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Original Article

Neutrophil, TLR2, and TLR4 expression in newborns at risk of sepsis

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Abstract

Background There is increasing evidence that toll-like receptors (TLR) play a key role in the mediation of systemic responses to invading pathogens during sepsis. Saliva is an important body fluid for detecting physiological and pathological conditions of the human body. Neutrophils are participants in the acute response against pathogens in many tissues, and their influx into the oral cavity may occur at any time.

Objective To compare mean neutrophils and the expression of TLR2 and TLR4 in saliva and blood of newborns at risk for sepsis to those of healthy newborns.

Methods This cross-sectional study was conducted from July to December 2011 in the Division of Neonatology, Department of Child Health, Ulin General Hospital, Lambung Mangkurat University Medical School, Banjarmasin. Case subjects were newborns with sepsis risk factors (30 infants), while 30 healthy infants were in the control group. Saliva and blood specimen examinations were performed in the Biomedical Laboratory of Brawijaya University Medical School, Malang. We used T-test for statistical analyses.

Results From saliva specimens, mean neutrophils were significantly higher in the case group than in the control group [14.43 (SD 12.21) % vs. 5.63 (SD 6.78) %, respectively, (P=0.021)]. In addition, mean TLR2 and mean TLR4 saliva levels were significantly higher in the case group than in the control group [TLR2: 64.97 (SD 26.42) % vs. 40.06 (SD 6.23) %, respectively, (P=0.011); TLR4: 1.5 (SD 1.61) % vs. 0.57 (SD 0.53) %, respectively, (P=0.044)]. From blood specimens, mean neutrophils were also significantly higher in the case group than in the control group [1.09 (SD 0.61)% vs. 0.21 (SD 0.09)%, respectively, (P=0.000)]. Similarly, mean blood TLR2 and TLR4 levels were significantly higher in the case group than in the control group [TLR2: 92.51 (SD 5.51) % vs. 81.74 (SD 11.79) %, respectively, (P=0.003); TLR4: 0.71 (SD 1.42) % vs. 0.12 (SD 0.06) %, respectively, (P=0.000)].

Conclusion There are significant increases in neutrophils, as well as neutrophil expression of TLR2 and TLR4 in the saliva and blood from newborns with sepsis risk factors compared to those of healthy newborns. **[Paediatr Indones. 2013;53:132-7.]**

Keywords: *TLR2, TLR4 neutrophil expression, sepsis neonatal, saliva, blood*

Sepsis is an interaction between microorganisms and their host, as a manifestation of an immunoinflammatory dysregulatory response.[1,2,3] Phagocytic cells play a role in the acute inflammatory response, due to their ability to efficiently ingest and kill a variety of pathogenic microorganisms. Professional phagocytes include neutrophils, monocytes, macrophages, eosinophils and dendritic cells. In this group, neutrophils are the most abundant and usually the first cells to arrive at the site of inflammation. They are a major cellular component of the natural immune response during acute infection.[4,5] The basic strategy of natural immunity is to recognize specific microbial

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molecular structures, known as pathogen-associated molecular patterns (PAMPs). Pathogen-associated molecular patterns consist of lipopolysaccharide (LPS) from Gram-negative bacterial cell walls, peptidoglycan (PGN), lipoteichoic acid (LTA), and teichoic acid (TA) from Gram-positive bacterial cell walls, lipoarabinomannan from mycobacterium, mannan from yeast, and viral double-stranded ribonucleic acid (RNA). These products of microbial metabolism are in the form of gene products or microbial metabolic pathway products. On the host side, the immune system or immune cells have receptors for PAMPs, known as pattern-recognition receptors (PRRs), of which TLRs are useful for providing a rapid response to invading microbes. Toll-like receptors bind to bacterial cell membranes or their soluble proteins, and are able to recognize a variety of PAMP molecular structures found in various pathogens.[5,6,7,8]

Toll-like receptors-2 can recognize various microbial components, such as lipoprotein / lipopeptides from a wide range of pathogens, PGN and LTA from Gram-positive bacteria and glycolipids from *Treponema maltophyllum*. Toll-like receptors-2 was also found to recognize LPS from non-enterobacteria, such as *Leptospira enterogans, Porphyromonas gingivalis* and *Helicobacter pylori*.[9,10] The LPS structure recognized by TLR2 is different from LPS derived from Gram-negative bacteria recognized by TLR4, mainly in the number of acyl chains in the lipid A component.[11] Toll-like receptors-4 is an essential receptor for LPS recognition.[12,13] In addition, TLR4 also recognizes some endogenous ligands, such as heat shock protein (HSP 60/70), fibronectin extract domain A, oligosaccharides of hyaluronic acid, heparin sulfate and fibrinogen; but the TLR4 activation requires a high concentration of endogenous ligand. In contrast, very small amounts of LPS are able to activate TLR4, as LPS is a potential immunoactivator.[13]

Bacterial isolation from body fluids (usually blood) is the standard and most specific method for sepsis diagnosis.[15] Saliva analysis may have physiological and pathological implications and may be useful to diagnose disease, especially in terms of origin, composition, function, and interactions with other organ systems. Although biological markers for saliva diagnosis of certain adult diseases have been identified, these results have not been widely used for early detection of neonatal sepsis.

The aims of this study were to assess neutrophil expression and neutrophil TLR2 and TLR4 expression in saliva and blood of newborns at risk of neonatal sepsis, compared to those of healthy newborns. A correlation of saliva and blood results would strengthen the evidence for using saliva as biomarker tool for identifying early-onset sepsis in newborns.

Methods

This cross sectional study was conducted from July to December 2011 in the Division of Neonatology, Department of Child Health, Lambung Mangkurat University Medical School and Ulin General Hospital, Banjarmasin. Laboratory tests were conducted at the Biomedical Laboratory of Brawijaya University Medical School, Malang. Saliva and blood specimens were taken from 60 newborns, of which 30 infants were at risk of sepsis and 30 infants were healthy and served as a control group. All subjects' parents provided written informed consent. Saliva specimens (3 mL each) were taken via suction from the oropharynx according to standard procedures for neonatal resuscitation. Blood specimens (3 mL each) were taken from the umbilical cord vein immediately after clamping and cutting. All samples were immediately sent to the Biomedical Laboratory at Brawijaya University Medical School, Malang.

Subjects in the sepsis risk group were included on the basis of having at least 1 major criteria or 2 minor criteria. Major risk criteria were membranes ruptured for > 24 hours, maternal fever with intrapartum temperature > 38 ^{0}C, chorioamnionitis, fetal heart rate persisting at > 160 times/minute or foul-smelling amniotic fluid. Minor risk criteria were membranes ruptured for > 12 hours, maternal fever with intrapartum temperature > 37.5 ^{0}C, low Apgar score (<5 at the 1st minute , <7 at the 5th minute), very low birth weight baby (VLBWB) of <1500 grams, gestational age < 37 weeks, multiple pregnancy, foul-smelling vaginal discharge, maternal urinary tract infection (UTI) or suspected untreated maternal UTI.

Blood specimens were prepared as follows: blood was mixed with 6 mL of lymphocyte separation medium (LSM) at a density of 1.077 g/mL in a 15-mL centrifuge tube, then carefully insert the 3 mL of blood in the tube by passing through on the wall of the tube and centrifuged at 1000 rpm for 30 min at

room temperature (RT), resulting in the formation of 4 layers, plasma, buffy coat ring, LSM, and erythrocytes. The ring buffy coat was transferred to a new centrifuge tube, washed with 10 mL of phosphate-buffered saline (PBS) at pH 7.4, centrifuged at 1000 rpm for 10 minutes and repeated 2-3 times. The supernatant was discarded and the pellet was resuspended in 2 mL of Roswell Pack Medium Institute (RPMI) medium containing 10% fetal bovine serum (FBS). A 500-μL sample was taken to test for neutrophil, TLR2, and TLR4 levels. Saliva specimens were prepared as follows: 0.9% NaCl was added 1:1 with the saliva, vortexed until homogeneous, and centrifuged at 1400 rpm for 10 minutes at RT. Pellets were washed twice with PBS at pH 7.4, and centrifuged. Supernatants were discarded and pellets were resuspended in 2 mL RPMI with 10% FBS. A 500-μl sample was taken to test for neutrophil, TLR2, and TLR4 levels. The remaining sample was centrifuged at 1000 rpm for 5 minutes, supernatants discarded, and pellets stained with fluorescein isothiocyanate (FITC) for cluster differentiation 64 (CD 64), phycoerythrin (PE) for TLR2 and peridinin-chlorophyll-protein complex (PerCP) for TLR4. Samples were incubated for 15 min at RT in the dark, followed by the addition of 300μL of PBS and 2% FBS. Flow cytometry was performed with BD FACS *Calibur Cell Quest Pro* mode.

Variables observed were the expression of TLR2, TLR4, and double-positive TLR2-TLR4 neutrophils in saliva and blood. Saliva and blood data from measured variables were analyzed using the Kolmogorov-Smirnov test for normality.

Results

We included 30 newborn infants with risk of sepsis and 30 healthy newborn infants as a control group, the baseline characteristics of our subjects described on **Table 1** and **Table 2.**

As shown in **Table 3**, there was a significantly higher mean R2 (neutrophil) level in saliva from the case group than from the control group. Similarly,

Table 1. Sex and Birth delivery

Characteristics	Control group n=30	Case group n=30
Sex		
Male	12	13
Female	18	17
Birth delivery		
Spontaneus delivery	16	15
Sectio caesarea	13	11
Vacuum extraction	1	4

Table 2. Major & Minor risk criteria in the sepsis risk group (n=30)

Risk criteria	n
Membranes ruptured for > 12 hours & Foul-smelling vaginal discharge	8
Membranes ruptured for > 24 hours	7
Foul-smelling vaginal discharge & Maternal UTI or suspected untreated maternal UTI	7
Foul-smelling vaginal discharge & gestational age < 37 weeks	3
Chorioamnionitis	1
Membranes ruptured for > 24 hours & maternal fever with intrapartum temperature > 37.5^0C	1
Membranes ruptured for > 24 hours & Gestational age < 37 weeks	1
Foul-smelling amniotic fluid & Maternal UTI or suspected untreated maternal UTI	1
Gestational age < 37 weeks & Maternal UTI or suspected untreated maternal UTI	1

Table 3. Mean systemic immune responses in the saliva between two groups

Variables	Control group	Case group	P value
	Mean (SD) %	Mean (SD) %	
R-2	5.63 (6.78)	14.43 (12.21)	0.021
TLR2	40.06 (23.6)	64.97 (26.42)	0.011
TLR4	0.57 (0.53)	1.5 (1.61)	0.044
TLR2, TLR4	8.69 (7.06)	23.02 (22.46)	0.026

P value < 0.05 was considered to be significant by T-test

Table 4. Mean systemic immune response in blood between the two groups

Variables	Control group	Case group	P value
	Mean % (SD)	Mean % (SD)	
R-2	0.21 (0.09)	1.09 (0.61)	0.000
TLR2	81.74 (11.79)	92.51 (5.51)	0.003
TLR4	0.12 (0.06)	1.42 (0.71)	0.000
TLR2-TLR4	2.85 (2.08)	7.17 (5.06)	0.005

P value < 0.05 was considered to be significant by T-test

the mean TLR2 in saliva was significantly higher in the case group than in the control group, as well as the mean TLR4 in saliva. In contrast, TLR2-TLR4 coexpression was not significantly different between the case and control groups.

As shown in **Table 4**, there was a significantly higher blood mean R2 (neutrophils) in the case group than in the control group. Similarly, the blood mean TLR2 in the case group was significantly higher than in the control group, as well as the blood mean TLR4 in the case group was significantly higher than in the control group. In addition, the blood mean TLR2-TLR4 was significantly higher in the case group than in the control group.

Discussion

Neutrophils are the first line of phagocytic cells in the natural immune system. Normal newborns generally have higher levels of neutrophils than in children and adults.[16] In this study, neutrophil levels in saliva and blood from the case group were significantly higher than those of the control group. Similar to other neonatal sepsis studies,[17,18] we found significantly increased neutrophil levels in subjects at risk of sepsis, as evidenced by the elevation of neutrophil CD64, a very sensitive marker. Bhandari *et al*. reported that the combination of CD64 with an absolute neutrophil amount had a high negative predictive value (93%) and sensitivity (95%) for sepsis markers.[18]

There have been several reports on TLR2 and TLR4 expression in newborn babies. Vieman *et al*. conducted a TLR2 and TLR4 expression study on granulocytes and monocytes from the peripheral veins of 20 healthy adults and from the umbilical cord veins of 85 newborns (32 septic infants and 53 healthy infants). They found that TLR2 expression in healthy newborns was slightly lower than in healthy adults, while TLR4 expression was similar in both groups. However, there was a TLR2 significant up-regulation in the group of sepsis newborns compared to healthy newborns, based on C-reactive protein (CRP), IL-6 and IL-8 levels. TLR4 expression was not different in septic and healthy newborns.[19] Consistent with our results, TLR2 expression was significantly higher in the case group compared to the control group, but we found that TLR4 expression was also significantly elevated in the sepsis group. In contrast, infants with respiratory syncytial virus (RSV) bronchiolitis had a significant decrease in neutrophil TLR4 expression.[20]

Saliva has been shown to have many benefits,[21,22] such as containing antimicrobial compounds and biomarkers of infectious diseases,[23,24] malignancy,[25,26] and neutrophil levels. Saliva also may be used to determine the success of bone marrow transplants.[27] In our study, the saliva specimens from the case group had significantly increased mean neutrophils compared to that of the control group. Although neutrophils participate in the acute response against microbial pathogens in various tissues, their influx into the oral cavity occurs at any time. Neutrophil influx may be a result of chemoattractants found in the oral environment, including microorganisms, toxins, chemokines, and cellular degradation products.[28] Similarly, case group blood specimen results had significantly increased mean neutrophils compared to that of the control group. Furthermore, saliva neutrophil levels were much higher than that of blood in the case group, suggesting that neutrophil activation starts early in saliva. We also found significantly higher TLR2 expression in saliva and blood from the case group compared to those of the control group, consistent with an animal study by Williams *et al*. and a neonatal sepsis study by Vieman *et al*.[19,29] As such, TLR2 expression may be used as an early marker of sepsis.[30]

There was a correlation between systemic immune response in the saliva and in the blood. Neutrophil levels and TLR2 expression in both blood and saliva were significantly higher in newborns with sepsis risk factors than in healthy newborns, neutrophil levels and TLR2 expression might be useful as early markers of neonatal sepsis. Further research is needed to establish the usefulness of saliva measurements as an early-onset neonatal sepsis biomarker. A better understanding of TLR biology may unveil novel therapeutic approaches for sepsis, to decrease morbidity and mortality caused by neonatal sepsis.

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