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Research Report

# Expression analysis of CD63 in salivary neutrophils and the increased level of *Streptococcus mutans* in severe early childhood caries

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***ABSTRACT***

***Background:*** *Severe early childhood caries (S-ECC) and decay exfoliation filling teeth (def-t) >6 is a destructive disease that afflicts teeth, including maxillary anterior teeth. In Indonesia, the prevalence of this disease is still high, for instance in Semarang 2007, the rate reached 90.5% in urban areas and 95.9% in rural areas for early childhood caries which is caused by Streptococcus mutans (S. mutans). Neutrophils are effector cells of innate immunity which become the main component of the very first line of defense against microbes.* ***Purpose:*** *This study analyzed the effect caused by the change of CD63 expression on the surface of salivary neutrophils and the increased level of S. mutans in S-ECC.* ***Method:*** *This study employs observational analytic and cross sectional approach by using T test analysis technique for forty cases of early childhood that had been divided into two groups, first group of twenty children positively diagnosed as S-ECC and second group of twenty children negatively diagnosed as the control group. The sample's result of gargling with 1.5% NaCl was used for neutrophils isolation and analysis function of salivary neutrophils phagocytosis by using flow cytometry test, while the sample of saliva was used to isolate S. mutans and calculate the level of S. mutans.* ***Result:*** *The expression of CD63+ salivary neutrophils in S-ECC was lower (2.32% ± 0.57) than in caries-free (2.67% ± 0.46), while the level of S. mutans showed that the level was not higher than in S-ECC (9.78 ± 2.22)x105 CFU/ml compared to in caries-free (5.13 ± 1.86)x105 CFU/ml.* ***Conclusion:*** *The low expression of CD63 in salivary neutrophils can lead to the increased level of S. mutans in S-ECC.*

***Keywords:*** *Salivary neutrophils; Streptococcus mutans; S-ECC*

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## INTRODUCTION

Early childhood caries (ECC) is caries experienced by younger children and is a serious problem in all over the world, particularly in developing countries.[1] The prevalence of dental caries occurred to children from minority ethnics in China is very high, such as the prevalence in Zhuang, Bonan, Dai, Dongxiang, Korea, Tibet.[2] If ECC is not treated seriously, it can thrive and cause dental caries on the entire teeth in a short period of time which is known as severe early childhood caries (S-ECC), it will affect the physical and mental health[3] and it will increase the risk for the subsequent caries on permanent teeth[4] that will not be able to handle by a mere restorative treatment.

The classical etiology of ECC involves bacteria, diet, and host affected by the interaction of sociological and environmental factors, while the existence of cariogenic microbes, the frequency of consuming foods and drinks, oral hygiene, educational level of parents, family income, knowledge of oral health and the child's behavior is proven to be the main cause in ECC.[4]

In the recent years, the role of neutrophils has changed dramatically in which neutrophils become the main component of the first line of defense against microbes.[4]

Neutrophils do not only act as microbes exterminator with phagocytosis, releasing reactive oxygen species (ROS) and antimicrobial peptides but also as a regulator to the activation of immune response.[5] Neutrophils also evidently produce cytokine, chemokine and growth factor until become the main contributor in the pro-inflammatory cytokine production on the infected areas.[6]

The important function of neutrophils in exterminating microbial pathogens is phagocytosis, which significantly more effective due to the opsonization process by antibody and complement on the microbial surface. Phagocytosis on microbes can generate oxidative burst process to produce reactive oxygen species (ROS) with degranulation of cytoplasmic granules in phagosome contains antimicrobial peptides and proteases comprised microbes.[7] Azurophilic granule (primary granule) contains antimicrobial proteins such as defensin, elastase, cathepsin dan proteinase-3 and also contains CD63 in its membrane. *Streptococcus mutans* (*S. mutans*), the bacteria that cause caries, can activate neutrophils host until it produces antimicrobial peptide (AMPs) in the form of human neutrophil peptide (HNP) 1-3. Besides functioning as antimicrobial, it also acts as chemoattractant and immunomodulatory. HNP 1-3 AMPs function as natural antibiotics which give the first line of defense with wide spectrum of various bacteria.[8]

Various preventions of dental caries have been done, for example by brushing the teeth properly, fluoridation by topical application, and vaccines manufacturing that still has not shown any expected results until today.[9] Therefore, this study was aimed to analyze the change of expression of CD63 salivary neutrophils as the effector cells of innate immunity towards the increased level of *S. mutans* in S-ECC.

## MATERIALS AND METHODS

The sample of this study was obtained from saliva and gargling result with 1.5% NaCl of kindergarten children aged 4 to 6 years old in Surabaya. Examination of dental caries was done in advance by measuring the def-t index, and then the subjects were divided into two groups: caries-free group and S-ECC group with def-t index higher than 6. Before the sample was taken, the questionnaire were distributed and the inform consent were signed by the parents respectively.

The sample was taken from the saliva without stimulation as much as 2 ml by using expectorate within the falcon tube 5 ml during school hours between 08.00 up to 10.00 a.m. to determine the level of *S. mutans* and 5 minutes later the children were instructed to gargle with 1.5% NaCl which then accommodated in the 50 ml falcon tube to determine the expression of CD63 on the surface of salivary neutrophils. The sampling was done by the researcher and the trained personnel using standard protocol. The subjects of this study were not allowed to eat, drink, chew gum, or brush their teeth for 60 minutes before the sampling. After the sample was collected, it was frozen at -80°C to be analyzed.[10]

*S. mutans* isolation was done by taking saliva sample from preschool children identified either as severe caries (def-t >6) or caries-free performed in the following instructions: biochemical isolation and characterization from *S. mutans*. Saliva sample then were diluted in brain heart broth (BHI), after incubated for 24 hours, sample was planted on gelatin medium triptone yeast cystein (TYC). The colonies assumed as *S. mutans* then were sub-cultured to be biochemically tested by using mannitol fermentation, raffinose, sorbitol, salicin, esculin and arginine.

Isolates were identified as *S. mutans* when it is positive to mannitol fermentation, raffinose, sorbitol, salicin, esculin and is negative to arginine and subsequently is confirmed by Gram staining and negative catalase test. Isolates of *S. mutans* were stored at -80°C.10 Based on its morphology, all colonies of *S. mutans* on gelatin TYC were calculated using the formula: number of colonies x dilution factor x 50 (1 ml volume) = CFU/ml with minimum detection level 1 x $10^3$ CFU/ml.

Profile measurement of neutrophil cells employed CD63 antibody which marks the active neutrophils. CD63 within primary granule membrane were expressed on the surface of neutrophils membrane because of azurophilic granule fusion and plasma membrane which increased due to the stimuli given to neutrophil cells in which the signs can be measured with flow cytometry using the method modified by Bjornsson.[11] The sample used was the result of gargling with 1.5% NaCl which its neutrophils had been isolated. The suspension of isolated neutrophil cells then was inserted into microtube filled by 500µl PBS. The cell suspension was then centrifuged at 2500rpm speed, for 5 minutes at 4° C temperature. The pellets obtained were subsequently stained with extracellular antibodies 50µl (Biolegend antihuman α-CD63PE), and Biolegend α-PI PE conjugated with antibody ratio: PBS is 1:200.

Cells that have been added with antibody were then stored at 4° C temperature for 30 minutes. The suspension cells were then added with 1ml PBS and were centrifuged at 2500rpm speed, for 5 minutes at 4° C temperature. Biolegend Cytofix Cytoperm was then added to the pellets as much as 100µl and was homogenized until it well blended. The incubation was done subsequently at 4° C temperature without light for 20 minutes. After incubation, cells were then added with 1mL Biolegend Washperm once and subsequently were centrifuged at 2500 rpm speed, for 5 minutes at 4°C temperature. The obtained pellets were then coupled with intracellular antibodies, including BD antihuman α-CD64 PerCP conjugated, and then suspension cells were inserted into cuvette flow cytometer, and then added with PBS as much as 300µl, and mounted on nozzle BD FACS Calibur to do running with flow cytometer machine. The sample was then analyzed by flow cytometry (FACS Calibur flow cytometry, BD Bio Sciences, San Jose, CA).

Neutrophil gate was identified by density and size with side angle light scatter and then continued with forward angle light scatter. This compensation was achieved by employing FITC and PE labeled with individual antibodies.

The result was shown as mean fluorescence intensity (MFI). FACS Calibur of Becton Dickinson with Cell Quest software Program was used for the analysis.

## RESULTS

The result of *S. mutans* number calculation from S-ECC group and caries-free group using colony counter and the result of activated salivary neutrophils (CD64+) analysis showed that CD63+ was expressed in ECC-free and S-ECC.

The calculation result of the number of *S. mutans* saliva from Triptone Yeast Cystein Gelatin medium using colony counter tested by t 2 independent samples showed the significance value smaller than α. This means that there were significant differences in the *S. mutans* numbers between the two groups. Based on the mean value, it was known that the number of *S. mutans* in caries-free children ($5.14 \times 10^5 \pm 1.86 \times 10^5$ CFU/ml) was significantly lower than in children with severe caries ($9.78 \times 10^5 \pm 2.23 \times 10^5$ CFU /ml) (Table 1 and Figure 1).

The result of analysis using flow cytometry activated salivary neutrophils that express CD63+ after given comparative test using t 2, independent sample showed that the significant value was lower than α. This means that there was a significant difference in CD63+ expression between the two groups. Based on the mean value, it was confirmed that salivary neutrophils that expresses CD63+ in ECC-free was higher (2.67% ± 0.46) than in S-ECC (2.32% ± 0.57) (Table 2 and Figure 2).

## DISCUSSION

ECC is a multifactorial disease that occurs as a result of a series of interactions between vulnerable hosts, cariogenic bacteria, cariogenic diet and behavior. Dental caries is not caused by exogenous bacteria, but is caused by the irregularities in ecology so that commensal oral bacteria become pathogenic after the disruption of the immune system and homeostasis of the body which later develop into dental caries. The important role in the homeostasis of the oral cavity and the prevention of dental caries depends on the content of immune component in saliva.[12]

*S. mutans* have integral role as the etiology regarding to the occurrence of ECC which is an infectious and contagious disease,[13] so that *S. mutans* is considered an important predictor as cariogenic bacteria because it is acidogenic (able to produce acid) and aciduric (able to survive in acidic environment).[14]

In saliva, neutrophils are the most prominent first line of defense from the immune cells for defense against microbial pathogens. The importance of neutrophils in host immune system in patients with neutropenia or defect in neutrophils function leads to the tendency for serious infection to happen.[15]

Neutrophils recruitment process, transmigration, phagocytosis, and activation are highly coordinated to prevent or eliminate infection in human. In the infected area, neutrophils bind and engulf microbes through a process known as phagocytosis. Neutrophils recognize

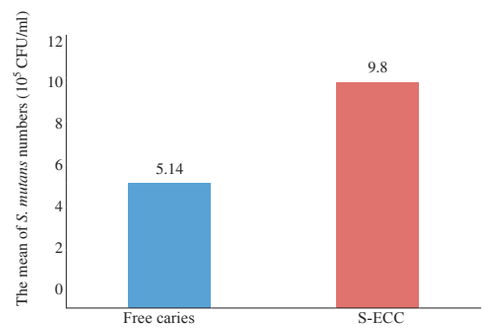


**Figure 1.** The mean and standard deviation of S. mutans numbers on saliva calculated by colony counter in S-ECC and caries-free ($10^5$ CFU/ml).

**Table 1.** The mean and standard deviation of the number of S. mutans in saliva calculated by colony counter in S-ECC and caries-free ($10^5$ CFU/ml)

| Groups | N | mean ± standard deviation | 95% CI | p Value |
|---|---|---|---|---|
| Caries-free | 20 | 95.13 ± 1.86 | 394.789,36–632.210,64 | p < 0.0001 |
| S-ECC | 20 | 9.78 ± 2.22 | 834.661,22 –1.119.338,78 | (p < α) |

**Table 2.** The mean and standard deviation of activated salivary neutrophils (CD64+) that express CD63+ in ECC-free and S-ECC (%)

| Groups | N | mean ± standard deviation | 95% CI | p Value |
|---|---|---|---|---|
| Caries-free | 20 | 2.67±0.46 | 2.37 – 2.96 | p < 0.040 |
| S-ECC | 20 | 2.32±0.57 | 1.96 – 2.68 | (p < α) |

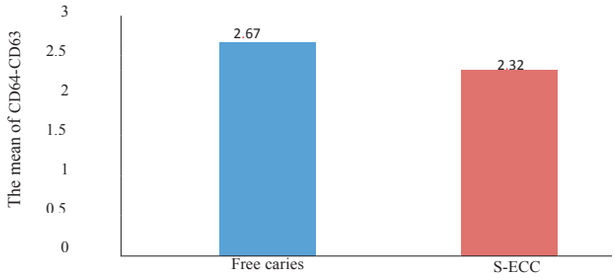


**Figure 2.** The mean and standard deviation of activated salivary neutrophils (CD64+) that express CD63+ in ECC-free and S-ECC (%).

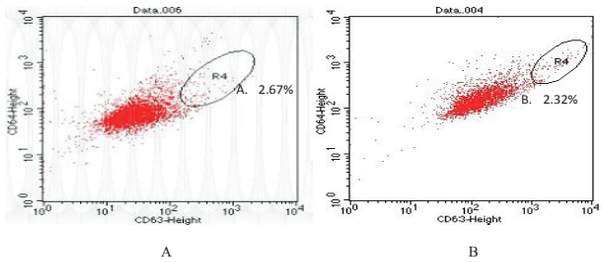


**Figure 3.** Activated salivary neutrophils (CD64+) that expresses CD63+ detected by flow cytometry in ECC-frees (A) and in S-ECC (B).

the bound-surface or free molecule secreted by bacteria, including peptidoglycan, lipoprotein, lipoteichoic acid (LTA), lipopolysaccharide (LPS), CpG containing DNA, and flagellin. This pathogenic molecule is known as pathogen associated molecular pattern (PAMPs), interacts directly to a number of pathogen recognition receptors (PRRs) which is expressed on the surface of cells, including toll like receptors (TLRs).[16] *S. mutans* as the main etiology agent of ECC because it has several mechanisms to colonize on the tooth surface and under particular condition to be cariogenic species which signifies the highest within the biofilm environment of oral cavity;[17] thus, indicating the existence of causal link between dental caries and the high number of *S. mutans*. Several studies suggested that the development of dental caries is preceded by an increase in colonization of *S. mutans*,[12] whereas the other researchers said that the increased level of *S. mutans* in saliva is an indication of the increased risk of dental caries.[18,19]

Phagocytosis is a process which is mediated by active receptor, the internalization of cell to the microbes and is subsequently followed by the rearrangement of cytoskeletal, the enlargement of neutrophil plasma membrane around the microbes and the formation of membrane-bound vacuoles called phagosome. In phagosome neutrophils release a variety of antimicrobial proteins and intracellular enzymes that function to kill microbes.

Primary granules (azurophilic) contains many antimicrobial compounds, such as myeloperoxidase (MPO), defensin like human neutrophil peptide 1-3 (HNP 1-3), lysozyme, azurocidin, and serine proteinase elastase, cathepsin G, proteinase 3, esterase N. Azurophilic granules are the one that associated with phagocytic vesicles which then release the content in phagosome which contains phagocytized microbes.[20] Neutrophil proteins in the primary granules (azurophilic) are alarmins which is a molecule that can activate antigen precenting cells (APC) and stimulate innate dan adaptive immunity responses.[21]

Based on the results of this study (Table 1) of salivary neutrophils suggested that the expression level of CD63+ in S-ECC was lower than the expression level of CD63+ in caries-free children with the average value in S-ECC is lower (2.32% ± 0.57) than the expression level of CD63+ in caries-free children (2.67% ± 0.46). There is a chance of the low expression level of CD63 in salivary neutrophils in S-ECC is caused by *S. mutans* which have been internalized by neutrophils through a phagocytosis process that mediated through FcαR (CD89) or CR1 (CD35) may be able to develop three strategies of defense system to avoid intracellular killing, firstly, escaping out of phagosome, secondly, blocking the fusion of phagosome-lisosome, and thirdly, using a mechanism that allows survival in phagolysosomes. There is also a chance of the low expression level of CD63 in salivary neutrophils in S-ECC is caused by the deficiency of proteins elastase and cathepsin G.[22] Less active neutrophils will release fewer neutrophil extracellular traps (NETs) that work to kill extracellular microbes because it contains lactoferrin, cathepsin and enzymes which are highly toxic for microbes. In addition, NETs also facilitate the phagocytosis process.[23,24] *S. mutans* level in S-ECC is higher than *S. mutans* level in caries-free maybe because of the pathogenic *S. mutans* is not optimal by removed.

It can be concluded that the low expression of CD63 in salivary neutrophils can lead to cause the increased level of *S. mutans* in S-ECC.

## REFERENCES

1. Bagramian RA, Garcia-Godoy F, Volpe AR. The global increase in dental caries. A pending public health crisis. Am J Dent 2009; 22(1): 3–8.
2. Zhang S, Liu J, Lo EC, Chu CH. Dental caries status of Dai preschool children in Yunnan Province, China. BMC Oral Health 2013; 13: 68.
3. Isaksson H, Alm A, Koch G, Birkhed D, Wendt LK. Caries prevalence in Swedish 20-year-olds in relation to their previous caries experience. Caries Res 2013; 47(3): 234–42.
4. Wigen TI, Wang NJ. Caries and background factors in Norwegian and immigrant 5-year-old children. Community Dent Oral Epidemiol 2010; 38(1): 19–28.
5. Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Review Immunol 2006; 6(3): 173-82.
6. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol 2011; 11(8): 519-31.
7. Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. Neutrophils in the innate immune response. Arch Immunol Ther Exp. 2005; 53(6): 505-17.

8. Yang D, Biragyn A, Hoover DM, Lubkowski J, Oppenheim JJ. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophilderived neurotoxin in host defense. Annu Rev Immunol 2004; 22: 181–215.
9. Trigg TE, Wright PJ, Armour AF, Williamson PE, Junaidi A, Martin GB, Doyle AG, Walsh J. Use of a GnRH analogue implant to produce reversible Longterm suppression of reproductive function in male and female domestic dogs. J Reprod Fertil Suppl. 2001; 57: 255-61.
10. Phattarataratip, E. The role of salivary antimicrobial peptides in shaping Streptococcus mutans ecology. Annu Rev Microbiol 2010; 57: 677-701.
11. Borjesson DL, Kobayashi SD, Whitney AR, Voyich JM, Argue CM, Deleo FR. Insights into pathogen immune evasion mechanisms: Anaplasma phagocytophilum fails to induce an apoptosis differentiation program in human neutrophils. J Immunol 2005; 174(10): 6364–72.
12. Tanzer JM, Livingston J, Thompson AM. The microbiology of primary dental caries in humans. J Dent Educ 2001; 65(10): 1028-37.
13. Thakur AS, Acharya S, Singhal D, Rewal N, Mahajan N, Kotwal B. A comparative study of Mutans Streptococci and Lactobacilli in mothers and children with early childhood caries (ECC), severe early childhood caries (S-ECC) and caries free group in a low income population. Oral Health Dent Manag 2014; 13(4): 1091-5.
14. Tinanoff N, Reisine S. Update on early childhood caries since the Surgeon General's Report. Acad Pediatr 2009; 9(6): 396-403.
15. Rosenzweig SD, Holland SM. Phagocyte immunodeficiencies and their infections. J Allergy Clin Immunol 2004; 113(4): 620–6.
16. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004; 4(7): 499–511.
17. Ramamurthy PH, Swamy HS, Bennete F, Rohini M, Nagarathnamma T. Relationship between severe-early childhood caries, salivary Mutans Streptococci, and lactobacilli in preschool children on low socioeconomic status in Bengaluru city. J Indian Soc Pedod Prev Dent 2014; 32(1): 44-7.
18. Beighton D. The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent Oral Epidemiol 2005; 33(4): 248–55.
19. Corby PM, Lyons-Weiler J, Bretz WA, Hart TC, Aas JA, Boumenna T, Goss J, Corby AL, Junior HM, Weyant RJ, Paster BJ. Microbial risk indicators of early childhood caries. J Clin Microbiol. 2005; 43(11): 5753–9.
20. Skubitz KM. Neutrophilic leukocytes. In: Greer JP, eds. Wintrobe's clinical hematology. 12th ed. Philadelphia: Lippincott Williams and Wilkins; 2009. p. 170-213.
21. Kobayashi SD, DeLeo FR. Role of neutrophils in innate immunity: a systems biology-level approach. Wiley Interdiscip Rev Syst Biol Med 2009; 1(3): 309–33.
22. Urban CF, Lourido S, Zychlinsky A. How do microbes evade neutrophil killing?. Cell Microbiol 2006); 8(11): 1687–96.
23. Phillipson M, Kubes P. The neutrophil in vascular inflammation. Nat Med 2011; 17(11): 1381–90.
24. Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. Trends Immunol 2009; 30(11): 513–21.