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Research Report

Salivary neutrophils isolation of severe early childhood caries patients with flow cytometry analysis using magnetic beads and CD177 marker

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ABSTRACT

Background: *Neutrophils are the first line of defense, not only serving as he killer of microbes through phagocytosis process, in which reactive oxygen species (ROS) and anti-microbial peptides were released, but also regulating activation of immune response. CD177 is a tidylinositol glycosylphosphate glycoprotein with a molecular weight of 58- 64-kDa exclusively found on neutrophils, neutrophilic metamyelocytes, and mielosit. CD177 expression, a protein on the cell surface with an average size ranging from 45% to 65%, is only found on subpopulations of neutrophils.* ***Purpose:*** *This study aims to analyze the effects of salivary neutrophil isolation using magnetic beads and CD177 marker on S-ECC patients.* ***Method:*** *The study is an observational analytic research with cross sectional approach using flow cytometry analysis on the S-ECC patients and the caries-free children who were asked to use mouthwash, NaCl 1.5%. For the isolation of neutrophils, magnetic beads labeled with FITC funds and $CD177^+$ marker were used.* ***Result:*** *There were 77.66% of salivary neutrophils expressing $CD177^+$ markers, successfully isolated in the S-ECC patients, while in the caries-free children there were 63.67% of salivary neutrophils.* ***Conclusion:*** *In the S-ECC patients, there were 77.66% of salivary neutrophils expressing CD177markers, successfully isolated, while in the caries-free children there were 63.67% of salivary neutrophils.*

Keywords: *magnetic beads; salivary neutrophils; S-ECC*

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INTRODUCTION

Dental caries in preschool children is a very serious health problem, requiring special attention since dental caries is a focal infection that causes a variety of systemic diseases, and it is not possible to recover the formation of tooth structure when a cavity/hole occurs. Dental caries is a disease that is irreversible, consequently, it needs to be cured since its impact is huge in children. For instance, it can cause difficulty in chewing, malnutrition, gastrointestinal disorders, growth disorders, especially weight and height, articulation disorders of speech, and impaired social and cognitive development. Dental caries can be considered as a continuous problem that burden children,[1] such as affecting the physical and mental health of the children and increasing the risk of dental caries to become the permanent one.[2]

Streptococcus mutans (*S. mutans*) are the primary etiologic agents of early childhood caries (S-ECC) since they have some mechanisms to colonize the tooth surface, and under certain conditions, they can alter into cariogenic species significantly higher in the oral biofilm environment. As a result, it indicates a causative relation between dental caries and the increasing of *S. mutans*. In other words, the increasing of the number of *S. mutans* in saliva can be an indication of the increasing of dental caries prevalence.[3] In the S-ECC cases, the increasing of the number of *S. mutans* can cause the migration of neutrophils out of the bloodstream into oral mouth to perform phagocytosis against microbial pathogens in an effort of homeostasis.

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In recent years, the perspective about neutrophils has changed dramatically. Neutrophils are considered as a key component of the first line of defense against microbes.[3] Neutrophils not only act as the killer of microbes through phagocytosis process, in which reactive oxygen species (ROS) and anti-microbial peptides were released, but also regulate the activation of immune response.[5] Neutrophils, moreover, can produce a variety of cytokines, chemokine, and growth factors. Therefore, neutrophils can be considered as the major contributor to the production of proinflammatory cytokines on infection area.[6]

Furthermore, neutrophils isolated from saliva by a nylon filter sequentially with 20 and 11µm nylon filter that is often used nowadays still have not been able to get the maximum results. Meanwhile, the latest method using magnetic beads labeled with CD177 and analyzed using flow cytometry can be considered as one alternative method for the isolation of neutrophil cells.

CD177 is a tidylinositol glycosylphosphate glycoprotein with a molecular weight of 58-64-kDa contained exclusively in neutrophils, neutrophilic metamyelocytes, and mielosit.[7,8] CD177 expression is only found on subpopulation of neutrophils, a protein on the cell surface with an average size ranging from 45% to 65%.[9] Based on the above information, the researcher wants to isolate and analyze salivary neutrophils with such method in order to obtain optimal neutrophil cells from saliva of severe early childhood caries (S-ECC) patients and caries-free children.

MATERIALS AND METHODS

Students of kindergartens selected as sampling sites in the area of Surabaya were examined for dental caries by measuring the index of the decay- exfoliation filling (def-t). The children were divided into two groups, namely caries-free group and severe caries group with def-t more than 6. All the subjects in the sample aged between 4 to 6 years. Prior to the sampling process, questionnaire sheet and informed consent were distributed to the parents of those students.

Sampling process was conducted by researcher and trained personnel using a standard protocol. Subjects should not eat, drink, chew gum, or brush teeth for 60 minutes prior to the sampling process. After collected, the samples were stored at -80° C for analysis.[10] Salivary neutrophils were obtained by asking the subjects to rinse their mouth with 10 mL of sterile 1.5% NaCl solution for 30 seconds, and then expectorated in a sterile glass. This procedure was repeated four times. The samples were subsequently centrifuged at 450 g for 15 minutes at a temperature of 4° C. Pellets obtained from the centrifugation result were mixed with 2 ml of RPMI medium. Neutrophil cells then were identified using human neutrophils enrichment kit of Easy Sep brand in with the following methods: the cell suspension with a concentration of 5 x 10^7 cells/ml was placed in a polystyrene EasySep® magnet tubes sized 5 ml (12 x 75 mm). Falcon ™ 5 ml polystyrene tube (Becton Dickinson, catalog # 352 058) was then added with 50 ul/ml of EasySep® neutrophil cell cocktail (e.g. for 2 ml of cells, 100 ul of cocktail is added). It was stirred well and incubated at 4° C for 10 minutes. Three Mix EasySep® Nanoparticles were then used to ensure whether the cells were in a homogeneous suspension by conducting pipetting five times.

Afterward, nanoparticles were added into 100 uL of cells/ ml (2 ml of cells were added into 200 ul of nanoparticles). It was then stirred well and incubated at 4° C for 10 minutes. 2.5 ml of the cell suspension was then put on the tube (without cap) to the magnet for 5 minutes. The next stage, EasySep® magnet was removed in a single motion sequence, i.e. reversing the magnet and the tube. Cells that were not needed on magnetic beads already labeled were remained and bound in the tube. The tube was in the inverted position for 2-3 seconds, and then returned to the upright position. After that, the empty tube was taken from EasySep® magnet and replaced with a new tube containing supernatant fraction placed on the magnet, and then left for 5 minutes. Cells in the new tube was then ready for use.

Analysis of cell suspensions using flow cytometry was performed on a fluorescence activated by cell FACScan analyzer (Becton Dickinson). At least 25,000 events were analyzed for each sample of salivary neutrophils. Each sample of salivary neutrophils was identified to get profile of those neutrophils using FSC and SSC based on the size and granular respectively in the neutrophil suspension. Cells that had been identified their profile were then analyzed, and positive staining for neutrophil marker was conducted to define events beyond the level of fluorescence. Neutrophils that were more than 70% of the isotope and matched with staining control were studied. The percentage of neutrophil cells was determined by subtracting isotope positive staining cells-matched with positive staining antibody cells. Meanwhile, the percentage of fluorescent neutrophil cells was determined by gating on both of the cells reacting negatively to propidium iodide which had been labeled. Back gating for FSC was then compared to SSC plot to verify the morphology of cells stained positive.

RESULTS

Figures 1 and 2 show the isolation results of salivary neutrophils cultured on Hank's balanced salt solution (HBSS) media observed using Olympus inverted microscope with a magnification of 200x on days 1 and 3. They were prepared based on cell sorting method using human neutrophils enrichment kit of Easy sept brand and analyzed with flow cytometry using CD177 marker.

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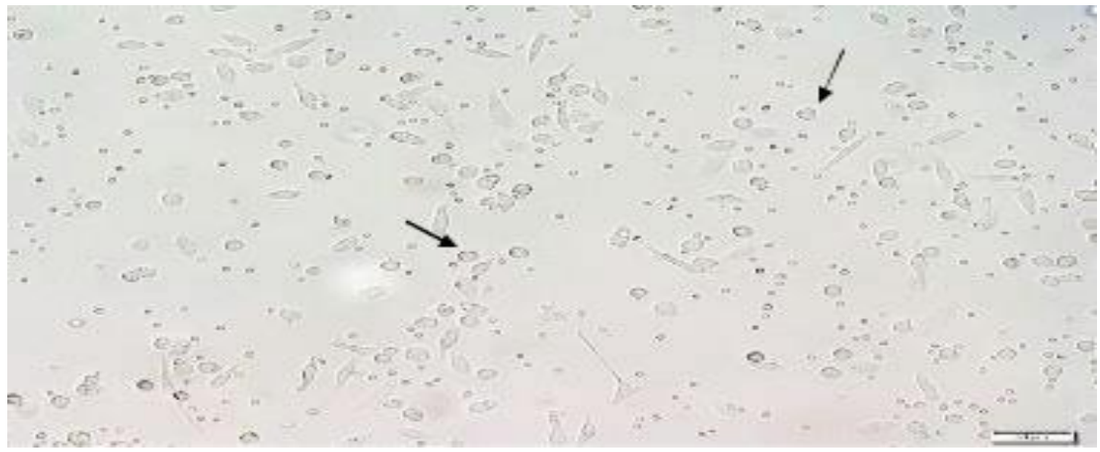

Figure 1. Neutrophil cell culture on day 1 after incubation (arrows show the possibility of neutrophil cells, but must be confirmed by further tests).

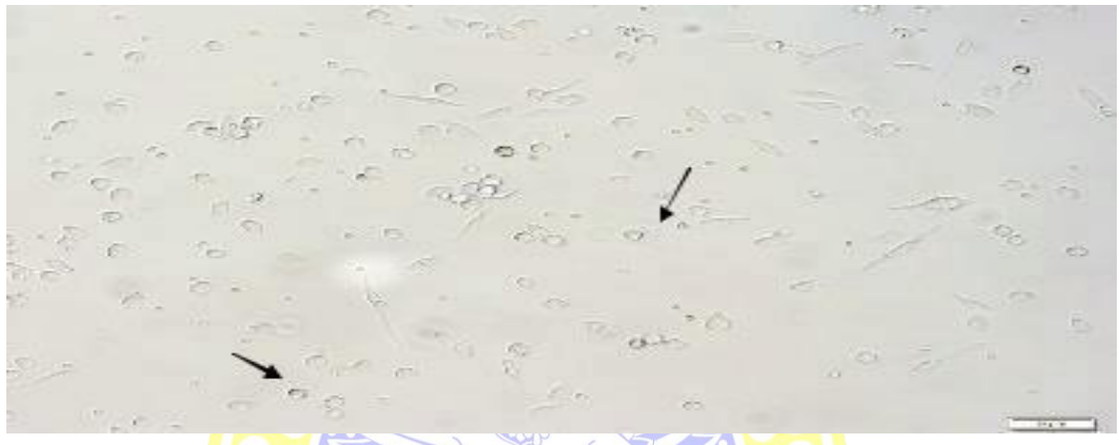

Figure 2. Neutrophil cell culture on day 3 after incubation (arrows show the possibility of neutrophil cells, but must be confirmed by further tests).

Figures 3 and 4 show the isolation of neutrophils on day 1 and 3, using flow cytometry which had previously been prepared using beads magnitude followed by manipulation using human neutrophils enrichment kit of Easy Sep brand. The results of analysis using flow cytometry show that the number of salivary neutrophils isolated in the caries-free children was 63.67% (Figure 3), while that in the S-ECC patients was 77.66% (Figure 4).

DISCUSSION

Neutrophils are important effector cells participating in innate immune response playing an important role in the first line of host defense against invading pathogens. Neutrophils isolated from the oral cavities of both the caries-free children and the S-ECC patients using magnitude beads analyzed by flow cytometry labeled with CD177. Based on the analysis, the percentage of neutrophil cells expressing CD177 in the caries-free children was 63.67%, while that in the S-ECC patients was 77.66%. This indicates that the isolation was accurate enough to get neutrophil cells of saliva since in addition to neutrophils, there are a lot of innate immunity cells in saliva, such as eosinophil, basophils, macrophage, and others.

Human body has cells that function to the defense collectively forming immune system.[11] Under normal condition, the number of neutrophils is very high in circulation, reached 60-70% of circulating leukocytes. The number of neutrophils in case of inflammation increases rapidly to more than 90%. Under the influence of several factors associated with humoral and cellular signals, the neutrophils will migrate to the site of injury or infection that serves as members of anti-pathogens and damaged cells.

After being at the site of infection, immune cells, such as neutrophils, monocytes, macrophages, dendritic cells, and mast cells are able to produce specific anti-microbial peptides, such as proteases and reactive oxygen radicals to

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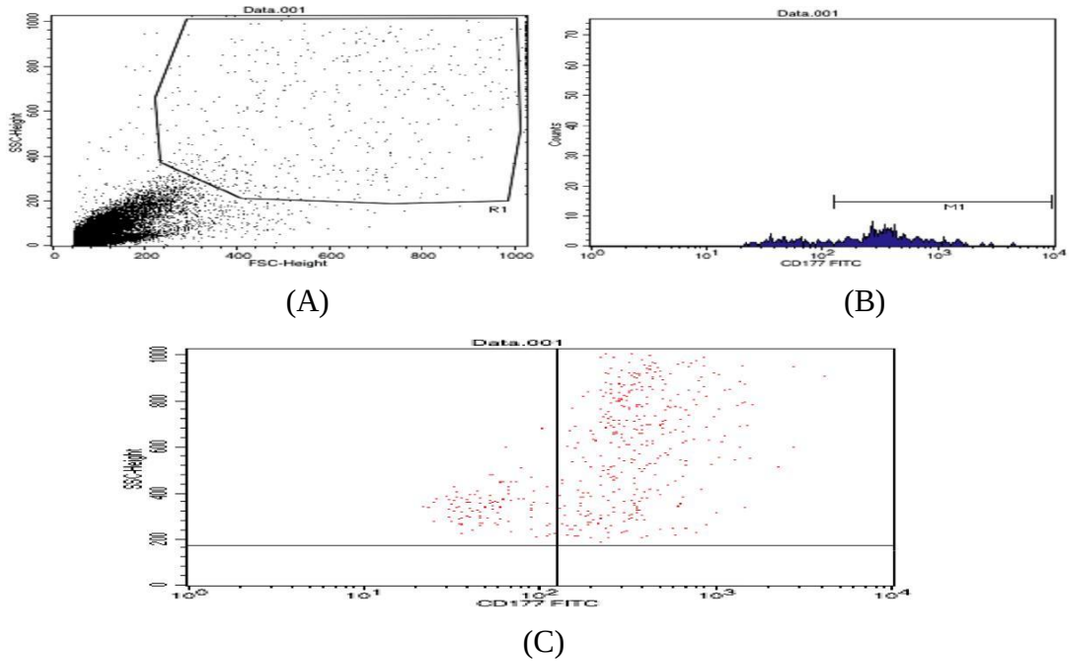

Figure 3. The isolation of salivary neutrophils using labeled magnitude beads and CD177 marker analyzed using flow cytometry in the caries-free children was 63.67%. (A) Getting neutrophils by SSC and FSC Height; (B) Histogram of neutrophils expressing CD177; (C) Neutrophils expressing CD177.

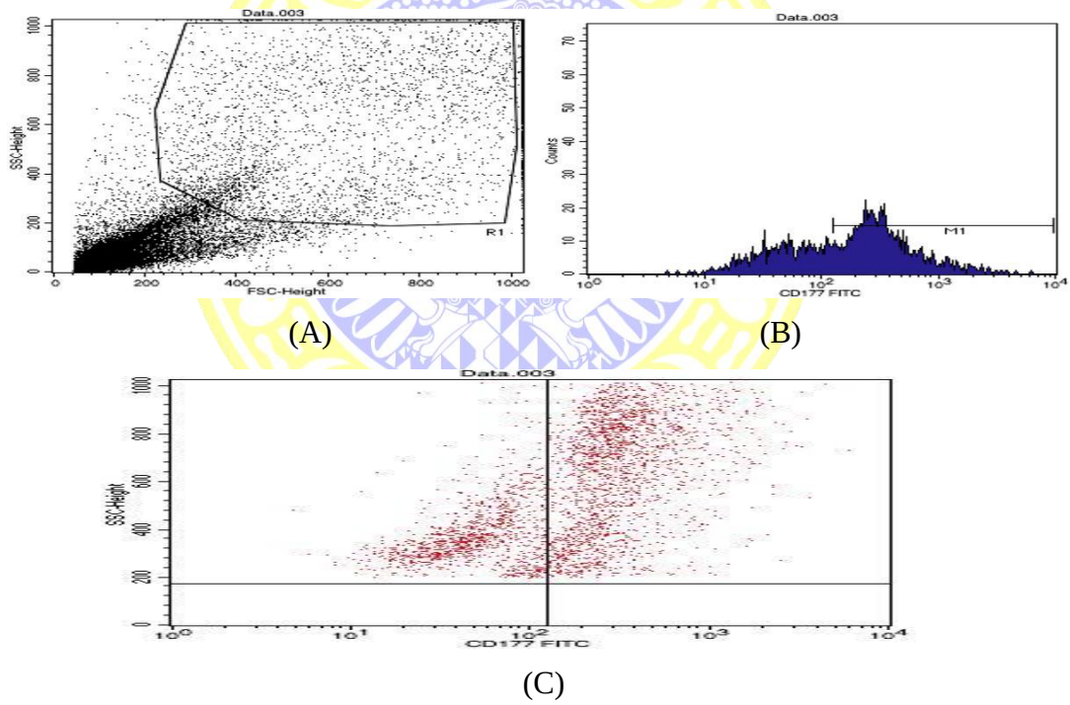

Figure 4. The isolation of salivary neutrophils using labeled magnitude beads and CD177 marker analyzed using flow cytometry in the S-ECC patients was 63.67%. (A) Getting neutrophils by SSC and FSC Height; (B) Histogram of neutrophils expressing CD177; (C) Neutrophils expressing CD177.

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facilitate the murder of microbes by disturbing bacterial membrane and metabolism.[12] In the human oral cavity, there are actually about 300 to 500 species of microbes mostly consisted of commensal and opportunistic bacteria. The relation between bacteria in the oral cavity and the host dynamically configured is considered as the balance of bacterial virulence factors and host-immune system strength.

In saliva, neutrophils are the first line of defense as the most prominent of immune cells for defense against pathogenic microbes. The importance of neutrophils in the host immune system of patients with neutropenia or defects in neutrophil function can lead to a tendency for the occurrence of serious infections.[13]

Recruitment, internal migration, phagocytosis, and activation processes of neutrophil are highly coordinated to prevent or eliminate infection in humans. In the area of infection, neutrophils bind and immerse microbes through a process, known as phagocytosis. Neutrophils recognize surface-bound or free molecules secreted by bacteria, including glycan peptide, lipoprotein, lipoteichoic acid (LTA), lipopolysaccharide (LPS), DNA containing CpG, and flagellin. This pathogen molecule is known as pathogen-associated molecular pattern (PAMPs), interacting directly with a number of pathogen recognition receptors (PRRS) expressed on the surface of cells, including toll like receptors (TLRs).[14] In conclusion, the number of neutrophils in S-ECC patients successfully isolated was 77.66%, while that in the caries-free children was 63.67%.

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