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Methylglyoxals, Thiocyanate, and Hydrogen Peroxide in saliva of newborns at risk of sepsis: Methylglyoxals as a promising biomarker of early-onset neonatal sepsis

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ABSTRACT

Introduction: Sepsis represents a severe problem for newborns. The relatively high incidence rate of 1 to 10 cases per 1000 live births, with even higher rates in low-birth-weight neonates. Given its high incidence, identifying markers to optimize the early diagnosis and therapeutic interventions is highly desirable. This study investigates three oxidative markers taken from saliva, methylglyoxal (MG), Thiocyanate (SCN⁻) and hydrogen peroxide (H₂O₂), as early-onset neonatal sepsis markers.

Methods: This study was conducted from June to August 2014, and saliva specimens were taken from 30 newborns of mothers with one major risk factor or two minor risk factors (case group) and 30 healthy newborns for the control group. Saliva levels of MG were determined by calculating the percentage of MG absorbance compared with the carbonyl compounds. Data were analyzed by using Kolmogorov-Smirnov and Student's t test.

Results: We found a significant increase of SCN⁻, and MG level case group (28.91 mmol and 0.01%, respectively) compared to the control group (19.49 mmol and 0.005%, respectively) p-value = 0.03. In contrast, the H₂O₂ in the case group (32.56 mmol) was lower than the control group (37.47 mmol), with a p-value = 0.04.

Conclusion: We found the increased levels of SCN⁻, MG and the decreased levels of H₂O₂ in neonates' saliva with the risk of sepsis. MG measurements as a routine diagnostic for suspected sepsis should be taken into consideration.

Keywords: methylglyoxal, Thiocyanate, hydrogen peroxide, early-onset neonatal sepsis.

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INTRODUCTION

Neonatal sepsis or septicemia is clinical syndromes during the first month of life caused by systemic responses to infection and supported by discovering microorganisms in the blood cultures.¹ Sepsis is a leading cause of neonatal mortality, especially in developing countries. It is estimated that mortality caused by sepsis is about 30-50% of all neonatal deaths.² Indonesian perinatal mortality data reported that 29.9% of deaths occurred on the first day of life and 75.6% at one week after birth.³ Babies who are born with risk factors have a greater possibility to have sepsis.¹ In terms of sepsis timing, early neonatal sepsis occurs within the first three days of life.² Several obstetric and neonatal factors have been associated with an increased risk of

neonatal infection. The presence of any of those factors alone is not an indication for a complete sepsis workup or antibiotic therapy. Nonetheless, the presence of a set of risk factors should significantly enhance the suspicion of neonatal sepsis.⁴

Various pathomechanisms have been proposed in neonatal sepsis. Oxidative stress due to the general inflammatory response has been pointed as the seed for sepsis development.⁵ Oxidative stress is mediated by several enzymatic reactions, originally intended to establish a defense system against microbial invasion.⁶ This enzymatic defense is orchestrated by endogenous enzymes through the various scavenging reactions of reactive oxygen species (ROS) and nitrogen metabolites derived from polymorphonuclear neutrophils (PMN), the well-known process called the respiratory burst.^{6,7}

Hydrogen peroxide (H₂O₂) as the latter product during respiratory burst will be transformed into antimicrobial compounds by peroxidase enzymes found in blood and saliva.⁸⁻¹⁰ Peroxidase catalyzes the oxidation of halide and pseudohalide ions such as iodide (I⁻), bromide (Br⁻), chloride (Cl⁻) and thiocyanate (SCN⁻) as its cosubstrate in the presence of H₂O₂, leading to the production of a much more bactericidal and fungicidal agent.^{9,11} SCN⁻ is the primary substrate for lactoperoxidase (LPO), and the closely related salivary peroxidase (SPO), the preferred substrate for myeloperoxidase (MPO).¹²

Although clinical reports are consistent with ROS involvement in neonatal sepsis and its complications, strangely, not much conclusive evidence supports the beneficial effect of antioxidants treatment in sepsis.^{5,13} It may indicate that other

mediators of cellular stress, such as reactive carbonyl species (RCS), play a much larger role. Research has shown that various, heterogeneous groups of RCS emerged as essential mediators of cellular dysfunction. These compounds are relatively stable and quickly diffuse out of the cell. Thus, these molecules can exert an Effect far from the formation site. Like many other oxidants, several of the most potent RCS is derived from the glycolysis process.^{14,15} Among many, Methylglyoxal (MG) is a highly reactive electrophilic α,β -dicarbonyl aldehyde compound, one of several compounds of the RCS group, produced endogenously during glycolysis.¹⁶⁻¹⁸

During sepsis, glycolytic contribution to energy production increases about 10% as a compensatory mechanism to sustain the energy supply.¹⁹ Glycolytic enhancements might be related to or stimulated by mediators produced during the systemic inflammatory response.²⁰ The Glycolysis inevitably produces methylglyoxals (MGs) as a by-product, and the rate of formation is correlated with the rate of glycolysis. In advance, MG is a pro-oxidant and an AGEs precursor.^{15,21} MG also induces hydrogen peroxide production in neutrophils and platelets.²²

From those pathological conditions, it can be assumed that the energy generation pathways, inflammatory, and oxidative processes have such relationships. However, there has been no research that relates these forms of metabolic stress in neonatal sepsis. Therefore, this study aimed to measure MGs, SCN^- , and H_2O_2 level in the saliva of newborns at risk of sepsis, the main objective was to investigate the used of MG as biomarkers in neonates with septicemia risk which, can be used as an early marker for early-onset neonatal sepsis.

METHODS

This study is a prospective cross-sectional study approved by the Research Ethics Committee Faculty of Medicine Lambung Mangkurat University / Ulin General Hospital (No.012/KEPK-FK UNLAM/EC/IV2015). Saliva specimens were taken from 30 newborns at risk of sepsis and 30 healthy newborns (control group) in which their parents had signed the informed consent. The study was conducted from

June to August 2014 at the Neonatology Division of Ulin General Hospital Banjarmasin. Samples were examined in the Biochemistry Laboratory at the Faculty of Medicine Lambung Mangkurat University, Banjarmasin. The inclusion criteria were newborns from mothers with a major risk factor or two minor risk factors. Major risk factors were rupture of membranes > 24 hours, intrapartum fever ($>38^\circ\text{C}$), chorioamnionitis, a persistent fetal heart rate > 160 beats/minute, and foul-smelling amniotic fluid. Minor risk factors were rupture of membrane > 12 hours, intrapartum fever ($> 37.5^\circ\text{C}$), low Apgar score (< 5 at the 1st minute, < 7 at 5th minute), very low birth weight babies (<1500 grams), gestational age < 37 weeks, multiple pregnancies, foul-smelling vaginal discharge, maternal urinary tract infection (UTI) / suspected untreated UTI. Exclusion criteria are babies born outside Ulin General Hospital Banjarmasin, newborns with severe asphyxia, newborns with major congenital abnormalities, low birth weight (<2500 grams).

Protein Carbonyl Assay

The modified dinitro-phenylhydrazine (DNPH) method was used to measure the Carbonyl compounds. From each test solution, a 0.5 ml solution was. It was divided into two tubes, 0.25 ml in volume in each tube. The two tubes are a sample tube (A) and a blank/non-sample tube (B). Each of the sample tubes (tube A) was given a drop of 1 ml of DNPH, and each blank tube (tube B) were 1 ml HCl 2.5 M. All the tubes were incubated for 45 minutes at room temperature and protected from light, then shaken with vortex for 15 minutes. A milliliter of trichloroacetic acid (TCA) 20% was added into each tube (A and B) and then incubated for another 5 minutes. Tubes were recentrifuged (1400 rpm) for about 5 minutes to separate the supernatant. The pellets were further centrifuged and washed three times with the addition of 1 ml ethanol-ethyl acetate mixture. The final step was to measure carbonyl compounds by adding 1 ml of urea (9M) and then incubated and shaken for 10 minutes at 37°C . The solutions were centrifuged again for 5 minutes at 1400 rpm. The absorbance of the solution was measured at $\lambda = 390$

nm. Protein breakdown was measured by using the following formula.

$$C = A/(e.b)$$

(A : absorbance,

e : extinction coefficient $22000 \text{ mM}^{-1}\text{cm}^{-1}$,

b : 1 cm)

Methylglyoxal (MGs) Assay and measurement

A total of 25 μL of the homogenate was added to 350 μL DNPH (0.1% DNPH in 2 N HCl), and then 2.125 ml distilled water was added. It is incubated for 15 minutes at 37°C , then 1.5 ml NaOH 10%. Absorbance was measured at $\lambda = 576 \text{ nm}$.²³ MGs level was determined by calculating the percentage of MGs absorbance compared with the carbonyl compounds. The formula to determine the MGs level is presented below.

$$\text{MGs level (\%)} = \frac{\text{MGs absorbance}}{\text{Carbonyl absorbance}} \times 100 \%$$

Hydrogen Peroxide Assay

Five milliliters of phosphate buffer (pH 7 and concentration of 0.01 M) was mixed with 1 mL of saliva and was homogenized slowly. A milliliter of the mixture was taken and was mixed with 2 mL dichromat/glacial acetate. It was heated for 10 minutes in boiling water to remove the blue precipitate and produce a green solution. The absorbance was measured at $\lambda = 570 \text{ nm}$. A Standart curve was used to determine how much of the remaining H_2O_2 when the reaction was stopped by acetic acid.

Thiocyanate Assay

Standart solutions contain 800 μL HCl 0,1 N was mixed with 100 μL FeCl_3 0,1 M and 100 μL KSCN 0,1 M. The mixture was centrifuged at 1000 rpm for 10 minutes. Absorbance was measured at $\lambda = 450 \text{ nm}$ (A_0). Samples solution contain 800 μL HCl 0,1 N was mixed with 100 μL FeCl_3 0,1 M and 100 μL saliva. The mixture was centrifuged at 1000 rpm for 1 minute. Absorbance was measured at $\lambda = 450 \text{ nm}$ (A_1). The following formula measured thiocyanate level (C_1) in saliva:

$$C_1 = \frac{A_1 \times C_0}{A_0}$$

Statistical Methods

Data from both groups were analyzed statistically for comparison. The normal distribution of the variables was tested with the Kolmogorov-Smirnov test. The mean comparison of the control group variables with the case group was analyzed with Student's t test (if the requirement was met, otherwise the equivalent non-parametric test was used). P-values of 0.05 or less were considered statistically significant.

RESULTS

Subjects' characteristics in both groups were presented in Table 1. Risk factors of 30 subjects in the case group were presented in Table 2. Each subject may have more than one risk factor.

Overall, there were significant differences between the mean of the variables measured in the newborn at risk of sepsis (case group) and the control group. We found mean Thiocyanate (SCN⁻)

level in the control group is 19.49 mmol, and in the case group mean SCN⁻ level is 28.91 mmol. It showed that the SCN⁻ level in the case group was higher than the control. From Kolmogorov Smirnov, data have normal and homogeneous distribution. To assess the difference between both groups, data were analyzed using an independent t-test, and the result showed a significant difference between the groups ($p = 0.03$). In contrast, the mean H₂O₂ level in the control group was higher (37.47 mmol) than in the case group (32.56 mmol). From the Kolmogorov-Smirnov test, the data was known to have normal distribution. The statistical test results using independent t-test showed a significant difference between the groups ($p = 0.04$). Lastly, the mean MG level in the control group is lower (0.005 %) than in the case group (0.01 %). From the Kolmogorov-Smirnov test, data have normal and homogeneous distribution. The independent t-test showed that there is a significant difference between the

groups ($p = 0.03$). The mean level of SCN⁻, H₂O₂ and MG in both groups is presented in Figure 1.

DISCUSSION

The saliva defense mechanism is the first line against harmful microorganisms.^{7,9} Saliva defense system inhibits microorganisms through various in vivo activity of salivary antimicrobials.²⁵ One of those salivary microbial is an enzymatic defense mechanism which is played by the respiratory burst.^{6,7} From this study, there was a significant difference between thiocyanate (SCN⁻) level between the groups where the saliva of neonates at risk of sepsis has higher levels of SCN⁻. We also found decreased levels of H₂O₂ in the saliva of neonates with the risk of sepsis. This result indicates increased respiratory burst activity in the saliva of neonates with the risk of sepsis. High levels of Thiocyanate are required to activate LPO and MPO during sepsis. Under physiological condition, biological fluids such as saliva, breastmilk and tears only contain millimolar levels of SCN⁻.²⁵ The levels of H₂O₂ was found lower in case groups. The capitalization of H₂O₂ causes it into antimicrobial end products.

Respiratory burst produced by the antimicrobial system through ROS formation, which catalyzed by NADPH-oxidase. This reaction produced a superoxide radical that was then further modified to H₂O₂ and hydroxyl radicals (OH) by superoxide dismutase (SOD). H₂O₂ will then be transformed into antimicrobial compounds by peroxidase enzymes in saliva. The lactoperoxidase (LPO) and myeloperoxidase (MPO) are the two principal components of the peroxidase system in saliva and work together to catalyze the oxidation of thiocyanate ions (SCN⁻) by H₂O₂.^{8,9} The resulting product of the reaction is the more potent bactericidal and fungicidal compound such as OSCN⁻, HOSCN, O₂SCN⁻, and O₃SCN⁻.^{10,25,26} Salivary LPO-SCN-H₂O₂ has been proven to produce a positive unidirectional antimicrobial system through the various antibacterial mechanism of action.^{6,9-11}

Another finding in this study was the difference in MG level between the control and case groups. MG levels were found

Table 1. Characteristics of subjects

Characteristic	Control group (n=30)	Case group (n=30)
Sex		
Male	11	12
Female	19	18
Delivery		
Spontaneous delivery	15	16
Breech extraction	2	3
Caesarian section	12	10
Vacuum extraction	1	1

Table 2. Risk factors of subjects

Risk factors	n
Membranes ruptured for > 24 hours	9
Membranes ruptured for > 24 hours & Foul-smelling vaginal discharge	5
Foul-smelling vaginal discharge & Maternal UTI or suspected untreated maternal UTI	5
Membranes ruptured for > 12 hours & Maternal fever with intrapartum temperature > 38.5 °C	4
Foul-smelling vaginal discharge & Gestational age < 37 weeks	3
Membranes ruptured for > 24 hours & Gestational age < 37 weeks	2
Foul-smelling amniotic fluid & Maternal UTI or suspected untreated maternal UTI	2

Figure 1. Mean levels of SCN⁻, H₂O₂ and MG in control and case group

Figure 2. Respiratory burst, glycolysis and formation of MG in sepsis, novel approach of MG as a neonatal sepsis marker.^{15,28,29,34,36}

higher in the saliva of newborns at risk of sepsis. In sepsis, inflammatory cytokines induced glucose uptake and glycolytic flux in activated macrophages directed towards supplying ATP for respiratory burst.¹⁹ It was due to the dependency of neutrophils on glycolysis rather than oxidative metabolism for ATP generation to maintain the NADPH oxidase-driven respiratory burst required for killing certain bacteria.²⁸ This finding suggests that the increased respiratory burst activity in neonatal sepsis leads to increased glycolytic flux as the cellular energy source for respiratory burst against microbial.

The rate of glucose uptake and glycolysis were thought to be influenced positively by mediators produced during the systemic inflammatory response in sepsis.²⁰ Furthermore, it is also believed that any metabolic pathways that stimulate glycolytic activity may also increase the formation of MG, including the glycolysis pathway under physiological conditions and the activated polyolfructose pathway during the hyperglycemic conditions. The latter metabolic activity is one of the clinical features in patients with sepsis.²⁹ As the glucose metabolism happens, MG is formed in a significant amount (approximately 0.1% of glucose flux) as a normal by-product resulted from the fragmentation of the triosephosphate glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP).²¹ These MG then underwent various reactions, as it readily reacts with lipids, nucleic acids, lysine and arginine residues of proteins to form AGEs with downstream inflammatory effects.^{17,30-32}

Direct changes in protein function due to MG modification triggers an inflammatory response at the cellular level.³³ In sepsis, modification of different proteins by MG, particularly in mitochondria, increased the oxidative load, further aggravating cellular dysfunction and inflammation.^{15,34} Oxidative modifications of proteins are more stable than lipids, making proteins suitable for oxidative stress markers.³⁵ The schematic metabolism and mechanism of respiratory burst, MG, and MG-derived AGEs driven cell dysfunction or inflammation or both in human sepsis is presented in Figure 2. Due to these biochemical characteristics, MG is believed to be a potential reliable marker in neonatal sepsis. Studies showed that MG is a better biomarker to predict survival in patients with septic shock than the routine and established biomarkers like C-reactive protein (CRP), procalcitonin (PCT), IL-6, and sCD14-ST.^{15,36}

CONCLUSION

In this study, we found the increased levels of SCN⁻, MG and the decreased levels of H₂O₂ in neonates' saliva with the risk of sepsis. Increased SCN⁻ and MG levels followed by a decreased level of H₂O₂ in this study suggest that MG

metabolism is an important and generally overlooked biochemical pathway related to oxidative stress in early-onset neonatal sepsis pathomechanism. It also suggests that there is a relationship between these two forms of metabolic stress marker.¹⁵ Therefore, MG measurements in routine diagnostics of neonates with suspected sepsis should be taken into consideration. Further investigations are required to determine whether MG's diagnostic and prognostic value can be confirmed and validated to be used clinically.

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CONFLICT OF INTEREST

All authors declare no conflict of interest

AUTHOR CONTRIBUTION

All authors have contributed substantially during all phases of the study, including drafting and revising the manuscript, giving final approval, and agreeing to be accountable.

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