Research Square

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In Vitro Model stimulated by exposure to single and combined stress factors for Necrotizing Enterocolitis Running title: In Vitro Model stimulated by single and combined factors for NEC

chuchu gao
Children's Hospital of Soochow University
Zongtai Feng
The Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou Municipal Hospital)
Lixia Wang
The Second Affiliated Hospital of Soochow University
Zuming Yang
The Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou Municipal Hospital)
Sannan Wang
The Affiliated Suzhou Hospital of Nanjing Medical University (Suzhou Municipal Hospital)
Shenglin Yu (wm1971@sina.com)
Children's Hospital of Soochow University

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Abstract

Purpose

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease in neonates, with high death rate. The pathogenesis of NEC is particularly complex, mainly involving inflammation and hypoxic damage. In vitro cell model is an indispensable tool to study the pathogenesis of NEC. This study explored the effects of different stress factors on intestinal injury in vitro.

Methods

IEC-6 cells were stimulated by exposure to different stressors, including lipopolysaccharide (LPS), cobalt chloride ($CoCl_2$), and a combination of both. Cell viability was detected by CCK-8 assay. The expression of inflammatory cytokines (IL-6 and TNFα) at the gene and protein levels were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and enzyme-linked immune-sorbent assay (ELISA). While the expression of tight junction proteins (Claudin-1 and zonula occludens [ZO]-1) were evaluated by qRT-PCR and western blotting, respectively.

Results

The decrease in IEC-6 cell viability was observed after stimulation by $CoCl_2$ alone or in combination with LPS, but not after stimulation with LPS alone. The expression of IL-6 and TNFα increased in each group, especially in the combined stimulation group. After stimulation with $CoCl_2$ alone or in combination with LPS, a decrease in Claudin-1 was observed, but an increase was detected after stimulation with LPS alone. ZO-1 decreased in both mRNA and protein levels after combined stimulation.

Conclusion

The combined stimulation of $CoCl_2$ and LPS on IEC-6 cells could simultaneously induce severe inflammation and barrier damage, which may better simulate the pathological process of NEC. Further research is needed to determine whether this in vitro model can be used to study the pathogenesis of NEC.

Introduction

Necrotizing enterocolitis (NEC), defined as an acute necrotizing intestinal disease caused by a variety of perinatal factors, is the most common and devastating gastrointestinal pathology affecting prematurity [1]. The pathogenesis of NEC remains incompletely understood. Multiple risk factors have been recognized, including hypoxia and infection [2, 3]. Despite the rapid development of neonatal healthcare,

the prevention and treatment of NEC remains a global problem [4, 5]. Therefore, the in vitro model of this disease is very important to study the pathophysiological mechanism of this disease [6].

Continuous renewal and repair of intestinal epithelial cells is essential to maintain intestinal homeostasis [7]. Several intestinal epithelial cell lines have been used as classical intestinal models to simulate the pathogenesis of NEC in vitro [8]. Different stimulation methods have been proved to induce intestinal epithelial cell damage, mainly including LPS, TNFα, H_2O_2 and hypoxia [9–12]. However, few studies have compared the effects of single and combined administration on intestinal epithelial cell lines. One study showed that the use of LPS or H_2O_2 alone would not damage the regeneration of intestinal epithelial cells, while the use of multiple stress factors would damage their regeneration, as observed in NEC [13]. Recently, a new apical-out NEC in-a-dish model was reported based on hypoxia combined with LPS or TNFα. It was found that single exposure to LPS, TNFα or hypoxia did not reduce the integrity of epithelial barrier [14].

In this study, we compared the effects of LPS, $CoCl_2$ (a chemically hypoxia mimetic agent) and the combination of both on the injury of IEC-6 cells, in order to provide a preliminary basis for the mechanism study of NEC in vitro model.

Methods

Cell culture

The rat small intestinal crypt epithelial cell line-6 (IEC-6) was purchased from FuHeng Biotechnology Co., Ltd (China). According to the supplier's recommendation, cells were cultured in DMEM/ HIGH GLUCOSE medium (HyClone, USA) supplemented with 10% fetal bovine serum (Gibco, USA) at 37°C, 5% CO_2 conditions in an incubator.

Cell Counting Kit-8 (CCK-8) assay

IEC-6 cells were seeded into 96-well plates (BD Falcon, Corning Inc., Corning, NY) at a density of 0.8×10^4 cells per well for about 48 h to form a confluent monolayer. Then, cells were exposed to different concentrations of lipopolysaccharide (LPS; from *Escherichia coli* 0111: B4; Sigma–Aldrich) and cobalt chloride ($CoCl_2$; Sigma–Aldrich) individually or together for 24 h. Subsequently, cell viability was detected using a CCK8 assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions.

Quantitative Reverse Transcription-polymerase Chain Reaction (Qrt-pcr)

To examine the expression level of mRNA, total RNA was extracted from IEC-6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversely transcribed to cDNA using the All-in-one First Strand cDNA synthesis SuperMix kit (Novoprotein, China). qRT-PCR was conducted with SYBR qPCR SuperMix (Novoprotein, China). The expression levels of mRNA were normalized to β-actin copies and then

calculated using the $2^{-\triangle\triangle Ct}$ method. The sequences of forward (F) and reverse (R) primers for each gene were listed in Table 1. All experiments were performed in triplicate.

Enzyme-linked Immunosorbent Assay (Elisa)

The supernatant of cell culture was collected by centrifugation at 1000×g for 15 min. The levels of IL-6 and TNFα were detected using ELISA kits (RK00020, ABclonal, Wuhan, China; P16599, CUSABIO, Wuhan, China) following the manufacturer's instructions. The optical density of each well was determined using Multifunctional microplate reader (TECAN Infinite 200 Pro, Switzerland) set to 450 nm.

Western Blot Analysis

The whole protein of IEC-6 cells was extracted using RIPA buffer (Beyotime, Shanghai, China) with protease inhibitor cocktail (ab271306, Abcam). The protein concentration in solution was determined by the BCA protein assay kit (Beyotime, Shanghai, China) and 5 × loading buffer (Beyotime, Shanghai, China) was added to prepare protein samples. The protein of each sample was separated by SDS-PAGE electrophoresis and then transferred onto a PVDF membrane (Millipore, MA, USA).

The membrane was blocked in blocking solution (5% non-fat milk in TBST buffer) for 1 h at room temperature and then incubated with different antibodies on the shaker at 4 ℃ overnight. The antibodies comprise Claudin-1 (28674-1-AP, Proteintech, Hubei, China), ZO-1 (21773-1-AP, Proteintech, Hubei, China) and β-actin (AC026, ABclonal, Wuhan, China). Subsequently, the membrane was washed by TBST buffer and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The specific protein bands were visualized using ECL detection reagents (Merck Millipore, USA) and Syngen GeneGnome XRQ system (SYNGENE, UK).

Statistical analysis

GraphPad Prism version 8.3.0 was used to analyze all data. Consistent with normal distribution, the data were described as mean ± standard deviation and compared between groups using one-way analysis of variance (ANOVA). While non-normal distribution of quantitative data were expressed as median with interquartile range and compared between groups using non-parametric Mann-Whitney *U* test. The difference with a *p*-value < 0.05 was statistically significant.

Results

Cell viability of IEC-6 following administration of LPS, $CoCl_2$ individually or together

To determine the proper concentration of LPS and $CoCl_2$, we used CCK8 assay to detect IEC-6 cells viability. As Fig. 1 shows, different concentrations of LPS (10, 25, 50, 100, 200, 400 μg/ mL) did not cause significant cytotoxicity (Control: 1.28 [1.17–1.34], 10 μg/ mL LPS: 1.31 [1.06–1.53], 25 μg/ mL LPS: 1.44 [1.18–1.52], 50 μg/ mL LPS: 1.62 [1.36–1.66], 100 μg/ mL LPS: 1.66 [1.37–1.69], 200 μg/ mL LPS: 1.59 [1.29–1.77], 400 μg/ mL LPS: 1.46 [1.42–1.49]) (Fig. 1A). However, the viability of IEC-6 cells significantly decreased after stimulation by $CoCl_2$ alone (Control: 1.59 ± 0.19; 50 μM $CoCl_2$: 1.81 ± 0.14; 100 μM $CoCl_2$: 1.69 ± 0.04; 200 μM $CoCl_2$: 1.48 ± 0.11; 400 μM $CoCl_2$: 1.02 ± 0.12, $p < 0.001$; 800 μM $CoCl_2$: 0.27 ± 0.02, $p < 0.0001$; 1600 μM $CoCl_2$: 0.29 ± 0.01, $p < 0.0001$]) (Fig. 1B) or in combination with LPS (Control: 2.01 ± 0.02; 50 μM $CoCl_2$ + 10 μg/ mL LPS: 2.01 ± 0.05; 100 μM $CoCl_2$ + 10 μg/ mL LPS: 1.98 ± 0.03; 200 μM $CoCl_2$ + 10 μg/ mL LPS: 1.81 ± 0.04, $p < 0.001$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 1.33 ± 0.05, $p < 0.0001$) (Fig. 1C). Based on the above results, we selected 10 μg/ mL LPS and 400 μM $CoCl_2$ as the concentration for follow-up study of single or combined stimulation.

mRNA levels of inflammatory cytokines and tight junctions in IEC-6 cells exposed to LPS, CoCl2 individually or together

qRT-PCR was conducted to evaluate the mRNA levels of inflammatory cytokines (IL-6 and TNFα) and tight junctions (Claudin-1 and ZO-1) in IEC-6 cells exposed to different treatment. The relative gene expression of IL-6 significantly increased in each group, especially in the combined stimulation group (10 μg/ mL LPS: 4.81 [4.76–4.87]; 400 μM $CoCl_2$: 42.03 [36.64–43.79], $p < 0.0001$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 75.63 [57.92–75.63], $p < 0.0001$) (Fig. 2A). Similarly, the relative gene expression of TNFα significantly increased in each group, also especially in the combined stimulation group (10 μg/ mL LPS: 4.80 ± 0.67, $p < 0.0001$; 400 μM $CoCl_2$ 1.94 ± 0.62, $p <$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 4.98 ± 0.41, $p <$ 0.0001) (Fig. 2B). The relative gene expression of Claudin-1 showed a significantly decrease after stimulation with $CoCl_2$ alone or in combination with LPS, but a significantly increase after stimulation with LPS alone (10 μg/ mL LPS: 1.81 [1.81–1.90], $p < 0.0001$; 400 μM $CoCl_2$: 0.18 [0.15–0.19], $p < 0.0001$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 0.13 [0.10–0.14], $p < 0.0001$) (Fig. 2C). While, the relative gene expression of ZO-1 was most significantly down-regulated in the combination group (10 μg/ mL LPS: 0.79 [0.73–0.95], $p < 0.0001$; 400 μM $CoCl_2$: 1.09 [0.94–1.09], $p < 0.05$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 0.54 [0.54–0.56], $p < 0.001$) (Fig. 2D).

Secretion Of Il-6 And Tnfα In Iec-6 Cells Exposed To Lps, Cocl2 Individually Or Together

Using ELISA, we detected the levels of IL-6 and TNFα proteins secreted into the culture supernatant. Compared to the control group, the relative protein expression of IL-6 in different stimulation groups increased significantly, especially in the combined stimulation group (Control: 4.80 ± 2.56; 10 μg/ mL LPS: 29.32 ± 3.16, $p < 0.05$; 400 μM $CoCl_2$: 54.29 ± 12.69, $p < 0.001$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 88.66 ± 8.10, $p < 0.0001$) (Fig. 3A). Similarly, the relative protein expression of TNFα significantly increased in

each stimulation group, also especially in the combined stimulation group (Control: 1.65 ± 0.36; 10 μg/ mL LPS: 12.21 ± 1.63, $p < 0.01$; 400 μM $CoCl_2$: 5.41 ± 0.84; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 23.58 ± 5.44, $p < 0.0001$) (Fig. 3B).

Protein levels of Claudin-1 and ZO-1 in IEC-6 cells exposed to LPS, CoCl2 individually or together

Immunoblotting was used to investigate the protein levels of Claudin-1 and ZO-1 in IEC-6 cells. As Fig. 4 shows, after stimulation with $CoCl_2$ alone or in combination with LPS, a significant decrease in Claudin-1 was observed (400 μM $Cocl_2$: 0.25 ± 0.07, $p < 0.05$; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 0.29 ± 0.05, $p < 0.05$), but a significant increase was detected after stimulation with LPS alone (10 μg/ mL LPS: 1.75 ± 0.38, $p < 0.05$). ZO-1 protein level showed a decreasing trend in each stimulation group, especially after combined stimulation (10 μg/ mL LPS: 0.85 ± 0.29; 400 μM $CoCl_2$: 0.75 ± 0.23; 400 μM $CoCl_2$ + 10 μg/ mL LPS: 0.68 ± 0.17), but with no statistical significance.

Discussion

Since NEC mainly occurs in premature infants, we selected IEC-6 cell lines as model cells in this study. They are non-transformed intestinal epithelial cells derived from rat intestinal crypts and have immature crypt-like phenotype [8, 15]. Epidemiological studies have shown that hypoxia and infection are significant risk factors [2, 3, 16]. Simulated hypoxia is an important basis for the study of NEC in vitro [12, 17]. Physical hypoxia inevitably requires expensive special hypoxia culture equipment and consumes a large amount of hypoxia gas [18], we chose $CoCl_2$ to stimulate IEC-6 cells in our study to induce chemical hypoxia, which may better simulate the pathological characteristics of NEC with chronic hypoxia. On the other hand, the induction of inflammation is also a key to the study of NEC mechanism [19]. LPS has been proved to induce increased TLR4 expression in IEC-6 cells, which can activate the natural immune process and cause inflammation [20]. Therefore, we chose LPS to stimulate IEC-6 cells in our study to induce inflammation. Then, we chose single or combined factors to stimulate IEC-6 cells and observe the reactivity.

Different from most previous literature reports, our study showed that the application of LPS alone at concentrations ranging from 10 μg/ mL to 400 μg/ mL for 24 h did not reduce the viability of IEC-6 cells. On the contrary, compared with the control group, the viability of cells in the LPS group increased to a certain extent, although this increase did not reach statistical significance. This suggested that LPS alone may only induce mild and reversible cell damage. Similarly, after 24 h of stimulation by $CoCl_2$ alone, the viability of IEC-6 cells was also increased to a certain extent at low concentrations ranging from 50 μM to 100 μM. However, at high concentrations ranging from 200 μM to 1600 μM, cell viability decreased in a dose dependent manner. According to the use concentration of LPS reported in the previous literature [21], we chose to combine the specified LPS concentration at 10 μg/ mL with the gradient concentration of $CoCl_2$. The viability of IEC-6 cells decreased to 66.17% when LPS combined with 400 μM concentration of $CoCl_2$. Therefore, we determined the concentrations of the two stimuli for subsequent studies.

Inflammatory reaction and intestinal barrier destruction are key characteristics of NEC [2, 22]. We compared the expression of inflammatory factors at mRNA and protein levels in IEC-6 cells stimulated by single application of LPS and $CoCl_2$ or both. The results showed that IL-6 and TNFα were increased in different degrees in each group. Among them, the expression of IEC-6 and TNFα in the combined stimulation group was the highest, suggesting that the combination of LPS and $CoCl_2$ can better simulate the intense inflammatory immune response of NEC. Intestinal barrier integrity is maintained by tight junctions composed of transmembrane, scaffold and adaptor proteins [23]. The decrease of Claudin family proteins and ZO-1 expression has been widely reported to be highly related to the impairment of intestinal barrier function [24, 25]. We further detected the expressions of Claudin-1 and ZO-1 at mRNA and protein levels. Inconsistent with many other studies, LPS alone did not result in the significant reduction of Claudin-1 and ZO-1 in IEC-6 cells. Even interestingly, Claudin-1 levels was found to be higher than those in the control group. This indicates that the inflammatory response induced by LPS alone may not be strong enough to destroy the integrity of the intestinal barrier, and may even promote the regeneration of intestinal epithelium. At the same time, we found that Claudin-1 and ZO-1 decreased in $CoCl_2$ alone or in combination with LPS stimulation. It suggested that hypoxia may be an indispensable factor leading to the destruction of intestinal barrier.

To our knowledge, the effects of single and combined stimulation on IEC-6 cells have not been reported in vitro. This study showed that the combined stimulation of LPS and hypoxia can induce severe inflammatory reaction in IEC-6 cells and damage intestinal epithelial cell barrier. NEC is a complex multifactorial disease involving a variety of environmental and pathophysiological factors [26]. Consistently, the experimental NEC in vivo model is based on the combined induction of multiple factors [13, 14], which supports the view that multiple stimuli may be required to simulate the pathogenesis of NEC in vitro model. This study has some limitations. First of all, the occurrence of NEC involves many types of intestinal epithelial cells [8, 27]. We only compared the stimulation effects of single factor and combined factors on IEC-6 cells, which cannot represent the responsiveness of other types of intestinal epithelial cells to different stimuli. In addition, there are various ways to stimulate intestinal epithelial cells in vitro. We chose LPS and $CoCl_2$ to simulate inflammation and hypoxia, but did not study about other stimulation modes, such as TNFα, IFNγ and H_2O_2.

In conclusion, our study provided some basis for stimulating intestinal epithelial cells by multiple factors to better simulate NEC in vitro model, which may be beneficial to further research on the mechanism of NEC and explore potential prevention or treatment methods.

Declarations

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Author contributions

GCC, FZT and WLX had primary responsibility for the study design, data analysis and manuscript preparation. YZM prepared the draft manuscript. WSN helped in the data analysis. YSL contributed to the study design and provided valuable suggestions for conducting the experiments. All authors read and approved the final manuscript.

Consent for publication

No applicable.

Availability of data and materials

The data and materials supporting the conclusions of the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflicts of interest.

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Figures

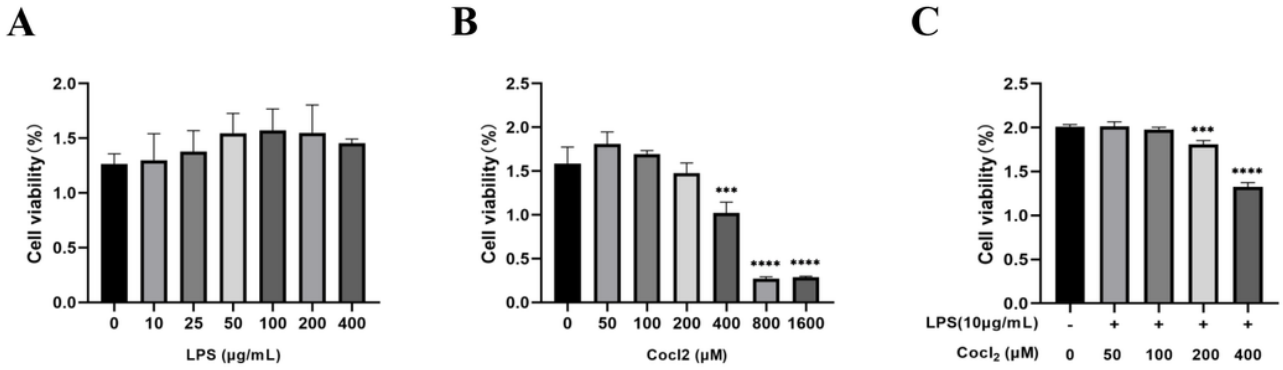

Figure 1

Cell viability of IEC-6 following administration of LPS, $CoCl_2$ individually or together. (a, b) Cells were exposed to different concentrations of LPS and $CoCl_2$ individually for 24 h. ***$p < 0.001$, ****$p < 0.0001$, compared with the control group. (c) Cells were exposed to different concentrations of $CoCl_2$ combined

with a specific concentration of LPS for 24 h. ***$p < 0.001$, ****$p < 0.0001$, compared with the control group.

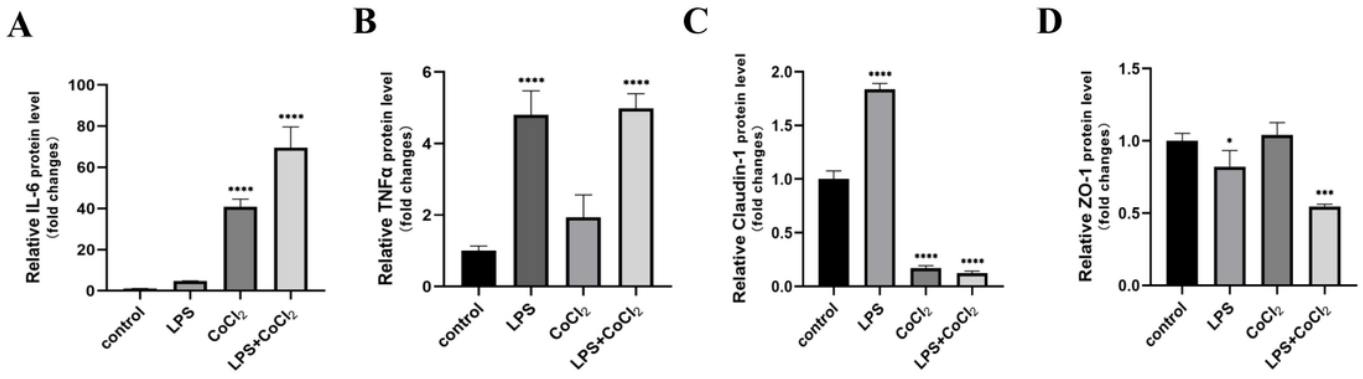

Figure 2

mRNA levels of inflammatory cytokines and tight junctions in IEC-6 cells exposed to LPS, $CoCl_2$ individually or together. The mRNA expressions of IL-6 (a), TNFα (b), ZO-1 (c) and Claudin-1 (d) in different groups. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, compared with the control group.

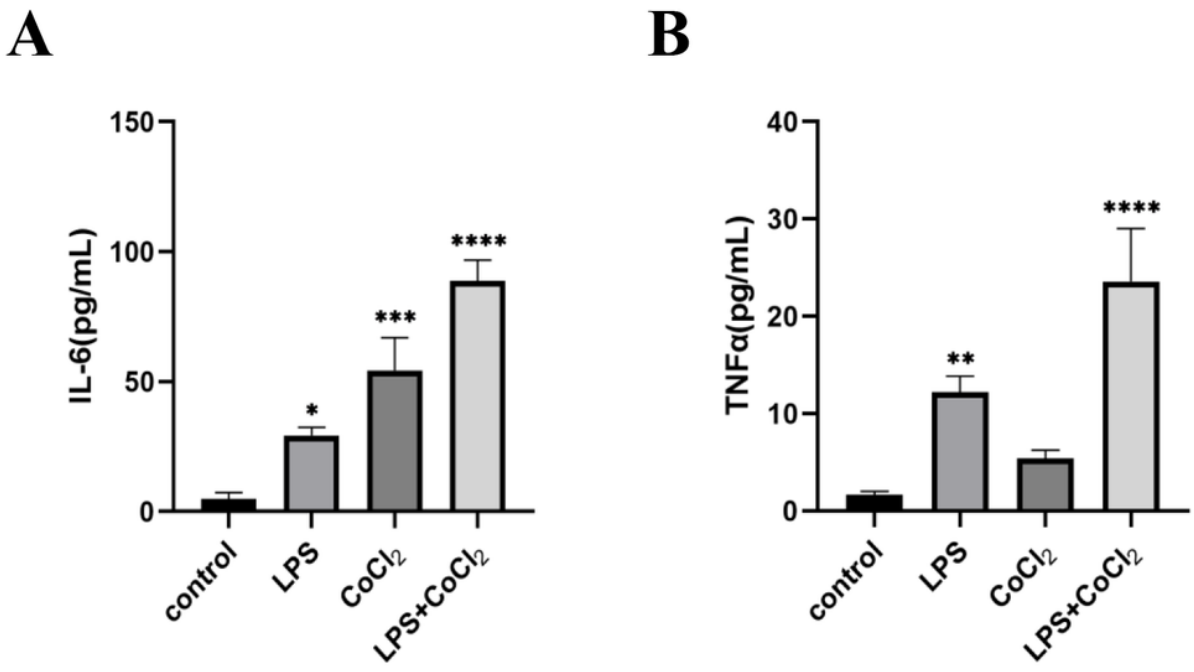

Figure 3

Secretion of IL-6 and TNFα in IEC-6 cells exposed to LPS, $CoCl_2$ individually or together. Relative expressions of IL-6 (a) and TNFα (b) from cell supernatant in different groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, compared with the control group.

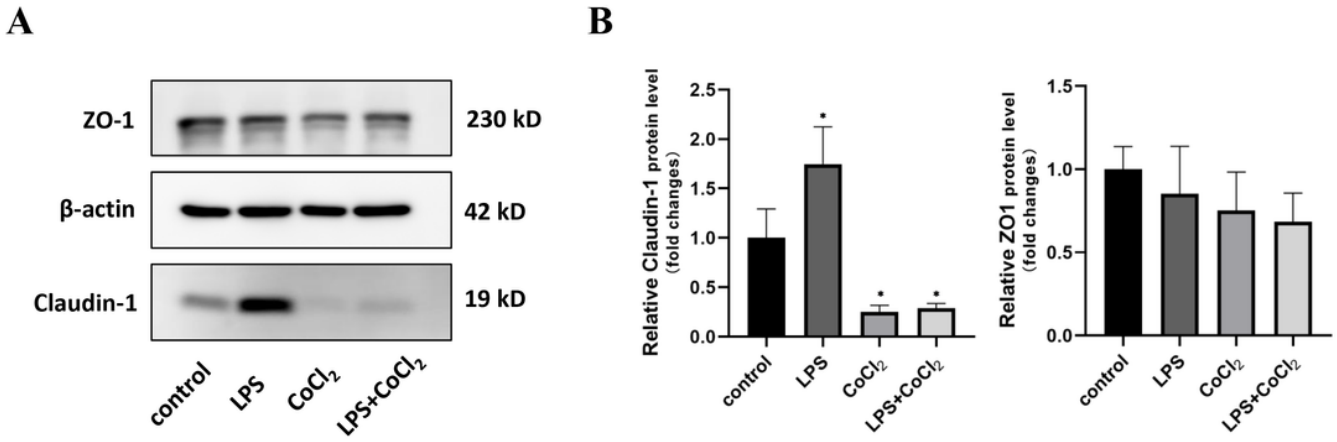

Figure 4

(a)Protein levels of Claudin-1 and ZO-1 in IEC-6 cells exposed to LPS, $CoCl_2$ individually or together. Representative immunoblotting analysis of Claudin-1 and ZO-1 in different groups. (b, c) Quantification analysis of Claudin-1 and ZO-1 in different groups. *$p < 0.05$, compared with the control group.