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Causes of immune dysfunction in hyperbilirubinemia model rats

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ABSTRACT

Objective: To explore the causes of immune dysfunction in neonatal rats with hyperbilirubinemia. **Methods:** A total of 60 newborn SD rats were equally randomized into normal saline (NS) group, LPS control group, bilirubin control group, low-dose group and high-dose group. After anesthesia, 0.1 mL NS was given to the NS and LPS control group and different doses of bilirubin for the other groups; 1 h later, the NS and bilirubin control group received the intraperitoneal injection of 0.05 mL NS and 1mg/kg LPS for the other groups. After 5 or 24 hours of model establishment, spleens were collected for detecting the expression levels of MyD88 and p-TAK1 protein and the spleen cells apoptosis by immunohistochemistry and TUNEL method. After 24 hours of model establishment, serum inflammatory factors levels and T cell subsets distribution were determined by ELISA and flow cytometry. **Results:** In contrast to low-dose bilirubin, high-dose bilirubin could induce spleen cells apoptosis in coordination with LPS. After 5 hours of model establishment, compared with NS group, MyD88 expression level in low-dose group elevated while p-TAK1 level in high-dose group reduced ($P < 0.05$). In high-dose group, inflammatory factors levels and CD8⁺ T cells percentage were all higher than LPS control and NS group ($P < 0.05$), while CD4⁺ T cells percentage was lower than NS group ($P < 0.05$). **Conclusions:** High-concentration plasma bilirubin in coordination with LPS could inhibit NF- κ B signal pathways activation and aggravate inflammatory reaction, thus caused immunosuppression with inflammation cascade, which resulted in the immune dysfunction.

1. Introduction

Jaundice, also called hyperbilirubinemia, refers to the stained yellow of sclera and skin caused by the increased production or acatharsia of bilirubin[1]. In neonatal period, because of the over-production of bilirubin, liver could not intake and transform bilirubin efficiently accompanying with the particular enterohepatic circulation of bilirubin, which results in the common incidence of hyperbilirubinemia. Children with severe jaundice showed the reduced CD4⁺ cells, CD8⁺ cells, CD4⁺/CD8⁺ and CD4⁺/Fas with decreased IgG, IgA and IgM, which implied that hyperbilirubinemia

could inhibit the children's immune function[2]. Bilirubin has potential toxicity, free bilirubin can gather and damage the brain, heart, kidney, stomach and intestine, lung and immune tissues and cells through the biomembrane[3]. When the concentration of free bilirubin increased, it could form stable bilirubin free radical, leading to the lipid peroxidation injury[4].

Neonate jaundice is related to the increasing blood unconjugated bilirubin (UCB) that is from the disintegrated red blood cells. Bilirubin can damage nervous tissue and evoke neurological dysfunction[5]. Moreover, increasing bilirubin can also lead to multiple dysimmunity, including humoral immune abnormalities, T cell subsets imbalance, and abnormal cytokine production and so on. For example, bilirubin could inhibit CD4⁺ cellular immune response through various ways and high-concentration bilirubin could induce CD4⁺ cell apoptosis[6]. However, bilirubin is not

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always to impair the tissues in vivo, instead, it could exert physical protection effect through its antioxidant properties[7].

Currently, there are no more profound and comprehensive studies about the relationship of immune function and bilirubin. In the study, we simulated the inner environment of human body through establishing the hyperbilirubinemia model in neonatal SD rats, in order to observe how the bilirubin influencing immune system under multiple regulatory mechanisms and explore the causes of immune dysfunction in neonates with jaundice.

2. Materials and methods

2.1. Experimental animals and grouping

A total of 60 SD neonatal rats were selected without differentiating male or female, aging 7-8 days and weighing 12.0-15.0 g. They were divided into 5 groups ($n=12$) randomly: normal saline (NS) group, LPS control group, bilirubin control group, low-dose group and high-dose group; each group was divided into two sub-groups: 5h sub-group and 24h sub-group ($n=6$).

2.2. Establishment of rats model with jaundice

After anesthesia, newborn SD rats were fixed, their necks were disinfected, and jugular vein exposed. 0.1 mL NS was given to the rats in NS group and LPS control group; bilirubin control group, low-dose group and high-dose group were given bilirubin through intravenous injecting at 15 mg/kg, 15 mg/kg and 50 mg/kg, respectively. 1 h later, NS group and bilirubin control group received the intraperitoneal injection of 0.05 mL NS and the other groups received the injection of LPS (1 mg/kg). After all the processes, all the newborn rats were sent back to their mothers.

After 10 min, 30 min, 60 min, 120 min, 300 min and 480 min of administration, the experimental rats were anesthetized for collecting blood samples. After the centrifugation at 3 000 rpm for 5 min, the plasma was obtained and plasma bilirubin content was determined by automatic biochemical analyzer.

2.3. Determination of myeloid differentiation factor 88 (MyD88) expression and transforming growth factor- β -activating kinase 1 (TAK1) phosphorylation and spleen cell apoptosis

After 5 h and 24 h of administration, animals were sacrificed to take out the spleen. The spleens were firstly fixed by 4% paraformal at 4 °C for 6-12 h. Then spleen tissue paraffin sections were made for detecting MyD88 and p-TAK1 protein expression by immunohistochemistry. Image-Pro Plus6.0 software was used to

determine the integrated optical density (IOD), 3 horizons were randomly selected and the average IOD represented the target protein expression level.

Apoptosis rate of spleen cells after 24 hours of administration was detected by TUNEL method.

Apoptotic index (AI)=(apoptotic number/400) \times 100%.

2.4. Determination of serum cytokines levels and T cell subsets distribution

After 24 hours of administration, the animal blood sample was collected and centrifuged at 1 500 rpm for 5 min. Then, IL-6 and IL-8 levels in serum were detected by ELISA; the percentage of CD4⁺ and CD8⁺ cells and the ratio of CD4⁺/CD8⁺ were analyzed by flow cytometry.

2.5. Data statistics analysis

SPSS16.0 software was used to process the data. Data in normal distribution was symbolized by mean \pm SD otherwise data was symbolized by the median. When homogeneity of variance was satisfied One-Way ANOVA was used to analyze otherwise the Kruskal-Wallis Test would be used. When $P<0.05$, the difference was statistically significant.

3. Results

3.1. Bilirubin level at different time points after administration

Figure 1 showed that after 1 hour of administration, the 95% confidence intervals of bilirubin concentration in low-dose group and high-dose group were 104.8-120.2 μ mol/L and 195.8-238.1 μ mol/L, respectively, accordant with the expected range of bilirubin level [the bilirubin level of newborn children with jaundice was about 102.6 μ mol/L (6 mg/L)]. So it was thought that the rats model of hyperbilirubinemia was established successful.

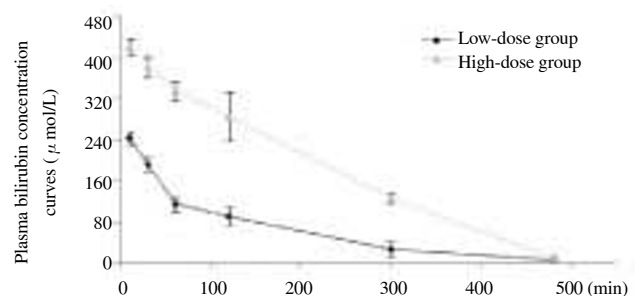


Figure 1. Bilirubin concentration-time curves of low-dose and high-dose group.

3.2. Expressions levels of MyD88 and p-TAK1 and spleen cells apoptosis

Results in Figure 2 showed that single injection of bilirubin or LPS both could induce the increasing spleen cells apoptosis rate ($P<0.05$), high-dose bilirubin (50 mg/kg) could induce the apoptosis in coordination with LPS ($P<0.05$), while low-dose bilirubin (15 mg/kg) did not display the synergistic effect. Compared with NS group, MyD88 expression level of low-dose group raised obviously and p-TAK1 level of high-dose group reduced ($P<0.05$) after 5 hour of administration (Figure 3).

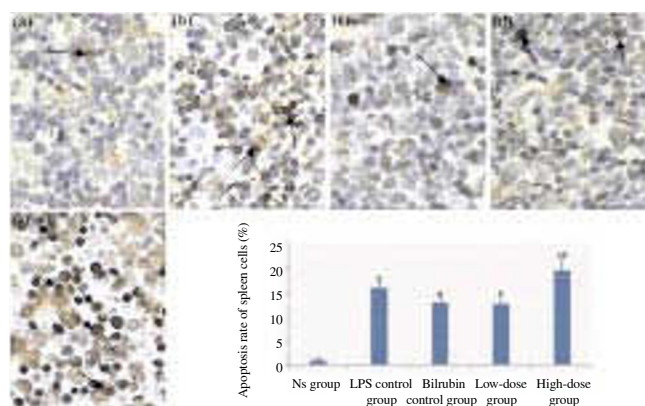


Figure 2. Spleen cells apoptosis after 24 hours of administration.

a: NS group; b: LPS control group; c: bilirubin control group; d: low-dose group; e: high-dose group.

Note: *compared with NS group, $P<0.05$; #compred with LPS control group, $P<0.05$. The next figure is same.

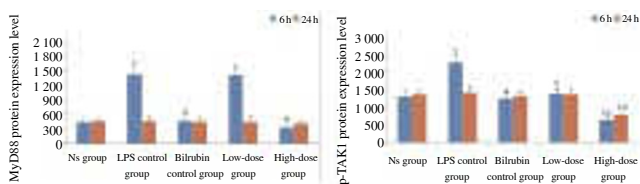


Figure 3. Expression levels of MyD88 and p-TAK1 protein after 5 or 24 hours of administration.

3.3. Serum cytokines levels and T cell subsets distribution

Cytokines levels in LPS control group and high-dose group were both higher than those in NS group, those in high-dose group were also simultaneously higher than LPS control group ($P<0.05$) while cytokines levels in low-dose group were lower than LPS control group ($P<0.05$) (Figure 4). These results indicated that high-dose bilirubin (50 mg/kg) could induce inflammatory reaction in coordination with LPS while low-dose bilirubin (15 mg/kg) could inhibition the inflammatory reaction induced by LPS.

Moreover, in high-dose group, percentage of CD4⁺ T cells was lower than NS group and percentage of CD8⁺ T cells was higher than NS group and LPS control group ($P<0.05$), which implied that

severe immune dysfunction existed in rats of high-dose group.

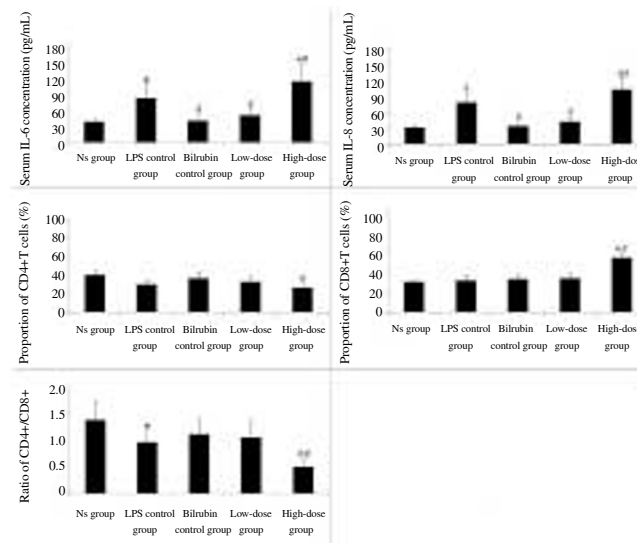


Figure 4. Content of serum IL-6, IL-8 and plasma CD4⁺, CD8⁺ T cells.

4. Discussion

In the study, we induced the slight inflammatory reaction by LPS injection, meanwhile, we injected different doses of bilirubin to observe the synergistic effect of bilirubin and LPS on inflammation and immune system. Our results showed that LPS could induce the up-regulation of MyD88 and p-TAK1 protein expression while high-dose bilirubin abolished the up-regulation. High-dose bilirubin could aggravate the inflammatory reaction in coordination with LPS while low-dose bilirubin alleviated the inflammation. Besides, rats of high-dose group showed severe immune dysfunction.

Many studies showed that high-concentration bilirubin could lead to the injury of multi-systems and multi-organs especially in neonates[8,9]. Berns *et al* separated the cortical neurons of Wistar rat embryos for primary culture, and then high-concentration bilirubin was added to imitate hyperbilirubinemia and their results showed that high-concentration bilirubin can increase the neurons apoptosis[10]. In the study of substances causing cerebral injury after intracerebral hemorrhage, Lakovic *et al*[11] found that 0.5 mmol/L bilirubin treatment damaged the function and the structure of myelinated axons and bilirubin oxidation products may be responsible for the toxicity of bilirubin.

MyD88 is the crucial adapter protein of Toll-like receptors (TLRs) signal pathways. It transfers the LPS inflammatory signals recognized by Toll-like receptor 4 (TLR4) into cells[12]. In the body, LPS firstly activates TLR4 through pre-processing way, then it activates the downstream factor including MAPK, p38 and ERK through MyD88, which leads to the activation and translocating into nucleus of NF- κ B and consequently induces the inflammation cascade. TAK1 is the member of MAP3K family, its elevated activity could activate NF- κ B signal pathway and trigger cascade, thus TAK1 regulates multiple effect genes expression. TAK1 also participates in regulating NF- κ B signal pathway mediated by B cell receptor, and it is necessary for B cells to secrete various

immunoglobulins[13]. In the study, high-dose bilirubin inhibited the MyD88 and p-TAK1 expression, which indicated that high-concentration plasma bilirubin could restrain signal transduction mediated by NF- κ B. On the other hand, NF- κ B is closely related to innate immune function[14]. When Bhakar et al researched NF- κ B transcriptional activity, they found the mice immune organs atrophied and significant neurons death after blocking the activation of NF- κ B[15]. Bureau *et al* blocked NF- κ B in silent lymphocytes, granulocytes and monocytes through special medicine, which resulted in the increasing apoptosis of lymphocytes and granulocytes[16]. Moreover, NF- κ B also takes part in the expression regulation of important immune receptors and immunoglobulins and the antigen presentation[17-19]. Herbst *et al* identified inhibition of a newly discovered macrophage TLR9-BTK-calcineurin-NFAT signalling pathway as a key immune defect that leads to organ transplant-related invasive aspergillosis[20]. In our study, because high-dose bilirubin obviously restrained the up-regulation of MyD88 and p-TAK1 expression, the activation of NF- κ B and NF- κ B signal pathway were inhibited to a great extent, and eventually the initiation of immune response was restrained[21,22]; meanwhile, high-concentration bilirubin induced high level of inflammatory factors causing interactive activation with immune cells and inflammation cascade, and inflammation cascade in coordination with immunosuppression induced by high-dose bilirubin produced immune dysfunction ultimately.

In conclusion, in neonatal rats with hyperbilirubinemia, high-concentration bilirubin aggravated inflammatory reaction induced by LPS and produced inflammation cascade; meanwhile, it in coordination with LPS inhibited NF- κ B signal pathways activation and caused immunosuppression which resulted in the immune dysfunction in synergy with inflammation cascade eventually.

Conflict of interest statement

We declare that we have no conflict of interest.

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