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Original research paper

Sodium butyrate attenuate hyperglycemia-induced inflammatory response and renal injury in diabetic mice

MAN YAN [1,a]
YAN-YAN ZHANG[2,a]
YUE XI[1]
LONG-KUN DING[1]
CHANG SUN[1]
LI-JUAN QU[3]
XIN QIAN[1]
JING-WEN XU[4]
WEN SUN[4*]
LIANG WU[1*]

[1] *School of Medicine, Jiangsu University Zhenjiang, Jiangsu, 212013, China*

[2] *Testing Center, Yangzhou University Yangzhou, Jiangsu, 225009, China*

[3] *Dental Treatment Center, Shanghai Ninth People's Hospital, Shanghai Shanghai, 200000, China*

[4] *Department of Critical Care Medicine Jurong Hospital Affiliated to Jiangsu University, Zhenjiang 212444, China*

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ABSTRACT

The activation of the monocyte-macrophage system and the damage to the renal and pancreatic tissue are common complications in patients with diabetes induced by hyperglycemia. This study aimed to evaluate the effect and mechanism of butyrate (NaB), a metabolite of intestinal flora, on inhibiting the inflammatory response of human monocyte-macrophages (THP-1 cells) induced by high glucose and the damage of pancreatic and renal tissue in diabetic mice. The results showed that high concentration glucose significantly up-regulated the expressions of IL-1β, TNF-α, and NLRP3 in THP-1 cells and mouse spleen, and that NaB could inhibit the overexpression of those genes. The abundance of Beclin-1, LC3B and reactive oxygen species (ROS) in THP-1 cells is increased due to the high glucose concentration, and NaB can inhibit the genes responsible for upregulating the expression. In diabetic mice, vacuolar degeneration of renal tubules was observed. Then we observed that some of the epithelial cells of the renal tubules were exfoliated and some formed tubules. NaB could alleviate these pathological lesions, but NaB cannot alleviate pancreatic injury. Our results indicated that NaB could be used for the prevention and adjuvant treatment of diabetic kidney injury.

Keywords: high concentration glucose, diabetic nephropathy, butyrate, inflammation

Type 2 diabetes (diabetes mellitus type 2, T2DM) is a common chronic metabolic disease. The prevalence of T2DM in China reaches 10.4 % on average, and that over the age of 60 is already as high as 20 %. The essence of T2DM is a "chronic inflammation" characterized by insulin resistance and progressive pancreas beta-cell death (1), and uncontrolled inflammation is an important cause of diabetic nephropathy and other complications (2). Hyperactivity of macrophages during hyperglycemia leads to overexpressions of pro-

[a] These two authors contributed to this work equally.
* Correspondence; e-mails: sunwen19851021@163.com; wlujs@ujs.edu.cn

-inflammatory cytokines IL-1β and TNF-α and oxidative stress. Increased pro-inflammatory cytokines and reactive oxygen species can directly activate NLRP3 inflammasome (3), which leads to various complications in diabetic patients including diabetic nephropathy and generalized body small vascular inflammation (4). All these complications are the main cause of death (5).

Long-term studies have found that dietary fiber intake can prevent and decrease the need for treatment of complications such as diabetic nephropathy, but the exact mechanism is still unclear (6). Dietary fibers can be fermented in the human colon to produce short-chain fatty acids (SCFAs) (7). The SCFAs are a subset of organic fatty acids with the number of carbon atoms less than or equal to 6. SCFAs usually exist in an ionic state in the human intestines. Acetate (NaAc), propionate (NaPc) and butyrate (NaB) account for more than 90 % of the total SCFAs. The anti-inflammatory activity of NaB is the most attractive (8). NaB is the main energy source of intestinal mucosal epithelial cells, which can promote the proliferation, differentiation and maturation of mucosal cells and maintain the integrity of intestinal mucosal barrier structure. At the same time, NaB also acts as a deacetylase inhibitor, which can improve histone acetylation, inhibit the proliferation of tumor cells and promote the aging and apoptosis of tumor cells (9). Recent studies have also found that NaB can promote the proliferation and activation of regulatory T cells and affect the transformation of M1-type macrophages (pro-inflammatory) to M2-type macrophages (anti-inflammatory) by regulating the immune balance of Th1 and Th2 (10). NaB has been used as an adjunct therapy in clinical inflammatory diseases such as inflammatory bowel disease. However, there is still a lack of research on inhibiting inflammatory response in T2DM by NaB. In this study, we investigated the inhibitory effect of NaB on macrophage activation induced by high concentration glucose and the effect of treatment with NaB on diabetic kidney and pancreatic injury.

EXPERIMENTAL

Cell culture and inflammation induction

Human monocyte-macrophage (THP-1) were purchased from Shanghai Cell Bank and maintained in our laboratory. THP-1 cells were cultured in a 37 ° and 5 % CO_2 environment in RPMI 1640 medium (Biological Industries, Israel) supplemented with 10 % fetal bovine serum (Biological Industries). The low-glucose medium and high-glucose RPMI 1640 medium were prepared by adding a certain concentration of glucose solution into the glucose-free RPMI 1640 medium (Biological Industries). Sterile phosphate buffer solution (PBS) was used to dissolve sodium butyrate (NaB, Sigma-Aldrich, USA), and it was filtrated in order to prepare a 100 μmol L^{-1} NaB solution. For the study, cells were inoculated in 6-well plates with 1×10^6 cells in each well.

The experiment was designed to test three different settings: low glucose concentration (L-glucose, 5.5 mmol L^{-1}) group, convention glucose concentration (M-glucose, 11.1 mmol L^{-1}) group, and high glucose concentration (H-glucose, 33.3 mmol L^{-1}) group. With the addition of NaB group in each experimental setting (L-glucose + NaB group, M-glucose + NaB group and H-glucose + NaB group), L-glucose group, M-glucose group and H-glucose group cells were cultured with the corresponding concentration of culture medium. The treatment of THP-1 cells by NaB followed Xia's method (11). All of the above NaB

pre-treated group cells were cultured in the conventional glucose concentrations medium with a final concentration of 100 μmol L^{-1} NaB for 24 h, then replaced the medium with corresponding glucose concentrations with a final concentration of 100 μmol L^{-1} NaB, and continued to be cultured. At the end of the experiment, the total RNA and total protein were extracted for subsequent research.

Construction of diabetic mice

The diabetic mice were prepared by referring to the method reported by Wang *et al.* (12). Our study's animal experiment methods were according to ARRIVE guidelines and approved by the management committee, and used laboratory animals, Jiangsu University (No. UJS-IACUC-AP-2020032009). Six-week-old female ICR mice were purchased from the Animal Center of Yangzhou University and fed in the barrier system of the Animal Center of Jiangsu University. Mice were divided into the normal control (NC) group, the diabetes group and the NaB group, with each group containing 6 mice. The mice in diabetes and NaB group were fed with a high-fat diet consisted of 66.5 % basal diet + 10 % lard oil + 20 % sucrose + 2.5 % cholesterol + 1 % sodium cholate (Serve Life Science, Shanghai, China). From the 5th week, 50 mg kg^{-1} day^{-1} streptozotocin (STZ, Sigma-Aldrich, USA) was intraperitoneally injected into mice for 5 consecutive days in order to induce diabetes. The fasting blood glucose of the mice higher than 11.1 mmol/L was considered a successful diabetic model 72 h later, and then the mice were fed a high-fat diet for 8 weeks. Huang's (13) method was used to treat mice with NaB. The NaB group was intraperitoneally injected with 100 mg kg^{-1} of NaB every two days at the beginning of the experiment and the NC group received sterile phosphate buffer injection of equal amount and frequency. In the 12^{th} week of experiments, all mice were sacrificed and the animal tissues, including serum, spleen, pancreas and renal cortex were collected.

Quantitative PCR (qPCR) detection

The total RNA of THP-1 cells and animal tissues were extracted by the Trizol method and reversed into cDNA for the qPCR detection. The total reaction system was 10 μL, including 5 μL of SYBR Green Master premix (Vazyme, China), 0.2 μL of upstream and downstream primers (10 μmol L^{-1}) and 1 μL of cDNA template. The reaction process included initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 3 s, annealing at 58 °C for the 20 s, and extension at 72 °C for 30 s, with a total of 40 cycles. Using GAPDH as an internal reference, the relative gene expression was calculated by formula $2^{-\Delta\Delta Ct}$. The PCR primers' sequences are shown in Table I.

Western blotting analysis

Western blotting was performed according to Lin *et al.* (14). The proteins were extracted by RIPA lysate (Beyotime Biotechnology, China) containing 1.2 μL PMSF (Beyotime Biotechnology) and boiled with 5 × loading buffer for 10 min. All samples were subjected to SDS-PAGE electrophoresis, transferred to PVDF membranes (0.45 μm, Thermo Fisher Scientific, USA), then blocked with 5 % skim milk powder for 1 h at room temperature. The rabbit antibodies (Boster Biological Technology co. Ltd., China), including NLRP3 (1:250), Caspase-1 (1:1000), Beclin-1 (1:500), LC3B (1:100) and β-actin (1:5000) were diluted with TBST buffer and incubated at 4 °C overnight. The PVDF membranes were washed 3 times

Table I. The qPCR primers sequence

Gene	Primer sequences（5'→3'）
Hum_GAPDH	F: CATCACTGCCACCCAGAAGACTG
	R: ATGCCAGTGAGCTTCCCGTTCAG
Hum_IL-1β	F: CCTGTCCTGCGTGTTGAAAGA
	R: GGGAACTGGGCAGACTCAAA
Hum_TNF-α	F: AATGGCGTGGAGCTGAGA
	R: TGGCAGAGAGGAGGTTGAC
Hum_NLRP3	F: AACAGCCACCTCACTTCCAG
	R: CCAACCACAATCTCCGAATG
Mum_GAPDH	F: AATGGATTTGGACGCATTGGT
	R: TTTGCACTGGTACGTGTTGAT
Mum_IL-2	F: ACACCTTTAATTGGTCAACACGA
	R: CCTGCTACGTTCTCTACCTCT
Mum_TNF-α	F: GAACTGGCAAAAGGATGGTGA
	R: TGTGGGTTGTTGACCTCAAAC
Mum_NLRP3	F: ATTACCCGCCCGAGAAAGG
	R: CATGAGTGTGGCTAGATCCAAG

in tris buffered saline with tween (TBST) buffer and incubated with the goat anti-rabbit IgG-HRP (Boster Biological Technology co. Itd.) with the dilution of 1:10000 for 1 h. The ECL chemiluminescence kit (Millipore, USA) and the Image J software were used to analyze and calculate the expressions of target proteins.

Flow cytometric analysis

The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe kit (Beyotime) was used to detect the intracellular ROS concentration according to Zou's method (15). After the cells were washed 3 times with sterile PBS buffer, the DCFH-DA probe was diluted with serum-free RPMI 1640 medium at 1:1000. The cells were re-suspended in the diluted DCFH-DA probe suspension and incubated for 20 min at 37 °C. The cell suspension was mixed every 5 minutes so that the probe was in full contact with the cells. Finally, the cells were washed with a serum-free cell culture medium 3 times to remove the DCFH-DA probe that did not bind to the cells. The cells were re-suspended with sterile PBS buffer according to the kit instructions. The intracellular ROS expression was detected by Flow Cytometer (BD Biosciences, USA).

Enzyme-linked immunosorbent assay (ELISA) analysis

According to Shen's method(16), the concentrations of IL-1β in the serum of mice were detected by an ELISA kit (Meimian, Yancheng, China). The serum samples were added to

the plates and incubated at 37 °C for 30 min, and then washed 3 times. Finally, the HRP-goat anti-human IgG antibody was added and incubated at 37 °C for 30 min. An enzyme-labelled instrument determined the absorbance value of each well at 450 nm. The concentration of IL-1β was counted by standard curve.

Hematoxylin-eosin (HE) staining

The HE staining followed the method reported by Cui (17). The renal cortical and pancreas tissues were fixed with 4 % paraformaldehyde for 24 h, dehydrated by 70, 75, 80, 90 and 95 % ethanol. The anhydrous was ethanol-treated transparently with xylene and finally embedded in paraffin wax. The embedded tissues were sectioned with a thickness of 4 μm, dewaxed and hydrated again, and stained with hematoxylin and eosin. After dehydration, the slides were sealed with neutral gum and dried overnight. The histopathological morphology was observed under an optical microscope.

Statistical analysis

SPSS 22.0 statistical software was used for data analysis, and all data were tested for homogeneity of variance. ANOVA was used for the analysis of homogeneity of variance, and the Kruskal-Wallis test was used for non-parametric statistical analysis of non-variance homogeneity data. The statistical graph was generated by GraphPad Prism 7 software, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

NaB inhibits the expressions of pro-inflammatory cytokines in THP-1 cells and spleen induced by high glucose

The THP-1 cells' mRNA expressions of IL-1β, TNF-α and NLRP3 in the H-glucose group significantly increased than that in the L-glucose and M-glucose groups ($p < 0.05$), and there was no significant difference between the L-glucose and M-glucose groups ($p > 0.05$). Only in the group treated with a high concentration of glucose (33.3 mmol L^{-1}), NaB significantly inhibited the mRNA expressions of IL-1β, TNF-α and NLRP3 ($p < 0.05$) (Fig. 1a). After treatment with different concentrations of glucose, the protein expressions of NLRP3 and Caspase-1 significantly increased in the H-glucose group, and NaB significantly inhibited the expressions of NLRP3 and Caspase-1 in the H-glucose group ($p < 0.05$). (Fig. 1b). Compared with the L-glucose group, the ROS expression significantly increased in the H-glucose group in 3 and 6 h. In 3 and 6 h, NaB can significantly inhibit the ROS expression when treated with a high concentration of glucose (33.3 mmol L^{-1}) ($p < 0.05$) (Fig. 1c).

Compared with the NC group, mRNA expressions of TNF-α and NLRP3 in the diabetes and NaB group significantly increased, amRNA expression of IL-2 significantly decreased ($p < 0.05$) in mice spleen. Compared with the diabetes group, the TNF-α and NLRP3 mRNA expressions significantly decreased, and IL-2 mRNA expressions significantly increased in the NaB group ($p < 0.05$). (Fig 2). In mice serum, compared with the NC group, the concentration of IL-1β significantly increased in the diabetes and NaB groups ($p < 0.05$); compared with the diabetes group, the concentration of IL-1β significantly decreased in the NaB group ($p < 0.05$) (Fig. 2b).

The pathogenesis of diabetes is still unclear. Recent studies have shown that the activation of NLRP3 inflammasome is involved in the pathological process of diabetes and its various complications (18–19). NLRP3 inflammasome is a member of nod-like receptors (NLRs), which activates caspase-1 and regulates the secretion and maturation of IL-1β and IL-18 in response to DAMPs, PAMPs, ROS, ATP, pore-forming toxins and various particles (20–21). Previous studies have shown that NaB inhibits inflammatory responses by inhibiting NF-κB and NLRP3 signalling pathways to reduce the release of pro-inflammatory cytokines from immune cells (21). In our study, we found that high glucose upregulated

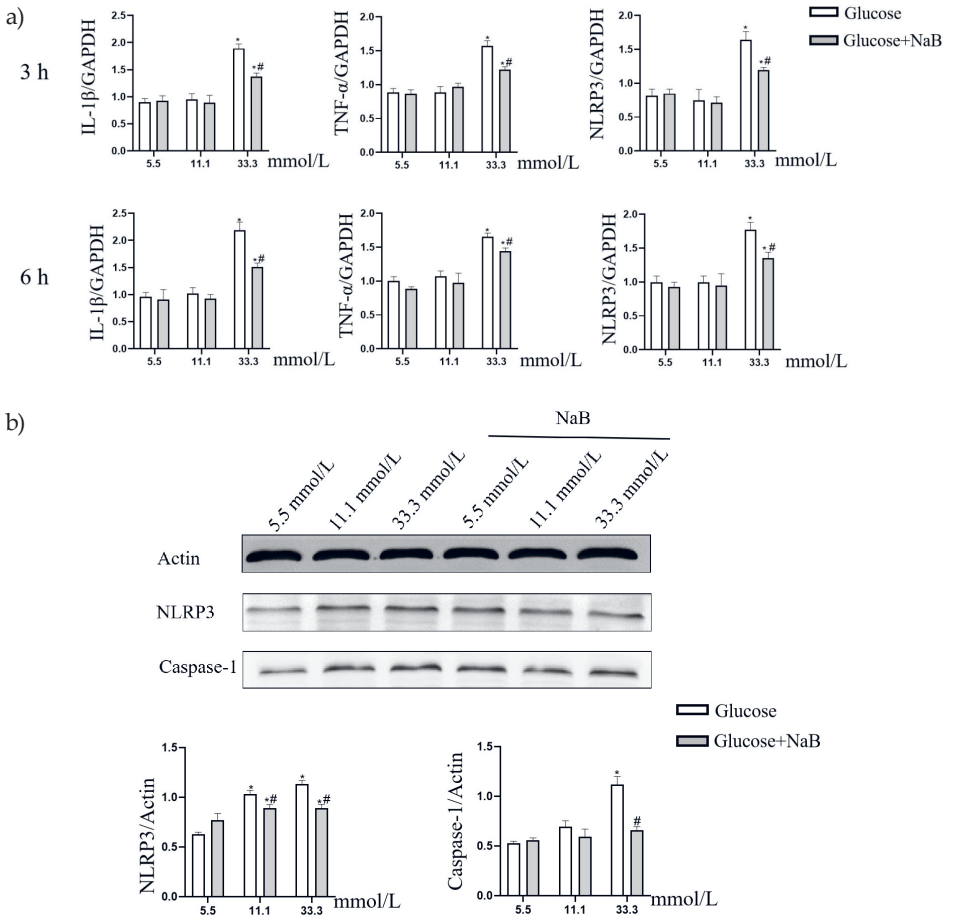

Fig. 1. The expression of pro-inflammatory factors in THP-1 cells. THP-1 cells were pre-treated with 100 μmol L^{-1} NaB for 24 h, and then treated with L-glucose (5.5 mmol L^{-1}), M-glucose (11.1 mmol L^{-1}) and H-glucose (33.3 mmol L^{-1}) for 3 and 6 h. a) mRNA expressions of IL-1β, TNF-α and NLRP3 in THP-1 cells; b) protein expressions of NLRP3 and Caspase-1 in THP-1 cells; c) ROS expression in THP-1 cells; * compared with the L-glucose group, $p < 0.05$; # compared with the same concentration of the glucose group.

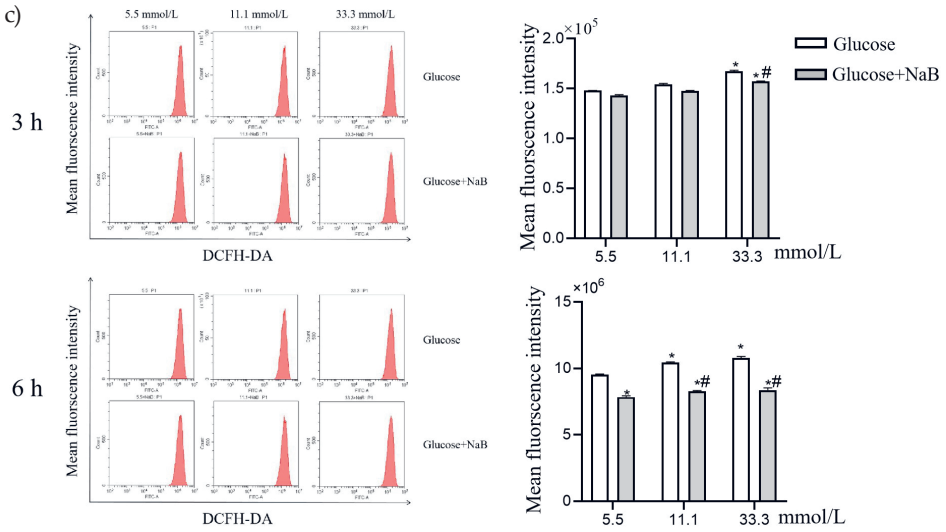

Fig. 1. Continued.

the expression of ROS and IL-1β in THP-1 cells, activates the NLRP3 immune complex, and significantly increased the expression of inflammatory cytokines in mice. After NaB intervention, THP-1 cell proinflammatory cytokines IL-1β and TNF-α and NLRP3 inflammatory complex reduced significantly. NaB can also inhibit the expression of IL-1β, TNF-α and NLRP3 in mice. Our results indicate that NaB could inhibit inflammation both *in vivo*

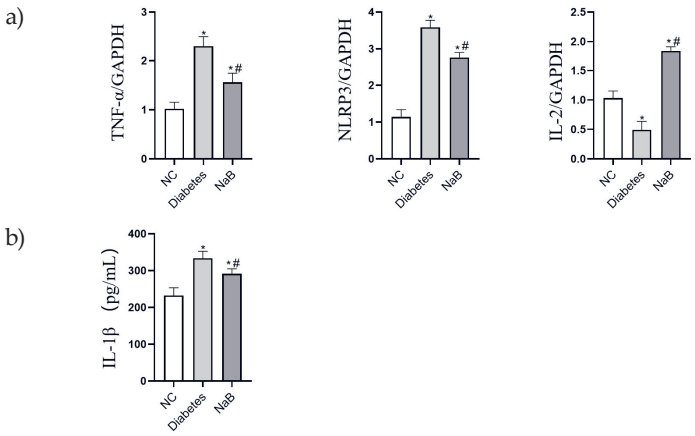

Fig. 2. The expressions of pro-inflammatory factors in mice spleen and serum. The ICR mice were fed a high-sugar and fat diet and intraperitoneally injected with streptozotocin to induce diabetes. The NaB group was intraperitoneally injected with 100 mg kg^{-1} of NaB every two days at the beginning of the experiment. The animal experiment lasted eight weeks. a) mRNA expressions of TNF-α, IL-2 and NLRP3 in mice spleen; b) IL-1β expression in mice serum. * compared with the NC group, $p < 0.05$; # compared with the same concentration of the diabetes group.

and *in vitro*, and significantly inhibit the activation of the NLRP3 inflammasome, thereby reducing the release of pro-inflammatory cytokines such as IL-1β and TNF-α.

High concentration glucose can induce autophagy, but NaB can reverse it

Compared with the L-glucose group, the expressions of autophagy-related protein Beclin-1 and LC3-II significantly increased in the M-glucose and H-glucose groups in 24 and 48 h ($p < 0.05$). At 24 h, THP-1 cell autophagy-related proteins Beclin-1 and LC3-II were not significantly inhibited by NaB ($p > 0.05$). However, NaB could significantly inhibit the expressions of autophagy-related proteins in THP-1 cells at 48 h ($p < 0.05$) (Fig. 3).

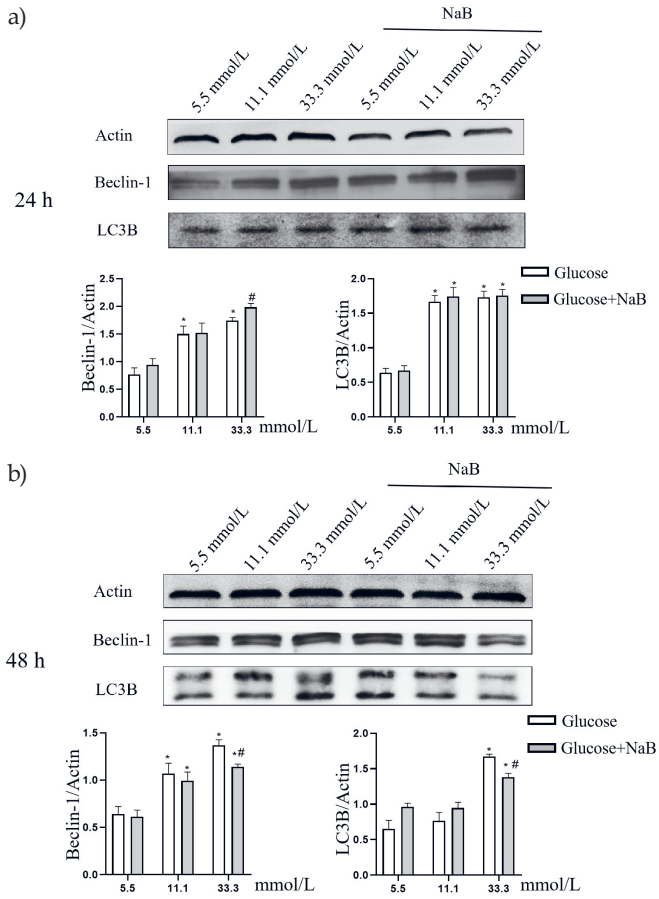

Fig. 3. The expressions of Beclin-1 and LC3B in THP-1 cells. THP-1 cells were pre-treated with 100 μmol L^{-1} NaB for 24 h, and then treated with L-glucose (5.5 mmol L^{-1}), M-glucose (11.1 mmol L^{-1}) and H-glucose (33.3 mmol L^{-1}) for 24 and 48 h. The expressions of autophagy-related protein Beclin-1 and LC3B were determined by Western blotting. a) 24 h; b) 48 h. * compared with L-glucose group, $p < 0.05$; # compared with the same concentration of glucose group.

Autophagy is closely related to T2DM. In this study, we found that high concentration glucose could up-regulate monocyte-macrophage autophagy, while NaB could significantly reverse the occurrence of monocyte-macrophage autophagy. LC3 and Beclin-1 are two important proteins in autophagy, and the increase in their expressions indicates the increase in autophagy. As a defence mechanism in the human body, autophagy can remove ageing or incapacitated organelles, avoid inducing oxidative stress and endoplasmic reticulum stress, and prevent insulin resistance and hyperinsulinemia caused by the accumulation of reactive oxygen species in mitochondria (22–23). But excessive autophagy can cause the reduction of a number of immune cells to depress the body's immunity (24). In this study, the autophagy level of macrophages pretreated with NaB was reduced, which may be a protective response of cells to inflammation and oxidative stress. It could avoid the decrease in the number of macrophages caused by autophagy in diabetic patients, thus maintaining the immunity of patients. However, there are still few studies on the use of NaB in the human body, and the improvement of chronic inflammatory response in diabetic patients by NaB remains to be studied further.

NaB can reverse diabetic kidney injury but no improvement effect was observed relating to pancreatic injury

After HE was stained, it was found that the renal corpuscles of the NC group mice were complete in structure and the renal follicle cavity was visible without hyperplasia and other lesions. The renal tubules were surrounded by simple cuboid epithelium, and the lumen

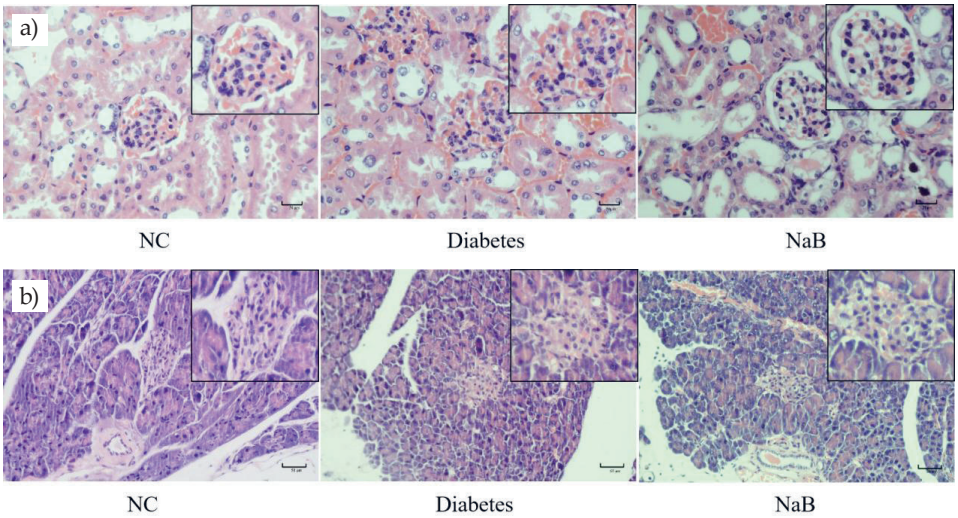

Fig. 4. a) HE staining results of mouse kidney tissue. The ICR mice were fed a high-sugar and fat diet and intraperitoneally injected with streptozotocin to induce diabetes. The NaB group was intraperitoneally injected with 100 mg kg^{-1} of NaB every two days at the beginning of the experiment. NaB can reverse diabetic kidney injury; b) HE staining results in mouse pancreatic tissue. The ICR mice were fed a high-sugar and fat diet and intraperitoneally injected with streptozotocin to induce diabetes. The NaB group was intraperitoneally injected with 100 mg kg^{-1} of NaB every two days at the beginning of the experiment. NaB can not reverse the pancreatic injury.

was regular. In the diabetes group, the volume of glomerulus increased, the mesangial matrix increased, and the epithelial cells of renal tubules were vacuolated; some epithelial cells of renal tubules shed to form the renal tubular type. In the NaB group, the epithelial cells of renal tubules shed less, and the tubule type decreased, as shown in Fig. 4a.

The NC group of mice pancreas tissue microstructure was normal and abundant; size was uniform, islet shape was regular, the boundary was clear, no swelling and congestion. Local acinar cells in the diabetes group were vacuolated, irregularly shaped, unevenly distributed and disorderly arranged in the islet. The islet cells were denatured and reduced. Compared with the diabetes group, the pancreas structure of the NaB group did not improve significantly (Fig. 4b).

CONCLUSIONS

Hyperglycemia in diabetic patients is an important factor in inducing systemic inflammation, which can increase the expression of many pro-inflammatory cytokines and cause various diabetic complications (25–27). Monocyte-macrophages are an important component of the human immune system. Pro-inflammatory cytokines secreted by activated monocyte-macrophages can effectively kill pathogens. Still, the overexpression of pro--inflammatory cytokines can cause damage to normal tissues and organs, resulting in complications such as diabetic nephropathy (27). Our results indicated that NaB could significantly reduce the inflammatory response of monocyte-macrophages induced by high concentration glucose, inhibit the expressions of pro-inflammatory cytokines in the spleen of diabetic mice, and have a protective effect on renal injury in diabetic mice.

The results showed that NaB could significantly reduce macrophage inflammation induced by high concentration glucose and protect against kidney injury in diabetic mice. The mechanism may be related to ROS production by inhibiting NLRP3 inflammasome activation. This study provides a new idea for preventing and treating diabetes and its complications in diabetic kidney disease.

Ethics approval and animal guidelines. – All the animal experiment methods in our study were in accordance with ARRIVE guidelines and approved by the management committee and use of laboratory animals, Jiangsu University (No. UJS-IACUC-AP-2020032009).

Conflicts of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualization, W.L. and S.W.; methodology, Y.M. and Z.Y-Y., X.Y. and D.L-K; analysis, Q.X.; investigation, X.L-J. and X.J-W.; writing, original draft preparation, Y.M.; writing, review and editing, W.L. and S.W. All authors have read and agreed to the published version of the manuscript.

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