International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 2 Issue 1 // January 2013 // PP.18-22

Neutrophil Phagocytosis Activity Compared To Myeloperoxidase, Hydrogen Peroxidase And Lactoferrin Levels In Saliva Of Newborn Baby With Sepsis Risk Factors To Detect Early-Onset Neonatal Sepsis

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Abstract: *Neonatal sepsis, often called neonatal sepsis or septicemia is a clinical syndrome in the baby's first month of life due to a systemic response to infection with the bacteria discovery in blood cultures. Phagocytes are the important factors that play a role in acute inflammatory response due to its ability to destroy various pathogens efficiently. In sepsis there is a decrease in phagocytosis activity due to qualitative and quantitative neutrophils changes. Neutrophil has the ability to phagocyte bacteria and insert it in a cellular compartment (phagosome) which role as a cytotoxic agent. Neutrophils that phagocyte bacteria, will burst of oxygen consumption (respiratory burst), and produced reactive oxygen species as bactericidal.*

Saliva analysis has been an important resource for evaluating the saliva condition physiological and pathologically, as a tool to diagnose disease, especially the origin, component, function, and interaction with other organ systems. Neutrophil plays an active role in acute response to microbial pathogens in variant tissues. Neutrophils influx occurred due to the availability of chemoantractant factors in the oral environment such as microorganism, toxins, chemokines, and cellular degradation products.

Keywords: *neutrophils, respiratory burst, early-onset neonatal sepsis*

I. Introduction

Neutrophil plays a role as one of the frontline of body's defense against infection. These cells use bactericidal pathways that dependent or independent oxygen as a weapon to eliminate infectious agents. Oxygen-dependent mechanism involves production of bactericidal reactive oxygen compounds. The mechanism oxygen-independent involves chemotaxis, phagocytosis, degranulation, and the release of lysis enzyme and bactericidal peptides [1]. In response to bacterial pathogens entrance, neutrophils will move into the infected tissue, then activate to form a reactive oxygen compounds. This event called respiratory burst involving the NADPH oxidase activation. At the respiratory burst, there was a rapid uptake of molecular oxygen and transformation into reactive oxygen compounds, which on one side is a representation of the host defense mechanisms in the inflammatory site, on the other hand the possibility damage close to healthy tissue. It is important that relevant reactive oxygen compounds physiological concentrations able to modulate the redox-sensitive signaling cascade and improve immunological cellular function [2]. Reactive oxygen compounds can trigger oxidative damage to macromolecules, leading to lipid peroxidation, amino acid chains oxidation, cross links protein formation, polypeptide chain oxidation forming protein fragmentation, DNA strands ruptured. Furthermore, through the degranulation process, occured secretion of several chemical compounds, especially MPO (myeloperxidase) and LTF (lactoferrin), respectively azurofilik granules and specific granules [3].

MPO is an enzyme that contains heme secreted by phagocytic cells after the respiratoty burst system activation. MPO is expressed mainly by neutrophils and monocytes in small quantities and it is very important to determine further process of hydrogen peroxide. MPO is usually used as a marker of neutrophils accumulation in tissues and a marker of neutrophil activity when its measured in plasma [4]. Most of the hydrogen peroxide produced by neutrophils will be consumed by the MPO. Hydrogen peroxide substrate is also derived from in vivo variety sources, including the leukocyte NADPH oxidase, xanthine oxidase, nitric oxide synthase (NOS) and various isoenzymes [5].

LTF is able to inhibit the growth of gram-positive and gram-negative bacteria. LTF also can inhibit the metabolism through low molecular weight synthesis by chelation process (siderophores), or by the production of specific lactoferrin receptors that facilitate the iron released from protein [6]. LTF major role in host defense mechanisms is a bacteriostatic that inhibit the bacteria proliferation through iron sequestration

capabilities. Besides bacteriostatic, LTF also serves as bactericidal, the bactericidal effect is derived from laktoferisin B activation as a proteolytic peptides molecule derived from the LTF N terminal. Bactericidal effect is due to the ability of LTF to break down the outer surface of bacterial membrane resulting changes in membrane permeability. Membrane damage happend because the LTF incorporation with membranes will inhibit lipopolysaccharide through a modulated process by cations (Ca2 +, Mg 2 +, or Fe 2 +) [7].

Although the benefit of research and biological markers development for certain diseases in diagnosis in adults salivary have been conducted, but no research reported the role of saliva in neonatal sepsis.

II. Material And Methode

This study is a prospective cross-sectional study, the study has received approval from the Ethics Committee, the saliva and blood samples were taken from 15 newborns with sepsis risk that met the inclusion criteria, saliva and blood samples were taken from 15 newborns with no risk as control, from the parents who have signed informed consent. The study was conducted in July to December 2011, in the Division of Neonatology Section of Child Health Ulin General Hospital / Faculty of Medicine, Lambung Mangkurat University Banjarmasin, All samples was examined in Biomedical Laboratory Faculty of Medicine Brawijaya University Malang. Inclusion criteria: Newborn baby with neonatal sepsis risk is born of mothers who meet the following criteria: 1 major risk or 2 minor risks [8]. Major risks include: rupture of membrane > 24 hours; maternal fever while intrapartum temperature> 38^0C; chorioamnionitis; fetal heart rate settled> 160 x / min; amniotic smell. The minor risks: rupture of membranes> 12 hours; maternal fever when intrapartum temperature> 37.5^0 C; low Apgar score (minute-1 <5, the 5^{th} minute <7), very low birth weight infants (<1,500 grams) ; gestational age <37 weeks: a multiple pregnancy; vaginal discharge in women who were not treated; women with urinary tract infection (UTI) / suspected UTI untreated. Exclusion criteria: Infants with risk factors for sepsis were born outside of Ulin Hospital; infants with severe congenital abnormalities; infants with severe asphyxia; baby is very very low birth weight.

Isolation of neutrophils: by using the method Gasparoto with modification [9]

Blood samples: Enter briefly, 6 ml Histopaque 1119 into 15 ml round bottom tube, then 6 ml whole blood werer layered over the gradient, centrifuged 1000 rpm in 30 min. The second band, which contained the neutrophils, was aspirated and washed twice with cold RPMI 1640. Centrifuge 1000 rpm 10 minutes and repeat 2-3 times, supernatant removed, the pellet plus 2 cc RPMI medium containing 10% FBS.

Saliva samples: Saliva plus 3 ml 1:1 with 0.9% NaCl, then vortexed until homogeneous, centrifuge 1400 rpm 10 min, pellets were washed with PBS pH 7.4 2 times and then centrifuged, the supernatant was discarded and the pellet taken added 2ml RPMI 10% FBS, homogenized.

Phagocytosis test

Pellets that are mixed media RPMI put in culture TC 24 well. Incubate 1 hr 37^0C 5% CO_2 incubator. Treatment plus 15 ml bacterial suspension (OD 1 = 10^6), Incubate 30 minutes, and 60 minutes 37^0C 5% CO2 incubator. Each well pipetted then centrifuged 1000 rpm for 5 min. Supernatant was discarded, pellet was washed PBS pH 7.4 was repeated 2 times. Pellet made smear the glass object, giemsa staining 10%.

Data analysis

• Comparison of the MPO, H_2O_2, LTF levels, respectively blood and saliva samples between case and control groups were tested using independent samples t test

• Comparison of the phagocytosis activity and neutrophil number, respectively blood and saliva samples between case and control groups were tested using independent samples t test

The measurement of MPO, H_2O_2 and Lactoferrin

• Measurement of MPO; Measuring Human MPO using Instant ELISA BMS2038INST eBioscience.

•Measurement of H_2O_2; Measurement of H_2O_2 using Hydrogen Peroxide Assay Kit K265-200.

• Measurement Lactoferrin; measurements using Max Human Lactoferrin ELISA Assay Kit EL2011-1.

III. Results And Discussion

Table 1. t test comparison results of saliva in cases and control group

Variable	Control group	Case group	p-value
	Mean ± SD	Mean ± SD	
MPO	38.74±10.57	75.23±13.18	0.000
H_2O_2	10.92±2.53	14.17±5.29	0.041
LTF	37.13±4.11	40.37±2.74	0.017

Description : MPO : Myeloperoxidase; H_2O_2 : Hidrogenperoxida ; LTF = Lactoferrin

From **Table 1.** There is a significant difference / significantly ($p < \alpha$) mean MPO (Myeloperoxidase) in the saliva from the control group (38.74±10.57) while in the case group (75.23±13.18). It is indicate that in neonatal sepsis patients saliva showed a significant improvement MPO levels than in normal patients, based on the mean increased. Similary, the H_2O_2 mean in saliva control group (10.92±2.53), in the case group (14.17±5.29),

indicating there is a significant difference ($p < \alpha$). In LTF between the control group (37.13±4.11) and the case group (40.37±2.74) also shows a significant difference ($p < \alpha$).

Table 2. t test comparison results of blood in cases and control group

Variable	Control group	Case group	p-value
	Mean ± SD	Mean ± SD	
MPO	360.95±142.72	623.52±97.55	0.000
H_2O_2	10.71±2.28	13.17±2.71	0.012
LTF	33.35±3.29	35.57±2.09	0.035

Description : MPO : Myeloperoxidase; H_2O_2 : Hidrogenperoxida ; LTF = Lactoferrin

From **Table 2.** There is a significant difference ($p < \alpha$) mean MPO (Myeloperoxidase) in the blood from the control group (360.95 ± 142.72) with the case group (623.52 ± 97.55). The same thing happened in the mean blood levels of H_2O_2 in the control group (10.71 ± 2.28) with the case group (13.17 ± 2.71) showed significant difference ($p < \alpha$). It is clear that the blood of neonatal sepsis patients showed elevated levels of H_2O_2 than in normal patients, showed in the higher value of mean case compared to control.

Table 3. Comparison of neutrophils number and phagocytosis levels in saliva

Variable	Control group	Case group	p-value
	Mean ± SD(%)	Mean ± SD(%)	
Neutrophil	44.75±6.76	81.72±6.77	0.000
Phagocytosis (30min)	33.97±9.34	49.55±7.69	0.000
Phagocytosis (60min)	65.27±7.70	75.64±6.53	0.000

Description : *p-value* > 0.05 no significant difference; *p-value* < 0.05 there is a significant difference

Table 3. shows that there is a very significant difference ($p < \alpha$) the mean number of neutrophils in saliva between the control group (44.75 ± 6.76) and the case group (81.72 ± 6.77). Similarly, the mean phagocytosis (30 min of observation) in saliva between the control group (33.97 ± 9.34) and the case group (49.55 ± 7.69) showed there is a very significant difference ($p < \alpha$). It is clear that in the saliva of patients showed increased phagocytosis of neonatal sepsis than in normal patients, seen in the case of the mean case > mean control.

Also evident in **Table 3**. indicate that there is a very significant / significant ($p < \alpha$) mean phagocytosis (observation 60 minutes) in saliva between the control group (65.27 ± 7.70) and the case group (75.64 ± 6.53). Suggesting that the salivary neonatal sepsis patients showed an increase in phagocytosis level than in normal patients, seen in the case of the mean case > mean control. Furthermore, the mean phagocytosis level in 30 minutes of observation case group (49.55 ± 7.69) compared to 60 minute of observation case group (75.64 ± 6.53) shows there is also a very significant difference ($p < \alpha$).

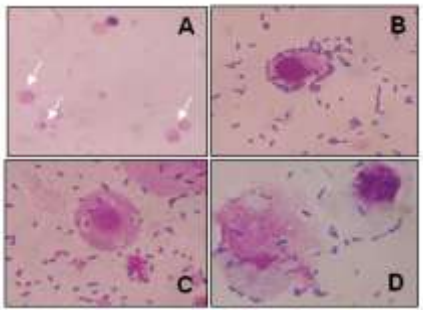

Figure 1. Neutrophils and the bacteria phagocytosis and adhesion in saliva from a microscope with magnification 1000 x, (A). neutrophils (arrows) without exposure to bacteria, (B) and (C). neutrophils with *E. coli* exposure 30 min, (D) neutrophils to bacterial exposure *E. coli* 60 minutes.

Fig 1. shows the activity of neutrophils taken from saliva samples. There are no phagocytic activity in control because there is no exposure to the bacteria, while in the bacteria exposure there were the bacteria phagocytosis and adhesion activity. In **Fig 2**. clearly visible *E. coli* in the neutrophils phagosome view using SEM.

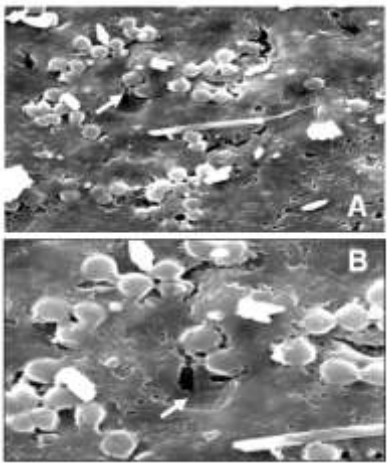

Figure 2. Neutrophils salivary by bacteria exposure to scanning electron microscope (SEM) ; (A) from a microscope with magnification 20,000 x, (B). from a microscope with magnification 40,000 x. The arrows indicate *the E. coli* inside the phagosome.

Table 4. Comparison of neutrophils number and phagocytosis levels in blood

Variable	Control group	Case group	p-value
	Mean ± SD	Mean ± SD	
Neutrophil	19.28±13.26	56.14±5.27	0.000
Phagocytosis (30min)	23.61±11.24	39.04±7.49	0.000
Phagocytosis (60min)	46.13±17.63	72.84±8.02	0.000

Description : *p-value* > 0.05 no significant difference; *p-value* < 0.05 there is a significant difference

Table 4. shows that there is a very significant difference ($p < \alpha$) neutrophil mean in blood between the control group (19.28 ± 13.26) and the case group (56.14 ± 5.27). It is clear that the blood of neonatal sepsis patients showed significant improvement compared to neutrophil normal patients neutrophil, based on the mean grade increased.

Similarly, the mean phagocytosis (the first 30 minutes of observation) in blood between the control group (23.61 ± 11.24) and case group (39.04 ± 7.49) shows there is a very significant difference ($p < \alpha$). It is clear that the patient's blood showed the higher phagocytosis level from neonatal sepsis than in normal patients, seen in the case of the mean case > mean control. Also evident in mean phagocytosis (the first 60 minutes of observation) between the control group (46.13 ± 17.63) and case group (72.84 ± 8.02) that there is a very significant difference ($p < \alpha$).

Assesing the neutrophils quality in blood and saliva samples indicated by measuring the MPO, H_2O_2, and LTF production. From the research, we are able to obtained a significant comparisons between the case and control groups. In case group showed higher production levels of MPO, H_2O_2, and LTF compared to control group. This is consistent with previous measurements, that an increase in the neutrophils number and neutrophil phagocytosis level will activate the signal transduction and increasing the production of MPO, H_2O_2, and LTF used to eliminate pathogenic bacteria.

In the phagocytosis analysis, bacteria exposed to different periods of time (30 minutes and 60 minutes), the rate of phagocytosis of neutrophils in the span of 60 minutes is higher than the 30 minutes. This suggests that the longer neutrophils exposed to a type of bacteria, the ability to memfagosit bacteria is increasing and becoming more active neutrophils.

It is been proved that saliva has many benefits [10,11], which are contain antimicrobial compounds, and it's use as biomarkers of infectious diseases [12,13], malignancy [14,15], and neutrophil levels in saliva may also indicate successful bone marrow transplant [16].

IV. Conclusion

Comparison of neutrophils number and phagocytosis level in saliva is higher in case group than control group. That is mean that the production levels of MPO, H_2O_2, and LTF in case group higher than control group. Also in the neutrophil phagocytosis quality in the span of 60 minutes is higher than the 30 minutes. Further research is needed to enhance the advantage of using saliva as an early-onset neonatal sepsis biomarker. A

continous adequate therapy in neonatal sepsis hopefully will be able to reduce the numbers of morbidity and mortality of neonatal sepsis.

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