Investigating the Antimicrobial Impact of Ampicillin-Gentamicin in Neonatal Saliva via Chlorinative Stress Mechanisms

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ABSTRACT

Clinical diagnosis of sepsis in neonate is difficult, because many signs of sepsis are nonspecific. There are several salivary biomarkers of stress as objective indicators of stress reactions.This study was designed to investigate the effect of ampicillin-gentamicin treatment to chlorinative stress parameters insaliva of newborn at risk of sepsis. Four chlorinative stress parameters were used, hydrogen peroxide (H_2O_2) levels, myeloperoxidase (MPO) activity, advanced oxidation protein products (AOPPs) levels, and chlorinative index (CI). This study was performed in May until September 2016. Saliva samples were taken from 26 newborns at risk of sepsis treated in Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia. All newborns were given ampicillin-gentamicin three until ten days. The H_2O_2 levels, MPO activity, AOPPs levels, and CI were measured from the saliva taken from both pre and post treatment. Statistical analysis of the parameters were obtained from before and after the ampicillin-gentamicin treatment, using Mann-Whitney test. The used ofampicillin-gentamicin for treatment of newborns at risk of sepsis showed significant decrease in the H_2O_2 levels, MPO acitivity, AOPPs levels, and significant increases in the CI. From this results, it can be concluded that Ampicillingentamicin could reduce the chlorinative stress in newborn at risk of sepsis.

Keywords: Ampicillin, Chlorinative Index, Gentamicin, Neonatal Sepsis.

INTRODUCTION

Neonatal sepsis is a systemic response to infection which trigger by bacteremia in the first four weeks of life. It is one of the three major causes of neonatal deaths worldwide. It contributes in 30 to 50% of neonatal deaths in most of developing countries. Neonatal sepsis is a diagnosis which is often found when newborns were treated and reffered to hospital[1].

The sign of neonatal sepsis are nonspesific and similar with other non-infectious conditions. Because of that, some clinicians used “Suspected sepsis” to diagnoses neonatal sepsis before the blood culture results. Patients with suspected neonatal sepsis have non spesific laboratory signs. Although a normal physical examination in the neonatus, bacteremia can occur in the absence of clinical signs[2].

Therapy in both suspected and neonatal sepsis is antimicrobial treatments, and each health facility has a different protocol in the use of antibiotic as initial therapy in neonatal sepsis. Various therapy in both conditions use a broad spectrum of antibiotics such as ampicillin combine with aminoglycosides, and third-generation of

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cephalosporins, and also meropenem. As a WHO recommendations, neonatal sepsis treatment in a hospital setting usualy through parenteral antibiotic therapy and supportive care, which has shown positive impacts[4]. There are some difficulties in performing blood sampling in neonatal[5]. One of the current measurement of sampling and less invasive biological fluids is using the saliva as a diagnostic media, for the purpose of clinical and basic research[6]. Because saliva contains various molecules such as hormones, enzymes, immunoglobulins, proteins and oxidative stress parameters. There are several salivary biomarkers of stress as objective indicators of stress reactions[5].

The inflammatory response which occurs in sepsis is characterized by mitochondria damage due to the formation of free radicals. When the production of ROS and antioxidants is not balance, and antioxidants have been overwhelmed, this leads to oxidative stress[6]. Tissue damage due to reactive oxygen species is a major cause of oxidative stress, will activate neutrophils[7]. Neutrophils have mechanism of phagocytosis and capable to ingesting microorganisms or particles,they can internalize and kill many microbes and secretion reactive oxygen species and hydrolytic enzymes. It contains primary granules of several proteolytic enzymes and a wide range of bactericidal proteins including myeloperoxidase (MPO)[8,9,10]. MPO can be found in a number of affected tissue injury and sepsis because of the spread of neutrophils, thereby MPO can be used as a marker of neutrophil activity and can be detected in the blood and saliva[11]. MPO is a strong oxidizing agent and acts as destruction of bacteria and resulting tissue necrosis and apoptosis. When neutrophil functioning, NADPH oxidase in the cell membrane becomes active, resulting in a compound of hydrogen peroxide (H_2O_2)[8,9]. H_2O_2 is a reactive oxygen species, which can be consumed

by MPO to oxidize chloride ions into reactive strong non-radical oxygen species is HOCl[10]. The existence of HOCl as chlorinative stress, happen when endogenous antioxidant capacity was not able to reduce hypochlorite anion reactivity[10]. One indicator of chlorinative stress is chlorinative index, the ratio between the levels of H_2O_2 and MPO activity[9].

Advance Oxidation Protein Products (AOPPs) is a marker of chlorinative stress and oxidative damage to proteins,as a result of the reaction between protein amine groups and HOCl. Recently, AOPPs can be to healdiseases, so that the examination of AOPPs is a referable therapeutic success[12]. Since the chlorinative stress could be involved in the pathomechanism of neonatal sepsis and indicator therapy for antibiotics in sepsis,our present study aimed to measure the effects of the ampicillin-gentamicin applications on chlorinative stress mechanism in saliva of newborn at risk of sepsis.The involvement ofchlorinative stress by measuring H_2O_2levels, MPO activity, AOPPs levels, and calculate the CI in the saliva of neonatal sepsis patient, who has ampicillin-gentamicin as a treatment.

MATERIAL AND METHODS

This study is a prospective cross-sectional study. Saliva samples were collected from 26 newborns at risk of sepsis. The samples were collected in the level II and Neonatal Intensive Care Unit (NICU), Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia from May until September 2016. Laboratory tests were conducted at Medical Chemistry/Biochemistry Department, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin. Data were primarily collected to determine the effect of antibiotics application through chlorinative stress mechanism in saliva of newborn at risk of sepsis. In this present study, the data from the original study were used. This study was approved by the ethics committee of University of Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia.

Experimental section

Saliva samples were taken from newborn at risk of sepsis, the sepsis risk group must have at least 1 major criteria or 2 minor criteria for sepsis as per ACOG guidelines, and all newborns were given ampicillin-gentamicin three until ten days. Major risk criteria were premature ruptured of membranes (PROM) for > 24 hours, maternal fever with intrapartum temperature > 38^{o}C, chorioamnionitis, fetal heart rate persisting at > 160 times/min or bad smelling of amniotic fluid. Minor risk criteria were PROM for > 12 hours, maternal fever with intrapartum temperature > 37.5^{o}C, low Apgar score (<5 at the 1st min , <7 at the 5th min), very low birth weight baby (VLBWB) of <1500 gr, gestational age < 37 weeks, multiple pregnancy, bad smelling of vaginal discharge, maternal urinary tract infection (UTI) or suspected untreated maternal UTI. Saliva samples were taken twice, before first antibiotic ampicillin-gentamicin and after antibiotic ampicillingentamicin therapy. Sampling for the first ampicillingentamicin were collected from saliva specimens (3 ml each) from the oropharynx according to standard procedures for neonatal resuscitation,and the second time were collected when the ampicillin-gentamicin was given completely, saliva samples were taken again by giving 3-4 ml NaCl slowly by using 1 cc syringe at the bottom of the patient's tongue and then pulled back 3-4 cc of saliva. Samples then were divided into 2 groups. Group 1 for pre ampicillin-gentamicin therapy (n=26) and group 2 (T1) for post ampicillin-gentamicin therapy.

H_2O_2 level analysis

H_2O_2 level was calculated by the FOX2 method with slight modification. Solutions measured spectrophotometrically at λ = 505 nm. Standard and test solutions consisted of 1 M H_2O_2 200 µL and 200 µL serum, respectively, with the addition of 160 µL phosphate buffer solution pH 7.4, 160 µL $FeCl_3$ (251.5 mg $FeCl_3$ dissolved in 250 ml distilled water) and 160 µL o-fenantroline (120 mg o-phenantroline dissolved in 100 ml distilled water) for both solutions. The composition of the blank solution was identical to the test solution, except for the absence of $FeCl_3$ in the blank. Subsequent to preparation, all solutions were incubated for 30 minutes at room temperature, then centrifuged at 12,000 rpm for 10 minutes, and the absorbance of the standard (As), test (Au) and blank (Ab) solutions measured at λ=505 nm, using the supernatant of each solution. [13]

MPO activity analysis

MPO activity was measured spectrophotometrically using o-dianisidine (Sigma-Aldrich) and H_2O_2. In the presence of H_2O_2 as oxidizing agents, MPO catalyses the oxidation of o-dianisidine yielding a brown coloured product, oxidized o-dianisidine, with a maximum absorbance at 470 nm. One unit (U) of MPO activity was defined degrading 1 µmol of H_2O_2 per minute at 25^{o}C. [13]

AOPPs level measurements

AOPPs measurement were made by spectrophotometric methods as describe by Witko-Sarsat et al., with small modification. Briefly, AOPPs were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions which in the presence of potassium iodide at 340 nm. In test wells, 200 ml of plasma diluted 1/5 in phosphate buffer solution were placed on a 96-well microtiter plate and 20 ml of acetic acid was added, in standard wemp/lls, 10 ml of 1.16 mol Kalium iodide was added to 200 ml of chloramine-T solution (0–100 mmol/l) followed by 20 ml of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm on the microplate reader against a blank which containing 200 ml of phosphate buffer solution, 10

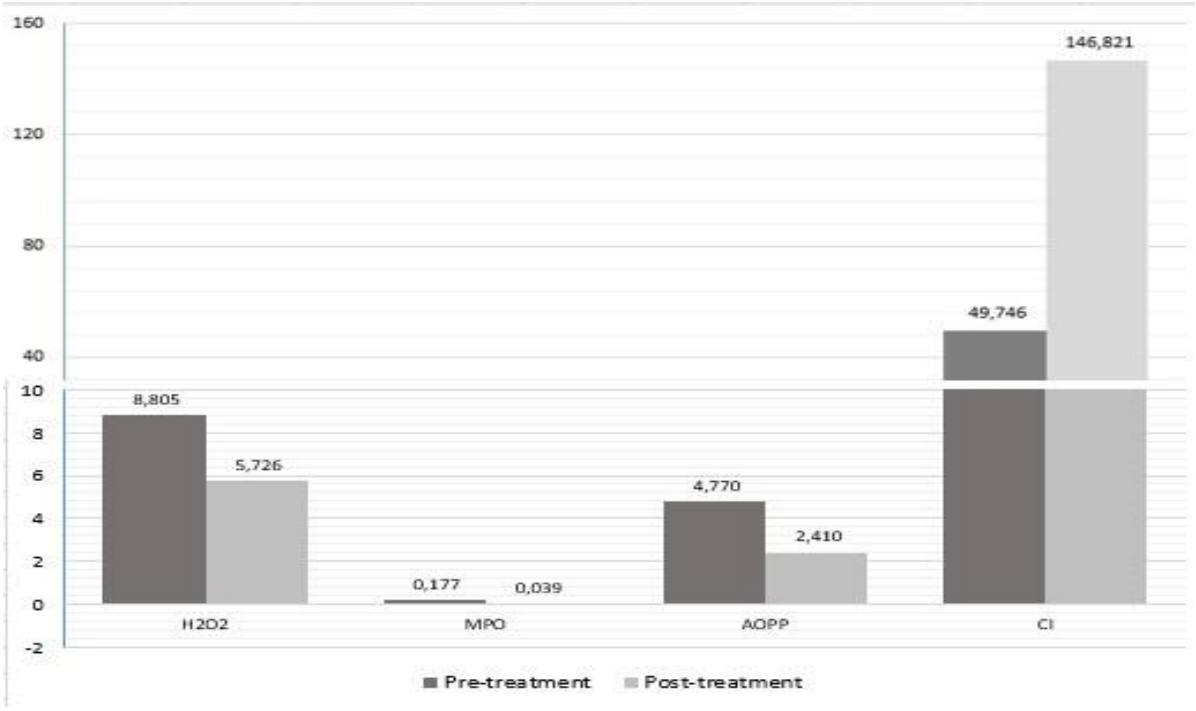

Figure 1: Chlorinative stress parameters in Pre and post ampicillin-gentamicin treatment.

ml of potassium iodide,and 20 ml of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/l. AOPP concentrations were expressed as µmol L^{-1} of chloramine-T equivalents[14].

CI analysis

CI was a ratio between H_2O_2 levels and MPO activity. CI was calculated following to equation: [13]

$$CI = = \frac{Hydrogen\ peroxide\ level}{MPO\ activity}$$

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0. Data were checked for normality (Shapiro–Wilk normality test) and homogeneity of variance (Levene's test). Then, data was divided in to two assumptions. The normaland homogeneity distributed data were run with one-way analysis of variance (ANOVA) and followed by post hoc Tuckey HSD test. The nonnormaland /or non-homogeneity distributed data were run with Mann-Whitney U test. P-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

This study aimed to determine the effect of first line antibiotic ampicillin-gentamicin against chlorinative stress, which is characterized by four parameters, such as H_2O_2 levels, MPO activity, AOPPs levels, and CI. The research show that H_2O_2 was produced in newborns saliva at risk of sepsis, and with ampicillin-gentamicin administration obtained an impairment of H_2O_2(Figure 1). Mann-Whitney test results show that there are a significant difference of H_2O_2 levels between pre and post-treatment of antibiotics ($p<0.05$).

The figure 1 also show that antibiotics treatment can reduce the MPO activity and AOPPs level significantly. The results indicated that the chlorinative stress pathomechanism may be involve on newborn at risk of sepsis.

The effect of ampicillin-gentamicin on CI is also presented in figure1. The CI level seems to be increased from 49.746 before the antibiotics treatment to 146.821 after antibiotics treatment.. Statistical analysis test result shows that there is a significant difference of CI between pre and postreatment of antibotics (Mann-Whitney test result; $p<0.05$). This result suggest that ampicillin-gentamicin could reduce the chlorinative stress in newborn at risk of sepsis.

Neonatal sepsis is a systemic inflammatory response to microorganism and oxidative stress as one of the pathophysiology. An increase number of neutrophils in sepsis has an important role in the immune sytem as a physiological response to inflamation[15]. Oxidative stress resulting from the imbalance between ROS and antioxidant defense mechanisms[15,16]. It forms a reactive oxygen species named H_2O_2[17,18] these could be formed enzymaticely by SOD[19]. Myeloperoxidase is enzyme secreted by phagocytes and contribute to tissue damage occurs during sepsis. MPO acting on hypochloric acid and chloramines and lead a condition called chlorinative stress[9,18]. The increasing of ROS will damage the bacterial protein component and forming AOPPs. AOPPs curently widely used as a marker of disease. Pathophysiology AOPPs can provide valuable information regarding source and development of sepsis and its relationship with chlorinative stress[20]. The results of this study demonstrated that in newborn at risk of sepsis, there were detected four parameters of the chlorinative stress which indicates the involvement of chlorinative stress as one of the mechanisms of neonatal sepsis. Chlorinative stress plays a crucial role as mediator of the systemic

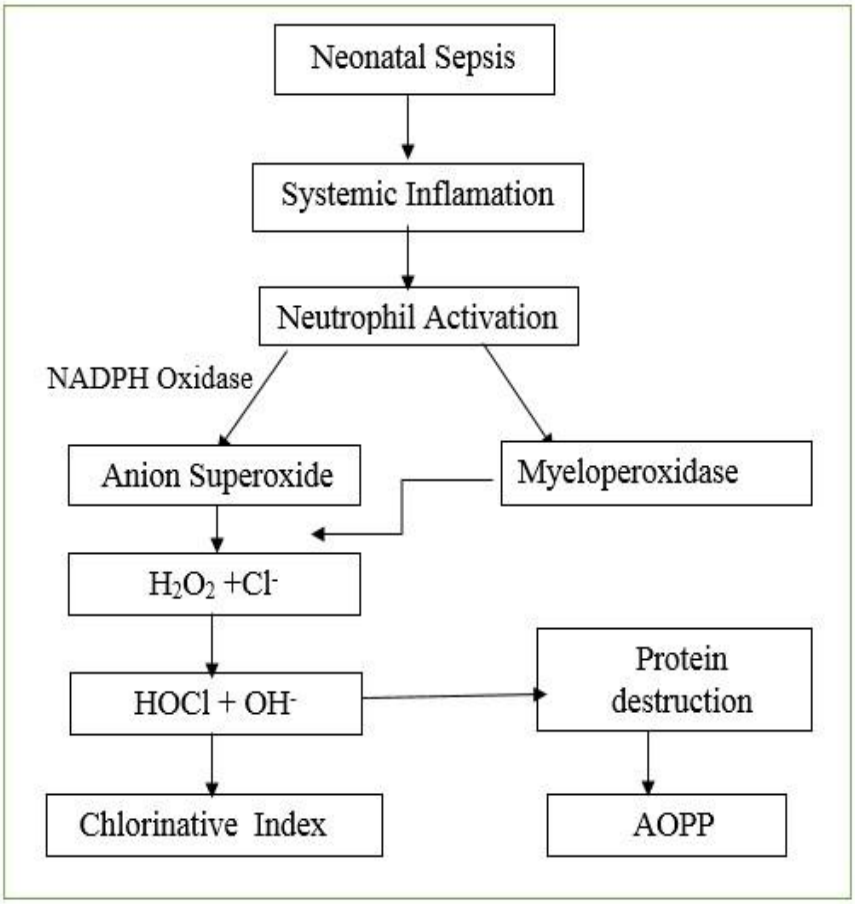

Figure 2: Chlorinative stress in neonatal sepsis.

inflammatory response in neonatal sepsis, and signify potential targets for the treatment in neonatal sepsis. Recently we see many researches about antimicrobial lethality. The mechanism is enhanced by the limitation of genes that protect against damaging reactive species and ROS contribute to lethality, and the oxidative stress was detected in bacteria treated with antibiotics. Ampicillingentamicin as a bactericidal antibiotic has a mechanism of bactericidal antibiotic-mediated cell death. Bactericidal antibiotics are associated with the occurrence of ROSinduced hydroxyl radical (OH •)[21.22,23]. The presence of ROS, bactericidal antibiotics can work making lethal cellular damage in bacterial cells[22].

H_2O_2 could be involved in the antibacterial action and strengthen the effects of oxidative DNA damaging proteins, and lipids. This condition will increase H_2O_2 concentrations in bacterial cell that will have a destruction effect for bacterial living in neonatal at risk of sepsis[24,25]. The impairment of H_2O_2 significantly after the administration of antibiotics in neonatal sepsis showed that the decrease in this parameter can be a good biomarker of inflammatory response in neonatal sepsis. The Involvement of H_2O_2 in the antebacterial action due to activation of neutrophil, also increases the activity of MPO. The ampicillin-gentamicin mechanism which have primary damage resulting in cell death can blocking accumulation of MPO. This result also similar to El Gammasy et al. which stated that the MPO activity increased in neonates with sepsis than in healthy neonates, and in infants with sepsis who followed treatment until cured obtained, MPO activity in line with the improvement of the neonatal clinical state[10].

CI is the quotient between H_2O_2 and MPO. H_2O_2 and MPO capability in using chloride give a role in a powerful antimicrobial agent[26]. Ampicillin-gentamicin, likely mechanism stated before, can lowering the ratio between H_2O_2 and MPO so the index of chlorinative increasingly which indicates the infection has subsided. MPO activity is form HOCl as chlorinated oxidant and trigger the formation of AOPPs. Because of AOPPs are formed during the chlorinative stress condition due to MPO presence, then the decline of AOPPs will have a similar mechanism to decrease the activity of MPO, which is the involvement of resulting bacterial cell death response[27]. Antibiotic drug-target interact not only with a common mechanism, but also in new insight complicity of chlorinative stress. This result indicated treatment using antibiotics can improve newborn at risk of sepsis with a new mechanism antibiotic therapy. Giving ampicillin gentamicin proven to improve chlorinative index and indicates effectiveness of therapy in neonatal sepsis

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