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*Shri B. M. Shah College of
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AuthorId=15763373500

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ashokakabari@yahoo.com

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Research Article

Procalcitonin, IL-1 β , HSP10 and Resolvin D2 Mechanism as Sepsis Biomarkers in Sepsis Model

MUHAMMAD VITANATA AFRIJANTO¹, USMAN HADI^{1*}, YOES PRIJATNA DACHLAN²¹Department of Internal Medicine, Faculty of Medicine, Universitas Airlangga/Dr. Soetomo Teaching Hospital, Surabaya 60131, Indonesia²Department of Parasitology, Faculty of Medicine Universitas Airlangga/Dr. Soetomo Teaching Hospital, Surabaya 60131, Indonesia

*Corresponding Author

Email ID: usmanhadi@sby.centrin.net.id¹

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ABSTRACT

Background: The use of various biomarkers in sepsis has an important role in helping diagnosis and decision making therapy quickly and accurately. This study aimed to explain the mechanism of procalcitonin, IL-1 β , RAMPs markers such as HSP10 and resolvin D2 as biomarkers of sepsis in the sepsis model of *Escherichia coli* infection given with meropenem antibiotics.

Methods: An experimental study using the *E. coli* sepsis model in male *Rattus norvegicus* was conducted in 2 stages. The first step was to observe a model of sepsis in rats and determine the time of sepsis and the second step was to compare levels of procalcitonin, HSP10, IL-1 β , and resolvin D2 with different dosage of germs and different antibiotic time of administration.

Results: The time of sepsis was occurred between 8 and 10 hours. There were differences in *E. coli* exposure with IL-1 β levels ($p = 0.03$), procalcitonin ($p = 0.084$), HSP10 ($p = 0.000$), and resolvin D2 ($p = 0.024$). There were differences in levels of procalcitonin and resolvin D2 between groups at 24, 48 and 72 hours and between the treatment of antibiotics at different times and without antibiotic administration. While there were differences the levels of IL-1 β and HSP10 between groups at 24 and 72 hours, but there was no difference at 48 hours antibiotic treatments.

Conclusion: Resolvin D2 and HSP10 have a slightly different pattern at 24 and 72 hours compared to procalcitonin and HSP10. Thus, it has the potential to be developed as a new biomarker in sepsis.

Keywords: sepsis, biomarker, Procalcitonin, IL-1 β , HSP10, Resolvin D2

INTRODUCTION

Sepsis is a systemic inflammatory disease with a high risk of mortality. Sepsis is still big problem and needs to improve the management [1]. Excessive uncontrolled inflammation and an inappropriate immune response are special characteristics that make it difficult to identify good targets for treatment and treatment. Delayed diagnosis and initiation of antibiotic therapy have been reported to increase mortality [2, 3]. The use of various biomarkers in sepsis has an important role in helping diagnosis and decision making therapy quickly and accurately [4, 5]. One marker that has been recognized for the diagnosis and prognosis of sepsis is procalcitonin. In addition, procalcitonin can also be used as a guide to continue or stop antibiotic therapy [6–8]. Although various efforts through the management of sepsis and biomarker research have been developed to reduce mortality,

sepsis mortality is still high, sepsis by 30%, severe sepsis by 50%, and septic shock by 80% [9].

Procalcitonin is a biomarker that has been extensively studied for early diagnosis of sepsis and is superior to CRP and IL-6 [10]. In bacterial sepsis, procalcitonin is produced by various neuroendocrine cells, and the main site of production is in the liver. Procalcitonin is induced directly by LPS and or indirectly by pro-inflammatory cytokines such as IL-1 β , IL-2, IL-6, and TNF α [11–13]. The kinetic profile of procalcitonin is better than CRP because it starts to increase 3-4 hours after the onset of systemic infection and reaches a peak between 8 to 24 hours. The role of procalcitonin in sepsis is still not fully known, but it is known that the presence of inflammation will trigger the release of procalcitonin through two processes, either directly or indirectly. Toxins and LPS will directly trigger procalcitonin release, and various pro-

inflammatory cytokines such as IL-1 β , IL-6, and TNF α indirectly affect procalcitonin production [14]. In addition, other studies states that procalcitonin have good accuracy to predict bacterimia and fungal bloodstream infection [15].

Endogenous mediators known as DAMPs (danger associated molecular patterns), for example, are HSP (heat shock protein), fibrinogen and HMGB-1 (high mobility group box-1 protein). DAMPs and PAMPs activate inflammation, followed by activation of Caspase-1, the release of cytokines IL-1 β and IL-18, and the occurrence of Pyroptosis. Inflammasome is a multiprotein complex in mononuclear phagocyte cytosols, dendritic cells and other cell types proteolytically producing the active form IL-1 β from the pro-inactive precursor IL-1 β . The formation of inflammatory complexes includes NLRP3 (a NOD like pattern receptor receptor), a protein adapter, and the enzyme caspase-1, stimulated by microbial products, cell damage-associated molecules and crystals [16]. HSP27, HSP10, and α -crystallin are included in RAMPs as regulators and resolution of acute inflammation [17]. Resolvin D2 in studies in rats reduces leukocyte infiltration, inflammatory cytokines, and bacteria. Minimizing inflammation might slow resolution because of inappropriate induction of anti-inflammatory and pro-resolving pathways. Some new mediators do not simply or easily reduce inflammation but contribute to the exact time of resolution [18].

Preclinical studies provide real hope for the therapeutic aspects of sepsis; among them are sepsis models in rats that have similar mediators and molecular variations in sepsis patients. Studies in mouse models compared to other animals have many advantages, especially the problem of deep mechanisms that are difficult for us to understand in humans because of ethical issues. So that further evaluation of research on the model is needed to be applied to humans. Animal models used in the study of sepsis are non surgical models, surgical, implantation, toxemia, live bacteria models, cecal ligation puncture (CLP), and colon ascendent stent peritonitis (CASP) [19]. This study aimed to explain the mechanism of procalcitonin markers, IL-1 β markers, RAMPs markers, such as HSP10, and resolvin D2 as biomarkers of sepsis in the sepsis model of *E. coli* infection given meropenem antibiotics.

METHODS

This was an experimental study using the *E. coli* sepsis model in male rats (*Rattus norvegicus*) wistar strains. The study was conducted in 2 stages. The

first step was to look for a model of sepsis in rats and determine the time of sepsis. Male rats were divided into 2 groups, carried out randomly to get treatment as the sepsis model group was given with *E. coli* 0.5 mc Farland 0.1 ml, and the control group was given with NaCl 0.9% 0.1 ml intravenously and evaluated every 2 hours to take IL-1 β and TNF α levels by ELISA, measured temperature increases, and complete blood levels. Descriptive analysis of IL-1 β and TNF α was to determine the time of occurrence of known sepsis from levels increasing from normal to 2 times the SD (standard deviation), increasing in leukocyte levels $>10.5 \times 10^3/\mu\text{l}$, and increasing in rectal temperature $>38.5^\circ\text{C}$.

Thus, the second step was to calculate and compare levels of procalcitonin, HSP10, IL-1 β and resolvin D2 with different dosage of germs and different antibiotic time. Adult male *Rattus norvegicus* Wistar strain with 190-200 grams body weight which met the inclusion criteria included in this study. Male rats were divided into 6 groups. Group 1 was sepsis group with *E. coli* 0.5 mc Farland 0,1 ml, and Group 2 was sepsis group with *E. coli* 1 mc Farland 0,1 ml. Group 3 was control group given by NaCl 3% 0.1 ml. Group 4 was sepsis model group with *E. coli* 0.5 mc Farland which given by meropenem antibiotic at hour-2. Group 5 was sepsis model group with *E. coli* 0.5 mc Farland given by meropenem antibiotic at hour-10, and group 6 was sepsis model group with *E. coli* 0.5 mc Farland given by meropenem antibiotic at hour-22. Blood sampling was taken and evaluated at 24, 48, and 72 hours after administration of *E. coli*. Procalcitonin, IL1- β , HSP10, and resolvin D2 were examined by the ELISA.

The statistical test was done in stages: the normality test was used Shapiro Wilk when data were normally distributed. To observe the differences between groups, we used ANOVA or Kruskal Wallis or Mann Whitney. The study was conducted in the Animal Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. The examination of IL-1 β , TNF α , procalcitonin, HSP10 and resolvin D2 was conducted at the Biology Services Unit of Faculty of Science and Technology and Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia.

RESULTS

Determining Sepsis Time by Using Temperature, Complete Blood Markers, TNF α , IL1- β

This study determined the time of sepsis using TNF α , and IL1- β . We evaluated every 2 hours in 24 hours.

This sample study was *Rattus novergicus* Wistar strain with mean weight 170.55 grams. This study obtained the time of sepsis between 8 and 10 hours

where the increased level of temperature increased of leukocytes. Animals began to be lazy to move and did not want to eat. When IL-1 β was used, these events increased 2 times. While using TNF α , there has been an increase of 2 times at the 2nd hour, but the temperature has not been followed (Table 1).

Table 1: The IL-1 β level, TNF α level, temperature in first stage of study

Mean IL-1 β pg/mL	Hour	Mean temperature $^{\circ}$ C	Mean TNF α ng/L	Mean leucocyte $10^3/\mu$ l
277.50	0	36.2	464.514	8.3
328.17	2	36.5	774.190	13.9
346.23	4	37.1	637.466	15.6
491.28	6	37.7	470.241	13.8
512.45	8	38.7	395.409	14.9
544.46	10	38.5	437.363	23.4
932.99	12	38.8	693.453	21.3
1201.91	24	38.0	548.052	20.5

Calculating and Comparing the Levels of IL-1 β , procalcitonin, HSP10, and Resolvin D2 with Different Dosages of Germs and Different Times of Antibiotic

This study found that there were differences in *E. coli* exposure with IL-1 β levels ($p = 0.03$), procalcitonin ($p = 0.084$), HSP10 ($p = 0.000$), and resolvin D2 ($p = 0.024$). There were differences in levels of procalcitonin and resolvin D2 between

groups at 24, 48, and 72 hours between the treatment of antibiotics at different times and without antibiotic administration. Meanwhile, there were differences the levels of IL-1 β and HSP10 between groups at 24 and 72 hours, but there was no difference at 48 hours between the different antibiotic treatments and without antibiotics (Table 2).

Table 2: IL-1 β , Procalcitonin, HSP10, and Resolvin D2 Levels

Variable	Mean	Standard Deviation	Median	Minimum	Maximum
IL-1β					
Hour 24	1167.17	765.31	769.96	390.17	3003.51
Hour 48	1791.61	700.44	1464.56	354.01	3079.06
Hour 72	1246.85	640.62	1373.38	216.56	2173.87
Procalcitonin					
Hour 24	880.95	490.80	724.79	351.84	2195.76
Hour 48	1175.61	366.31	1144.90	360.37	2136.00
Hour 72	893.86	659.84	718.40	187.88	2026.63
HSP10					
Hour 24	14.83	7.94	7.83	3.02	31.29
Hour 48	14.49	7.85	13.74	3.09	35.39
Hour 72	10.61	8.24	9.12	1.10	36.07
Resolvin D2					
Hour 24	1669.93	161.49	1658.65	1916.74	1327.74
Hour 48	1587.24	107.33	1574.82	1812.40	1361.71
Hour 72	1649.77	115.84	1670.39	1885.83	1391.71

Analyzing the Relationship of IL-1 β , Procalcitonin, HSP10 and Resolvin D2 Levels in the Sepsis Model with Different Doses and Timing of Meropenam Antibiotic Administration

After seeing the results of the biomarker statistical test, the researchers wanted to see the dynamics of each treatment group in the development of the four biomarkers in the 24th, 48th and 72th hour evaluation. In the 24-hour analysis, the most significant difference in resolvin D2 levels was in the low-dose *E. coli* exposure group without antibiotics and the sepsis group given antibiotics at the 10th hour ($p = 0.000$). The next most significant difference was procalcitonin levels in the low-dose *E. coli* group without antibiotics and the administration of antibiotics at the 10th hour ($p = 0.022$). In the 48th hour analysis, there was the most significant difference in procalcitonin levels in the low-dose *E. coli* group without antibiotics and the low-dose *E. coli* group given with antibiotics at the 22nd hour ($p = 0.04$). Then, there were differences in resolvin D2 levels in the low-dose *E. coli* exposure group without antibiotics and the sepsis group with low-dose *E. coli* given with antibiotics at the 10th hour ($p = 0.003$). In the 72-hour analysis, the most significant difference was resolvin D2 levels between the sepsis group exposed to low-dose *E. coli* without antibiotics and the sepsis group given with antibiotics at the 22nd hour ($p = 0.000$). Then, there were differences in resolvin D2 levels between the sepsis group given with a low dose of *E. coli* and antibiotics at the 10th hour and the antibiotic group at the 22nd hour ($p = 0,000$). In addition, HSP10 levels were also the most significant difference in the low-dose *E. coli* group without antibiotics and the *E. coli* group given with antibiotics at the 22nd hour ($p = 0.027$). Procalcitonin levels also showed a significant difference between groups at the 72nd hour between low-dose *E. coli* without antibiotics and low-dose *E. coli* with antibiotics at the 10th hour ($p = 0.000$).

DISCUSSION

The results of the study showed that there were differences in the levels of procalcitonin and resolvin D2 between groups at 24, 48 and 72 hours between the different antibiotic treatments and without antibiotics. Meanwhile, at IL-1 β and HSP10 levels, there were differences between groups at 24 and 72 hours, but there was no difference at 48 hours between the different antibiotic treatments and without antibiotics. Procalcitonin is a marker used as a guide to start and stop antibiotics in sepsis, having a similar

pattern in all groups with IL-1 β . Resolvin D2 and HSP10 have a slightly different pattern at 24 and 72 hours compared to procalcitonin and HSP10. In sepsis, bacteria show that the gene 1 calcitonin mRNA is organized more neatly than other inflammatory markers such as the TNF α gene, IL-1 β , whereas mature calcitonin does not increase during sepsis, but the discovery of procalcitonin as part of increased calcitonin is expected to be a critical marker of cases [11]. This study supports the previous research that resolvin D2 is a lipid mediator stimulating macrophages via alternative pathways to stimulate T cells to activate the anti-inflammatory pathway [18].

The sepsis model in rats given antibiotic meropenam was carried out using *E. coli* isolate ATCC 25922 with a dose of 0.5 mc Farland (equivalent 1.5x10⁸/ml) intravenously 0.1 ml compared with NaCl 0.9%. A literature mentions the making of an intravenous sepsis model using *E. coli* doses of 4-5x10⁸ [20]. Some researchers have criteria according to the facilities they have and by calculating cytokine levels. Bone criteria from sepsis use parameters, temperature, pulse, breath count, and leukocytes. Meanwhile, SSC provides guidelines for increasing procalcitonin 2SD [6]. We set an estimated 8 hours; as for the basis used by researchers is to use temperatures above 38.5°C, the number of leukocytes above 10.5x10³/ μ l and increasing levels of TNF α , and IL-1 β 2 times above normal. TNF α The clinical signs of experimental animals observed to determine sepsis are piloerection, hyperpnea and decreased motor activity in the first 6 hours [21]. In this study, there were also the same signs in the first 6 hours. Leukocyte levels have increased starting at 2 hours, while the new temperature increased at 6 hours, and IL-1 β increased 2 times above normal at 8 hours. Up to a 24-hour evaluation, no deaths were found. Previous studies using *E. coli* ATCC 11775 given intraperitoneally and evaluated at 0, 24, 48 and 168 hours [21], whereas this study did intravenously.

According to research, animals show calcitonin which has been successfully isolated in brain tissue, spleen, spinal gastric tissue, lungs, liver, and fat [22]. Other studies evaluated LPS and procalcitonin levels in mice for 10 days. The results obtained are procalcitonin appearing on days 3 and 4, while LPS appeared on day 7. This shows that besides LPS, there are other factors influencing the increase in procalcitonin levels [23]. Research on human samples showed that the group with suspected sepsis with positive procalcitonin had high leukocyte levels, which was a mean of 14.3 and in the

suspected group of sepsis with negative procalcitonin which had leukocyst levels of 9.3 [24]. A 2012 study using LPS at a dose of 1 mg/L in mice, and TNF α , IL-6 and IL-1 β cytokine levels were examined after 24 hours of administration. Significantly, all three cytokines increased compared to controls. Examination of phosphorylation pathway specifically increases ERK $\frac{1}{2}$, JNK and P38 MAPK levels after 30 minutes of LPS administration [25]. Increased IL-1 β after administration of *E. coli* and decreased *E. coli* after administration of antibiotics showed that the inflammatory process was activated and inhibited by various pathways of pyroptosis, caspase1, TLR4 DAMPs NLRP3 also occurred in the sepsis model of this study. In this study, researchers did not use LPS but *E. coli* ATCC bacteria. Variations in antibiotic administration and variations in the dose of *E. coli* exposure in this study provide information and strengthen previous research that the inflammatory pathway shown by IL-1 β levels also occurs in sepsis models of *E. coli*, and the timing of antibiotic administration can provide the best information on reducing levels IL-1 β when it is a target for therapy. Previous study reported that significantly giving HSP10 can reduce LPS levels. The study evaluated that the administration of HSP10 can also reduce levels of cytokines IL-10, IL-4, IFN γ [26]. In this study, HSP10 levels increased higher in the group without antibiotics. Precisely in the low dose *E. coli* dose group, there was an increase in HSP10 levels at 12 and 24 hours compared to the control and high *E. coli* dose. Previous studies also gave HSP10 at a dose of 100 μ g given 30 minutes before administering LPS at a dose of 10 μ g. There was a significant difference marked by a decrease in TNF α and an increase in IL-10 [26]. This study can also provide information that variations in antibiotic administration, ie early administration, when the sepsis marker is being delivered, and late administration, can provide additional information or strengthen research on HSP10. The increase in HSP10 in the group without antibiotics at 24 hours was most likely the HSP10 response in reducing *E. coli*. Provision of antibiotics provides information on the possible role of antibiotics in helping the work of HSP10 in reducing *E. coli*, or HSP10 markers can be a marker of resolution.

This study provides information that early antibiotic treatment accelerates resolvin D2 levels back to normal. The late administration of antibiotics actually causes resolvin D2 levels to go down at 72 hours. These results are in line with previous studies using sepsis models in mice with the CLP technique and comparing groups given resolvin D2 therapy.

Death occurred starting at 24 hours and almost 90% at 48 hours in the CLP group only. Meanwhile, the CLP group who were given resolvin D2 therapy could survive 80% until 36 hours and 60% from 48 hours to 120 hours [18]. These results are also in line with other studies that conducted research on giving resolvin D2 injection drastically can reduce inflammatory cytokines associated with poor prognosis namely IL-6, IL-1 β , IL-23, and TNF α . There is a decrease in bacteria, leukocytes, and PMN in the peritoneum. Giving resolvin D2 and resolvin D1 therapy has the potential to reduce leukocytes associated with the occurrence of microbial peritonitis from the 15th minute to the 60th minute. Without the administration of resolvin D1 and resolvin D2 therapy, leukocytes continue to increase in the 15th minute, reaching a peak in the 60th minute. The study used a sepsis model in experimental animals with the CLP model. In that study, administration of resolvin D2 injection increased the survival of the sepsis model with CLP [27].

After seeing the evaluation of the four biomarkers and treatment groups by distinguishing the time of antibiotic administration, so if we have been using procalcitonin as a guide to starting and stopping antibiotics, then giving antibiotics at the 10th hour is the best. The IL-1 β biomarker that we use as a mechanism for inflammasomes also has the same pattern as procalcitonin. This is in accordance with previous studies that toxins and LPS will directly trigger the release of procalcitonin, and various pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF α indirectly affect procalcitonin production [14]. Procalcitonin levels begin to increase 4 hours after the onset of systemic infection and reach a peak between 8 to 24 hours [28]. HSP10 in humans is encoded by the nuclear HSPE1 gene (geneID 3336) on chromosome 2q33.1. Intracellular proteins such as HMGB1 and IL-1 β can secrete extracellular signals from HSP10 secretion [29]. LPS via TLR4 activates NF- β , which further stimulates pro IL-1 β with the help of cathepsin to activate NLRP-3 (inflammasome) stimulating Pyroptosis through the active caspase-1 pathway and so can secrete IL-1 β and IL-18 [30]. The existence of microbes that damage the tissue activates PMN; there is complete phagocytosis which will occur PMN apoptosis and stimulate the formation of SPM, then stimulate macrophages to inhibit edema. The other pathway which is not phagocytosis complete results in the formation of ROS captured by LTB4 receptors to secrete cytokines and chemokines, results in diapedesis, adhesion in blood vessels resulting in edema or extravasation and involves lipid

mediators DHA and EPA to produce resolvin stimulate macrophages and inhibit edema. The occurrence of edema as an inflammatory response begins within a few minutes after germs enter, followed by PMN infiltration along with the emergence of SPM synthesizing lipoxins, resolvins, protectin, and maresin within a few hours to days [31]. After observing the dynamics of the four biomarkers in sepsis and statistically obtained differences in each group affected by *E. coli* doses and differences in the time to start antibiotics and without antibiotics, there is the potential for developing other biomarkers in sepsis other than procalcitonin.

This study has several limitations. The study design was limited to experimental animals so they could not evaluate for more blood sampling. Blood collecting was only a maximum of 3 times, including when animals were sacrificed. Provision of antibiotics could not be in accordance with the half-life required because it was limited to the time of giving outside working hours and experts who di.

CONCLUSION

The time of sepsis occurred at 8-10th hour. There were differences in levels of procalcitonin and resolvin D2 between groups at 24, 48, and 72 hours between the treatment of antibiotics at different times and without antibiotic administration. Meanwhile, there were differences the levels of IL-1 β and HSP10 between groups at 24 and 72 hours, but there was no difference at 48 hours between the different antibiotic treatments and without antibiotics. Procalcitonin is a marker used as a guide to start and stop antibiotics in sepsis having the same pattern in all groups with IL-1 β . Resolvin D2 and HSP10 have a slightly different pattern at 24 and 72 hours compared to procalcitonin and HSP10. Thus, it has the potential to be developed as a new biomarker in sepsis.

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