Expression Of Serum Cytokines Profile In Neonatal Sepsis

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Abstract

Objectives: Sepsis remains a major cause of neonatal death. In order to better characterize the inflammatory response during neonatal sepsis, we compared the differences in cytokines and chemokines between children with neonatal sepsis and full-term neonates without infection.

Methods: We enrolled 40 full-term neonates with sepsis and 26 full-term neonates without infection as controls between October 2016 and June 2018. Forty cytokines /chemokines in serum were analyzed using the Luminex Bead Immunoassay System.

Results: Our results showed that serum IL-6, IL-8, TNF-α, IL-1β, MIF, CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27, and CX3CL1 levels were significantly increased in neonates with sepsis compared to those in the control group (all adjusted $p<0.1$). The levels of serum CCL20, and IL-17 were higher in late-onset sepsis (LOS) than those in early-onset sepsis (EOS) (all adjusted $p<0.1$). Conversely, serum CXCL16 was lower in LOS than that in EOS (adjusted $p<0.05$).

Conclusions: Our findings revealed that excessive pro-inflammatory cytokines might be involved in neonatal sepsis. In addition, chemokines significantly increased the recruitment of immune cells after infection to participate in the anti-infection defense of neonates, but this could lead to damage.

What Is Already Known On This Topic

The clinical features of neonatal sepsis include systemic inflammatory response syndrome. Previous studies have shown that serum TNF-α, IL-1β, IL-6, IL-8, CXCL10, CXCR4 and CXCL12 levels rapidly and significantly increase in neonatal sepsis. These cytokines can be used as biomarkers of neonatal sepsis.

"What this study adds"

Research on the cytokine/chemokine difference between early-onset sepsis (EOS) and late-onset sepsis (LOS) is limited. In addition, there is little work to assess the significant changes in innate immunity and adaptive immunity during neonatal sepsis. Our results found that while the recruited immune cells participate in the anti-infection defense in neonates, they might also cause damage.

Introduction

Neonatal sepsis is a systemic infection by bacteria, viruses, or fungi, which is characterized by life-threatening organ dysfunction [1]. Despite advances in the management of neonates and the new generation of antibiotics, sepsis is still the leading cause of neonatal deaths with more than one million deaths worldwide each year [2–4].

After infection, pathogen-associated molecular patterns are recognized by sentinel immune cells through several classes of pathogen recognition receptors (e.g., toll-like receptors) [5–7]. Activation of these receptors can stimulate the release of inflammatory mediators, including cytokines and chemokines.

Therefore, the clinical features of neonatal sepsis include systemic inflammatory response syndrome. Several studies had demonstrated that serum pro-inflammatory TNF-α, IL-1β, IL-6, and IL-8 levels are rapidly and strikingly elevated in neonatal sepsis [8–10]. Moreover, in previous studies, the levels of serum CXCR4 and CXCL12 in neonatal sepsis were found to be significantly higher than those in controls [11, 12]. Another study showed that the level of CXCL10 was increased in the blood and peritoneum in a murine model of neonatal polymicrobial sepsis [13]. Furthermore, these cytokines served as valuable biomarkers for the diagnosis of neonatal sepsis [8–12]. However, studies on the cytokines/chemokines differences between early-onset sepsis (EOS) and late-onset sepsis (LOS) were limited. Moreover, little work had been done to assess the dramatic changes in innate immunity and adaptive immunity during neonatal sepsis. Therefore, in the present study, to better characterize the inflammatory response during neonatal sepsis, we systematically analyzed cytokine/chemokine profiles in neonatal sepsis.

Materials And Methods

Subjects and ethics statement

Neonates who were hospitalized for sepsis at the neonatal intensive care unit of the Second Affiliated Hospital of Wenzhou Medical University, between October 2016 and June 2018, were eligible to participate in this study. Written informed consent was obtained from the parents or legal guardians. A total of 40 full-term neonates with sepsis were enrolled in this study, and 26 full-term neonates without clinical manifestations or maternal risk factors for infection were included as the control group. Neonates with congenital malformations, those had treatment prior to samples collection, and those who had undergone surgery were excluded. The study was approved by the Ethical Committee of the Second Affiliated Hospital of Wenzhou Medical University (Registration code: LCKY2018-65).

The Diagnostic Criteria Of Neonatal Sepsis

Confirmed neonatal sepsis was defined as a positive blood culture accompanied by the presenting signs and symptoms. Suspected neonatal sepsis was defined as the presence of laboratory findings suggestive of infection (neutrophilia/neutropenia, thrombocytopenia, elevated C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR)) in combination with at least three of the following symptoms and signs without other causes: temperature instability (core temperature $\geq$ 38.5 or $\leq$ 36°C); respiratory symptoms (apnea, tachypnea with respiratory rate > 60 per minute, cyanosis, need for high ventilator settings or oxygen); cardiovascular symptoms including hypotension (blood pressure < fifth percentile for age), tachycardia (heart rate > 160 beats per minute), bradycardia (heart rate < 80 beats per minute), or poor perfusion; neurological symptoms (hypotonia, hyporeflexia, irritability, lethargy, and seizures); gastrointestinal symptoms (poor feeding, abdominal distension, green or bloody residuals, and vomiting). EOS was defined as onset in the first 72 hours after birth, and LOS was defined as onset after the first 72 hours of life.

Sample Collection

A total of 2 mL of venous blood was collected from all participants prior to treatment and was centrifuged at 3000 rpm for 15 min. In addition, sera were collected from 15 neonatal sepsis after treatment (3 EOS and 12 LOS). The sera were stored at -70°C until analysis.

Quantification Of Serum Cytokines And Chemokines

Forty cytokines and chemokines, including CCL21, CXCL13, CCL27, CXCL5, CCL11, CCL24, CCL26, CX3CL1, CXCL6, GM-CSF, CXCL1, CXCL2, CCL1, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, CXCL10, CXCL11, CCL2, CCL8, CCL7, CCL13, CCL22, MIF, CXCL9, CCL3, CCL15, CCL20, CCL19, CCL23, CXCL16, CXCL12, CCL17, CCL25, and TNF-α, were analyzed using the Luminex Bead Immunoassay System (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. Serum IL-17 level was measured using human IL-17 Quantikine ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL). Categorical variables were expressed as frequencies and percentages. Continuous variables were presented as median and interquartile range(IQR). The Mann-Whitney U test or Kruskal-Wallis H test was used to compare the serum cytokine and chemokine levels between different groups. Difference before and after treatment were analyzed using the Wilcoxon test. For correlation analysis, Spearman's correlation coefficients were calculated. For all comparisons, $p<0.05$ was considered statistically significant.

Results

1. Clinical characteristics of the enrolled neonates

A total of 66 full-term neonates were enrolled in this study, including 40 neonates with sepsis (10 EOS and 30 LOS) and 26 uninfected neonates as controls. The body temperature was 38.1°C (IQR 37.13°C-38.65°C) and 36.8°C (IQR 36.5°C-37.2°C) in neonates with sepsis and those in the control group, respectively ($p<0.0001$). In 13 neonates with positive blood culture, the following pathogens were isolated: *Escherichia coli* (seven cases), *Streptococcus lactis* (four cases), *Klebsiella* (one case), and *Enterococcus faecium* (one case). The levels of serum C reactive protein (CRP) and serum amyloid protein A (SAA) in neonates with sepsis were 9.11 mg/mL (IQR 6.54 mg/mL-10.13 mg/mL) and 29.38 mg/mL (IQR 4.83 mg/mL-36.24 mg/mL), respectively, which were significantly higher than those in the control group (both $p<0.0001$). The neutrophil counts of the neonates with sepsis and those in the control group were 9.06×10^9/L (IQR 5.34×10^9/L-14.84×10^9/L) and 6.14×10^9/L (IQR 4.29×10^9/L-7.76×10^9/L) ($p=0.018$), respectively. There were no significant differences in the monocyte, lymphocyte, and platelet counts between the two groups (all $p>0.05$). Detailed information of the clinical characteristics was shown in **Table 1.**

Table 1 The clinical characteristics of the enrolled neonates

Variables	Neonate sepsis (n=40)	Control (n=26)	*p* value
Age, days	12(3.25-19.75)	10(7-16.25)	0.672
Male gender, n (%)	25(62.5)	13(68.4)	0.775
Temperature (°C)	38.1(37.13-38.65)	36.8(36.5-37.2)	<0.0001
Blood culture, n (%)	13(32.5)	nd	/
Early of sepsis, n (%)	30(75)	/	/
WBC(x10^9/L)	17(11.68-21.13)	12.1(9.9-14.7)	0.005
Neutrophils(x10^9/L)	9.06(5.34-14.84)	6.14(4.29-7.76)	0.018
Monocytes(x10^9/L)	1.49(0.90-2.05)	1.26(0.96-51.46)	0.189
lymphocytes(x10^9/L)	4.07(3.14-5.53)	4.22(3.43-5.02)	0.942
Platelets(x10^9/L)	316.5(249-436)	262(227-345)	0.076
CRP (mg/ml)	9.11(6.54-10.13)	0.35(0.18-0.69)	<0.0001
SAA (mg/ml)	29.38(4.83-36.24)	0.38(0.04-3.86)	<0.0001

Note: nd, not done; WBC, White Blood Cell; CRP, C reactive protein; SAA, serum amyloid protein A. Values are given as median (interquartile range).

2. Levels of serum cytokines and chemokines in neonatal sepsis

We compared serum cytokine and chemokine levels between neonates with sepsis and those in the control group. Since the level of CCL15 was not in the quantitative range of the assay and exceeded the highest calculated concentration, we did not analyze it further. As shown in Figure 1, the levels of the pro-inflammatory cytokines, IL-6, IL-8, TNF-α, and IL-1β, were significantly higher in neonates with sepsis than those in the control group (all $p<0.05$). There was no difference in the levels of IL-4, IFN-γ, IL-17, IL-2, IL-10, GM-CSF, and IL-16 between the two groups (all $p>0.05$). Serum MIF level was remarkably increased in neonates with sepsis, and was 6,270 pg/mL (IQR 4,615 pg/mL-11,820 pg/mL) and 4,947 pg/mL (IQR 3,204 pg/mL-6,767 pg/mL) in the sepsis and control groups, respectively ($p=0.0223$). Serum CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27, and CX3CL1 levels were significantly increased in neonates with sepsis compared to those in the control group (all $p<0.05$). Interestingly, the levels of serum CXCL5, IL-2 and IL-4 in female were higher than those in male **(Table S1)**.

Subsequently, we further analyzed the differences in these cytokines and chemokines between the EOS and LOS groups. There were 10 EOS and 30 LOS. The ages of EOS and LOS were 2 days (IQR,1day-3days) and 17days (IQR,11.75days-21.25days), respectively (*p<0.001*). The body temperature was 37.15℃ (IQR, 36.63℃-37.5℃) and 38.45℃ (IQR, 38℃-38.73℃) in EOS and LOS, respectively (*p=0.0006*). All cases who were blood culture positive were LOS (Table 2). As shown in Figure 2, the levels of serum CCL20 and IL-17 were higher in LOS than those in EOS (both *p<0.05*). Conversely, serum IL-16 was lower in LOS than that in EOS (*p<0.05*).

Table 2 The clinical characteristics of EOS and LOS

Variables	EOS (n=10)	LOS (n=30)	*p* value
Age, days	2(1-3)	17(11.75-21.25)	<0.001
Male gender, n (%)	4(40)	21(70)	0.135
Temperature (℃)	37.15(36.63-37.5)	38.45(38-38.73)	0.0006
Blood culture, n (%)	0	13(43.3)	<0.001

Note: EOS, early of sepsis; LOS, late of sepsis.

Among the 40 neonates with sepsis, there were 13 neonates with positive blood culture and 27 neonates with negative blood culture. We compared the levels of serum cytokines and chemokines between neonates with positive and negative blood cultures. The levels of CXCL6, CCL8, and CCL23 in neonates with positive blood culture were significantly higher than those with negative blood culture (all *p<0.05*) (**Table S2**). There was no difference in the levels of other cytokines and chemokines between the two groups (data not shown).

3. The association between the levels of serum cytokines/chemokines and age in neonatal sepsis

We further analyzed the association between the levels of cytokines/chemokines and age among neonatal sepsis. As shown in **Table S3**, the levels of CCL21, CCL27, CCL22, CCL19, CXCL16, CCL17, IL-10, and IL-16 were negative correlation with their ages, and IL-17 was positively correlated with ages (all *p<0.05*).

4. Levels Of Cytokines And Chemokines After Treatment

Of 15 neonatal sepsis (3 EOS and 12 LOS) were treated with antibacterial therapy and were effective. The sera of the15 neonates with sepsis were collected after treatment. The point-in-time of collection was 5.5days (IQR, 2days-7.25days) after treatment. A total of 41 cytokines and chemokines were measured. As shown in Figure 3, IL-6, IL-8, TNF-α, IL-1β, CXCL13, CXCL16, CCL27, CCL3, CCL23, and CX3CL1 were significantly decreased after treatment (all *p<0.005*).

Discussion

Neonatal sepsis is a major risk factor for neonatal mortality [14]. Furthermore, the underlying pathological mechanisms remain unclear. In the present study, we systematically investigated the dynamic changes in cytokine and chemokine profiles in neonatal sepsis. In accordance with previous studies [8–10], the levels of the pro-inflammatory cytokines IL-6, IL-8, TNF-α, and IL-1β were significantly increased in neonates with sepsis. A moderate increase in cytokines plays a protective role and promotes antimicrobial immune responses, whereas excessive upregulation of pro-inflammatory cytokines is commonly associated with a severe and often fatal outcome due to multiple organ failure [15].

Chemokines are a family of cytokines that have the capacity to recruit leukocytes to pathogen invasion sites, which is essential for the host to defend against infections [16]. Our results showed that serum CXCL13, CXCL1, CXCL2, CXCL5, CXCL6, CXCL8, CXCL16, CCL27, CCL2, CCL8, CCL3, CCL20, CCL23, CCL27, and CX3CL1 levels were significantly increased in neonates with sepsis compared to those in the control group. *Manoura* et al also found that the levels of serum CXCL1 and CXCL5 were higher in neonates with sepsis [17]. Among these chemokines, CXCL1, CXCL2, CXCL5, CXCL6, and CXCL8 are potent chemoattractants for neutrophils. Neutrophils are the most abundant cells of innate immunity and play an important role in responding to bacterial, viral, and fungal infections [18]. Previous studies have shown that elastase and nitric oxide are upregulated in neonates with sepsis [19, 20]. In our study, neutrophil counts in neonates with sepsis were higher than those in controls. These results suggested that neutrophil-related chemokines were immediately synthesized and released after infection, which might have subsequently led to the recruitment of neutrophils as a defense mechanism against infections in the neonates.

The defense system in neonates is initially dependent on their innate immune system because adaptive immunity develops later in life [21]. Interestingly, in our study, the levels of Th17 cell-related cytokine/chemokines, IL-17 and CCL20 were significantly higher in LOS than those in EOS. The level of IL-17 was positively correlated with ages. The ages of LOS were higher than those of EOS. Taken together, our results indicated that the pro-inflammatory cytokines were initially released in neonates at the onset of infection, but adaptive immunity gradually developed in the later stage of infection.

The serum MIF level in neonatal sepsis was significantly higher than that in the control group. After treatment, MIF significantly decreased. The MIF of newborns is 10-fold higher than that of children and adults. *E. coli* and *Group B Streptococcus* induce MIF secretion by neonatal monocytes [22]. MIF plays an important role in promoting the production of inflammatory cytokines by monocytes [23]. Previous studies have indicated that MIF is correlated with the expression of pro-inflammatory markers and the dysregulation of pituitary and adrenal function, severity scores, and disease outcomes [24–27]. Therefore, MIF might play a critical role in the immune regulation of neonatal sepsis.

In our study, serum CX3CL1 levels were increased in neonates with sepsis. A previous study reported that the level of plasma CX3CL1 was elevated in a mouse model of CLP-induced sepsis [28]. The level of CX3CL1 in adult sepsis patients increased with the severity and number of organ dysfunction [29]. Nonsurvivors had sustained elevated CX3CL1 levels compared to survivors [29]. Taken together, these data suggest that CX3CL1 is a risk factor for sepsis outcomes. CX3CL1 acts through the CX3CR1 receptor and is a unique member of the CX3C chemokine family. CX3CR1 is mainly expressed on $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ monocytes, which represent an activated and a more mature "macrophage-like" subset. Furthermore, the cell population is greatly expanded in various infectious and inflammatory diseases and can be more than 50% of total monocytes during sepsis [30]. These data suggest that CX3CL1 recruits activated monocytes and can be involved in neonatal sepsis damage. However, the roles of CX3CL1 in neonatal sepsis need to be further studied.

Our study has some limitations. First, the subjects enrolled were relatively few; therefore, our findings need to be proven in future studies with a larger cohort. Second, gestational age is an essential factor for these cytokines and chemokines, whereas we only enrolled full-term neonates in our study.

In conclusion, our study suggested that excessive inflammation in neonatal sepsis might be involved in the damage associated with neonatal sepsis. Neutrophils, monocytes, and lymphocyte-associated chemokines increased significantly after infection. To summarize, while the recruited immune cells participate in the anti-infection defense in neonates, they might also cause damage.

Abbreviations

EOS: early-onset sepsis; LOS: late-onset sepsis; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; SAA: serum amyloid protein.

Declarations

Acknowledgments

We thank all the children and their families for participating in this study.

Authors' contributions

XQ conceived and designed the study; KM, CS, QYand HS collected data; KM, GB, LS, and TH had a phone follow-up; CS, WH, WG and YJ conducted the statistical analyses. KM, YZ, JM and XQ drafted the manuscript. All authors read and approved the final version of the manuscript.

Authors' information

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Availability of data and materials

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

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. Written informed consents have been obtained from the parents of subjects under 16 years of age for this study, and has got ethics approval and consent by Research Ethics Committee of the Second Afliated Hospital of Wenzhou Medical University. The Ethical Approval Number was *LCKY2018-65*.

Competing interests

Neither this paper nor any similar paper has been or will be submitted to or published in any other scientific journal. All authors are aware and agree to the content of the paper and to their being listed as an author on the manuscript. There is no conflict of interest or competing financial interests for all authors.

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Figures

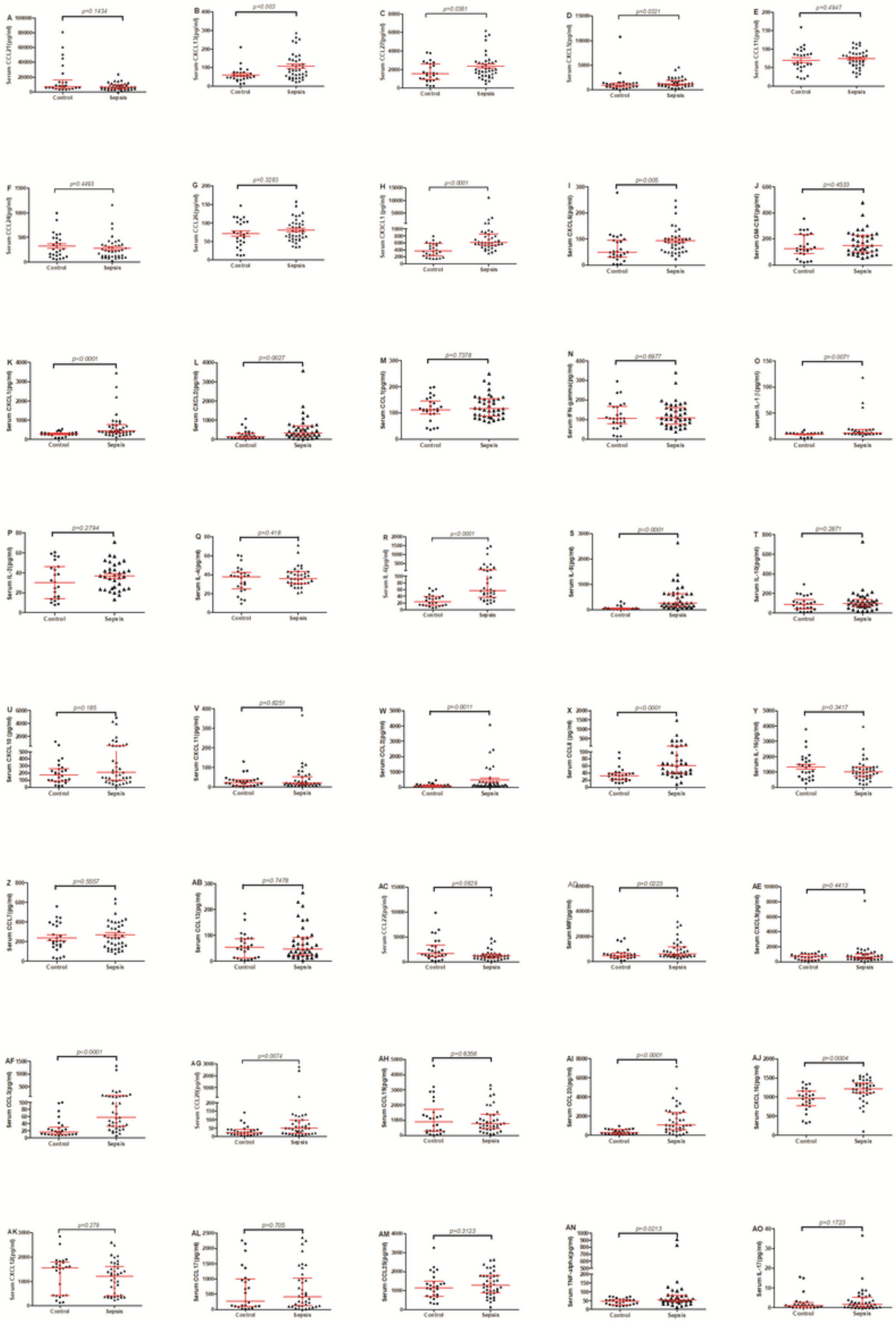

Figure 1

Comparisons of circulating levels of chemokines and cytokines in controls and neonatal sepsis. The numbers of controls and neonatal sepsis were 26 and 40. The difference between control and neonatal sepsis was assessed using the Mann-Whitney *U* test.

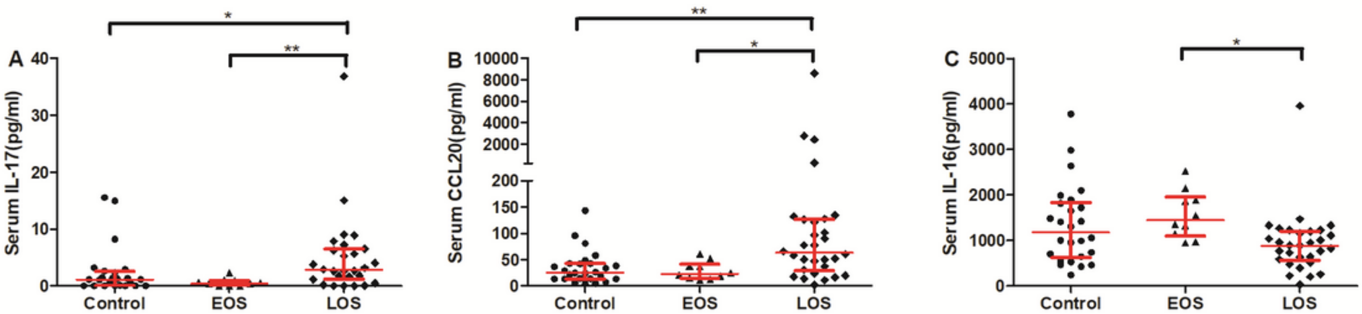

Figure 2

Levels of cytokines and chemokines between early neonatal sepsis(EOS) and late neonatal sepsis(LOS). The numbers of EOS and LOS were 10 and 30, respectively. The difference between control and neonatal sepsis was assessed using the Kruskal-Wallis *H* test.

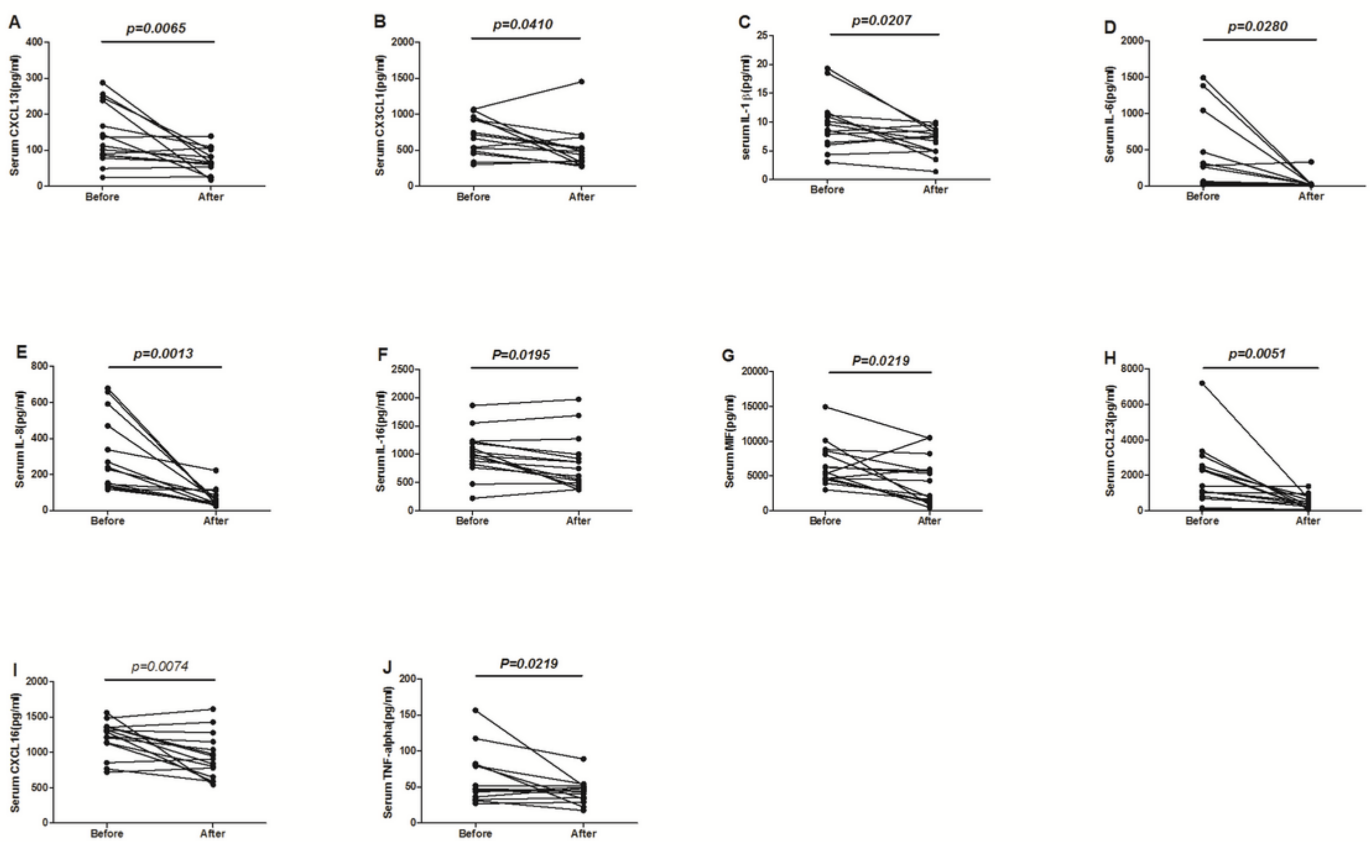

Figure 3

Levels of cytokines and chemokines before and after treatment in 15 neonates with sepsis. Differences before and after treatment were analyzed using the wilcoxon test.

Supplementary Files

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