Betaine attenuates LPS-induced downregulation of Occludin and Claudin-1 and restores intestinal barrier function

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Abstract

Background: The intestinal epithelial barrier, which works as the first line of defense between the intestinal environment and the parasitifer, once destroyed, it will cause serious inflammation or other intestinal diseases. Tight junctions (TJs) play a vital role to maintain the integrity of the epithelial barrier. Lipopolysaccharide (LPS), one of the most important inflammatory factors will downregulate specific TJ proteins including Occludin and Claudin-1 and impair integrity of the epithelial barrier. Betaine (Bet) has excellent anti-inflammatory activity but whether Bet has any effect on tight junction proteins, particularly on LPS-induced dysfunction of epithelial barriers remains unknown. Intestinal porcine epithelial cells (IPEC-J2) were used as an in vitro model, the purpose of this study is to explore the pharmacological effect of Bet on improving intestinal barrier function represented by TJ proteins.

Results: The results demonstrated that Bet enhanced the expression of tight junction proteins while LPS（1μg /m L）downregulates the expression of these proteins. Furthermore, Bet attenuates LPS-induced decreases of tight junction proteins both shown by WB and RT-PCR. The immunofluorescent images consistently revealed that LPS induced the disruption of tight junction protein Claudin-1 and reduced its expression while Bet could reverse these alterations. Similar protective role of Bet on intestinal barrier function was observed by transepithelial electrical resistance (TEER) approach.

Conclusion: In conclusion, our research demonstrated that Bet attenuated LPS-induced downregulation of Occludin and Claudin-1 and restored the intestinal barrier function.

Background

Intestinal barrier, a single layer of cells located in the inner wall of the intestine, is mainly composed by the enterocyte membranes and the tight junctions between the enterocytes[1]. The integrity of the intestinal barrier is critical for the health of humans and animals. Destruction of the intestinal barrier give rise to increased intestinal permeability, which in turn accelerates the translocation of pathogens or other harmful substances to the blood stream[2]. This disruption will also contribute to the progress of the necrotizing enterocolitis (NEC) and inflammatory bowel disease (IBD) [3, 4]. Thus, protecting the completeness of the intestinal barrier is an excellent strategy to prevent the progress of both IBD and NEC. Intestinal epithelial cells are closely bound by tight junctional complexes between the cells, which regulates the permeability of adjacent cells and is critical to the integrity of the epithelial barrier [5, 6] And one kind of the essential complex is TJ proteins, Claudin-1 and Occludin for instance.

Betaine (Bet), also called glycine betaine, is a kind of natural compound which is wildly distributed in organic organisms [7] and can be easily obtained from the plant beet. It is reported that Bet, as an effective organic osmotic substance, plays an important role in regulating cells' adaptation to adverse osmotic environment[8]. Besides, Bet also has the activities of anti-inflammatory and can improve intestinal function[9]. And some researchers find that Bet has osmotic protection properties that help protecting the proteins and enzymes of intestinal cells from environmental stress. Inclusion of Bet in the

diet have a beneficial effect on relieving physical reactions to heat stress in both poultry and growing-finishing pigs[10, 11]. However, the specific mechanisms regarding how does it improve the intestinal function remains elusive.

Lipopolysaccharide (LPS), an inflammatory stimulator, can destroy the intestinal barrier[12], which lead to the increase of intestinal permeability, the destroy of tight junction proteins and the inflammation of the gut [13–16]. Caco-2, the human colon cancer cell line is commonly used as an intestinal cell model. However, this approach has been questioning due to its abnormal characters including rapid growth and proliferation properties. Recently, differentiated IPEC-J2 cell line is recognized as a better cellular model to explore the role of the intestinal barrier[17]. Thus, in this study, IPEC-J2 cells were applied as a cellular model to evaluate the effects of Bet alone or combined with LPS on intestinal barrier function. The purpose of this study is to explore the mechanistic effect of Bet on improving intestinal barrier function, on the basis of Western blot, RT-PCR, TEER and Immunofluorescence.

Results

Effects of betaine on the expression of tight junction protein in IPEC-J2 cells

First, cells were treated with different concentrations of betaine (0–2 mmol/L) in order to determine its effect on TJ proteins. The results (Fig. 1A-B) clearly demonstrated that different concentrations of Bet can enhance the expression of Occludin and Claudin-1 with optimized concentration of 2 mmol/L. From the results we decided to choose the concentration of 2 mmol/L for the subsequent experiments. Next, we used LPS to treat the cells and the combination of Bet and LPS significantly restored Occludin and claudin-1 protein expression, compared with LPS alone.

Effects of betaine on gene expression of Claudin-1

To further confirmed the effects of betaine, RT-PCR was used to determine the mRNA expression level. We treated IPEC-J2 for 4 h with ctrl, LPS, LPS + Bet and Bet four groups. LPS reduced the mRNA expression level of Occludin and increased the expression of IL-6, indicating that LPS destroyed TJs and induced inflammation. And the expression of IL-6 and Occludin of LPS + Bet group was decreased almost to the same as the ctrl group, indicating that betaine could enhance the mRNA expression of Occludin and exert the function of anti-inflammation.

Effects of betaine on cell morphology

Based on the above results, we are interested in if there are any change of the cell morphology after different treated. Therefore, we culture the cells and then treated with ctrl, LPS(1 μg/mL), LPS + Bet and Bet alone for 24 h. Pour the culture medium out and then soak cells in PBS and take photos under microscope. It could be observed from the Fig. 3 that after different treatments, the cell morphology didn’t have an obvious change.

Immunofluorescence to detect the expression and localization of Claudin-1

To further observe and verify the effect of Bet on tight junction proteins, immunofluorescence was used since the results obtained from the immunofluorescence microscope are more visualized. After different processing, we observed the localization and expression of tight junction protein Claudin-1. The protein of control group is complete and well-connected while LPS destroyed it. When co-treated with Bet it was recovered, and excitingly Bet alone processing team has the best expression, which proved that betaine can enhance the expression of TJ protein.

Betaine increases the TEER in LPS-induced IPEC-J2 cells Differentiated IPEC-J2 cell was used as a cellular model to investigate the role of the intestinal barrier function, and TEER was used to measure the integrity of intestinal barrier function and as an indicator of intestinal barrier function. Previous studied has proved that LPS increased the gut permeability and disordered the intestinal barrier function. However, whether Bet plays any role in LPS-induced intestinal barrier dysfunction and permeability variation remains unclear. As indicated in Fig. 5, LPS caused the decline of TEER in 12 h and keep it to 72 h. In contrast, Bet treated alone group increased the TEER value obviously, and the TEER of LPS and Bet combined team was almost the same as control group, which fully proved that betaine is able to keep the intestinal permeability and improve intestinal barrier function.

Discussion

Betaine is a stable and non-toxic natural compound[18] and is widely distributed in organisms such as bacteria and mammals[19], especially abundant in the plant beet. Betaine is a elementary biochemical molecule which participates in the methionine/homocysteine cycle [20] and acts as a methyl donor during methyl-transformation [21, 22]. And it is also an osmotic pressure protector which is important to maintain the function of intestinal tissues. There are lots of credibility evidences show that betaine also have the function of anti-inflammation and improving intestinal barrier function[23]. Yang et al found that betaine alleviates monocrotaline-induced pulmonary hypertension in rat by restraining the inflammatory response, such as down-regulating the NF-κB signaling pathway or inhibiting the inflammatory reaction [24]. Olli. K demonstrated that betaine have the function to reduce the expression of inflammatory adipokines [25]. Besides, Wang et al found that betaine reinforced the activity of digestive enzymes, ameliorated the intestinal morphology, and enriched the intestinal microbiota of rats with high-salt stressed, which indicates it's positive effect on intestinal barrier function [8]. Betaine is involved in osmotic regulation of the duodenal epithelium of broiler chicks and has a positive effect on the movement of water across the intestinal epithelial cell in vitro. In addition, betaine supplementation reduced the challenge effect of coccidioides and had a positive effect on the nutrient digestibility and the feed conversion ratio[26, 27]. Dietary betaine can assemble in intestinal tissues and reinforces the structure of intestinal epithelium [28]. However how does betaine improve intestinal function is not clear and needed to

explore. It is well-known that tight junction is an essential constituent part of intestinal barrier and has a strongly association with intestinal inflammation. Tight junction proteins Occludin and Claudin-1 are necessary for tight junctions to maintain intestinal permeability and intestinal barrier function[13]. And the intestinal barrier function is even accommodated in some ways by tight junction proteins.[29–31]. There are evidences proved that LPS-induced inflammation destroyed the integrity of intestinal epithelial cells and tight junctions[17]. In the present research, we determined that betaine(0–2 mmol/L) improved the expression of TJ proteins and with the concentration of 2 mmol/L, betaine was able to relive the down-regulation of TJ proteins completely which is induced by LPS. Meanwhile, the results improved that Bet decreased the gene expression of IL-6 which also means Bet was able to withstand inflammatory. TEER (Transepithelial electrical resistance) has long been considered as a common useful indicator of intestinal epithelial cell permeability [32, 33]. And IPEC-J2 cell is a widely accepted model of intestinal cell lines [34]. The increase of TEER signifies the reinforce of intestinal barrier function and the decrease of permeability. Our results are conformed to it: the TEER of Bet and LPS co-treatment team is recovered almost to the same as ctrl group while LPS decreased the TEER, and Bet alone increased the TEER and enhanced intestinal barrier function. In summary, we can get the information from the results that betaine regulates intestinal barrier function via enhancing the expression of TJ proteins and relieving the LPS-induced destroy of TJs. The results lay a solid mechanism foundation for the daily supplement application of betaine.

Conclusion

In summary, our research expounded the important regulatory role of betaine in intestinal epithelial barrier function. Different concentrations of betaine improved the expression of tight junction proteins Occludin and Claudin-1. With the concentration of 2 mmol/L, betaine recovered the LPS-induced inflammation, the down-regulation of TJ proteins and the dysfunction of intestinal epithelial barrier. Therefore, betaine, a non-toxic natural compound, is a useful nutrient supplement to regulate intestinal barrier function and prevent intestinal inflammation.

Materials And Methods

Materials

Betaine and LPS were purchased from Sigma-Aldrich (Saint Louis, USA), and it was dissolved in PBS and prepared as a solution with a concentration of 100 mmol/L. Occludin and Claudin-1 were obtained from Cell Signaling Technology (Shanghai, China). All the reagents were stored at -20°C.

Cell culture

The intestinal porcine epithelial cell line (IPEC-J2 cells) was obtained from Animal Nutrition & Human Health Laboratory, Hunan Normal University. IPEC-J2 cells were cultured in DMEM with 10% of FBS and

1% of penicillin–streptomycin (Hyclone, Logan, UT, USA) at 37 °C, 5% of CO_2. The medium was refreshed every second day.

Western blot

Western blot (WB) was used to evaluate the expression of tight junction proteins in IPEC-J2 cells. The experimental steps are consistent with regular procedure. First, prepare polyacrylamide gel and then electrophoresis after adding the required samples. Next, cover the PVDF membrane with 5% milk for 1 hour after western transfer, and then wash the membranes for 3 times. Then incubate it with primary antibodies overnight. Wash the membrane 6 times and then incubate second- antibody for 1 hour the next day. Development was performed by chemiluminescence equipment after washing it again and the pictures were measured by Image J.

Morphological observation of cells

Inoculate 5×10^5 IPEC-J2 cells per well in 6-well plate and culture for 2 days(change the culture medium after inoculated cells for 24 h), then treated it with Ctrl, LPS(1 μg/mL), LPS + Bet(2 mmol/L) and Bet(2 mmol/L) alone for 24 h. Discard the culture medium and wash it for 2 times with PBS. Cover the cells with PBS and then take the picture under light contrast microscopy (Leica, DFC450C, Wetzlar, Germany).

Immunofluorescence

IPEC-J2 cell monolayers were incubated and cultured in 6-well plates. Then treated with ctrl, LPS(1 μg/ml), LPS + Bet(2 mmol/L) and Bet(2 mmol/L) for 24 hours. Wash the IPEC-J2 cell with PBS for three times, then fix it with paraformaldehyde (4%) no less than 20 minutes. Wash it again and then incubate with Triton-X-100(0.1%) for 10 minutes to permeate the cells. Then wash it for 3 times (10 min each) and block for 30 minutes with bovine serum albumin (BSA 1%). Next the cells were incubated with Claudin-1 overnight. Then wash it with PBS for 3 times and incubate the cells with second- antibody for 2 hours. Next wash the cells were for another 30 minutes and then cover the DAPI dye solution with a glass slide where the cells attached to. Then using inverted fluorescence microscope to take the microscopic images of the cells.

RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the expression of relative mRNA. First cDNA was extracted by conventional methods, and the PCR system was composed by 5.0μL of Green qPCR Mix, 0.3μL of Forward primer and Reverse primer, 4.2μL of double distilled water, and 0.2μL of cDNA. The final volume was 10μL. Then the housekeeping gene GAPDH was used as the standard control, and each sample was repeated for three times, then Real Master Mix SYBP ROX (5’ Prime) was used for quantitative real-time PCR.

Table 1
Sequences of Primers

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
GAPDH	GAAGGTCGGAGTGAACGGAT	CTGGCATTGACTGGGGTCAT
Occluding	TTGCCTGGGACGAGGCTATG	ATCCCTTTGCTGCTCGTGGA
IL-6	TGGATAAGCTGCAGTCACAG	ATTATCCGAATGGCCCTCAG

TEER

Infiltrate the 24-hole transwell plate (Costar, Coring Inc, NY, USA) with DMEM culture for 2 h or overnight before inoculating IPEC-J2 cells (1×10^5 cells per cm^2). After being adherence, cells were differentiated in the culture medium without serum for 7–10 days. When the resistance value reaches the desired value, treat the cells with ctrl LPS(1 μg/mL) LPS + Bet(2 mmol/mL) Bet 2 mmol/mL respectively. The way to measure the cell integrity is TEER, which used an epithelial voltage ohmmeter with chopstick electrodes (Millicell ERS-2, EMD Millipore, Billerica, MA). TEER measurements are operated in gnotobasis, take the plate out of the 37 °C cell incubator and put them in the operating floor for at least 30 min, disinfect the electrodes by 70% ethanol and then dry in the air for 30 s. Then wash the electrodes with culture medium, immersed the electrodes and make sure the shorter electrode is in the insert of the plate and the longer electrode is located between the outer wall and the filter insert. Ensure all the electrode tips are absolutely immersed by solution and the shorter one does not come into contact with the cells which are growing on the insert. And record the resistance value of 12 24 36 48 72 h.

Statistical analysis

All the statistical data were analyzed by the software GraphPad Prism version6. Data were expressed as mean ± SD and relative gene expressions were transformed into natural logarithm scale. One-way and two-way ANOVAs were used to compare the differences between different treatments. A p-value 0.05 was considered statistically significant.

Abbreviations

Bet Betaine

LPS Lipopolysaccharide

TJs Tight junctions

TEER Transepithelial electrical resistance

IPEC-J2 Porcine intestinal epithelial cells

Declarations

Abbreviations

Bet Betaine

LPS Lipopolysaccharide

TJs Tight junctions

TEER Transepithelial electrical resistance

IPEC-J2 Porcine intestinal epithelial cells

Acknowledgments

Not Applicable

Authors' contributions

CH directed the TEER and RT-PCR experiments. JW finished the main part of the experiments and was a major contributor in writing the manuscript. JB and YL analyzed the results of western blot. SY, CY and CY participated in writing the manuscript and searching related articles. All authors read and approved the final manuscript.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Competing interests

The authors declare no competing interests

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Figures

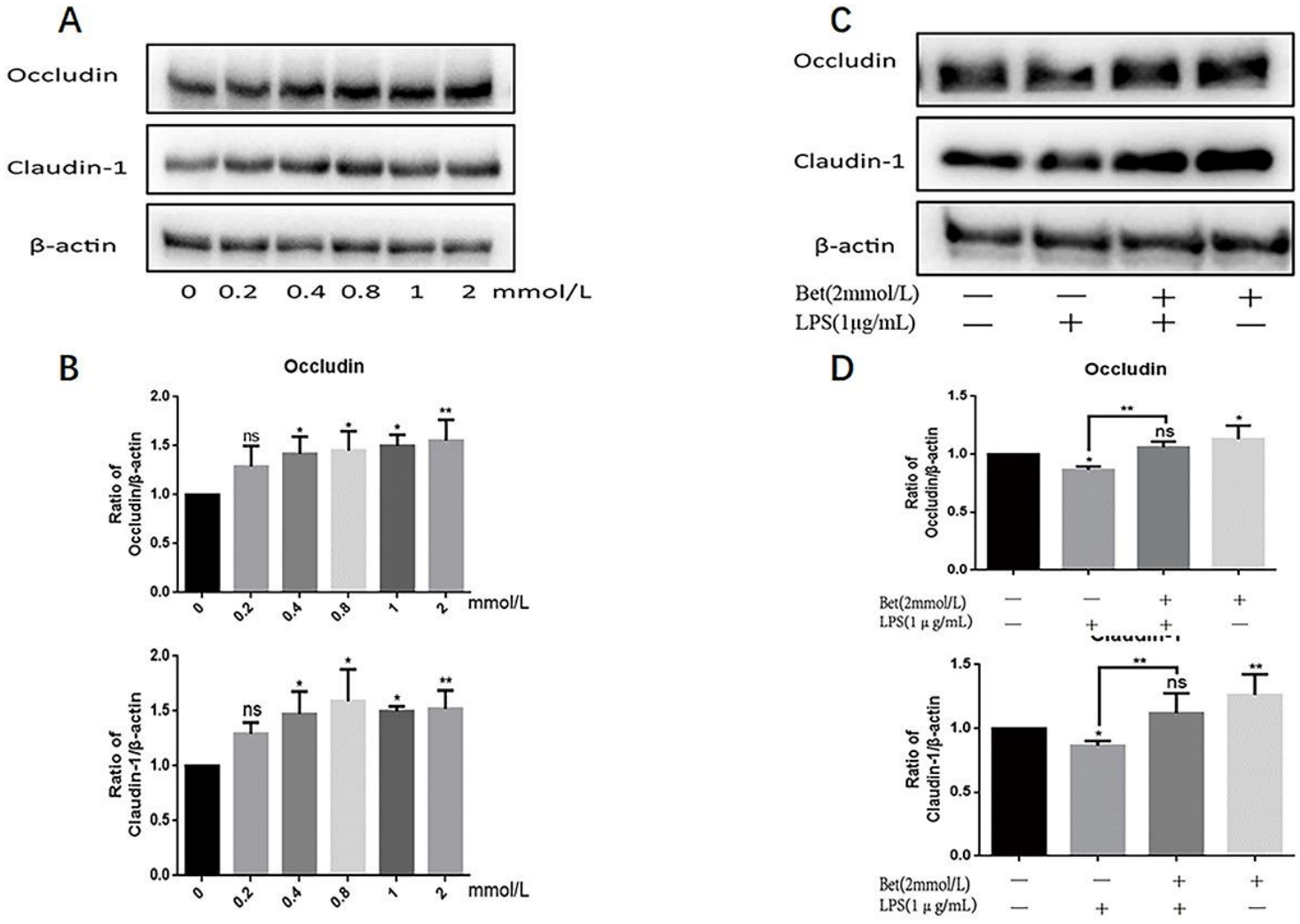

Figure 1

Bet increased the expression of TJ proteins. A showed the results of western blot about Occludin and Claudin-1. After treated for 24h, various concentrations of betaine (0-2mmol/L) improved the expression Occludin and Claudin-1 in IPEC-J2 cells. C confirmed that LPS down-regulate the protein levels of Occludin and Claudin-1, while Bet totally attenuated the downregulation and recovered it to a normal level. B and D are the statistical graphs of the density ratios of different proteins to β-actin calculated by Image J and analyzed by Graphpad Prism6 . (*P < 0.05, **P < 0.01 compared with control).

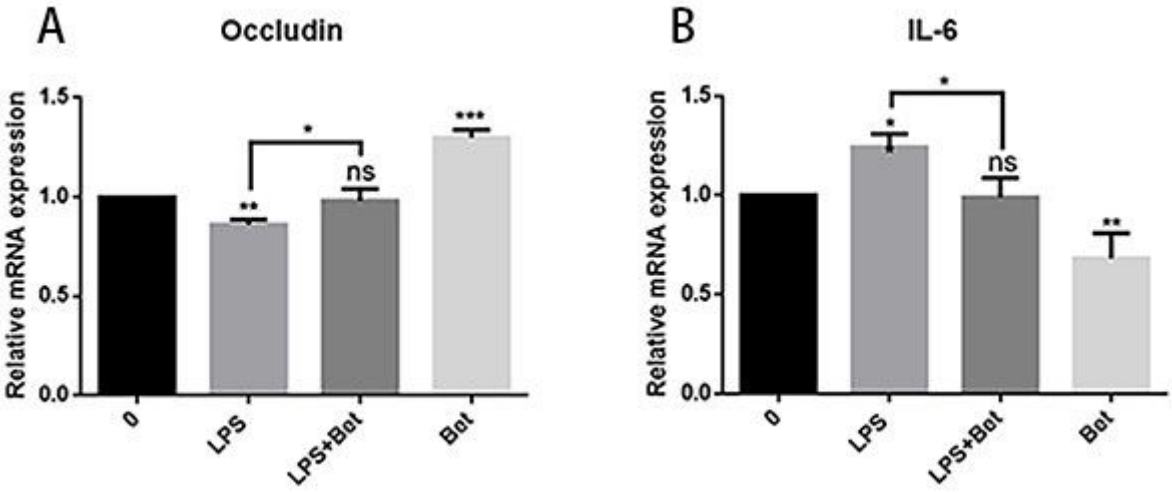

Figure 2

Bet suppressed inflammatory response and recovered the mRNA expression of TJ proteins. A confirmed that betaine（2mmol/L, treated for 4h）recovered the down-regulation of Occludin induced by LPS(1μg/mL), and enhanced the mRNA expression of Occludin. Picture B proved that LPS induced inflammatory while co-treatment of Bet and LPS made the IL-6 expression drop to normal levels. Besides, betaine alone reduced the IL-6 expression. (*P < 0.05, ** P < 0.01, ***P<0.001 compared with control).

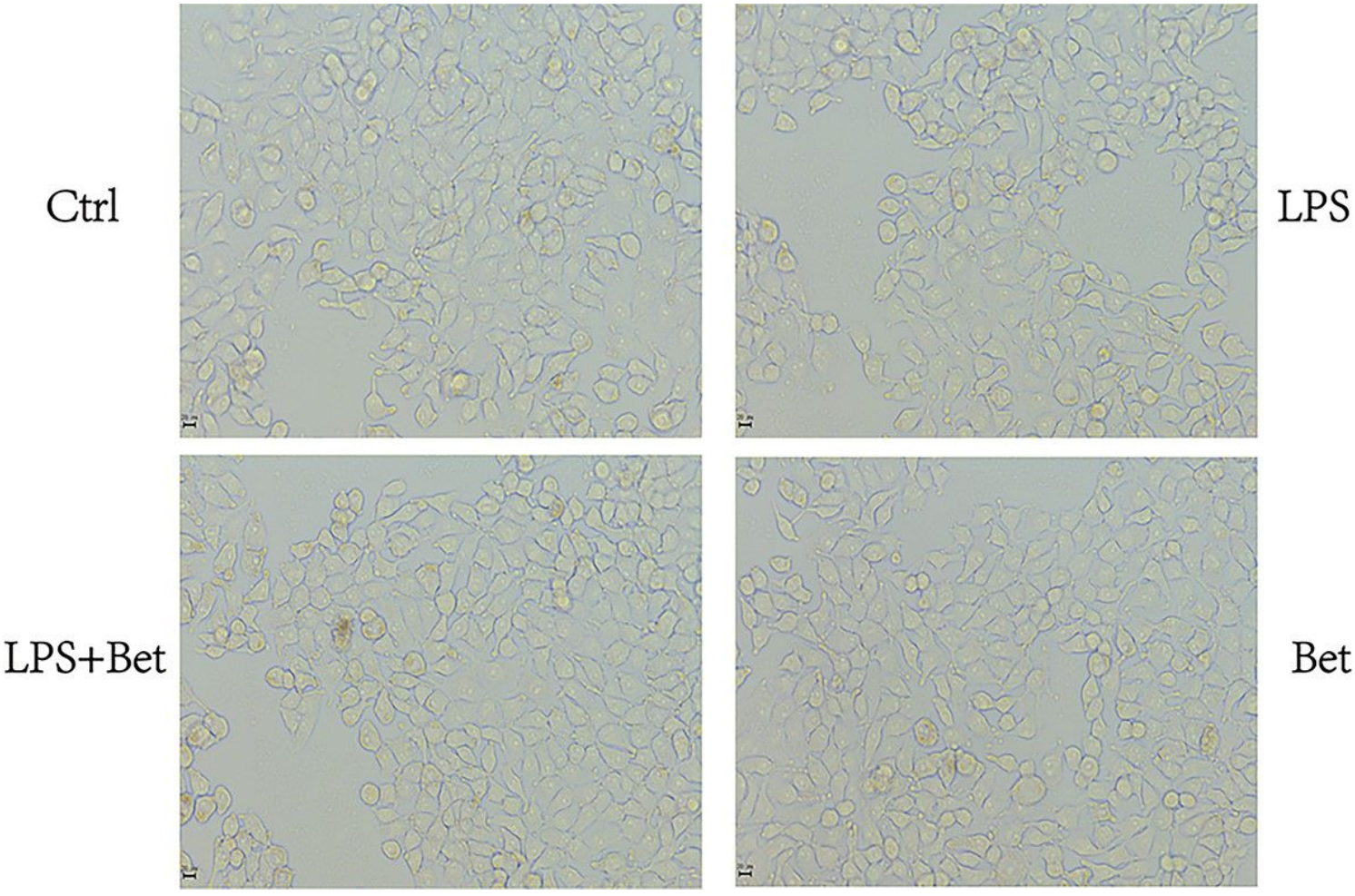

Figure 3

The observation of IPEC-J2 cells. There was no obvious difference between the four group, all the cells looked normal and complete.

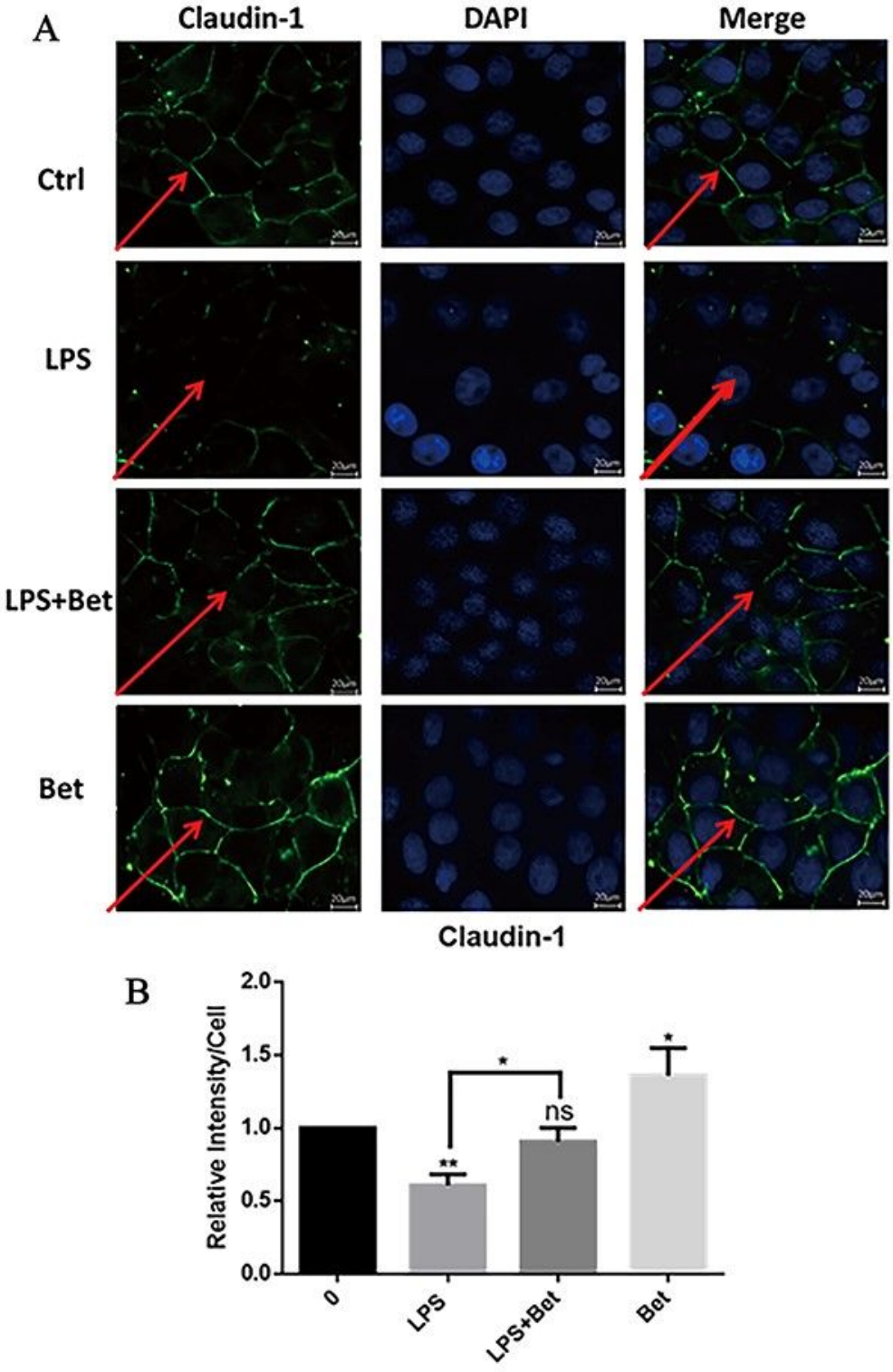

Figure 4

Bet reinforced the tight junctions between the IPEC-J2 cells. Picture A showed the result of immunofluorescence localization of the TJ protein Claudin-1. The TJ protein of control processing group was in good connection while the LPS treatment group was seriously destroyed. The LPS and betaine combined treatment group was much better than the LPS treatment group and nearly the same as the control group. And betaine alone treatment group had the best expression of Claudin-1. B was the semi-quantitative analysis of fluorescence intensity. (*P < 0.05, ** P < 0.01, compared with control).

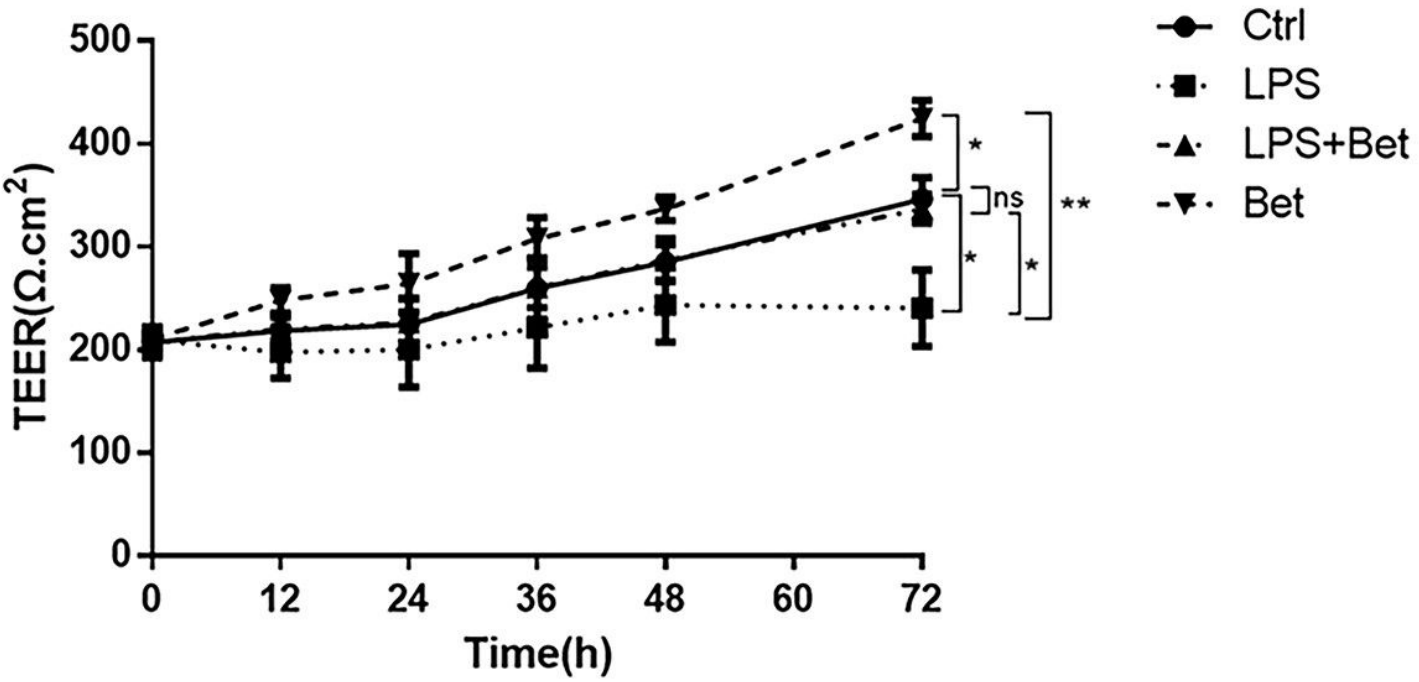

Figure 5

Intestinal barrier function and permeability were restored by Bet. The result of TEER measurement indicated that LPS(1μg/mL) decreased the TEER which represented the dysfunction of intestinal barrier and permeability, while combined with Bet, the TEER was relieved to normal. And Bet alone increased the TEER and all the effects continued to 72h. Attention, all the TEER value in 0h were very close and didn't have a big margin of error (Data are expressed by means ± SD, n=3, *p<0.05 ** P < 0.01, comparison between various groups by two-way ANOVA.).