Changes of Gut Microbiota in Diabetic Nephropathy and Its Effect on the Progression of Kidney Injury

Kedan Cai
  HwaMei Hospital, University of Chinese Academy of Sciences

Yanhong Ma
  Zhejiang University School of Medicine First Affiliated Hospital

Fanghao Cai
  Zhejiang University School of Medicine First Affiliated Hospital

Xiaohan Huang
  Zhejiang University School of Medicine First Affiliated Hospital

Liang Xiao
  Zhejiang University School of Medicine First Affiliated Hospital

Chenyu Zhong
  HwaMei Hospital University of Chinese Academy of Sciences

Pingping Ren
  Zhejiang University School of Medicine First Affiliated Hospital

Qun Luo
  HwaMei Hospital University of Chinese Academy of Sciences

Jianghua Chen
  Zhejiang University School of Medicine First Affiliated Hospital

Fei Han (✉ hanf8876@zju.edu.cn)
  The First Affiliated Hospital of USTC: Anhui Provincial Hospital   https://orcid.org/0000-0001-5363-7556

Abstract

Background: We aimed to illustrate gut microbiota and short chain fatty acid (SCFA) levels in diabetic nephropathy (DN) patients, and investigate the mechanism of sodium butyrate in diabetic mellitus (DM) rats.

Methods: Gut microbiota and serum SCFA levels were measured by 16S rDNA and GC-MS respectively. After being built by streptozotocin (DM rats), the DM rats were administered 300mg/kg sodium butyrate for 12 weeks (DM+BU rats). Gut microbiota, serum and fecal butyrate level were measured. RT-PCR, WB and transmission electron microscopy were performed to explore LC3mRNA or LC3B protein expression, and autophagosomes in kidney tissues. AMPK/mTOR protein expression in renal tissue were also measured.

Results: The gut microbial dysbiosis was found in DM and DN groups, and some SCFAs-producing bacteria were decreased in DN group. The serum butyrate concentrations were lower in SCFA-DN group compared with SCFA-HC group and SCFA-DM group in the other cohort. Serum butyrate level was positively correlated with eGFR. Sodium butyrate increased serum and fecal butyrate levels, and improved the enlargement of glomerular area and fibronectin and collagen Ⅳ expressions in renal tissues in DM+BU rats. The LC3 mRNA, LC3BⅡ/Ⅰ ratio and number of autophagosomes were increased in renal tissue of DM+BU rats. Higher p-AMPK/AMPK ratio and lower p-mTOR/ mTOR ratio were shown in renal tissue of DM+BU rats compared with DM rats.

Conclusions: We found the decrease in SCFAs-producing bacteria and low SCFAs concentrations in DN patients. Oral butyrate supplementation may improve kidney injury in DM rats, possibly by increasing autophagy via activating AMPK/mTOR pathway.

Introduction

Diabetic nephropathy (DN) is a common co-morbidity of diabetic mellitus (DM) and the major cause of end stage renal disease (ESRD) in the world. The prevalence of DM was reported to 11.2% recently in China[1]. About 35% of all diabetic patients may develop DN[2]. Intensive glycemic control and the use of sodium-glucose cotransporter-2 (SGLT2) inhibitors and renin-angiotensin-aldosterone system (RAAS) blockade may delay the progression of DN[3, 4]. However, it is necessary to study the mechanism and risk factors of DN progression and find more treatment strategies to delay disease progression to ESRD.

Recently, an intimate connection between gut and kidney has been proposed[5]. The gut microbiome may have obvious changes in construction in DM participants[6]. It was reported that patients with type 2 diabetes had a moderate degree of gut microbial dysbiosis characterized by decrease in the abundance of some universal butyrate-producing bacteria and increase of various opportunistic pathogens[6].

The short-chain fatty acids (SCFAs), mainly including acetate, butyrate and propionate were generated by gut microbiota[7]. In chronic kidney disease (CKD) patients, serum and fecal SCFAs levels were

significantly decreased, which may contribute to disease progression[8]. SCFAs can diffuse into bloodstream through intestinal mucosa[9]. In an ischemia/reperfusion model, SCFAs inhibited apoptosis to increase cell proliferation, and then improved the renal function[10]. Autophagy is a highly conserved cellular process to maintain intracellular homeostasis[11]. When cells encounter starvation or augmented energy demands, AMP-activated protein kinase (AMPK) (a key energy sensor) activates and downregulates mammalian target of rapamycin (mTOR) pathway, which may activate autophagy to maintain homeostasis[12]. In this study, we aimed to identify the changes of the intestinal flora and the serum SCFAs concentrations in DN patients. Then, we investigated the effects and mechanism of sodium butyrate (the most biological effective SCFA) on the progression of DN in streptozotocin induced diabetic rats. Our findings may provide further understanding of gut-kidney axis and a promising strategy in DN treatment.

Methods

The research protocols were conformed to the provisions of the Declaration of Helsinki and were approved by the Ethic Committee of the First Affiliated Hospital of Zhejiang University School of Medicine and HwaMei Hospital, University of Chinese Academy of Sciences. Written informed consents were obtained from the patients.

Participants and 16S ribosomal DNA gene sequencing

A total of 97 fecal samples were collected from 31 non-dialysis dependent DN patients (DN group), 32 T2DM patients without renal injury (DM group) and 34 healthy controls (HC group) in North Zhejiang Province, Eastern China from January 1, 2018 to December 31, 2018. T2DM were diagnosed according to the criteria of American Diabetes Association in 2017[13]. DN was diagnosed clinically based on the following criteria as persistent albuminuria > 300mg/24h in at least two of three consecutive 24-hour urine collections, presence of retinopathy, and no evidence of other kidney diseases[14]. The medical history, blood pressure, and biochemical results including assessment of fasting glucose and lipids, renal function and urinary albumin creatinine ratio (UACR) were collected. Estimated glomerular filtration rate (eGFR) was calculated by CKD-EPI formula.

For DN patients and DM patients, the exclusion criteria included (1) patients complicated with other kidney diseases, active infection, severe liver dysfunction, cancer and history or existence of gastro-intestinal or systemic diseases known to affect gut bacterial composition; (2) under immunosuppressive therapy; (3) after kidney allograft transplantation or reaching ESRD before renal biopsy; (4) receiving antibiotics or probiotics within 2 months before collecting samples; (5) with incomplete baseline data. The normal controls were from health examination centers in the same hospital, with no hypertension, diabetes, dyslipidemia, metabolic syndrome, cancer, abnormal liver or kidney function, or history of gastro-intestinal or systemic diseases known to affect gut bacterial composition. Fresh fecal samples were collected at hospital in the morning and kept frozen at -80℃ until usage. The gene sequencing of gut microbiota was performed using 16S ribosomal DNA sequencing technology. DNA extraction was

conducted using PowerSoil® DNA Isolation Kit (MO BIO). DNA concentration (1ng/μL) and purity were monitored on 1% agarose gels. The V3-V4 region of the bacterial 16S ribosomal DNA genes were amplified via PCR barcode-indexed primers, using KAPA HiFi™ HotStart ReadyMix (2X). The amplified PCR products were purified with Beckman DNA Clean Beads and quantified by the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). The enriched library was loaded in Ion 530™ Chip and sequenced on an Ion S5TM platform (Thermo Fisher Scientific, Waltham, MA) and 600bp single-end reads were generated, supplied by LC-Bio Technology Co., Ltd (Hang Zhou, China).

Sequencing analysis was performed by Uparse software (Uparse V8.1.1861, http://drive5.com/uparse/). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). The subsequent analysis of α-diversity and β-diversity were performed base on this output normalized data. The α-diversity metrics determines species richness and evenness within bacterial populations, including ace and Chao1 (microbial richness), and Shannon index and Simpson index (microbial diversity). All these indices in our samples were calculated with QIIME (Version 1.9.1). The β-diversity determines the shared diversity between bacterial populations. Non-Metric Multi-Dimensional Scaling (NMDS) was carried out using R programming language (Version 3.2.2) and Vegan package. The Linear Discriminant Analysis Effect Size (LEfSe) algorithm was used to identify the genomic features of taxa differing in abundance among three groups. A size-effect threshold of 2.0 on logarithmic LDA score was used for discriminative functional biomarkers.

Participants and measurement of SCFAs using gas chromatograph-mass spectrometry (GC-MS)

We enrolled a separate cohort of 27 T2DM patients without renal injury (SCFA-DM group) and 100 DN patients (SCFA-DN group) who received a renal biopsy in our center. The diagnosis of T2 DM or DN was according to the criteria proposed by the American Diabetes Association in 2017 [13] or the criteria by the Renal Pathology Society in 2010[15], respectively. The control group included 30 healthy controls from the same hospital (SCFA-HC group). The exclusion criteria for all the participants were the same as those listed above.

Blood samples were collected during fasting, centrifuged at 3500rpm for 5min after collection, then stored in aliquots at -80°C until usage. Each serum sample was sufficiently mixed with 50μL 50% sulfuric acid and 200μL ether, centrifuged at 12,000 rpm for 20 min at 4°C, then stood for 30 min at 4°C. The supernatant ether layer was filtered through anhydrous sodium sulfate and the solution was transferred to a glass vial for GC-MS analysis. The analysis was performed using the GC-MS 7890A-5975C (Agilent Technology, USA). A FFAP capillary column (30m×0.25mm×0.25μm) was used for chromatographic separation, and helium (1mL/min) was used as the carrier gas. These were conducted in Metabo-Profile Biotechnology (Shanghai) Co.,Ltd.

Animal experiments

Sixteen male Sprague-Dawley (SD) rats aged 8 weeks and weighed around 180-200g were purchased from Shanghai Super−B&K laboratory animal Corp.Ltd and maintained at Zhejiang Chinese Medical University Laboratory Animal Research Center with a SPF facility at (22 ± 2°C), 4070% humidity, with a 12/12h light/dark cycle and air exchange, and free access to food and water *ad libitum*. All animal procedures were approved by the Institutional Ethics Committee on Animal Use of the Zhejiang Chinese Medical University (NO.11346). After 2 weeks of adaptive feeding, rats were randomly divided to receive modeling of diabetes or control. Diabetes was induced by single injection of streptozotocin (55mg/kg) (n = 12). Blood glucose levels were measured one week after the injection to confirm the establishment of diabetic models. The control group was given the same volume of citric acid buffer (NC, n = 4). The diabetic rats were randomly divided to receive sodium butyrate (303410, Sigma, USA) (DM + BU, n = 6) or equal volume of distilled water (DM, n = 6) after three weeks of establishment of diabetic model. Rats in DM + BU group were administered 300mg/kg sodium butyrate by oral gavage once a day for 12 weeks.

The blood glucose levels were determined by a J&J OneTouch UltraVue Blood Glucose Meter every week. The concentration of blood urea nitrogen and 24h urinary protein was measured every four weeks. The blood urea nitrogen levels were measured by an automatic analyzer (Hitachi Tokyo, Japan). The rats were kept alone in metabolic cages to collect their 24h urine samples, of which 24h urinary protein concentration was quantitatively analyzed by an automatic analyzer (Hitachi Tokyo, Japan). All rats were sacrificed after 12 weeks of sodium butyrate or control intervention, with blood, urine, feces in cecum and colon, and renal tissues collected. Kidney weights were measured at the time of sacrifice.

Fresh fecal samples were collected at the time of sacrifice, then frozen until usage at -80°C. The gene sequencing of gut microbiota was performed using 16S ribosomal DNA (16S rDNA) sequencing technology described above. Each colonic fecal sample with 100mg was mixed with 0.9mL ultrapure water, crushed with a tissue grinder and then centrifuged at 12,000 rpm for 20 min at 4°C. The detailed method of measurement of serum and fecal SCFAs were same as described above.

LC3 mRNA was measured by real time PCR. Primers for LC3 was from Sangon Biotech (Shanghai, CN) Co., Ltd. The primer sequences were listed in Supplementary Table 3. The LC3B, Phospho-AMPK(Thr172), AMPK, Phospho-mTOR (Ser2448) and mTOR protein from the renal cortex were measured by western blot. The detailed information of primary antibodies was shown in Supplementary Table 4. Protein expression was observed using Image J.

The morphology of renal tissue stained with hematoxylin and eosin was visualized by the microscopy (Olympus VS120, Japan). The glomerular area was measured by Image-Pro Plus 6.0 software. Renal section was stained with specific primary antibody of fibronectin and Collagen-Ⅳ in the immunohistochemical staining. Protein expression was observed using Image J. Transmission electron microscopic (TEM) examination was performed to observe autophagy using a HITACHI 7650 electron microscope (HITACHI, Japan).

Statistical analysis

All statistical analyses were performed with SPSS v19.0 software or Prism 7.0 GraphPad Software. The results were expressed as means ± standard deviation for normally distributed continuous variables, median values (interquartile ranges) for non-normally distributed continuous variables and frequencies and percentages for categorical variables. The significance of differences between 3 groups were analyzed by one-way analysis of variance with Student-Newman-Keuls test or least post-tests when data were normally distributed. Comparisons of non-normally distributed continuous variables were performed using the Kruskal-Wallis test. For categorical variables, the chi-square test was used. Correlations between serum SCFAs and clinical data were analyzed by Pearson's R coefficient using psych package 1.9.12, and visualized by heatmap in corrplot package 0.84. A P-value < 0.05 was considered statistically significant.

Results

The gut microbial diversity and microbial composition in DN

Baseline clinical and biochemical characteristics of all participants in DM, DN and HC groups are shown in Supplementary Table 1. There was no significant difference of age among the three groups. Calculated by chao1 and ace indexes, the fecal α-microbial richness in DN group was higher than that in DM group (p = 0.0042, 0.0049 respectively), similar to HC group (p = 0.78, 0.80 respectively) (Fig. 1A-B). Whereas the evenness of gut microbiome shown by the community diversity calculated by Shannon and Simpson indexes, was similar in DN group and DM group (p = 0.50, 0.79 respectively) (Fig. 1C-D).

To identify specific bacterial taxa associated with DN, we compared fecal microbiome using LEfSe. The fecal microbial structure and their predominant bacteria showed the significant differences in taxa among the DM, DN and HC groups by a cladogram representative (all p < 0.05, Fig. 1E) and LDA score (p < 0.05, Fig. 1F). The LEfSe showed that the relative abundances of Proteobacteria, δ-proteobacteria and γ-probacteria, Moraxellaceae and its Acinetobacter, Pseudomonadales, Erysipelatoclostridium and Hungatella of Firmicutes, Desulfovibrionales and its Desulfovibrionaceae, Desulfovibrio in DN group were higher than those in HC group and DM group. The relative abundances of Clostridia and Clostridiales, Firmicutes, Ruminococcaceae and its Ruminococcaceae_UCG_013, Ruminococcaceae_UCG_014, Ruminococcaceae_UCG_003, Lachnospiraceae and its Lachnospira, Lachnospiraceae_NK4A136_group, Butyricicoccus and Eubacterium were lower in DN group than those in HC group. As we know, Ruminococcaceae, Butyricicoccus and Lachnospiraceae were the SCFA-producing bacteria.

The levels of serum SCFAs in DN patients

The baseline clinical and biochemical characteristics of all participants in SCFA-DM group, SCFA-DN group and SCFA-HC group are shown in Supplementary Table 2. The major SCFAs identified in the serum samples included acetate, propionate and butyrate. The serum concentrations of acetate, butyrate and total SCFAs were lower in SCFA-DN group than those in SCFA-HC group and SCFA-DM group (Table 1).

The correlation analysis showed serum butyrate level was positively correlated with eGFR level (r = 0.15, p = 0.04) and negatively correlated with urinary albumin creatinine ratio (UACR) level (r=-0.20, p = 0.02) (Fig. 2). Also, there were positive correlations between serum levels of total SCFAs, acetate, propionate and eGFR level (r = 0.35, p < 0.05; r = 0.35, p < 0.05; r = 0.17, p = 0.03; respectively)(Fig. 2).

Table 1
The levels of serum SCFAs among the SCFA-HC, SCFA-DM group and SCFA-DN group.

	SCFA-HC group	SCFA-DM group	SCFA-DN group	p-value
Total SCFAs(µmol/L)	114.60 ± 43.12	69.81 ± 48.95 [a]	50.54 ± 23.90 [ab]	< 0.001
Acetate(µmol/L)	95.81 ± 37.02	58.92 ± 47.38 [a]	41.20 ± 18.50 [ab]	< 0.001
Propionate(µmol/L)	8.11 ± 10.50	5.73 ± 2.66	3.74 ± 1.77[a]	< 0.001
Butyrate(µmol/L)	2.23 ± 3.12	2.27 ± 2.17	1.22 ± 0.58[ab]	0.002

[a] P < 0.05 compared with SCFA-HC group; [b] P < 0.05 compared with SCFA-DM group.

Treatment with sodium butyrate improved the renal injury in DM rats

The blood glucose levels in DM rats(n = 6) and DM + BU rats(n = 6) were significantly increased compared with the controls(n = 4), confirming that the streptozotocin-induced diabetic model was successfully established. The blood glucose level decreased in DM + BU rats compared with DM rats after treatment with sodium butyrate at 8 weeks and 12 weeks without any adverse sides(Fig. 3A). The ratio of kidney-to-body weight, serum nitrogen level and 24h urine protein in DM rats was significantly higher than those in controls (Fig. 3B-D). After treatment with sodium butyrate, the DM + BU rats deceased in the levels of nitrogen and ratio of kidney-to-body weight compared with DM rats (Fig. 3B-C). However, there was no decrease in 24h urine protein in DM + BU rats compared with DM rats (Fig. 3D). The glomerular area in DM rats was bigger than the control rats. After the treatment of sodium butyrate, the glomerular area was smaller in DM + BU rats than DM rats (Fig. 3E). The expression of fibronectin and Collagen Ⅳ by immunohistochemistry in renal tissue showed that the glomerular expressions of fibronectin and Collagen Ⅳ increased in DM rats compared with control rats. But these changes were inhibited by treatment of sodium butyrate in DM + BU rats (Fig. 3E-F). These results indicated sodium butyrate may be a new therapy for alleviating DN progression.

Change of gut microbiota after treatment of sodium butyrate in DM rats

A total of 670 OTUs were obtained at a 97% homology cutoff. To display microbiome space among samples, the β-diversity calculated by NMDS showed the distribution of fecal microbial community

tended to be significantly different among the three groups (Stress = 0.10) (Supplementary Fig. 1A). The results of sequence analysis in the gut microbiota of each group showed that the relative abundance of *Ruminococcaceae_UCG-014* in the cecum of DM + BU group was higher than that of DM group, while similar to that of NC group (Supplementary Fig. 1B), demonstrating treatment of sodium butyrate may restore the normal gut microbiota in DM rats.

The GC-MS analysis showed that the serum and fecal levels of butyrate significantly decreased in the DM rats compared with normal rats. Sodium butyrate intervention in DM + BU rats increased the serum and fecal butyrate levels (Supplementary Fig. 1C-D). There was positive correlation between serum butyrate and fecal butyrate levels (r = 0.636, *p* = 0.008) (Supplementary Fig. 1E).

Sodium butyrate induces autophagy via activating AMPK/mTOR pathway in DM rats

LC3-Ⅱ formation is recognized as a marker of the existence of autophagosomes in animal experiments[16]. RT-PCR analysis showed that LC3 mRNA level was lower in renal tissue of DM rats than the control rats; after the treatment of sodium butyrate, LC3 mRNA level was increased in renal tissue of DM + BU rats (Fig. 4A). The protein levels of autophagy-associated protein, LC3B Ⅰ and LC3BⅡ showed LC3B Ⅱ/Ⅰ ratio was up-regulated significantly in DM + BU rats compared with DM rats (Fig. 4B). TEM showed more autophagosomes in renal tissue of DM + BU rats compared with DM rates and control rates (Fig. 4C). These results demonstrated that the autophagy level increased in DM rats after the treatment of sodium butyrate.

AMPK is a trimeric protein complex composed of α, β and γ subunits[17], which downregulates mTOR and induces autophagy[18]. The protein levels of p-AMPK, AMPK, p-mTOR and mTOR showed significant higher p-AMPK/AMPK ratio and lower p-mTOR/ mTOR ratio in renal tissue of DM + BU rats compared with DM rats (Fig. 4B). We speculated that sodium butyrate may induce autophagy by activating AMPK/mTOR signal pathway.

Discussion

In the present study, we showed an obvious change in the richness of gut microbiota between DM patients and DN patients, and the altered bacterial community may play an important role in the progression of DN. Tao *et al.* also showed the similar shift of the richness of gut microbiota in DN patients[19]. SCFAs such as acetate, propionate, and butyrate are the major microbial products and important energetic and signaling molecules. It was reported that serum SCFA levels were lower in CKD patients than healthy controls[8]. In this study, the concentration of serum acetate, propionate, butyrate and total SCFAs were lower in DN group than those in NC group and DM group. The serum butyrate level was positively correlated with eGFR level, but negatively correlated with UACR level. It suggested that SCFAs may be a new therapy to delay the progression of DN.

The reduced level of serum butyrate was positively correlation with the decreased level of fecal butyrate in DM rats in our study. Yamamura *et al.* demonstrated that fecal SCFAs level was likely to be positively associated with serum SCFAs level, and the relative abundance of several SCFAs-producing bacteria were positively associated with fecal SCFAs even after adjusting for age and sex[20–22]. We speculated that the disordered gut microbiome and decreased certain SCFA-producing bacteria may result in the reduced serum SCFAs level in DN patients. Tao *et al.* showed that at the genus level SCFAs-producing bacteria *Prevotella_9* decreased in DN group[19]. In our study, the results revealed that DN patients had apparent dysbiosis in gut microbiota, especially the decreased relative abundance of Ruminococcaceae, Butyricicoccus and Lachnospiraceae, which were SCFAs- producing bacteria of the human gut in Eastern China. In addition, the relative abundance of butyrate-producing bacteria, Ruminococcaceae_UCG-014, was decreased obviously in DM rats compared with normal rats in our study.

In order to demonstrate the effects of SCFAs on DN, we selected sodium butyrate, one of the most biologically effective SCFAs from microbial fermentation in the gut, to treat DM rats. Although probiotic supplements can increase the butyrate level, they occasionally cause detrimental metabolic activities or produce host deleterious metabolites, and cause inappropriate immune responses and systemic infections [23]. Herein, oral butyrate supplement was used to increase the serum butyrate level. After treatment with sodium butyrate, the DM rats showed reduced kidney damage in our study. Furthermore, sodium butyrate improved gut dysbiosis, promoting expansion of the genera Ruminococcaceae_UCG-014. Other reported studies also observed improvements in kidney injury in other models upon the butyrate treatment[24, 25] *Li et al.* revealed fiber promoted expansion of Prevotella and Bifidobacterium, which increased fecal and systemic SCFA concentrations and reduced expression of genes encoding inflammatory cytokines, chemokines, and fibrosis-promoting proteins in diabetic kidneys[26]. The fecal microbial transplantation was shown to increase the level of fecal butyrate in DN mice, and also improved renal pathological injury[27]. The *In vitro* studies also showed sodium butyrate played protective effects on podocytes[26], tubular cells[28], endothelial cells[29] and mesangial cells under high glucose status.

Autophagy is a highly conserved cellular process to maintain intracellular homeostasis. Accumulated evidences have shown that activation of autophagy can prevent the progression of DN[11]. In this study, our results showed that LC3 mRNA level and LC3B▯/▯ ratio in DM group were significantly lower than those in control group, and there was little autophagosomes in renal tissue, which indicated that autophagy was inhibited in DM rats. This finding is consistent with the other study[30]. Then, upon the treatment with sodium butyrate, LC3 mRNA level and LC3B▯/▯ ratio, as well as the number of autophagosomes in DM + BU rats increased compared with DM rats. So, we firstly showed that sodium butyrate could trigger autophagy in kidneys of DM rats. Recently, it was reported that SCFAs could regulate autophagy in acute kidney injury by promoting the expression of Atg 7 in renal tubular epithelial cells to inhibit renal fibrosis and improve renal function[10]. In other diseases, sodium butyrate has been found to induce Atg 5-dependent autophagy in mouse neuroendocrine STC-1 cells, which was mainly characterized by increasing expression of LC3-▯ and decreasing expression of p62[31]. Other studies also found that sodium butyrate promotes autophagy to inhibit tumor growth in colorectal cells [32, 33] .

We further investigated the mechanism of sodium butyrate in regulating autophagy in DM rats. The AMPK/mTOR pathway is the classic pathway to regulate autophagy. Butyrate was reported to activate AMPK phosphorylation to improve renal injury in CKD animals[34]. Wang *et al.* proved that the AMPK/mTOR pathway mediated sodium butyrate induced autophagy in human bladder cancer cells[35]. Our study found that sodium butyrate dramatically increased p-AMPK/AMPK, with AMPKα phosphorylation at Thr172, and downregulated p-mTOR/mTOR, with mTOR phosphorylation at Ser2448. These findings confirmed that the AMPK/mTOR pathway may mediate sodium butyrate induced autophagy in kidneys of DM rats.

Also, we observed that oral sodium butyrate supplement in DM rats decreased blood glucose level significantly after 8 weeks and 12 weeks. It was in coincided with previous studies[36, 37]. Gao *et al* showed that there was a significant and positive effect of sodium butyrate on glucose metabolites and insulin resistance[36]. In a randomized double-blinded placebo-controlled trial, combination of butyrate and inulin significantly reduced fasting blood sugar and waist to hip ratio[38]. A study also demonstrated that butyrate-producing bacteria Clostridium butyricum CGMCC0313.1 (CB0313.1) improved diabetic markers of fasting glucose, glucose tolerance and insulin tolerance[39]. Butyrate-mediated glucagon like peptide-1 (GLP-1) secretion may be a major factor in the improvement of insulin sensitivity and diabetes[40]. GLP-1 has been demonstrated to decrease hepatic gluconeogenesis and promote insulin secretion[41]. The increased glucose transporter-4 (GLUT-4) level induced by butyrate in adipose tissue may be considered as one of the other factors to improve glucose metabolites[36]. GLUT4, expressed in skeletal muscle and adipose tissue, is a member of glucose transporter protein family to uptake the glucose in tissues[42, 43]. Taken together, this may be an extra effect for controlling hyperglycemia to alleviate the progression of DN.

There were several limitations in our study. Firstly, the direct correlation between serum SCFAs and gut microbiota would not be analyzed in the participants, as they were not from the same cohort. Secondly, the lifestyles or diets were not analyzed due to a lack of records. So, the association between gut microbiota composition and lifestyles or diets needs to be explored in further studies. Thirdly, gene knockout mice, germ-free mice and fecal microbiota transplantation (FMT) were not used in the animal study. Herein further studies on the underlying mechanism and interactions are needed.

In conclusion, we demonstrated the changes in composition of the gut microbiota, especially the decrease in SCFAs-producing bacteria in DN patients. In addition, we showed that serum concentrations of SCFAs were low in DN patients, and oral butyrate supplementation may improve kidney injury in DM rats, possibly by increasing autophagy via activating AMPK/mTOR pathway (Fig. 5). These findings provided further understanding of gut-kidney axis in diabetic nephropathy and may provide a new promising strategy.

Declarations

Funding

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request

Contribution statement

Research idea and study design: FH, KDC and YHM; sample collection: KC YHM, CYZ and PPR; data acquisition and analysis: FH, KDC, YHM; Experiments performed: FH, KDC, YHM, FHC XHH and LX; supervision or mentorship: FH, QL, JHC. FH and KDC wrote the first draft of the manuscript. FH reviewed and edited the manuscript. KDC and YHM contributed equally in the study. All the authors approved of the final version of the manuscript.

Ethical approval

The research protocols were conformed to the provisions of the Declaration of Helsinki and were approved by the Ethic Committee of the First Affiliated Hospital of Zhejiang University School of Medicine and HwaMei Hospital, University of Chinese Academy of Sciences.

Consent to participate

Written informed consents were obtained from the patients.

References

1. Li YZ, Di T, Shi XG, et al. Prevalence of diabetes recorded in mainland China using 2018 diagnostic criteria from the American Diabetes Association: national cross sectional study. BMJ. 369, m997 (2020).
2. Demmer RT, Zuk AM, Rosenbaum M, Desvarieux M, Prevalence of Diagnosed and Undiagnosed Type 2 Diabetes Mellitus Among US Adolescents: Results From the Continuous NHANES, 1999–2010. Am J Epidemiol. 178(7), 1106-1113 (2013).

3. Alicic RZ, Johnson EJ, Tuttle KR, SGLT2 Inhibition for the Prevention and Treatment of Diabetic Kidney Disease: A Review. Am J Kidney Dis. 72(2), 267-277 (2018).

4. Alicic RZ, Rooney MT, Tuttle KR, Diabetic Kidney Disease Challenges, Progress, and Possibilities. Clin J Am Soc Nephrol. 12(12), 2032-2045 (2017).

5. Anders HJ, Andersen K, Stecher B, The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. Kidney Int. 83(6), 1010-1016 (2013).

6. Qin JJ, Li YR, Cai ZM, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 490(7418), 55-60 (2012).

7. Høverstad T, T M, Short-Chain Fatty Acids in Germfree Mice and Rats1. J Nutr. 116(9), 1772-1776 (1986).

8. Wang SQ, Lv D, Jiang SH, et al. Quantitative reduction in short-chain fatty acids, especially butyrate, contributes to the progression of chronic kidney disease. Clin Sci (Lond). 133(17), 1857-1870 (2019).

9. Jacobson A, Lam L, Rajendram M, et al. A Gut Commensal-Produced Metabolite Mediates Colonization Resistance to Salmonella Infection. Cell Host Microbe. 24(2), 296-307 (2018).

10. Andrade-Oliveira V, Amano MT, Correa-Costa M, et al. Gut Bacteria Products Prevent AKI Induced by Ischemia-Reperfusion. J Am Soc Nephrol. 26(8), 1877-1888 (2015).

11. Liu N, Xu LQ, Shi YF, Zhuang SG, Podocyte Autophagy: A Potential Therapeutic Target to Prevent the Progression of Diabetic Nephropathy. J Diabetes Res. 2017, 3560238 (2017).

12. Alers S, Löffler AS, Wesselborg S, Stork B, Role of AMPK-mTOR-Ulk1/2 in the Regulation of Autophagy: Cross Talk, Shortcuts, and Feedbacks. Mol Cell Biol. 32(1), 2-11 (2011).

13. Association AD, Standards of Medical Care in Diabetes—2017 Abridged for Primary Care Providers. Clin Diabetes. 35(1), 5-26 (2017).

14. Hansen TK, Tarnow L, Thiel S, et al. Association Between Mannose-Binding Lectin and Vascular Complications in Type 1 Diabetes. Diabetes. 53(6), 1570-1576 (2004).

15. Tervaert TW, Mooyaart AL, Amann K, et al. Pathologic Classification of Diabetic Nephropathy. J Am Soc Nephrol. 21(4), 556-563 (2010).

16. Mizushima N, Yoshimori T, Levine B, Methods in Mammalian Autophagy Research. Cell. 140(3), 313-326 (2010).

17. Hamasaki M, Furuta N, Matsuda A, et al. Autophagosomes form at ER−mitochondria contact sites. Nature. 495(7441), 389-393 (2013).

18. Kim MY, Lim JH, Youn HH, et al. Resveratrol prevents renal lipotoxicity and inhibits mesangial cell glucotoxicity in a manner dependent on the AMPK−SIRT1−PGC1α axis in db/db mice. Diabetologia 56(1), 204-217 (2013).

19. Tao SB, Li LZ, Li L, et al. Understanding the gut−kidney axis among biopsy-proven diabetic nephropathy, type 2 diabetes mellitus and healthy controls: an analysis of the gut microbiota composition. Acta Diabetol. 56(5), 581-592 (2019).

20. Ren ZG, Li A, Jiang JW, et al. Gut microbiome analysis as a tool towards targeted non-invasive biomarkers for early hepatocellular carcinoma. BMJ. 68(6), 1014-1023 (2018).

21. Yamaguchi Y, Adachi K, Sugiyama T, et al. Association of Intestinal Microbiota with Metabolic Markers and Dietary Habits in Patients with Type 2 Diabetes. Digestion. 94(2), 66-72 (2016).

22. Feng Q, Liang SS, Jia HJ, et al. Gut microbiome development along the colorectal adenoma–carcinoma sequence. Nat Commun. 6, 6528 (2015).

23. Daliri EB, Tango CN, Lee BH, Oh DH, Human microbiome restoration and safety. Int J Med Microbiol. 308(5), 487-497 (2018).

24. Dong WP, Jia Y, Liu XX, et al. Sodium butyrate activates NRF2 to ameliorate diabetic nephropathy possibly via inhibition of HDAC. J Endocrinol. 232(1), 71-83 (2017).

25. Du Y, Tang G, Yuan WJ, Suppression of HDAC2 by sodium butyrate alleviates apoptosis of kidney cells in db/db mice and HGinduced NRK52E cells. Int J Mol Med. 45(1), 210-222 (2019).

26. Li YJ, Chen XC, Kwan TK, et al. Dietary Fiber Protects against Diabetic Nephropathy through Short-Chain Fatty Acid–Mediated Activation of G Protein–Coupled Receptors GPR43 and GPR109A. J Am Soc Nephrol. 31(6), 1267-1281 (2020).

27. Li Y, Su XH, Gao Y, et al. The potential role of the gut microbiota in modulating renal function in experimental diabetic nephropathy murine models established in same environment. Biochim Biophys Acta Mol Basis Dis. 1866(6), 165764 (2020).

28. Kobayashi M, Mikami D, Kimura H, et al. Short-chain fatty acids, GPR41 and GPR43 ligands, inhibit TNF-α-induced MCP-1 expression by modulating p38 and JNK signaling pathways in human renal cortical epithelial cells. Biochem Biophys Res Commun. 486(2), 499-505 (2017).

29. Gu JL, Huang W, Zhang WQ, et al. Sodium butyrate alleviates high-glucose-induced renal glomerular endothelial cells damage via inhibiting pyroptosis. Int Immunopharmacol. 75, 105832 (2019).

30. Barbosa Júnior AdA, Zhou H, HÜltenschmidt D, Totovic V, Jurilj N, Pfeife U, Inhibition of cellular autophagy in proximal tubular cells of the kidney in streptozotocin-diabetic and uninephrectomized rats. Virchows Arch B Cell Pathol Incl Mol Patho. 61(6), 359-366 (1992).

31. Qiao CM, Sun MF, Jia XB, et al. Sodium butyrate causes α-synuclein degradation by an Atg5-dependent and PI3K/Akt/mTOR-related autophagy pathway. Exp Cell Res. 387(1), 111772 (2020).

32. Chang YJ, Zhang JT, Yi M, et al. Sodium Butyrate Induces Endoplasmic Reticulum Stress and Autophagy in Colorectal Cells: Implications for Apoptosis. PLoS One. 11(1), e0147218 (2016).

33. Luo SL, Li ZY, Mao LZ, Chen SQ, Sun SX, Sodium butyrate induces autophagy in colorectal cancer cells through LKB1/AMPK signaling. J Physiol Biochem. 75(1), 53-63 (2018).

34. Gonzalez A, Krieg R, Massey H, et al. Sodium butyrate ameliorates insulin resistance and renal failure in CKD rats by modulating intestinal permeability and mucin expression. Nephrol Dial Transplant. 34(5), 783-794 (2019).

35. Wang FF, Wu HS, Fan MJ, et al. Sodium butyrate inhibits migration and induces AMPK-mTOR pathway-dependent autophagy and ROS-mediated apoptosis via the miR-139-5p/Bmi-1 axis in

human bladder cancer cells. FASEB J. 34(3), 4266-4282 (2020).

36. Gao F, Lv YW, Long J, et al. Butyrate Improves the Metabolic Disorder and Gut Microbiome Dysbiosis in Mice Induced by a High-Fat Diet. Front Pharmacol. 10, 1040 (2019).

37. Si X, Shang WT, Zhou ZK, et al. Gut Microbiome-induced Shift of Acetate to Butyrate Positively Manages Dysbiosis in High Fat Diet. Mol Nutr Food Res. 62(3), 1-27 (2018).

38. Roshanravan N, Mahdavi R, Alizadeh E, et al. Effect of Butyrate and Inulin Supplementation on Glycemic Status, Lipid Profile and Glucagon-Like Peptide 1 Level in Patients with Type 2 Diabetes: A Randomized Double-Blind, Placebo-Controlled Trial. Horm Metab Res. 49(11), 886-891 (2017).

39. Jia LL, Li DY, Feng NH, et al. Anti-diabetic Effects of Clostridium butyricum CGMCC0313.1 through Promoting the Growth of Gut Butyrate-producing Bacteria in Type 2 Diabetic Mice. Sci Rep. 7(1), 7046 (2017).

40. Yadav H, Lee JH, Lloyd J, Walter P, Rane SG, Beneficial Metabolic Effects of a Probiotic via Butyrate-induced GLP-1 Hormone Secretion. J Biol Chem. 288(35), 25088-25097 (2013).

41. Jin T, Weng JP, Hepatic functions of GLP-1 and its based drugs: current disputes and perspectives. Am J Physiol Endocrinol Metab. 311(3), 620-627 (2016).

42. Lizunov VA, Lee JP, Skarulis MC, Zimmerberg J, Cushman SW, Stenkula KG, Impaired Tethering and Fusion of GLUT4 Vesicles in Insulin-Resistant Human Adipose Cells. Diabetes. 62(9), 3114-3119 (2013).

43. Salt IP, Hardie DG, AMP-Activated Protein Kinase: An Ubiquitous Signaling Pathway With Key Roles in the Cardiovascular System. Circ Res. 120(11), 1825-1841 (2017).

Figures
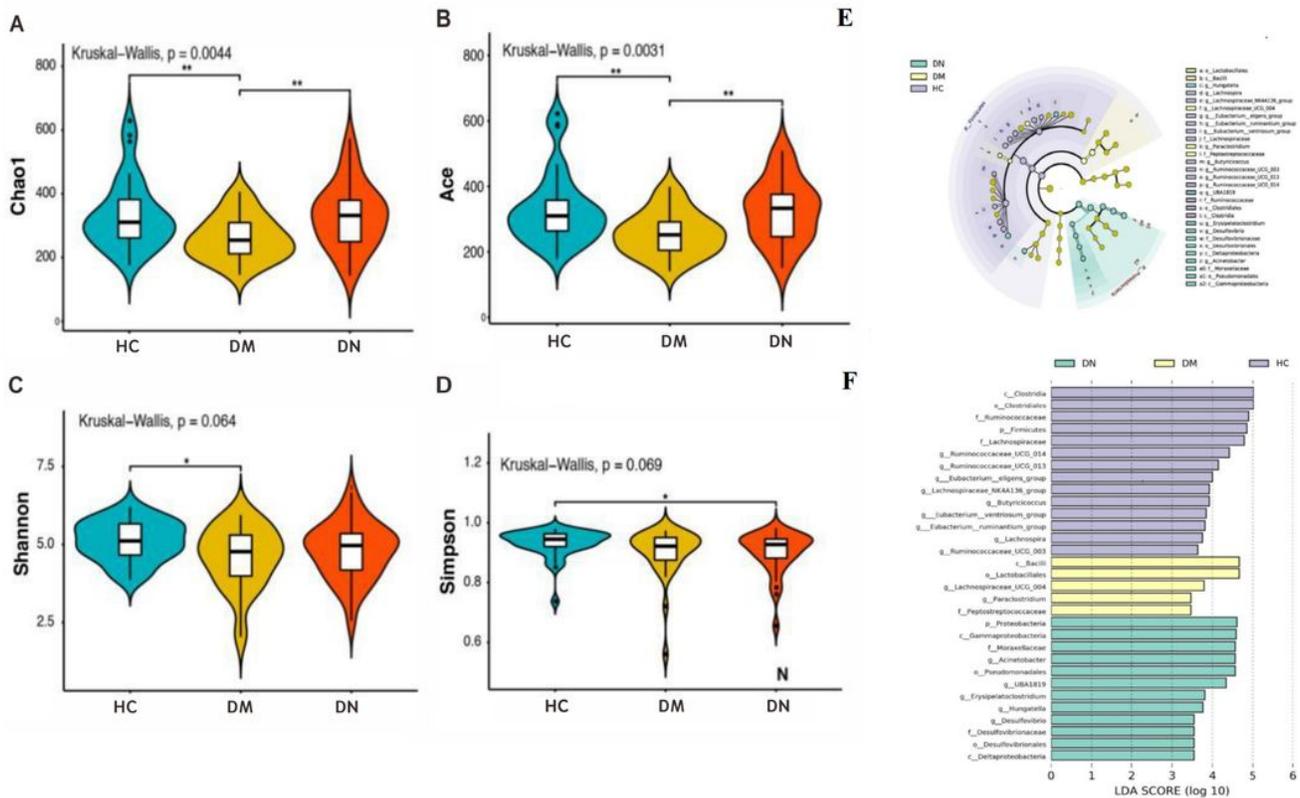
Figure 1

The fecal microbial α-diversity and differentially abundant taxa in DN group, DM group and HC group. (A) The fecal microbial richness calculated by ace among three groups. (B) The fecal microbial richness calculated by chao1 among three groups. (C) The community diversity calculated by Shannon index among three groups. (D) The community diversity calculated by Simpson index among three groups. LEfSe analysis was conducted to identify differentially abundant taxa, which are highlighted by the phylogenetic tree in cladogram format (E) and the LDA scores (F). Green color indicates an expanded taxa in the feces of DN compared with DM, while the yellow color indicates an increased taxa in the feces of DM compared with DN. *p<0.05,**p<0.01,wilcoxon rank-sum test.
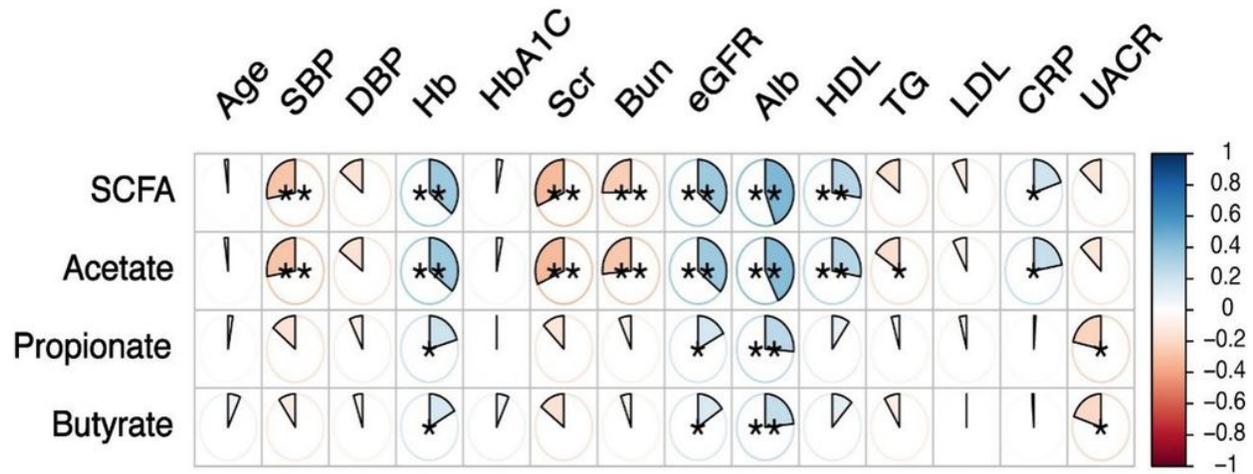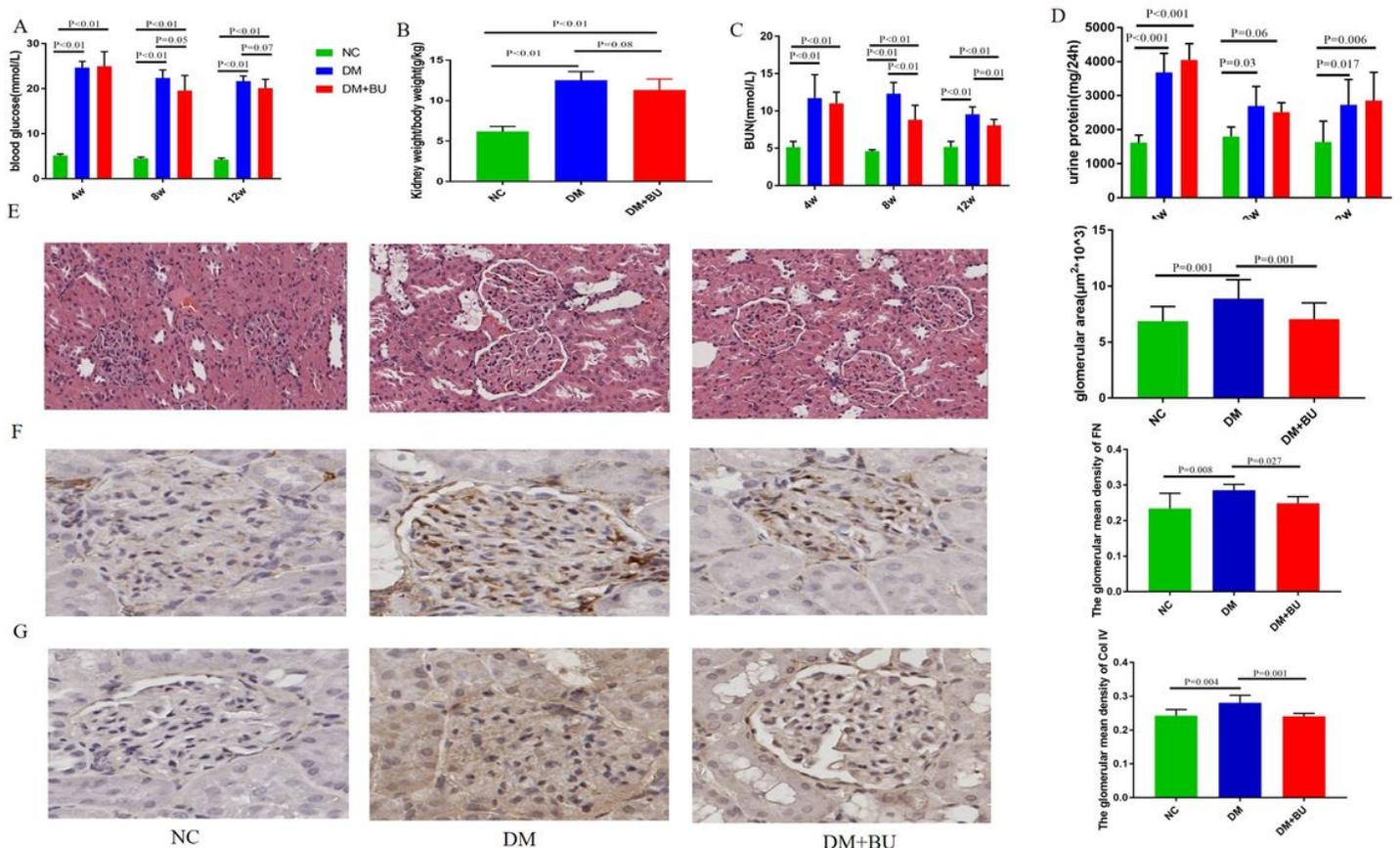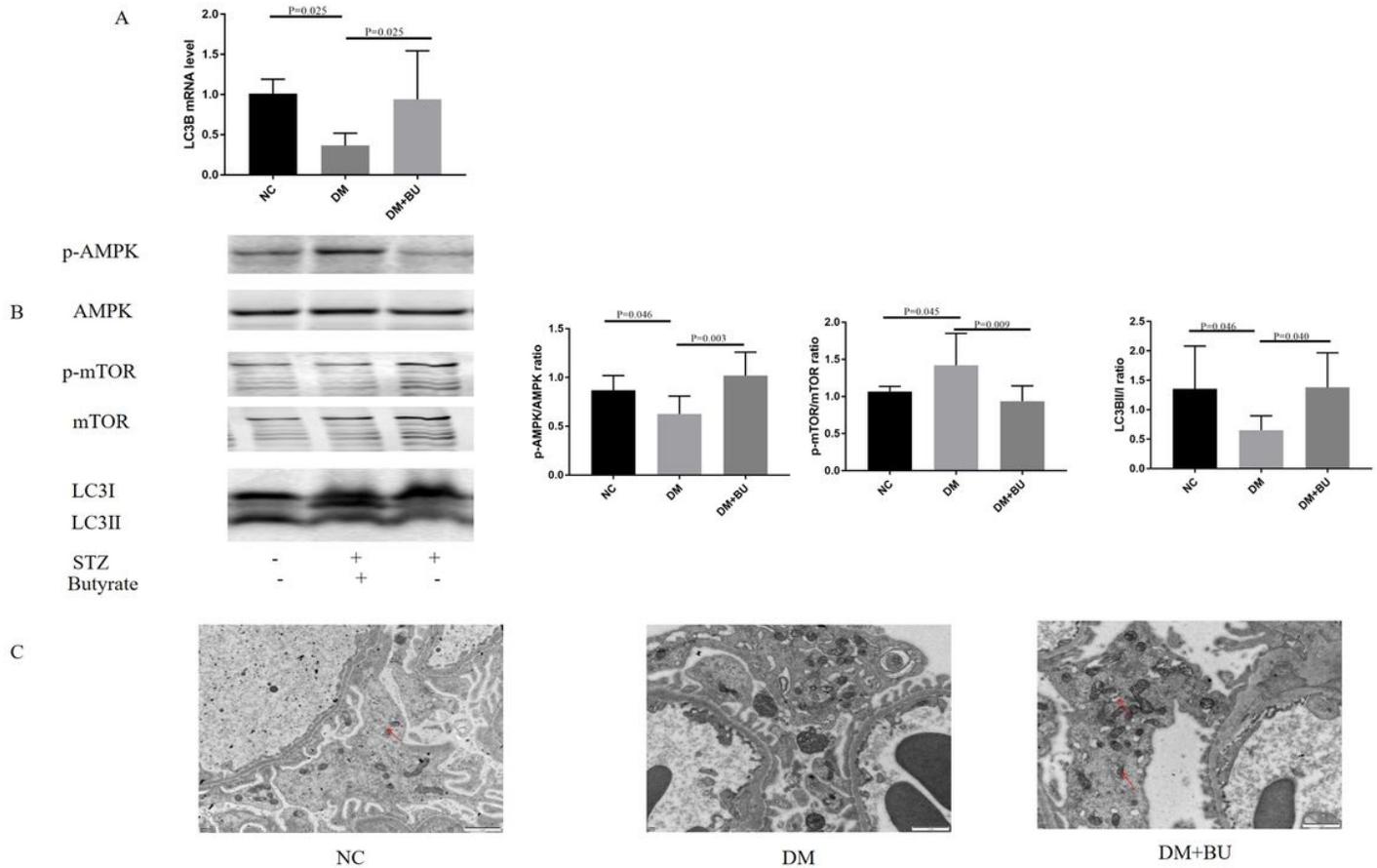
Figure 2

The correlations between serum SCFAs and clinical characteristic in SCFA-DN patients. *p<0.05,**p<0.01

Figure 3

The effect of sodium butyrate on blood glucose, renal function and pathological change(magnification ×200) of incipient DN.(A) Measurement of blood glucose in rats. (B)Measurement of renal weight/bodyweight in rats. (C) Measurement of serum BUN in rats. (D)Measurement of 24h-urine protein in rats.(E)Representative images of HE staining in renal tissues . (F)Protein expression levels of fibronectin in kidneys of rats measured by immunohistochemical staining (brown colour, original magnification *2). (G) Protein expression levels of Collagen in kidneys of rats measured by immunohistochemical staining (brown colour, original magnification *2)

Figure 4

Sodium butyrate affects autophagy via activating AMPK/mTOR signal pathway (A) The gene expression of LC3 mRNA in kidneys of mice detected by RT-PCR. (B) Measurement of p-AMPK, AMPK, p-mTOR, mTOR and LC3B protein expression in the kidney of mice by western blot. (C) Autophagosomes were observed under the TEM in renal tissues(scale bar = 1 μm) .
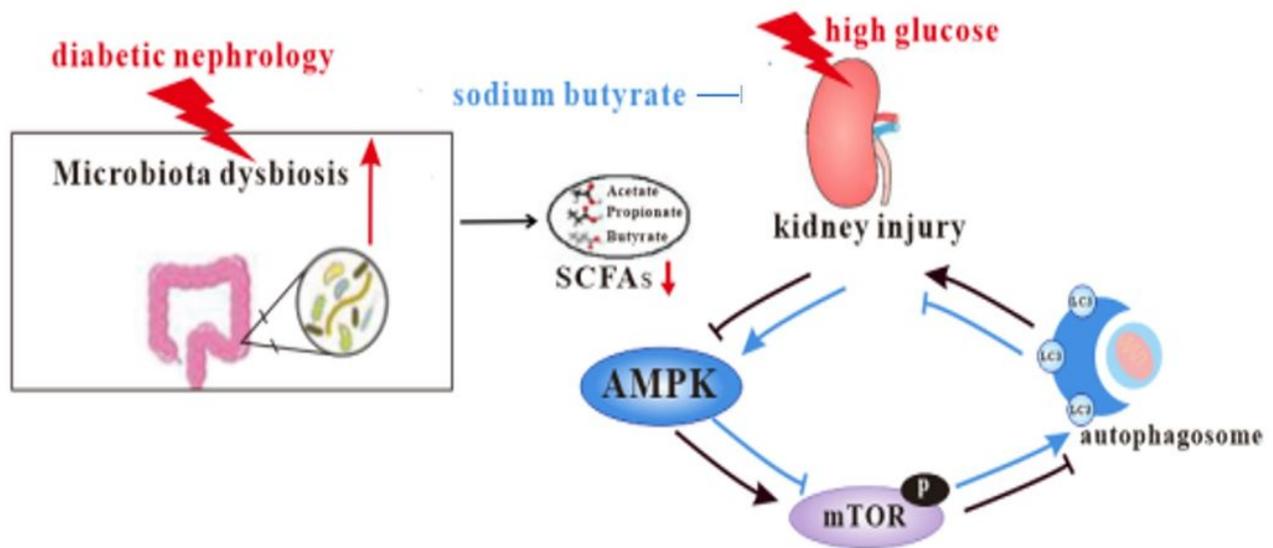
Figure 5

The study of intestinal micro-ecology in DN and its effect on the progression of the nephropathy. It revealed that there was the gut microbial dysbiosis in DN. Meanwhile, the serum total SCFAs, acetate, propionate and butyrate concentrations were significantly decreased in DN. The supplement of sodium butyrate might delay DN progression via AMPK/mTOR pathway to activate autophagy

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryfigure1.tif
- supplementarytable1.doc