Engineered Butyrate-producing Bacillus Subtilis Alleviated Ethanol-induced Intestinal and Liver Damage in Mice

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Abstract

Ethanol-induced intestinal and liver injury are closely associated with intestinal dysbiosis and altered short-chain fatty acid (SCFA) metabolites which is crucial for intestinal health. *Bacillus subtilis* (BS) strains with biotherapeutic potential can benefit the host through maintaining intestinal homeostasis and regulating systematic immunity via producing small molecules, although these molecules do not include butyrate. To combine the advantages of butyrate and BS, we evaluated the bioactivity of an engineered butyrate-producing *Bacillus subtilis* (BPBS) strain against ethanol exposure in a chronic-binge ethanol feeding mouse model. Our findings suggested that prophylactic BPBS supplementation restored eubiosis of the gut microbiota and intestinal barrier function, which obviously reduced bacterial translocation of microbial products especially lipopolysaccharide (LPS) to the circulatory system. Additionally, the decrease of serum LPS is responsible for the relief of hepatic inflammation via the Toll-like receptor 4 (TLR4) pathway, resulting in improved hepatic structure and function. Collectively, these results demonstrated that engineered BPBS intervention imparted novel hepatoprotective functions by improving intestinal barrier function and reducing systematic inflammation under ethanol exposure, as well as paving the way for further exploration of engineered probiotics in improving human health care.

Introduction

Currently, amounting evidence indicate that excessive alcohol consumption contributes to gastrointestinal and liver diseases, which are closely associated with the alterations of gut microbiota abundance and diversity (Boyle et al. 2018; Ceni et al. 2014). Of which, alcohol consumption significantly leads to increases in the phyla *Proteobacteria* and *Actinobacteria* and decreases in commensal probiotic bacteria such as *Faecalibacterium prausnitzii*, *Roseburia* spp. and *Bifidobacterium* that are major butyrate producers in the intestine (V. B. Dubinkina et al. 2017a; Yan et al. 2011; Y. Chen et al. 2011). Additionally, the overgrowth of *Proteobacteria*, especially *Enterobacteriaceae* (*Klebsiella*, *Enterobacter*, *Salmonella*, *Escherichia coli*, and *Shigella*), greatly increases the pathogenic potential of the intestinal microflora and aggravates alcohol-induced injury (G. Szabo 2015a; Betrapally et al. 2016). Collectively, ethanol consumption directly disorders the gut microbiota and intestinal barrier (Ferrere et al. 2017; Bjørkhaug et al. 2019; Shao et al. 2018), which exacerbates the development of alcoholic liver diseases (ALD) via inflammation induced by bacterial translocation from the intestine.

Excessive consumption of ethanol is known to greatly alter the metabolic status of gut microbiota, of which short-chain fatty acid (SCFA) shows obvious alterations, especially the significantly reduced production of butyrate (Roychowdhury et al. 2019). Accumulating evidence has demonstrated that butyrate, the most widely recognized metabolite of the gut microbiota, is closely related with human health (Parada Venegas et al. 2019; Kim et al. 2013; Kimura et al. 2020). Notably, butyrate plays a significant role in maintaining intestinal health, and its major mode of action is via mediation of signaling pathways involving nuclear factor kappa B (NF-κB) and inhibition of histone deacetylase (Inan et al. 2000; Hodin 2000). In addition, butyrate as an energy substance provides energy for colonic epithelial cells and even participates in maintaining intestinal homeostasis by facilitating tight junction assembly
and mucus secretion (Guilloteau et al. 2010; J. Chen and Vitetta 2018). Furthermore, butyrate is involved in energy metabolism by stimulating the secretion of insulin via g-protein-coupled receptor 43 (GPR43). In this context, butyrate as a feed additive has been developed and widely used to improve mammal and poultry health (Li et al. 2018; Piazzon et al. 2017; Bedford and Gong 2018).

With a deepening understanding of the relationship between the gut microbiota and human health, the gut microbiota has been recognized as a therapeutic target in some diseases, including obesity, enteric disease, ALD, and so on (Sonnenburg and Bäckhed 2016; Thaiss et al. 2018; Desai et al. 2016). Additionally, probiotics such as Lactobacillus, Bifidobacterium, Roseburia, and Bacillus, have been reported to be capable of reversing ethanol-associated intestinal barrier dysfunction by decreasing intestinal permeability and preventing bacterial translocation, as well as enhancing immune responses and reducing the inflammatory responses in the liver and intestine (Rondanelli et al. 2017; Grander et al. 2018; Chiu et al. 2015; Gu et al. 2019; Han et al. 2020). Notably, Bacillus subtilis (B. subtilis)-based probiotics are widely used as food ingredients and food additives to maintain the intestinal health of mammals and poultry; the beneficial attributes of B. subtilis are pertinent to its ability to produce small extracellular effector molecules and its cross-talk with hosts through the adhesion and attachment features (Khochamit et al. 2015; Compaoré et al. 2013; Elshaghabee et al. 2017). Intriguingly, another ingenious method of probiotic bioengineering has been adopted to precisely modulate and restore the effects of gut dysbiosis, such as the designed pyrroloquinoline quinone-secreting probiotic Escherichia coli Nissle 1917 and engineered IL-22-producing Lactobacillus reuteri (Jiang et al. 2017; Singh et al. 2014), but few studies have involved engineered butyrate-producing B. subtilis (BPBS), which has the potential for outstanding bioactivity in maintaining intestinal homeostasis.

In the present study, we investigated the beneficial effects of engineered BPBS that integrated the advantages of the B. subtilis and the central biological effect of butyrate in protecting mice from ethanol exposure, and elaborated the mechanisms of engineered BPBS supplementation in mitigating ethanol-induced intestinal and liver injury by restoring intestinal homeostasis and reducing hepatic inflammation with the assistance of butyrate.

Materials And Methods

Bacterial strains and cultures

The Bacillus strains used in the study are shown in Table 1. Wild-type B. subtilis sck6 (BS) and engineered butyrate-producing B. subtilis sck6 (BsS-RS06550, BPBS) was engineered by Dr. Liang Bai (Bai et al. 2020). Single colonies of BS and BPBS strains were cultured in LB broth at 37 °C overnight with shaking (200 rpm). Then, the precultures were diluted 1:100 in LB broth and grown at 37 °C with shaking (200 rpm) until the OD600 reached 0.5 ~ 0.6. Then, all the cultures were pelleted by centrifugation, diluted in fresh PBS solution and mixed thoroughly to obtain the appropriate bacterial density (B. subtilis: 5 × 10⁸ CFU/ml).
Table 1

*B. subtilis* strains used in this study

| Name | Strain | Characteristics | Source |
|------|--------|-----------------|--------|
| Wild-type *B. subtilis* (BS) | SCK6 | ErmR, 1A751 derivate, lacA::PxylA-comK | This study |
| Butyrate-producing *B. subtilis* (BPBS) | RS06550 | ErmR, 1A751 derivate, lacA::PxylA-comKΔskfA ΔsdpC ∆ butyryl-CoA: acetate: CoA transferase | This study |

**Construction of a mouse chronic-binge ethanol feeding model**

The animal experiments in this study were approved by the Ethics and Clinical Research Committee of Nankai University (Project IRM-DWLL-2016121). Six to eight-week-old male C57BL/6J mice (20–22 g) were purchased from Beijing Huafukang Incorporated (hfkbio, Inc., Beijing, China) and kept in a sanitary status of SPF. A chronic and binge ethanol feeding model was constructed in mice based on the Lieber-DeCarli diet (TROPHIC, Nantong, China). Two mice were housed in each cage, and all the mice were divided (12 mice in each group) into 4 groups as shown in Fig. 1a: pair-fed group (control), ethanol-fed group (EtOH-fed), ethanol-fed and BS supplementation group (EtOH + BS), ethanol-fed and engineered BPBS supplementation group (EtOH + BPBS). Briefly, after adaptation to the SPF environment for 2 days, all the mice were treated with the control diet for 7 days to acclimatize them to a liquid diet, and then, the ethanol-fed mice received ethanol (5% vol/vol) for 10 days, whereas the pair-fed mice received an isocaloric amount of maltodextrin. To investigate the effects of prophylactic BS intervention, the ethanol-fed mice were orally administered BS and engineered BPBS (approximately $1 \times 10^8$ CFU per mouse) in anaerobic PBS solution or vehicle alone (PBS solution) daily starting from liquid diet acclimatization. Body weights were measured every other day, and food intake was checked every day. On the last day, the mice received a single dose of ethanol via oral gavage (5 g/kg body weight) and were sacrificed by anesthetizing them with isoflurane (4%) after 9 h fasting for excision of tissue samples.

**Biochemical analysis**

Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured using the Infinity ALT Kit (Thermo Fisher Scientific). Serum LPS was determined using the Mouse Lipopolysaccharides (LPS) ELISA Kit (Cusabio, Wuhan, China). The serum levels of TNF-α, IL-1β, and IL-6, as well as the hepatic triglyceride (TG) and hepatic lipid peroxidation (MDA) level, were determined using a Mouse ELISA Kit (Solarbio, Beijing, China). All the assays were performed in triplicate according to the manufacturer’s instructions.

**Intestinal permeability assays**

To assay the intestinal permeability of the mice, fluorescein isothiocyanate (FITC)-dextran (4 kDa; Sigma-Aldrich) was orally administered (600 mg/kg body weight) 4 h before sacrifice. Blood samples were collected and subsequently centrifuged (4000 rpm, 4°C) for 15 min to isolate serum. Fluorescence was
recorded using a spectrophotometer (Tecan) at an excitation wavelength of 485 nm and emission wavelength of 528 nm.

**Real-Time qPCR**

Total liver and colon RNA were extracted by TRizol reagent (Invitrogen, USA), total RNA concentration was quantified using the NanoPhotometer N50 (Implen, Germany), and reverse transcription was performed using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio) on a Mastercycler nexus PCR machine (Eppendorf, Germany). Real-time qPCR was conducted on a LightCycler96 System (Roche, Switzerland) using TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio) and a cycling program of initial denaturation for 10 min at 95 °C, then 40 cycles of 10 s at 95 °C, 10 s at 62 °C and 10 s at 72 °C, followed by 95 °C for 60 s and a dissociation curve analysis. The primer sequences are listed in Table S1, and the relative gene expression was normalized to 18S and calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

**Western blot**

Total protein was extracted from the colon and liver tissues with RIPA buffer (containing 1% protease inhibitor and 1% phenylmethylsulfonyl fluoride) and then quantified by the BCA assay. The total protein was separated on a 10% SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and blocked with 5% skim milk, followed by immunostaining with primary antibodies against Occludin (1:1000, Abcam #EPR20992), NF-κB (1:1000, CST #4882) or TLR4 (1:1000, CST #14358). After incubation with a horseradish peroxidase-conjugated secondary antibody (1:10000, Solarbio), the immunoreactive proteins were stained with ECL Western Blotting Substrate. Images were captured using the ChemiDoc™ XRS System (Bio-Rad, USA). β-actin was used as an internal standard.

**Histopathological observation**

For the histological analysis, the liver and colonic tissues were stained with hematoxylin and eosin (H&E). Briefly, the tissues were fixed in 10% formalin, and paraffin-embedded sections were stained with H&E. The paraffin embedded tissues were sectioned, and then stained with AB-PAS.

**Fecal SCFA analysis**

Murine feces were collected and quickly frozen in liquid nitrogen, the frozen fecal samples were ground, and the level of SCFA was quantified by gas chromatography-mass spectrometry (GC-MS), as previously reported (David et al. 2014).

**DNA extraction and 16S rRNA amplification sequencing**

Total genomic DNA was extracted from 150 ~ 200 mg of fecal sample using the QIAamp PowerFecal DNA Kit (QIAGEN, Germany). The hypervariable V3-V4 region (341F and 805R) of the prokaryotic 16S rRNA gene was amplified and purified. The sequence was performed on the paired-end Illumina MiSeq PE300 (2 × 300 bp) platform at Novogene Corporation (Tianjin, China) according to the manufacturer’s
instructions. These raw sequences were processed following the QIIME (v1.9.1) pipeline (Caporaso et al. 2010), and the analysis of gut microbiota diversity and composition of fecal samples was determined.

**Statistical analysis**

All experimental results were obtained from at least three independent experiments, and the data were expressed as the means ± standard deviation (SD). One-way ANOVA or T-test was used to determine whether the groups were statistically significantly different ($P<0.05$). GraphPad Prism 8.0 (GraphPad Software) and R were used for all statistical analyses.

**Results**

**Engineered BPBS intervention ameliorates ethanol-induced injury in mice**

To explore the effects of BPBS intervention on ethanol-induced injury, we constructed a chronic-binge ethanol feeding mouse model based on a Lieber-DeCarli diet, as described previously (Bertola et al. 2013); the diagrammatic representation of the whole experiment is shown in Figure 1a. During the liquid diet acclimatization period, prophylactic intervention with BS or BPBS was administered by gavage daily until the end of the experiment, and then, the ethanol-containing diet was fed for 10 days. As expected, ethanol consumption significantly lowered the murine body weight after 5 days (Figure 1b) and notably increased the liver/body weight ratio (Figure 1c). However, supplementation with wild-type BS and BPBS significantly alleviated the decline in body weight gain and partially reduced the liver/body weight ratio, and the efficiency of BPBS intervention was greater than that of BS (Figure 1b, 1c). Additionally, the ethanol exposure stimulated the high expression of IL-6 in serum (Figure 1d), however, the serum IL-6 was significantly alleviated via the intervention of BS or BPBS. Collectively, prophylactic BPBS rather than BS supplementation is efficient to alleviate ethanol-induced injury in mice.

**BPBS intervention restores the ethanol-induced gut microbiota disorders in mice**

Chronic ethanol overconsumption is an important cues of gut microbiota dysbiosis, which may underlie the pathophysiology of ethanol-related morbidity (Bjørkhaug et al. 2019; Veronika B. Dubinkina et al. 2017b). Due to the notable health functions and inherent plasticity, gut microbiota has been suggested as an important target for the prevention of ethanol-related diseases (Bajaj 2019; Bajaj et al. 2018; Aden et al. 2019). In line with prior studies, the influence of ethanol feeding on gut microbiota was confirmed, and ethanol exposure remarkably reduced the gut microbiota abundance and diversity compared with that of the pair-fed group (Figure 2a-c). Meanwhile, the wild-type BS and BPBS intervention significantly increased the microbial diversity (Figure 2a) and obviously restored the microbial composition in the β-
diversity analysis (Figure 2c). Interestingly, BPBS supplementation is likely to work more efficiently in a murine ALD model than wild-type BS.

Next, we explored the taxonomic shifts in the bacterial community. At the phylum level, Firmicutes and Bacteroidetes were dominant in the fecal microbiota of the pair-fed group, whereas Firmicutes, Proteobacteria and Bacteroidetes were dominant in the ethanol-fed group (Figure 3a). Interestingly, the proportion of the family Enterobacteriaceae was significantly increased in the ethanol-fed group compared with the pair-fed group (Figure 3c), and more significant alterations were detected in the BS or BPBS intervention groups than the ethanol-fed group (Figure 3b). After the administration of BS or BPBS, the genera Bacillus and Ruminococcaceae were significantly increased (Figure 3d). Specific enrichment of the families Lachnospiraceae and Prevotellaceae was observed in the gut microbiota of the BPBS-fed group (Figure 3c). We further analysed the altered gut microbiota and found that gram-negative bacteria were enriched along with obvious potentially pathogenic phenotypes in the ethanol-fed group (Figure 3e, 3f). In contrast, the administration of BPBS aborted these potentially pathogenic phenotypes and improved the gut microbiota dysbiosis induced by ethanol consumption.

**BPBS intervention improves ethanol-disrupted intestinal barrier in mice**

Gut microbiota dysbiosis directly influences the physiological status of the intestine, and the improved gut microbiota contributes to facilitate host defence against hazardous substances or unfriend environments. We further investigated how prophylactic BPBS intervention mitigated ethanol-induced intestinal injury. Ethanol exposure notably disrupted intestinal barrier integrity with a high FITC concentration in the EtOH-fed group (Figure 4a). However, significantly reduced serum FITC levels were observed in the mice with BPBS supplementation compared to the EtOH-fed mice, suggesting a recovery of intestinal integrity. In accordance with the intestinal permeability, ethanol feeding resulted in a significant increase in serum LPS compared with that of the pair-fed group, while the BS and BPBS intervention significantly decreased serum LPS levels compared with those of EtOH-fed mice (Figure 4b), and BPBS supplementation worked better than wild-type BS. Overall, these results showed that the administration of BPBS mitigated the translocation of endotoxin from the intestinal lumen to circulatory system.

Intestinal barrier integrity is largely dependent on tight junctions, of which Occludin is the major component (Feldman et al. 2005). The protein expression of Occludin in the colon was determined, and ethanol feeding obviously decreased its expression (Figure 4d); however, the administration of BS and BPBS partially restored the protein Occludin expression compared with that of the EtOH-fed group. In line with the protein results, we observed the same restoration of Occludin gene expression in the BPBS group, along with the increased ZO-1 expression (Figure 4c), suggesting that BPBS administration could stimulate the expression of tight junction genes. Furthermore, histological analysis of the colon showed a damaged and thin mucosal layer after ethanol feeding in comparison with those of the pair-fed mice.
Figure 4e), whereas supplementation with BPBS significantly restored Muc2 gene expression (Figure 4c) and increased the secretion of mucins in intestine (Figure 4f). Moreover, ethanol feeding significantly cut down the butyrate yield of the gut microbiota and activated the inflammatory reaction in the colon, with increased *IL-6, IL-1β* and *TNF-α* gene expression compared with the pair-fed group (Figure 4g,4h); however, the administration of BPBS dramatically restored the butyrate contents in the intestine and decreased the excretion of inflammatory cytokines (Figure 4g,4h). Altogether, our results suggested that BPBS intervention replenished the butyrate yield of the gut microbiota and alleviated the inflammatory reaction in colon, as well as rebuilding intestinal barrier function through restoring the tight junction and mucin components.

**BPBS intervention attenuates ethanol-induced hepatic injury**

To determine how BPBS protects the liver against ethanol exposure, the biochemical and pathologic changes were further carried out. The development of hepatic injury induced by ethanol feeding was confirmed by obviously increased serum ALT, AST, hepatic triglycerides and MDA levels compared with those of the pair-fed mice (Figure 5a-5d). Daily administration of BPBS remarkably reduced the serum levels of ALT, AST and hepatic triglycerides (Figure 5a-5c). Moreover, obvious neutrophil infiltration in liver tissues after ethanol feeding was observed through H&E staining and was significantly alleviated by dietary BPBS supplementation (Figure 5e). Next, we quantified hepatic gene expression related to steatosis. Notably, the major liver functions of triglyceride synthesis and fatty acid uptake were disordered, with a significant increase in the expression of peroxisome proliferator activated receptor-γ (*PPAR-γ*) and transporter *CD36* for fatty acids (Figure 5f). Additionally, the decreased expression of *Fas, SCD1* and *Srebp-1c* induced by ethanol feeding were improved via dietary BPBS supplementation, which suggested that BPBS administration likely accelerated fatty acid synthesis in the liver and attenuated the hepatic function injury induced by ethanol consumption.

**BPBS intervention ameliorates ethanol-induced liver inflammation**

Ethanol consumption seriously damaged the intestinal gut integrity and thus accelerated gut bacterial translocation (especially LPS) in the bloodstream, which greatly triggered hepatic inflammation and contributed to the development of ethanol-induced liver diseases. Additionally, mounting evidence have shown that LPS induces organic inflammation based on a TLR4-dependent mechanism (Kayagaki et al. 2013; Hagar et al. 2013; Park et al. 2009). Interestingly, the increased serum LPS levels significantly stimulated the protein expression of TLR4 and nuclear factor-κB (NF-κB) in the liver (Figure 6a), along with an increased release of critical proinflammatory cytokines in serum, such as tumor necrosis factor alpha (TNF-α) and IL-1β (Figure 6c,6d). Accordingly, hepatic gene expression related to inflammatory cytokines, including *TNF-α, IL-1β, NF-κB* and monocyte chemoattractant protein-1 (*MCP-1*), was greatly enhanced in the ethanol-fed group compared to the pair-fed group (Figure 6b). However, after the
administration of wild-type BS and BPBS, the expression of hepatic TLR4 protein was remarkably decreased, along with concomitant significant decreases in the TLR4-regulated gene expression of \textit{NF-κB}, \textit{TNF-α}, \textit{IL-1β}, and \textit{MCP-1} in the treated group compared to the ethanol-fed group. Notably, BPBS intervention seemed to function better in ameliorating ethanol-induced hepatic inflammatory status via the LPS/TLR4 pathway.

**Discussion**

Emerging evidence have detailed the critical role of the gut microbiota in ethanol-induced liver injury via the gut-liver axis (Gao and Bataller 2011; Gyongyi Szabo 2015b). With a growing understanding of the gut microbiota and its metabolites, its crucial role and modes of action have been used to benefit human health, especially SCFA. Additionally, alterations of human genetics, environmental and dietary factors, and ethanol consumption support the development of alcoholic liver diseases (Yan et al. 2011; Mutlu et al. 2012). Unfortunately, there is still a shortage of effective strategies to address this problem except to stop drinking alcohol. However, efforts to explore the effects of specific probiotic strains on the recovery of alcoholic diseases are proceeding well. Here, we provide evidence of the beneficial effects of dietary supplementation with engineered BPBS in mitigating the intestinal and hepatic injury induced by ethanol in mice (Fig. 7).

Prior studies have shown that ethanol consumption significantly reduces fecal and cecal contents of butyrate by altering the gut microbiota and its metabolism (Guoxiang Xie et al. 2013b; G. Xie et al. 2013a; Barr et al. 2018). Here, ethanol feeding significantly increased the abundance of phylum Proteobacteria and reduced those of Firmicutes and Bacteroidetes, greatly enhancing the pathogenic potential of the intestinal flora composition. In the murine model, the ethanol-induced perturbation of gut microbiota was partially restored by the administration of engineered BPBS, which led to increased gut microbiota abundance and diversity. Additionally, several important clinical trials have demonstrated that \textit{B. subtilis}-based probiotic supplementation could improve a disrupted gut microbiota and even relieve the adverse effect of antibiotic-associated diarrhea (Horosheva et al. 2014; Takimoto et al. 2018). Other studies have provided evidence that the administration of BS significantly increased fecal and salivary secretory IgA concentrations in human subjects, which effectively stimulated immune responses to preserve the subjects from gastrointestinal tract infections. Overall, engineered BPBS intervention has great potential to restore a disordered gut microbiota and to activate immune responses against pathogenic infections.

Increasing LPS in the plasma initiated potent innate immune responses and led to cytokine production and inflammatory responses (Shi et al. 2014). Chronic inflammation maintains the mammalian body in a constant state of alertness, which has a negative influence on systemic tissues and organs, especially the liver (Furman et al. 2019). Supplementation with probiotics or synbiotics is emerging as a new therapeutic strategy to treat bowel and hepatic diseases, including ethanol-induced liver diseases (Gu et al. 2019). Dietary engineered BPBS supplementation ameliorated alcohol-induced systematic injury by directly maintaining intestinal barrier function, which greatly reduced the translocation of bacterial products to the blood and liver.
Ethanol exposure resulted in severe gut microbiota dysbiosis and subsequently disturbed the metabolic status of SCFAs, which eventually exacerbated the alcoholic disorders related to the intestinal barrier and liver (Roychowdhury et al. 2019). Among SCFAs, butyrate acts as a primary energy source for gut epithelial cells and participates in maintaining intestinal homeostasis (Ge et al. 2017). Prior studies have suggested that butyrate-producing bacteria could attenuate alcoholic fatty liver and other gut disorders, such as inflammatory bowel disease (IBD) (Seo et al. 2020; Geirnaert et al. 2017). Thus, the engineered BPBS combines the advantages of probiotic B. subtilis and butyrate, which notably alleviate ethanol-induced intestinal and hepatic injury, due to the increasing production of butyrate in the intestinal tract.

**Abbreviations**

ALD: alcoholic liver disease; BPBS: butyrate-producing Bacillus subtilis; BS: Bacillus subtilis; GPR43: g-protein-coupled receptor 43; IL-6: interleukin 6; LPS: lipopolysaccharide; PPAR-\(\gamma\): peroxisome proliferator activated receptor-\(\gamma\); TLR4: Toll-like receptor 4.

**Declarations**

**Ethics approval and consent to participate**

All the animal procedures were monitored by the Animal Care and Research Ethics Committee of the Nankai University.

**Availability of data and materials**

Data will be shared whenever it is required.

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**Authors' contributions**

Dr. F.W. and Dr. C.C. designed the experiments; Mr. X.L. and Miss. M.Z. performed experimental research and data analysis under the supervision of Dr. Y.D. and Dr. C.C., Dr. F.W. and Dr. C.C. wrote the manuscript text. All authors read and approved the final manuscript.
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Conflicts of Interest:

The authors declare no conflict of interest.

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