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Research Article

NEW MOLECULAR MECHANISM OF CEFTAZIDIME-INDUCED HUMAN RED BLOOD CELL HEMOLYSIS THROUGH THE PHOTOHEMOLYSIS REACTION

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ABSTRACT

Objective: The present study was undertaken to investigate the photohemolysis reaction through photosensitization reaction by ceftazidime as a photosensitizer in human red blood cell (RBC).

Methods: In this present study, human erythrocytes have used a sample. The sample then divided into six groups consisting of Group 1 (T1) served a negative control which consists of erythrocytes and buffers phosphate with pH 6.8; Group 2 (T2) served as a positive control which consists erythrocytes and buffers phosphate with pH 6.8 and exposed to UV-light; and Group 3, 4, 5, and 6 (T3, T4, T5, and T6) served as an experimental group which consists of erythrocytes, buffer phosphate with pH 6.8, ceftazidime with concentration 10%, 20%, 30%, and 40%, respectively, and also exposed to UV-light. UV-light exposure was done in 2 h. After the treatment period, the level of hydrogen peroxide (H_2O_2), conjugated diene (CD), advanced oxidation protein products (AOPPs), and percentage of RBC hemolysis (RBCH) were measured.

Results: The results of this present studies showed that ceftazidime significantly increases the levels of H_2O_2, CD, AOPPs, and percentage of RBCH during the UV radiation.

Conclusion: The present study demonstrated that ceftazidime acts as a photosensitizer and induced the photohemolysis reaction in human RBC. Furthermore, the hemolysis of RBC seems through the protein damage than lipid damage.

Keywords: Ceftazidime, Photohemolysis, Photosensitization, Photosensitizer.

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INTRODUCTION

Sunlight is known contain some continuous spectrum electromagnetic radiation. These radiation is differentiated into three parts such as ultraviolet, visible, and infrared [1]. Among these three main parts, UV light is the most important part especially for the human body [2]. UV light wavelength zone occurs between 100 and 400 nm. In general, these wavelength regions are classified into three parts, among others UVC radiation (200–290 nm), UVB radiation (290–320 nm), and UVA radiation (320–400 nm). Furthermore, the UV radiation that reaches the earth's surface contains about 5% of UVB and 95% of UVA radiation. However, because of the damage of the Protection ozone the UVB radiation was increased [1,2].

Both UVA and UVB could induce the phototoxicity reactions with the presence of chemical substances called photosensitizer [3,4]. Photosensitizer is a substance which could absorb light and change from a low energy ground state to an excited state. Furthermore, these excited states of photosensitizer can interact with oxygen to form singlet oxygen and another reactive oxygen species (ROS) [5]. However, this ROS could promote a further reaction to oxidized lipid and/or protein, and DNA resulted in cellular damage [5].

It is well known that many drugs could act as a photosensitizer. One of a common source is antimicrobials. The most widely studied antibiotic on this pathomechanism is quinolones. However, the previous report suggested another antimicrobial which is potentially as a photosensitizer, such as ceftazidime [6]. Ceftazidime is a broadspectrum antibiotics. It is the third generation of cephalosporin and semisynthetic. It has broad-spectrum activity against Gram-positive and Gram-negative. However, unlike most third-generation agents, it is active against *Pseudomonas aeruginosa*. This antibiotic is administered parenterally every 8–12 h (2–3 times a day), with daily dosages of 500–6,000 mg, determined by the indication, infection severity, and/or renal function of the patient [7].

It is well known, the administration of drugs such as antibiotics can induce the blood cell hemolysis. Although drug-induced hemolysis is less common than other types of adverse reactions, they are associated with significant morbidity and mortality [7]. Arndt [8] reported that there are some antibiotics could induce the blood cell hemolysis including ceftazidime. Previous reports indicated ceftazidime induced immune hemolytic anemia on red blood cells (RBC) only and RBCs with the addition of plasma in two of the four methods performed and in three of four methods performed, respectively [8]. However, there are 4 proposed mechanisms in the drug-induced hemolysis such as (1) hapten or drug adsorption mechanism; (2) innocent bystander mechanism; (3) RBC autoantibodies mechanism; and (4) nonimmunologic protein adsorption mechanism [9].

However, in this present study, we propose another mechanism which is photohemolysis mechanism. In this mechanism, ceftazidime acts as a photosensitizer and with the UV radiation-induced the photohemolysis reaction. To investigate this mechanism reaction, four parameters will be measured such as hydrogen peroxide (H_2O_2) as a one of photosensitization reaction product, conjugated diene (CD), and advanced oxidation protein products (AOPPs) as an impact of photosensitization reaction product formation, and the percentage

of RBCs hemolysis (RBCH) to assess the damage of RBCs during this reaction. To explain the mechanism of RBCH due to ceftazidime, we also correlated CD and AOPPs with the percentage of RBCH.

METHODS

Sample preparation and experimental section

The packed red cells with 250 ml volume were obtained from the Indonesian Red Cross in Martapura, Banjar District, South Kalimantan, Indonesia. Then, the samples were divided into 6 groups with each group performed four replications. Treatment 1 (T1) group served as a negative control which consists of 1 ml of erythrocytes and buffers phosphate with pH 6.8. Treatment (T2) group served as a positive control which consists of 1 ml of erythrocytes and buffer phosphate with pH 6.8 and exposed to UV-light for 2 h. Treatment 3, 4, 5, and 6 (T3, T4, T5, and T6) served as an experimental group which consists 1 ml of erythrocytes, buffer phosphate with pH 6.8, ceftazidime with concentration 10%, 20%, 30%, and 40%, respectively, and exposed to UV-light for 2 h. After treatment, each solution will be measured the H_2O_2, CD, AOPPs levels, and percentage of RBCH. (Experiments performed complied with the guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council and were approved by the Ethical Committee of the Faculty of Medicine, University of Lambung Mangkurat, Banjarbaru, South Kalimantan, Indonesia [ethical approval number; 403/KEPK-FK UNLAM/EC/VI/2017]).

H_2O_2 concentration analysis

The H_2O_2 level was calculated by the FOX_2 method with slight modification [10]. 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 PBS pH 7.4, 160 µL $FeCl_3$ (251.5 mg $FeCl_3$ dissolved in 250 ml distilled water) and 160 µL o-phenanthroline (120 mg o-phenanthroline dissolved in 100 ml distilled water) for both solutions. The composition of the blank solution was identical to that of the test solution, except for the absence of $FeCl_3$ in the blank. Subsequent to preparation, all solutions were incubated for 30 min at room temperature, then centrifuged at 12,000 rpm for 10 min, and the absorbance of the standard (As), test (Au), and blank (Ab) solutions measured at λ=505 nm, using the supernatant of each solution [11].

CD concentration analysis

CD concentration was assayed as previously described by Recknagel [12] with minor modifications. Briefly, the sample extracted with chloroform: methanol (2:1) at a ratio of 1:2.5 homogenate to the organic solvent mixture. The resulting mixture was centrifuged at 2000 rpm for 8 min. The organic phase containing the CD was taken, dried under nitrogen, solubilized in hexane, and rinsed with 0.003 N HCl. Samples were analyzed spectrophotometrically at 233 nm, the maximal absorbance for CD, and expressed per nanomole of phosphate. Total phosphates were quantified by incubating a portion of the dried organic phase for 3 h with 70% perchloric acid at 130°C, followed by the addition of ammonium molybdate and Fiske-Subbarow reagent. Samples or standards were then reheated to 110°C for 10 min, cooled, and analyzed spectrophotometrically at 820 nm. Data are expressed as optical density at 233 nm/nmol phosphate.

AOPPs concentration analysis

AOPPs concentration analyses were calculated by spectrophotometric methods. 200 µl of supernatant from the kidney homogenate was diluted with phosphate buffer solution and then placed on 96-test wells. Add 20 ml of acetic acid in each test well. For the standard, add 10 ml of 1.16 mol potassium iodide, 200 ml of chloramine-T solution (0–100 mmol/l), and 20 ml of acetic acid. Placed the standard mixture into standard wells. Then, read the absorbance of the mixture at 340 nm. The absorbance was read against a blank solution. A blank solution is a mixture between 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. AOPPs concentrations were expressed as mmol/l of chloramine-T equivalents [13].

RBCH percentage analysis

A suspension of erythrocyte (500 µl) within a micro-tube was incubated for the required times with an equal volume of the test sample mixture, prepared in the buffer at 37°C. After incubation, the mixture was spun in a microcentrifuge at 2600 rpm for 10 min, and 200 µl of the resulting supernatants was added to 4.5 ml of Drabkin's reagent. To assay for hemoglobin released, the absorbance of samples was assessed in 546 nm wavelength using spectrophotometer. Positive controls consisted of 500 µl of uncentrifuged mixtures of erythrocyte suspensions and 500 µl of buffer, which was added to 3 ml Drabkin's reagent to obtain a value for 100% hemolysis. A negative control, included to measure the level of spontaneous hemolysis, comprised 500 µl buffer mixed with 500 µl erythrocytes, and after centrifugation for 10 min, a 200 µl sample of supernatant was added to 3 ml of Drabkin's reagent. Hemolysis percentage for each sample was calculated by dividing sample's absorbance on positive control absorbance (complete hemolysis) multiplied by 100% [14].

Statistical analysis

The results were expressed as mean±SE for four replicates. Significance of mean differences of all parameters between treatment and control groups was statistically compared using one-way analysis of variance and followed by a *post hoc* Tukey's Honestly Significant Difference test for multiple range test.

To determine the mechanism of RBCH due to ceftazidime, the percentage of RBCH was correlated to CD and AOPPs concentrations.

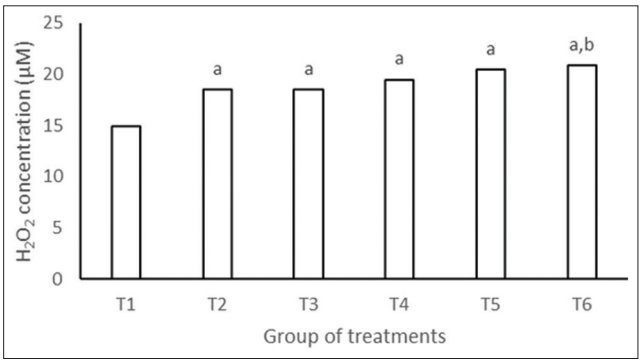

Fig. 1: It shows the effect of different concentration of ceftazidime during UV-light radiation on H_2O_2 concentration. Values are a mean±standard error of the mean of four replicates in each group of treatment. Statistical significance [a]p<0,05 in comparison with negative control group (T1), [b]p<0.05 in comparison with negative control group (T2)

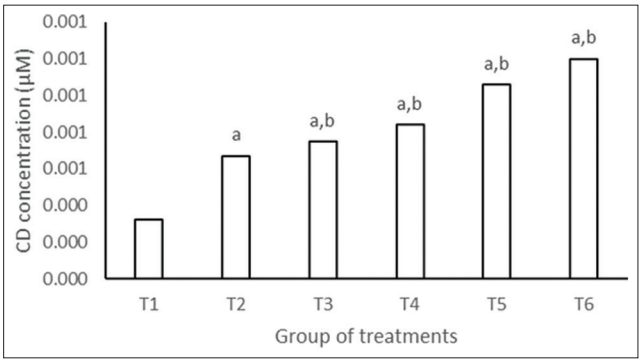

Fig. 2: It shows the effect of different concentration of ceftazidime during UV-light radiation on conjugated diene concentration. Values are mean±standard error of the mean of four replicates in each group of treatment. Statistical significance [a]p<0.05 in comparison with negative control group (T1), [b]p<0.05 in comparison with negative control group (T2)

The Pearson correlation test was used to analyze the relationship between those variables and the correlation coefficient (r) was derived to measure the strength of association between RBCH and both parameters that produced due to ceftazidime and during irradiation with UV. Significance was set at $p<0.05$. The software used for the data analysis was the Statistical Package for the Social Sciences version 16.0 and Microsoft Excel 2016 for Windows 10.

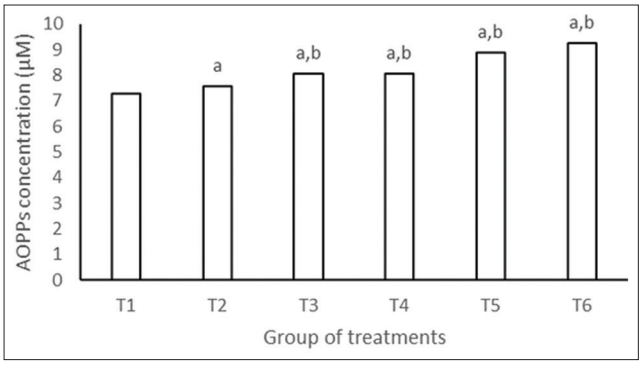

Fig. 3: It shows the effect of different concentration of ceftazidime during UV-light radiation on advanced oxidation protein products concentration. Values are a mean±standard error of the mean of four replicates in each group of treatment. Statistical significance [a]p<0.05 in comparison with negative control group (T1), [b]p<0.05 in comparison with negative control group (T2)

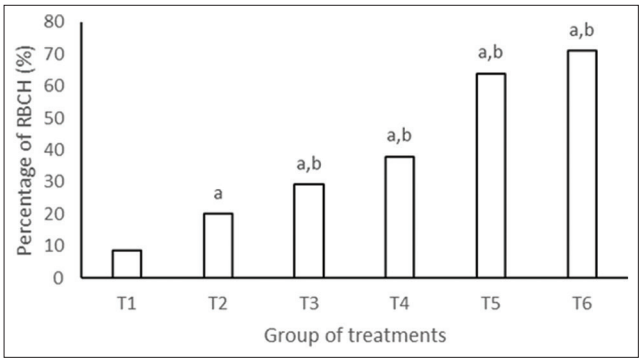

Fig. 4: It shows the effect of different concentration of ceftazidime during UV-light radiation on the percentage of red blood cells hemolysis concentration. Values are a mean±standard error of the mean of four replicates in each group of treatment. Statistical significance [a]p<0.05 in comparison with negative control group (T1), [b]p<0.05 in comparison with negative control group (T2)

RESULTS AND DISCUSSION

In this present study, we try to investigate that antibiotics like ceftazidime could induce blood cell hemolysis through the photohemolysis reaction. It was based on phototoxicity reaction which is started when blood cells are exposed to UV radiation and are photosensitized by ceftazidime. Ceftazidime in this reaction is called a photosensitizer. Ceftazidime could absorb photon from the UV-light which is electromagnetic spectrum and converted into chemical energy used in chemical reactions. These chemical reactions can lead to the formation of some ROS, such as H_2O_2 [6,15]. It can be seen from the result of this present study (Fig. 1).

According to Fig. 1, it can be seen that ceftazidime could increase the H_2O_2 formation. However, statistically, only the largest dose of ceftazidime could significantly increase the H_2O_2 level. To the best of our knowledge, there have been no investigations of the association between ceftazidime, UV-light radiation, and the formation of H_2O_2. Nonetheless, previous literature has suggested that antibiotics such as ceftazidime may trigger phototoxicity reactions and produce ROS [6].

As a result of increased levels of ROS during this photoxicity reaction, lipid and/or protein oxidation can occur. It was supported by the result of this present study. The result revealed that ceftazidime in all concentrations with UV-light exposure could significantly increase the level of CD and AOPPs (Figs. 2 and 3). This results also supported by another our previous reports that were indicated that ceftazidime could induce the formation of malondialdehyde and protein carbonyl during UV-light radiation [16].

Lipid and/or protein oxidation by ROS commonly used several biomarkers, such as CD and AOPPs. CD was reported as a marker for the early stage of lipid oxidation [17]. These compound formed at the initiation stage of lipid oxidation from the intramolecularly arrangement of lipid radical [18]. Furthermore, AOPPs was known as a product of the action of free radicals on proteins which is first described by Witko-Sarsat *et al*. AOPPs are defined as dityrosine containing cross-linked protein products and are considered to be reliable biomarkers to estimate the degree of protein oxidation [19].

Lipid and/or protein oxidation will promote a further reaction resulted in cell damage. From this point of view, cell damage that will occur in this study is damage to RBC. Fig. 4 shows that the percentage of RBCH is significantly increase in all concentration of ceftazidime. This result indicated that ceftazidime could induce hemolysis of RBC. To investigate which mechanism triggers RBCH, the levels of CD and AOPPs are correlated with percentages of RBCH. The results show in Fig. 5a and b.

According to Fig. 5a and b, both parameters have a significant strong positive correlation with the percentage of RBCH. However, if we look closely to the r value, AOPPs have a larger r value (0.992) than CD

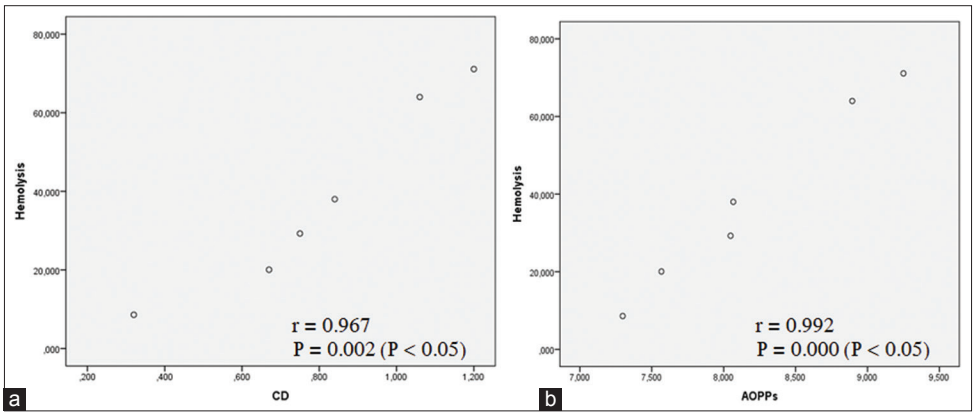

Fig. 5: It shows the correlation between percentage of red blood cells hemolysis and (a) conjugated diene and (b) advanced oxidation protein products concentrations by different concentration of ceftazidime during UV-light radiation

(0.967). These results prove that RBCH is more due to protein damage than lipid damage. To the best of our knowledge, there have been no investigations of the association between those all parameters by ceftazidime during UV-light radiation.

CONCLUSION

It can be concluded from the presented results that ceftazidime induced photohemolysis reaction in RBC. Moreover, from the results, the photohemolysis reaction might be followed by a photosensitization reaction, in which ceftazidime acts as a photosensitizer and uses UV-light to induce RBCH. Furthermore, from the results, it seems that the RBCH is more due to protein damage than lipid damage. Further studies are undergoing to clarify their molecular mechanisms.

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