (A) CAP combined with MET reduced blood lipid level in diabetic rats, (B) HE staining of liver, (C) The level of serum ALT and liver/weight ratio. CAP-MET(H): CAP combined with MET model group (n=6), CAP(H): CAP monotherapy model group (n=6), MET(H): MET monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). # $p < 0.05$ vs. MODEL group. ** $p < 0.01$ vs. CON group.

Figure 5

CAP combined with MET alleviate inflammatory in diabetic rats. (A) The levels of plasma inflammatory cytokines. (B) The mRNA expression of inflammation related genes in liver. (C) The mRNA expression of inflammation related genes in epididymal fat. (D) The mRNA expression of inflammation related genes in jejunum. (E) The mRNA expression of inflammation related genes in ileum. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6),

MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). * $p<0.05$ vs. CON group, ** $p<0.01$ vs. CON group, *** $p<0.001$ vs. CON group, # $p<0.05$ vs. MODEL group, ## $p<0.01$ vs. MODEL group, ### $p<0.001$ vs. MODEL group, + $p<0.05$ vs. CAP-MET(H) group, ++ $p<0.01$ vs. CAP-MET(H) group, +++ $p<0.001$ vs. CAP-MET(H) group.

Figure 6

CAP combined with MET increases the expression of intestinal tight junction molecules in diabetic rats. (A) The mRNA expression of Occludin gene in jejunum. (B) The mRNA expression of Occludin gene in ileum. (C) The expression of Occludin protein in ileum. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6), MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). * $p<0.05$ vs. CON group, ** $p<0.01$ vs. CON group, # $p<0.05$ vs. MODEL group, ## $p<0.01$ vs. MODEL group, + $p<0.05$ vs. CAP-MET(H) group.

A

B

C

D

Figure 7

The effect of CAP combined with MET on β -diversity of fecal microbiota in diabetic rats. (A) PCA and PCoA analysis between CAP-MET(H) groups and MODEL group. (B) PCoA analysis between CAP-MET(H) group and CAP(H) group. (C) PCoA analysis between CAP-MET(H) group and MET(H) group. T2D diabetic model rats (CAP-MET(H) group + MET(H) group + CAP(H) group + MODEL group, n=23), Normal normal rats (CAP-MET(N) group + MET(N) group + CAP(N) group + CON group, n=22), CAP-

MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6), MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). PCA: principal component analysis, PCoA: principal coordinate analysis, PC1: the first principal component, PC2: the second principal component, PC3: the third principal component. The dots in the graph represent each sample, the color represents the group, and the percentage represents the contribution of different principal components to the differences among samples.

Figure 8

The composition of fecal microbiota at genus level. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6), MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). **p<0.01 vs. CON group, ***p<0.001 vs. CON group, #p<0.05 vs. MODEL group, ##p<0.01 vs. MODEL group, ###p<0.001 vs. MODEL group, +p<0.05 vs. CAP-MET(H) group, ++p<0.01 vs. CAP-MET(H) group, &p=0.067 vs. CAP-MET(H) group.

Figure 9

The LefSe analysis in diabetic rats. (A) LefSe analysis of microbiota between CAP-MET(H) group and MODEL group. (B) LefSe analysis of microbiota between CAP-MET(H) group and MET(H) group. (C) LefSe analysis of microbiota between CAP-MET(N) group and CAP(H) group. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy model group (n=6), MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6). The different bacteria with LDA value > 4.0 were shown in the figure. The LDA value represents the influence degree on the difference between groups, and the color represents group.

Figure 10

Spearman correlation analysis of T2DM related indexes and the relative abundance of bacteria. (A) The heatmap of correlation coefficient. (B) The correlation analysis between the total relative abundance of beneficial bacteria and T2DM index. FBG: fasting blood glucose, FINS: fasting insulin, HOMA-IR: insulin resistance index, LPS: lipopolysaccharide, IL-1 β : interleukin-1 β , TNF- α : tumor necrosis factor α , IL-6: interleukin-6.

Figure 11

The analysis of KEGG functional pathways in diabetic rats. (A) KEGG pathway analysis of bacteria between MODEL group and CON group. (B) KEGG pathway analysis of bacteria between CAP-MET(H) group and MODEL group. (C) KEGG pathway analysis of bacteria between CAP-MET(H) group and MET(H) group. (D) KEGG pathway analysis of bacteria between CAP-MET(H) group and CAP(H) group. CAP-MET(H): capsaicin combined with metformin model group (n=6), CAP(H): capsaicin monotherapy

model group (n=6), MET(H): metformin monotherapy model group (n=6), MODEL: model control group (n=5), CON: normal control group (n=6).

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