The Mechanism of NF- κB/MAPKs-TLR4 Pathway in the Inhibitory Effect of Rhubarb Lingxian Formula on Inflammatory Response of Bile Duct Cells

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Research

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Abstract

Background: To explore the mechanism of NF- κB/MAPKs-TLR4 signal pathway in the inhibitory effect of rhubarb lingxian formula on inflammatory response of bile duct cells.

Methods: The chronic inflammatory state of bile duct in rats with primary choledocholithiasis was established by injecting LPS into common bile duct. Rat intrahepatic bile duct epithelial cells were isolated and purified. The proliferation activity of bile duct epithelial cells was detected by CCK-8. Blank group, LPS control group, LPS + NF-κB blocker (PDTC) group, LPS + p38MAPK blocker (SB203580) group, LPS + PTDC + SB203580 group, LPS + rhubarb lingxian group, LPS + PDTC + rhubarb lingxian group, LPS + SB203580 + rhubarb lingxian group and LPS + PTDC + SB203580 + rhubarb lingxian group were constructed, respectively. The NF- κB, MAPKs and TLR4 mRNA levels were detected by RT-qPCR, whereas NF-κB, MAPKs, TLR4, TNF-α and IL-6 protein levels were detected by immunohistochemical staining and Western blot.

Results: The mRNA and corresponding proteins of NF-κB and MAPKs in the LPS control group were noteworthily increased in contrast to the blank group ($P < 0.01$), which evidently lower in the LPS + rhubarb lingxian group than those presented in the LPS control group ($P < 0.01$). The activity of NF- κB and MAPKs channel in the LPS + PTDC + SB203580 group was suppressed simultaneously in comparison to the LPS control group, which further caused decreased TLR4 mRNA expression. Meanwhile, the TLR4 mRNA expression in LPS + traditional Chinese medicine group also decreased ($P < 0.01$). IL-6 and TNF- α protein expression in bile duct epithelial cells of rats in LPS control group increased apparently than those of blank group. In contrast to LPS control group, TNF- α and IL-6 protein expressions in each treatment group decreased in different degrees ($P < 0.01$), which decreased at most in LPS + X (X can be blank or blocker) group or LPS+ PTDC+ SB203580 group, followed by LPS + Y (Y is a single blocker), and finally by the LPS treatment group.

Conclusion: Rhubarb lingxian formula may inhibit the inflammatory reaction of bile duct epithelium in rats by down-regulating the activity of NF-κ B / MAPKs-TLR4 signal pathway.

Background

Primary hepatolithiasis (HL), a pervasive benign biliary tract illness in China, is characterized by long-term recurrent bile duct inflammation. Its etiology is complex and involves many various kinds of factors [1]. Inflammation, generally caused by various bacteria, is the reaction of correspond tissues to kind of not only mechanical but also chemical damage or infection. The bacterial infection induced inflammatory reaction is mostly caused by endotoxin. Endotoxin, also known as lipopolysaccharide (LPS), is an important part of outer membrane of Gram-negative pathogen. It participates in many important cellular reactions and exerts a critical function in inflammatory reactions [2]. LPS also launches an acute inflammatory reaction to bacteria. Endotoxin can stimulate different cell types [3, 4] to secrete interleukin

(IL)-8, IL-1β, IL-6, as well as tumor necrosis factor (TNF) -α, which is widely used to build an inflammatory cell model.

Toll-like receptor 4 (TLR4) is a receptor of LPS localized to the cell surface. The lymphocyte antigen 96 (MD-2), glycosyl-phosphatidylinositol (GPI)-anchored monocyte differentiation antigen CD14 (CD14), as well as lipopolysaccharide binding protein (LBP), participate in modulating the TLR4 activation. LBP transfers LPS to CD14 through combining with lipid A moiety of LPS, thereby ensuring and optimizing signal transmission via the TLR4 / MD-2 complex [5]. There are two signaling pathways launched by TLR4 activation. One pathway is to trigger NF-κB and mitogen-activated protein kinases (MAPKs) activity by recruiting as well as activating TollIL-1 receptor domain-containing adaptor protein (TIRAP), the main reactive protein of myeloid differentiation. The other one is regulated by TIR domain-containing linker molecule 2 (TRAM) and TIR domain-containing linker molecule 1 (TRIF), which requires internalized TLR4 to activate not only IκB kinase but also interferon regulatory factor 3 (IRF3), thereby induce type I interferon gene [6, 7]. These cascade transcription reactions stimulate the robust expression of a great deal of genes, and ultimately regulate the release of anti-inflammatory as well as inflammatory factors. Hence, TLR4-NF-κB / MAPKs signaling pathway serves significant parts in inflammation.

The rhubarb lingxian formula is based on the principle of dispersing stagnated liver qi for promoting bile flow, purgation and clearing gallstones. In this formula, rhubarb and crystallized sodium sulfate is beneficial for purgation, removing food stagnation, promoting bile flow, clearing gallstones. The combination of christina loosestrife, turmeric root tuber, Chinese thorowax root, orange fruit and hirsute shiny bugleweed herb has the functions of not only clearing heat and promoting diuresis, but also regulating qi-flowing for eliminating phlegm. Inner membrane of chicken gizzard, christina loosestrife and Chinese clematis root are all good for clearing gallstones. Chinese clematis root and magnetite are empirical medicine. Milkvetch root and liquorice root are utilized for benefiting qi, tonifying deficiency and protecting stomach qi.

We confirmed in the previous report the therapeutic effect of rhubarb lingxian capsule in prevention and treatment of HL, and found that it can alleviate the inflammatory injury in the bile duct. Thus, our study further focused on exploring the possible mechanism of NF- κB/MAPKs-TLR4 signal pathway in the inhibitory effect of rhubarb lingxian formula on inflammatory response of bile duct cells.

1 Materials And Methods

1.1 Animals

180 clean healthy SD rats, weighing 200-250g, were supplied by the Experimental Animal Center of Guangxi Food and Drug Inspection Institute. The experimental operation was carried out in the experimental operation room of the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine. All rats were classified at random into 9 groups, each with 20 rats: blank group, LPS (Sigma, USA) control group, LPS + NF-κB blocker (PDTC) group, LPS + p38MAPK blocker (SB203580) group, LPS

+ PDTC + SB203580 group, LPS + rhubarb lingxian (Jiangyin Tianjiang Pharmaceutical Co, Ltd. China) group, LPS+ PDTC+ rhubarb lingxian group, LPS + SB203580 + rhubarb lingxian group, and LPS + PDTC + SB203580 + rhubarb lingxian group.

1.2 Method

1.2.1 Bile duct cell isolation and culture

After anesthetizing the rats and removing most of the liver tissue, a complete bile duct tree for the separation of bile duct cells was obtained under a microscope. Free bile duct tree was grinded and bile duct cells were digested by adding 2.5 g / L pancreatin, 2 × 104 U / L Dnase I collagen digestion enzyme and 2 × 105 U / L Dnase IV collagen digestion enzyme. Then the cells were separated and purified with immunomagnetic beads and cultured in 1 × 10^5 /ml cells in flasks. After 6 days of culture, fluorescence microscopy was used to identify the concentration of bile duct epithelial cells. The fixing of cultured cells in methanol at -20 ° C for 20 min and washing with phosphate buffered saline twice (2 min / time) were conducted. Rabbit anti-mouse CK 19 (5 μg / ml, Santa Cruze, USA) was added to incubate overnight at 4 ° C. Alexa Flourr®594 donkey anti-rabbit IgG second antibody (1: 1000, Introvigen, USA) was add to incubate at 37 ° C for 30 min on the next day. Finally, DAPI staining (1 μg / ml) was used to investigate whether the purity of isolated rat bile duct epithelial cells was reached to 95% under a fluorescence microscope. Meanwhile, trypan blue staining exclusion method was used to determine the activity of epithelial cells. After staining, the survival rate of these cells was found to be above 90%. The cell densities were adjusted to 1 × 10^5 cells / ml. The cells were inoculated into a 50 ml plastic culture bottle coated with type I rat tail collagen in 37℃, 5% CO2 incubator.

1.2.2 Survival rate

Applying CCK-8 colorimetric method, cell viability was determined. Cells were cultured in 96-well plates in 5% CO2, 37˚C incubator overnight wherein the density was adjusted to 5 × 10^3 cells / well, and then stimulated with or without LPS (650, 1250, 2500, 5000 and 10000 ng / ml), rhubarb lingxian (0.5, 0.75, 1.0, 1.25 and 1.5 mg / ml), PDTC (2.5, 5, 10, 20 and 40 μmol / l), SB203580 (2.5, 5, 10, 20 and 40 μmol / l) for 24 h. Finally, incubations were completed for 1 hour at 37˚C after adding 10 μl CCK-8 into each well, and then the measurement of absorbance was achieved at 450 nm.

1.2.3 RT-PCR

The extraction of total RNA was conducted utilizing the Trizol method. The concentration was measured on an ultra-micro UV photometer to determine the RNA quality. The DNA purity with an OD260/OD280 ratio of 1.8–2.0 can meet the needs of subsequent RT-qPCR. After removing genomic DNA, first-strand cDNA was produced according to (Promega, USA) protocol. Resulting cDNA was amplified in a 20 μL

reaction mixture with the use of SYBR Green PCR Master Mix (Roche, USA), as well as 7500 Real-Time PCR System (Applied Biosystem). Primer 5.0 software was used to design primers for each gene and GAPDH (Table 1). Fold changes in expression were calculated with the use of 2−ΔΔCT method. GAPDH was utilized as reference gene for standardization. The mRNA expression levels were tested with 3 replicate wells, and the final results were averaged.

Table 1
The primer of target genes and GAPDH.

Genes	Primers
Myd88 Forward	CTAGCCTTGTTAGACCGTGA
Myd88 Reverse	GTCTGTGGGACACTGCTCT
TRAF6 Forward	GCCCATGCCGTATGAAGAGA
TRAF6 Reverse	ACTGAATGTGCAGGGGACTG
TAK1 Forward	CGCCATCGCAGGTCCTTAAC
TAK1 Reverse	GGCTGCATGCTGTGCAGGTA
IKKa Forward	AAGTTCGGTTTAGTAGCC
IKKa Reverse	CTTCCTTTAGCCCAGATA
NF-κB Forward	GAAGCAACAGCTCACGGAGGA
NF-κB Reverse	TGTTCTGGAAGTTGAGGAAGGCC
β-actin Forward	CACGATGGAGGGGCCGGACTCATC
β-actin Reverse	TAAAGACCTCTATGCCAACACAGT

1.2.4 Western blot

The bile duct epithelial cells were pretreated with 1 mg / ml rhubarb lingxian, PDTC and SB203580 for 2 hours in 36 well culture plates (5 × 10^5 cells / ml), and then stimulated with 500 ng / ml LPS for 12h. The protein extraction kit was utilized to isolate the total protein, whereas the BCA protein detection kit to determine protein concentration. An equal amount of protein (25 μg) were added, separated with 30% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (PVDF), which was sealed at room temperature with 5% nonfat milk for 1 hour, and subsequently incubated with mouse anti-β actin mAb (Zhongshan, China), NF-κB p65 (CST, USA), p38MAPK (CST, USA) and TLR4 (Proteintech, USA) antibody. The detection strip of the electrochemiluminescence kit. Image J v1.8.0 software, along with GraphPad Prism v.6 software was served for protein-level analysis.

1.2.5 Detection of inflammatory cytokines

The cells on a 36-well culture plate (5 × 10^5 cells / ml) were pretreated with 1 mg / ml rhubarb lingxian formula, PDTC and SB203580 for 2 hours according to grouping requirements, and then treated with LPS (500 ng / ml) for 12 hours. The collected cellular supernatant was adopted to detect serum TNF-α (Cusabio, China), IL-6 (Cusabio, China) and IL-8 (Cusabio, China) levels by ELISA kit.

1.3 Statistical analysis

All data were displayed as mean ± standard deviation (X ± S) and tested for normality homogeneity of variance prior to analysis. If the data showed in compliance with the test, the LSD method was used for comparison between groups; if not, the Dunnett's T3 method was used. The rank sum test is used if the variable doesn't obey normal distribution. P < 0.05 represents harboring statistical significances.

2 Results

2.1 Survival rate of rat bile duct epithelial cells

The results indicated that the maximum non-toxic concentrations of LPS, rhubarb lingxian, PDTC and SB203580 are 1250 ng / ml, 1 mg / ml, 10 μmol / l and 20 μmol / l, respectively. Thus, in following assays, varying compounds at non-toxic concentrations were applied to treat cells. See Figure 1.

2.2 MAPKs, TLR4 and NF-κB mRNA levels

Compared with blank group, NF-κB and MAPKs mRNA levels were markedly enhanced in the LPS control group ($P < 0.01$), indicating that LPS can induce rat biliary epithelial cells to increase the activity of MAPKs and NF-κB mRNA. NF-κB, together with MAPKs levels, was remarkably lower in the LPS + rhubarb lingxian group than those of the LPS control group ($P < 0.01$), whereas reduction degree was the same as those in the corresponding inhibitor group ($P > 0.05$), indicating that rhubarb lingxian reduced the activity of NF-κB and MAPKs mRNA. See Figure 2A and 2B.

In contrast to the LPS control group, both NF-κB and MAPKs channel activity were inhibited in the LPS + PTDC + SB203580 group, which could cause the decrease of TLR4 mRNA expression level ($P < 0.01$). In the LPS + rhubarb lingxian group, the TLR4 mRNA level also decreased ($P < 0.01$), indicating that the reduction of MAPKs and NF-κB channels might reduce TLR4 mRNA level. See Figure 2C.

2.3 MAPKs, TLR4 and NF-κB protein levels

In contrast to blank group, the NF-κB and MAPKs protein levels were noteworthily increased in the LPS control group ($P < 0.01$), indicating that LPS can induce rat biliary epithelial cells to secrete NF-κB and

MAPKs protein. Compared with the LPS control group, MAPKs and NF-κB levels in the LPS Chinese medicine group were notably reduced ($P < 0.01$), and the degree of reduction was not statistically varying from the corresponding inhibitor group ($P > 0.05$), indicating rhubarb lingxian reduced NF-κB and MAPKs protein levels. The LPS + PTDC + SB203580 group inhibited both NF-κB and MAPKs channel activity, which cause the TLR4 protein expression level to decrease ($P < 0.01$) in contrast to the LPS control group. Meanwhile, the TLR4 protein level also decreased in LPS Chinese medicine group ($P < 0.01$), indicating that the decreased activity of NF-κB and MAPKs channels can reduce TLR4 protein level. See Figure 3.

2.4 TNF-α and IL-6 expressions in each group

In contrast to the blank control group, TNF-α and IL-6 protein levels were apparently increased in LPS control group ($P < 0.01$), indicating that LPS can induce the release of inflammatory factors in bile duct epithelial cells, that is, modeling success. Meanwhile, in contrast to LPS control group, IL-6 and TNF-α protein expressions in each treatment group decreased to varying degrees with statistical significance ($P < 0.01$), indicating that different treatment methods in our study can inhibit the release of inflammatory factors in rat bile duct epithelial cells to a certain extent. See Figure 4.

3 Discussion

It has been confirmed that the formation of intrahepatic bile duct stones is related to biliary tract infection, biliary fibrosis, biliary stenosis, and bile hydrodynamic changes.
The relationship between infection and stones interrelated and mutually influence each other, causing long-term pain in patients. It can be seen that biliary tract infection and inflammation may be one of the main causes of calculi in the early stage of intrahepatic bile duct stones. This is mainly in part because the uneven surface of the biliary duct at early stage of inflammation causes the change of bile flow when it passes and further leads to biliary stasis, and partly because inflammation of the biliary tract can induce pathological remodeling of the bile duct, start the process of bile duct fibrosis, and lead to the occurrence of biliary stenosis [8,9]. In recent years, some scholars have suggested that intrahepatic bile duct stones and repeated biliary tract inflammation are actually the same disease. The former describes pathological changes, and the latter reflects clinical manifestations. It suggests that controlling long-term chronic biliary tract inflammation acts a pivotal part in treating this disease [10].

The rhubarb lingxian formula is based on the principle of dispersing stagnated liver qi for promoting bile flow, purgation and clearing gallstones. In this formula, rhubarb and crystallized sodium sulfate is beneficial for purgation, removing food stagnation, promoting bile flow, clearing gallstones. The combination of christina loosestrife, turmeric root tuber, Chinese thorowax root, orange fruit and hirsute shiny bugleweed herb has the functions of not only clearing heat and promoting diuresis, but also regulating qi-flowing for eliminating phlegm. Inner membrane of chicken gizzard, christina loosestrife and Chinese clematis root are all good for clearing gallstones. Chinese clematis root and magnetite are

empirical medicine. Milkvetch root and liquorice root are utilized for benefiting qi, tonifying deficiency and protecting stomach qi.

This study found that, in contrast to the LPS control group, the TNF-α and IL-6 levels in the biliary tract cells were significantly attenuated in the LPS Chinese medicine group ($P < 0.01$), indicating that rhubarb lingxian has an inhibitory effect on LPS-induced biliary epithelitis, which is consistent with our previous results [6]. Previous studies on the establishment of chronic hepatic injury rabbit cholelithiasis model, together with the research on the intervention effect of rhubarb lingxian formula, have found that rhubarb Lingxian formula can improve the pathological morphological structure of cells, gradually improve the cells from inflammatory injury state to physiological function level, and reduce IL-6 mRNA and protein expression [6]. Although rhubarb lingxian has a role in inhibiting biliary inflammatory reaction, the molecular mechanism and target for relieving biliary tract inflammation have not been elucidated.

It was previously reported that biliary tract infections are mainly dominated by Gram-negative bacteria. LPS, a major *toxic substance*, is an important part to construct cell wall of Gram-negative bacteria. Most of the endotoxin secreted *by the liver* into the bile still retains the complete molecular structure of LPS and has the biological characteristics of endotoxin [11]. This study found that when stimulated with LPS, the expression levels of NF-κB and MAPKs mRNA and their corresponding proteins in bile duct cells were significantly increased ($P < 0.01$). After treated with the corresponding channel inhibitors (one or multiple interventions) or rhubarb lingxian, the expression levels of NF-κB and MAPKs mRNA and their corresponding proteins were significantly reduced ($P < 0.01$). The reduction degree was consistent between the rhubarb lingxian group and other inhibitors group ($P > 0.05$). As for the expression levels of TLR4, the activity of NF-κB and MAPKs channels in the LPS + PTDC + SB203580 group was simultaneously inhibited in comparison to the LPS control group, which can cause the decreased expression of TLR4 mRNA to ($P < 0.01$). The expression level of TLR4 in rhubarb lingxian group also decreased ($P < 0.01$). In contrast to the LPS control group, the TNF-α and IL-6 protein levels in the bile duct epithelial cells of each treatment group decreased to varying degrees. It suggests that rhubarb lingxian can reduce the activity of TLR4-NF-κB / MAPKs channel, thereby further inhibiting the inflammatory response of bile duct. It was stated previously that LPS can activate downstream NF-κB and MAPK signaling pathways through the TLR4 signaling pathway to interactively amplify the inflammatory response [12,13], LPS also mediate the activation of fibroblasts, smooth muscle cells, epithelial cells, endothelial cells as well as monocytes, and induce the synthesis and release of cytokines before inflammation by acting on cell membrane receptors, and through intracellular signaling cascades to change the gene expression.

4 Conclusion

In short, this study suggests that rhubarb lingxian can effectively inhibit the inflammatory response of rat bile duct epithelium, which may be achieved by down-modulating the activity of TLR4-NF-κB / MAPKs channel.

Abbreviations

NF-κB blocker (PDTC); hepatolithiasis (HL); lipopolysaccharide (LPS); tumor necrosis factor (TNF) -α; Toll-like receptor 4 (TLR4); glycosyl-phosphatidylinositol (GPI); lipopolysaccharide binding protein (LBP); mitogen-activated protein kinases (MAPKs);

Declarations

Acknowledgments

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics statement

The experiment was often approved by the Ethics Committee of the First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine, and all patients participating in this study provided written informed consent in accordance with the “Helsinki Declaration”.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MZ and ML conceived and designed the study. YY and HW analyzed the data. BX and JC contributed to literature review. MZ and YY wrote the manuscript. XZ and YY reviewed and edited the manuscript. All authors read and approved the final manuscript.

Consent for publication

Written informed consent for publication was obtained from all participants.

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Figures

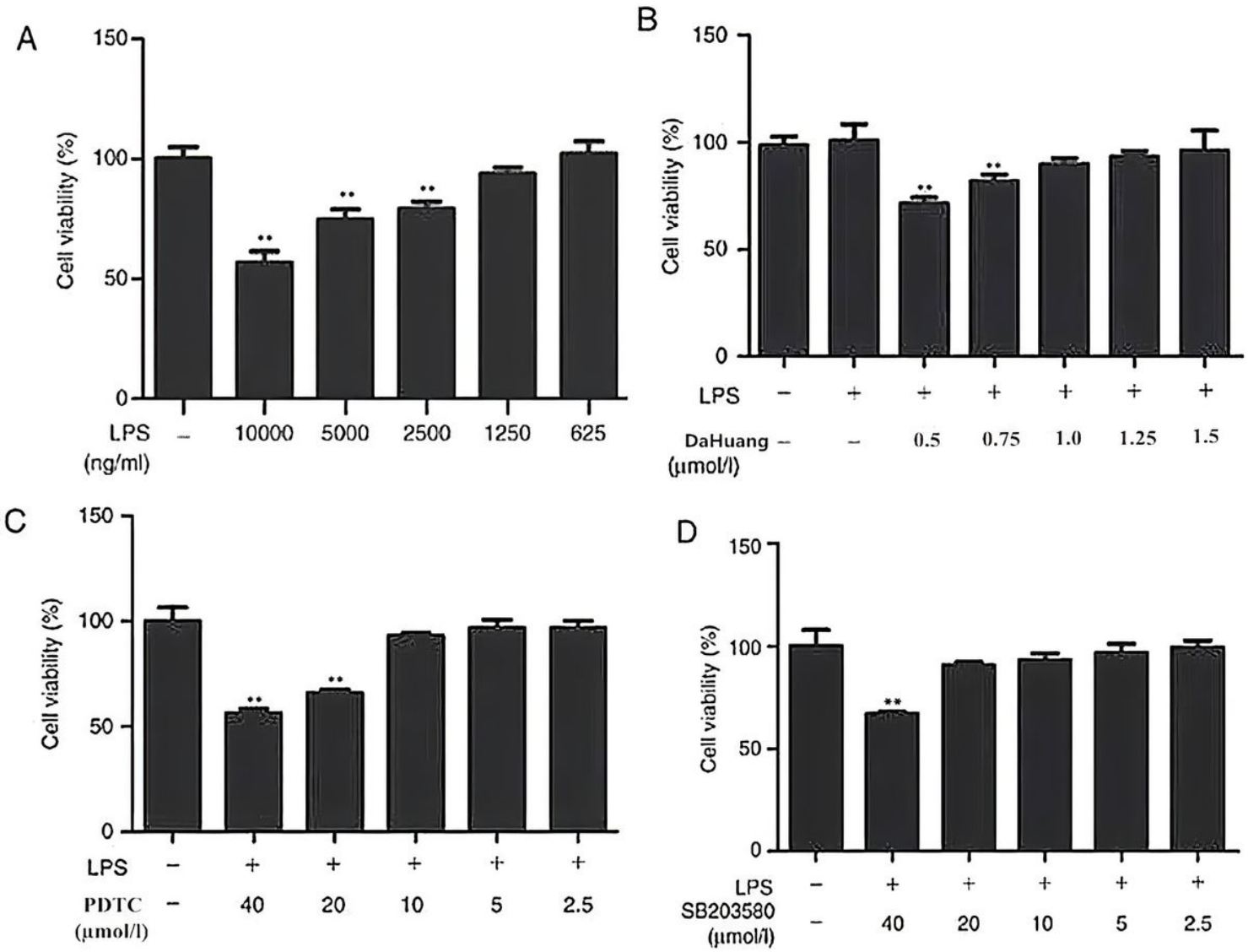

Figure 1

Survival rate of rat bile duct epithelial cells. Changes in cell viability after treatment with different concentrations of LPS (A), rhubarb lingxian formula (B), PDTC (C) and SB203580 (D). **P < 0.01.

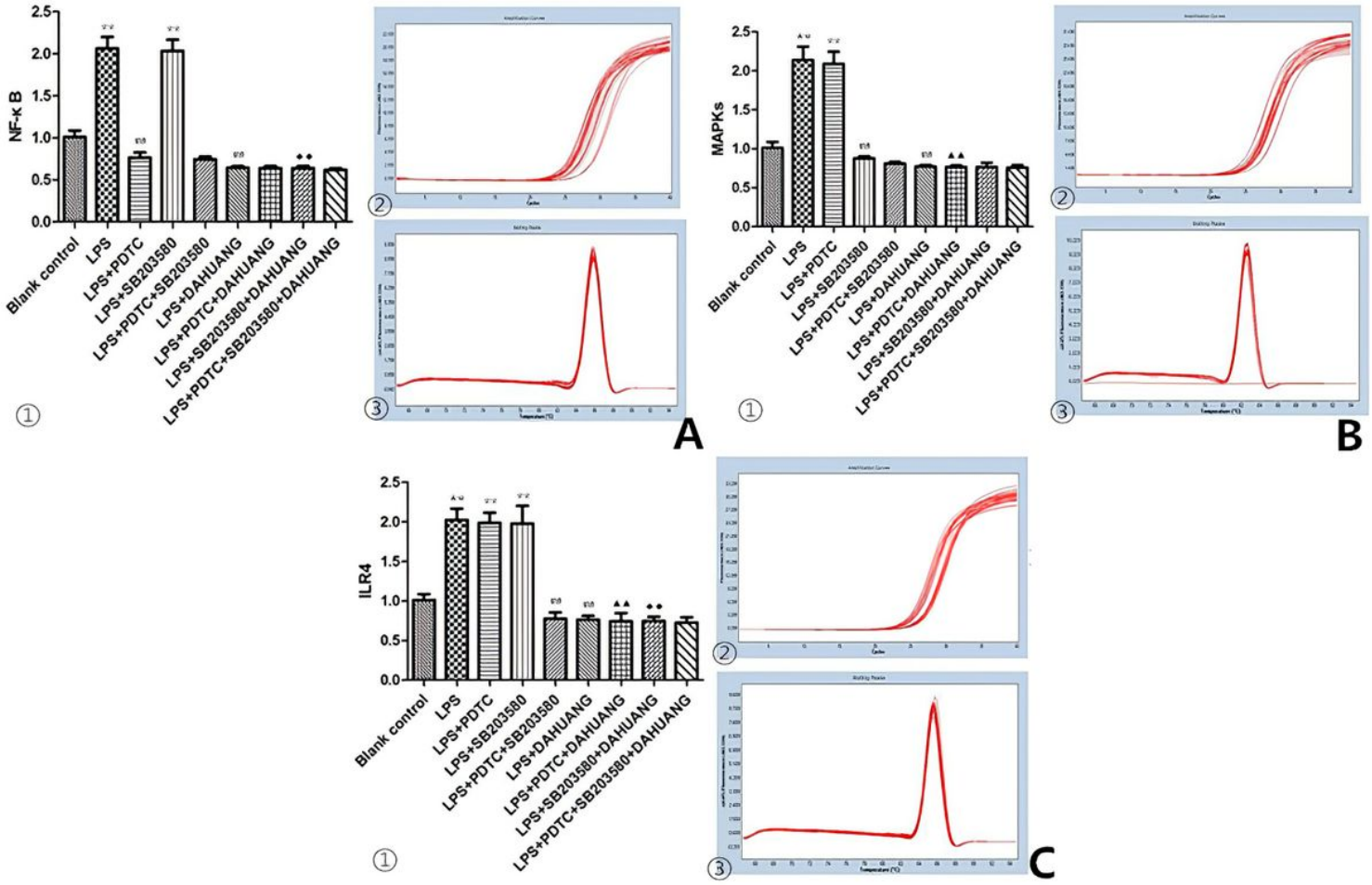

Figure 2

NF-κB (2A), TLR4 (2B) and MAPKs (2C) mRNA levels. □-□: corresponding mRNA expression quantitative plot, amplification curve and dissolution curve. In contrast to the blank group, **P < 0.01; in contrast to the LPS control group, ##P < 0.01; in contrast to the LPS + PDTC group, ▲▲P < 0.01; in contrast to LPS + SB203580 group, □□P < 0.01.

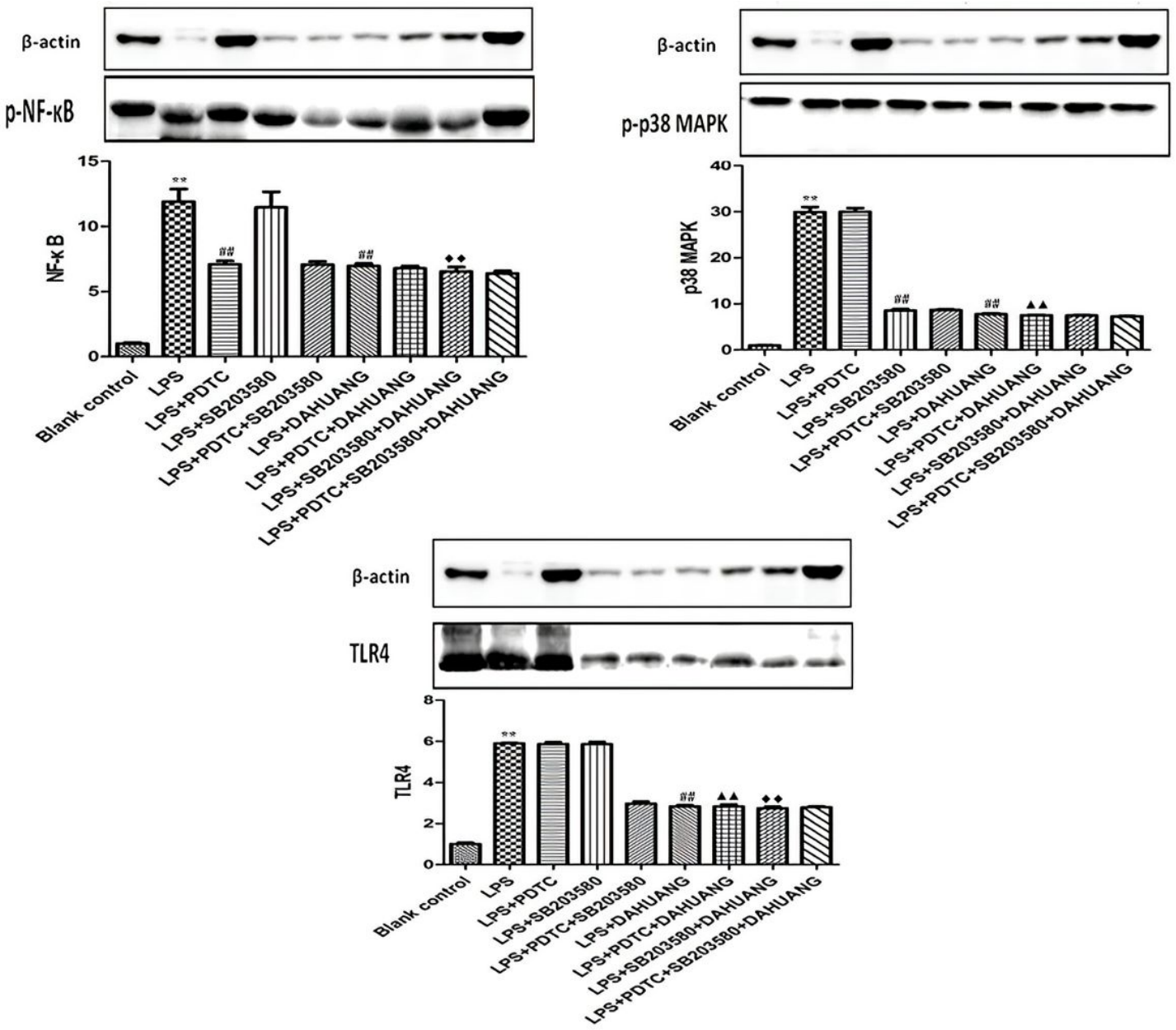

Figure 3

NF-κB, TLR4 and MAPKs protein levels. In contrast to the blank group, **P < 0.01; in contrast to the LPS control group, ##P < 0.01; in contrast to the LPS + PDTC group, ▲▲P < 0.01; in contrast to LPS + SB203580 group, ◆◆P < 0.01.

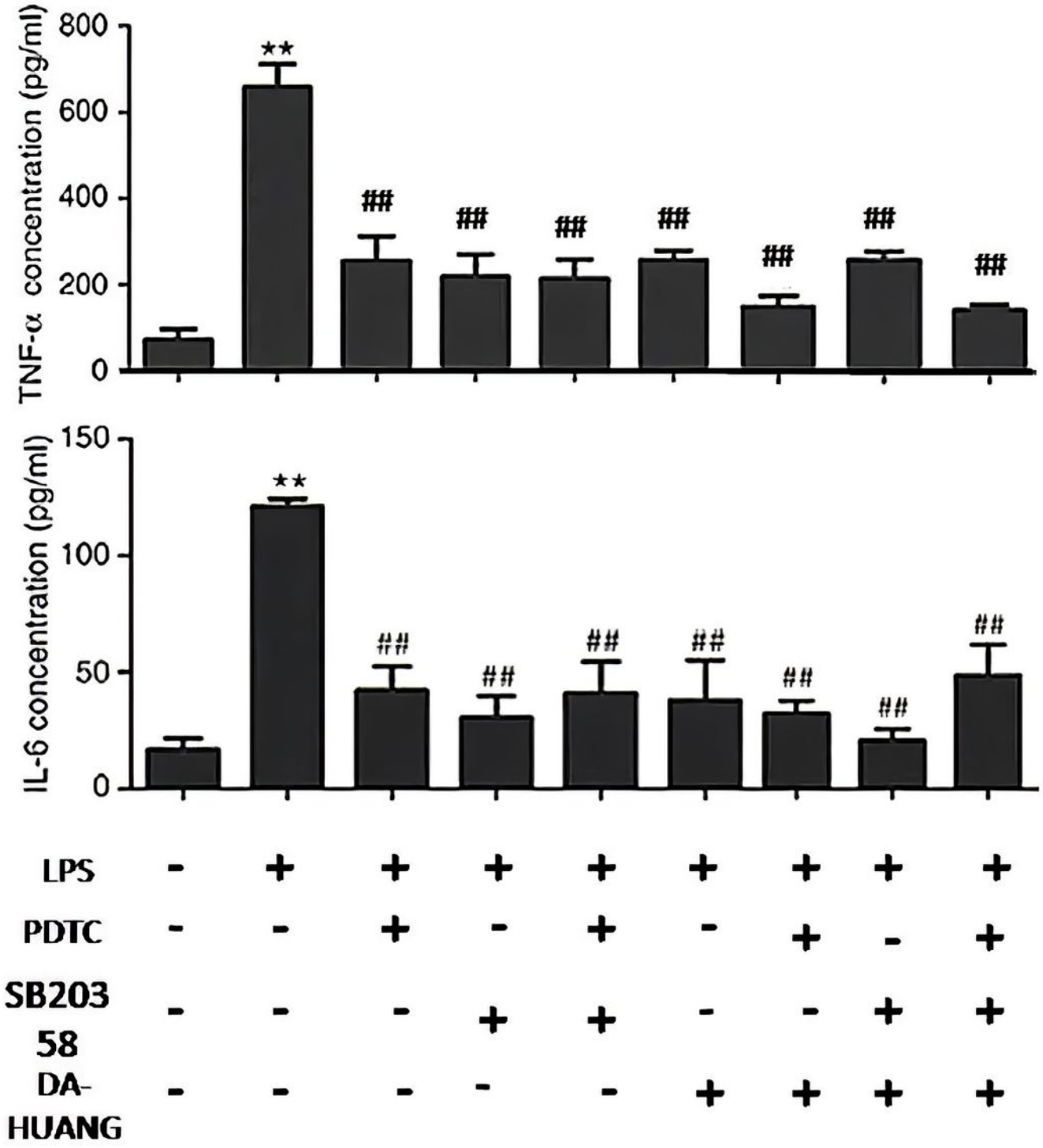

Figure 4

TNF-α and IL-6 expression. In contrast to the blank group, **P < 0.01; in contrast to the LPS control group, ##P < 0.01.