

## In-vitro Effects of Some Antibiotic Drugs on Saliva Thiocyanate and Oxidation Protein Products Levels on Newborn at Risk of Sepsis

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### ABSTRACT

In this present study, we try to investigate the effect of three antibiotics treatment to protein carbonyl (PC), advanced oxidation protein products (AOPPs), and thiocyanate (SCN) level in newborn at risk of sepsis. This present study was performed at February-June 2015. Saliva samples were taken from 20 newborns (5 from normal newborn and 15 from infants were a risk of sepsis) treated in Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia. Saliva samples then divided into four groups with; Control group which contains saliva only; T1 group which contains saliva+meropenem; T2 group which contains saliva+amikacin; and T3 group which contains saliva+diazole. Solutions then incubated at 37°C for 1 hours and then was prepared for PC, AOPPs, and SCN content analysis. The results showed that antibiotic treatments could decrease the PC and AOPPs, and increase the SCN levels. From this results, it can be concluded that all antibiotics treatment induced the production of ROS and SCN that will be used to kill bacteria. From this results also, can be concluded that antibiotics could reduce the oxidative stress in sepsis condition and activated MPO to produced HOSCN that known plays an important role to kill the bacteria.

**Keywords:** Antibiotic, AOPPs, neonatal sepsis, protein carbonyl, thiocyanate, saliva

### INTRODUCTION

Neonatal sepsis (NS) is a clinical syndrome characterized by systemic signs and symptoms of infection<sup>1</sup>. The sign of NS are nonspecific and similar with other non-infectious conditions. Because of that, some clinicians used "Suspected sepsis" to diagnoses neonatal sepsis before the blood culture results. It is well known that oxidative stress has been involved in the pathomechanism of NS<sup>2</sup>. Results of our previous study shows that there are significant differences on protein carbonyl (PC), advanced oxidation protein products (AOPPs), and hydrogen peroxide levels between healthy newborn and newborn at risk of sepsis<sup>1</sup>. Neonatal sepsis is treatable. Current recommendations for treating both NS and suspected sepsis is antimicrobial therapy<sup>3,4</sup>. It is well known that antimicrobial work by several common mechanisms, such as inhibit DNA, RNA, cell wall or protein synthesis. However, several reports show that antimicrobial not just works via common mechanisms. Our previous study shows that meropenem, amikacin, and diazole could decrease the affinity of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-catalase (CAT) complex. This will increase the level of an H<sub>2</sub>O<sub>2</sub> that that will be used to attack the bacteria in NS<sup>6</sup>. The increasing of an H<sub>2</sub>O<sub>2</sub> level, will lead to several consequences, including the activation of myeloperoxidase (MPO) enzyme. Myeloperoxidase is a heterodimeric, cationic and glycosylated haem enzyme<sup>7</sup>.

MPO use H<sub>2</sub>O<sub>2</sub> to catalyses the oxidation of halide (Cl<sup>-</sup>, Br<sup>-</sup>) and pseudohalide (thiocyanate ion, SCN<sup>-</sup>) to form HOSCN<sup>1,8</sup>. The halide and pseudohalide oxidation is known to play an important role in killing invading parasites and pathogens<sup>9</sup>.

Knowledge of antimicrobial work mechanism is essential in order to select appropriate antimicrobial treatment, especially in newborn at risk of sepsis. Considering the antibiotic work by several mechanisms, including oxidative stress and MPO activation. Still, there is no study in the literature examining the effect of antibiotic applications on those three parameters in newborns at risk of sepsis. Therefore, the present experimental study aimed to determine the effects of the antibiotic applications (meropenem, amikacin, and diazole) on PC, AOPPs, and SCN levels in newborns at risk of sepsis.

### MATERIAL AND METHODS

**Ethics Statements:** The study protocol and written consent forms were approved by both the Ethics Committee of Pediatric Department of Ulin General Hospital and by the Ethics Committee of the Faculty of Medicine of University of Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia. Full written consent forms were obtained from the parents of the newborns and all rules were respected.

**Samples collection:** Saliva samples were collected from 20 newborns, of which 15 newborns were at risk of sepsis and 5 from healthy newborns, admitted to Neonatal Intensive Care Unit (NICU) of Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia from February to June 2015. Subjects in the sepsis risk group must have at least 1 major criteria or 2 minor criteria for sepsis as per ACOG guidelines. Major risk criteria were premature ruptured of membranes (PROM) for > 24 hours, maternal fever with intrapartum temperature > 38°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°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 specimens (3 ml each) were taken via suction from the oropharynx according to standard procedures for neonatal resuscitation.

**Experimental models:** Samples were divided into 4 groups. 1 control group and 3 treatments group (T1, T2, and T3). Group 1: Control: Saliva only; T1: Saliva + Meropenem; T2: Saliva + Amikacin; and T3: Saliva + Diazole. Then, each of solutions was incubated at 37°C for 1 hour and undergo to CC, AOPPs, and SCN level analysis.

**Carbonyl compound content analysis:** protein carbonyl content was determined by the dinitrophenylhydrazine (DNPH) method. Samples (0.5ml) was pipetted into 1.5ml centrifuge tube and 0.5 ml of 10mM 2,4-DNPH in 2 M HCl was added and allowed to stand at room temperature for 1 hour, with vortexing every 10-15 min. Then, 0.5ml of 20% Trichloroacetic acid was added followed by centrifugation. The supernatant was discarded and the pellets were washed 3 times with 1 ml ethanol – ethyl acetate (1:1) to remove free reagent. The obtained precipitated protein was redissolved in 0.6 ml guanidine solution. Carbonyl content was calculated from maximum absorbance (390nm) using molar absorption coefficient of 21 mM<sup>-1</sup>cm<sup>-1</sup>.<sup>10</sup>

**AOPPs content analysis:** AOPPs measurement were made by spectrophotometric methods as describe by Witko-Sarsat et al., with slight modification. Briefly, AOPPs were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions that 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 wells, 10 ml of 1.16 mol potassium 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 containing 200 ml of phosphate buffer solution, 10 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 of chloramine-T equivalents<sup>11</sup>.

**SCN content analysis:** SCN concentration was measured spectrophotometrically as described by Aune and Thomas.

In brief, 50 µl of the sample was added to a mixture of 400 µl of 0.1 M HCl and 100 µl of 0.1 M ferric chloride. After centrifugation at 1000 g for 1 min, the absorbance of the supernatant due to FeSCN<sup>2+</sup> was measured at 450 nm<sup>12</sup>.

## RESULTS AND DISCUSSION

This present study which was undertaken to assess the effects of meropenem, amikacin, and diazole on the CC, AOPPs, and SCN levels in saliva of newborn at risk of sepsis. The result shows in figure 1, figure 2, and figure 3, for CC, AOPPs, and SCN levels respectively.

Figure 1 represented the mean values±standard error (mean±SE) of PC level. The result from figure 1 shows that antibiotic applications decrease the PC level. The difference between PC levels in the T1, T2, and T3 groups was significantly lower when compared with control group. PC is the result of amino acid modifications by ROS<sup>13</sup>. PC levels are the most frequently used as a biomarker for oxidative stress and protein oxidation<sup>14</sup>. It is well documented that the PC formation reaction is involved at the pathomechanism of several diseases, including NS. Our previous study result shows that the level of PC was significantly higher in newborn at risk of sepsis<sup>1</sup>. The result of this present study shows that all antibiotics applications can reduce the formation of PC during risk of sepsis condition. It is indicated that all antibiotics can reduce either ROS and PC. The decreasing of PC level by all antibiotics might be caused by the use of ROS to kill bacteria. The decreasing of ROS will reduce the PC level. The result of this present study also shows that the lowest PC level is in the T3 group. It is indicated that diazole has better antimicrobial activity than meropenem and amikacin in newborn at risk of sepsis.

Figure 2 represented the mean values±standard error (mean±SE) of the AOPPs level. AOPPs is tyrosine containing cross-linked protein products, a definition that is important as it excludes protein aggregates that are formed by disulphide bonds or amino acid modification as a result of oxidative stress<sup>15</sup>. AOPPs have been considered as novel disease-related biomarkers for oxidative stress. AOPPs is more specific oxidative stress marker than PC<sup>16</sup>. Serum concentrations of AOPPs are closely related to several diseases, including NS. According to our previous study, serum concentration of AOPPs was increased in newborn at risk of sepsis. The result from figure 2 shows that all antibiotics treatment decrease the AOPPs level. The difference between AOPPs levels in the T1, T2, and T3 groups was significantly lower when compared with control group. This result indicated that all antibiotic reduce the oxidative stress which can be seen from the decreasing of the AOPPs level. Because of AOPPs are produced during the oxidative stress condition, then the reason why the AOPPs level decreases is might be is the same as the reasons why the levels of PC is decreased. The result of this present study also shows that the lowest AOPPs levels were found in T1 and T3 group. This result indicated that meropenem and diazole have better antimicrobial activity than amikacin in newborn at risk of sepsis.

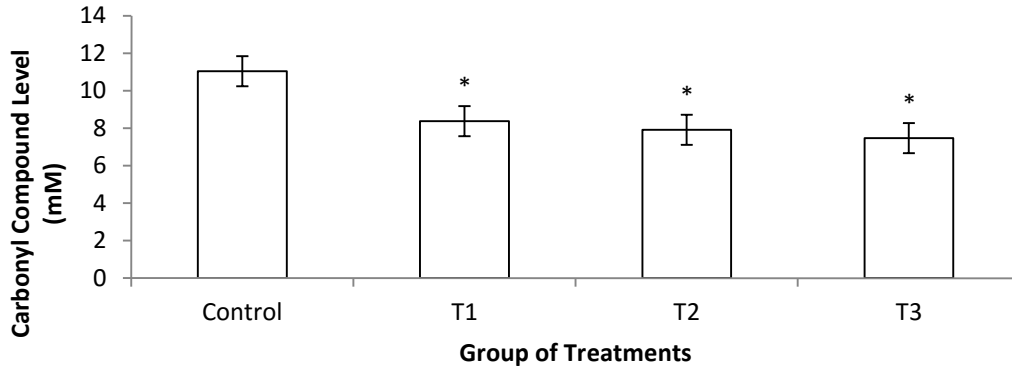


Figure 1. Comparison of PC content between a group of treatments. Data are presented as mean±SD. Statistical significance \*p<0,05 with in comparison with control group

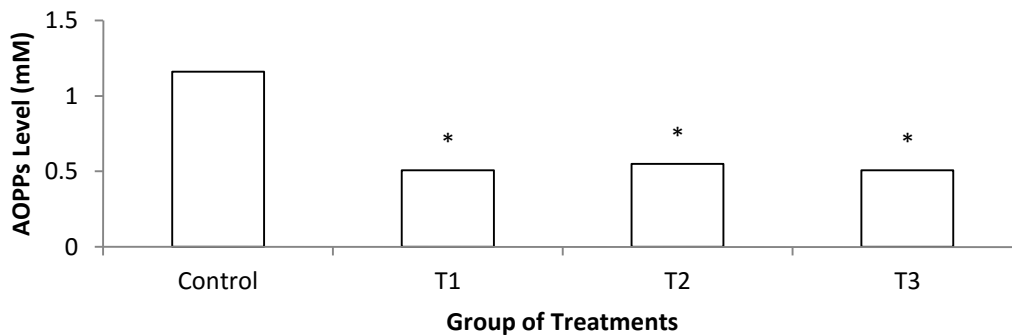


Figure 2. Comparison of AOPPs contents between a group of treatments. Data are presented as mean±SD. Statistical significance \*p<0,05 with in comparison with control group

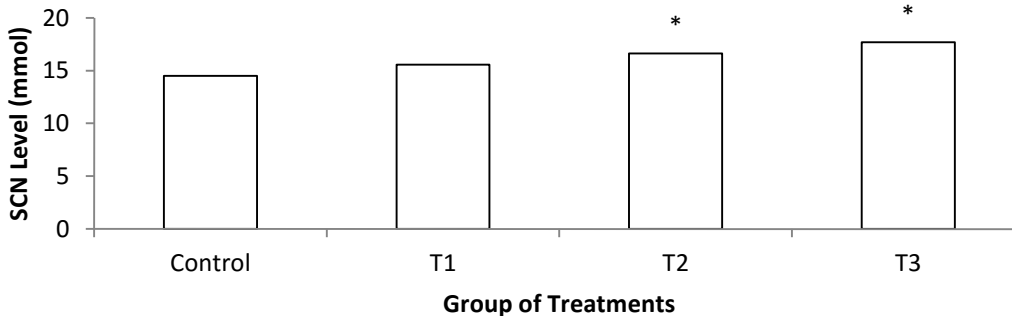


Figure 3. Comparison of SCN content between a group of treatments. Data are presented as mean±SD. Statistical significance \*p<0,05 with in comparison with control group

Figure 3 represented the mean values±standard error (mean±SE) of SCN level. SCN Result from figure 3 shows that antibiotic applications increase the SCN level. That data also shows that the highest SCN level was found in the T3 group and the lowest was found in the control group. The SCN levels were significantly higher in T2, and T3 groups in comparison with control, while in the T1 group was also higher, but non-statistically significant. is a physiological substrate for the mammalian heme peroxidases<sup>17</sup>. SCN together with H<sub>2</sub>O<sub>2</sub> generates hypothiocyanite acid (HOSCN)<sup>18</sup>. This reaction is catalyzed by MPO<sup>8</sup>. It is well documented in our previous study that meropenem, diazole, and amikacin could increase the concentration of H<sub>2</sub>O<sub>2</sub><sup>6</sup>. As discussed earlier that the increasing of the H<sub>2</sub>O<sub>2</sub> level by all antibiotics that

used in this study appears to play a key role in the mediation of antimicrobial effects. However, the results of this study indicated this antimicrobial activity of all antibiotics seems not just by the direct effect of H<sub>2</sub>O<sub>2</sub> production but also have indirect effect to increasing the SCN level. The increasing of both SCN and H<sub>2</sub>O<sub>2</sub> by all antibiotics that used in this experimental study can activate MPO to form HOSCN. HOSCN is known act as a potent and selective oxidizer of nucleophilic thiols that inhibits and kills multiple species of bacteria, viruses and fungi<sup>19</sup>. In conclusion, the present study demonstrated that antibiotic applications (meropenem, amikacin, and diazole) will decrease the PC and AOPPs and increase the SCN levels. Meropenem and diazole have a greater proportion to decrease the PC and AOPPs and increase the

SCN levels. Together these observations lead us to conclude that the antibiotics induced the production of ROS that will be used to kill bacteria. The use of this ROS then reduces the formation of PC and AOPPs in newborn at risk of sepsis. Also, the increasing of ROS including H<sub>2</sub>O<sub>2</sub> can increase the level of SCN which together can promote a further reaction to form HOSCN by MPO. The HOSCN that produced by antibiotics application also can be used to kill bacteria in newborn at risk of sepsis. This results indicated that meropenem, amikacin, and diazole could reduce the risk of sepsis in newborn with meropenem and diazole shows a better effect than amikacin.

#### CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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