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Acid pH of Saliva in Dental Caries of Pre-school Children repressed phagocytic capacity of neutrophil

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ABSTRACT

In Indonesia dental caries is one of the most important issues in children's health. Caries is one of common dental diseases in children who consume sugar-rich diets. Caries cause decalcification of tooth enamel and dentin. The bacteria involved in caries can converge on the dental pulp tissue and spread to other organs. Under adverse conditions, complications such as phylogenetic osteomyelitis and bacterial endocarditis can occur. This study aimed to investigate the effects of oral cavity pH on neutrophil and lymphocyte cell activity in order to eliminate *S. mutans*. Neutrophil and lymphocyte cells were isolated from the saliva of 30 preschool children, with and without caries, from the city of Surabaya, Indonesia. The dental condition was verified by a dentist based on the def-t index. Obtained results indicate that the number of activated neutrophils (CD11b⁺CD35⁺) from children in the caries group was significantly higher than the caries-free group at an acidic pH, but was unchanged at an alkaline pH. The activated neutrophils triggered naïve T cells to become effector T cells (CD4⁺), which produce cytokines to respond the infection. However, the increased CD11b⁺CD35⁺ were unable to enhance the phagocytic activity of neutrophils in the caries group, especially in acidic pH conditions. An acidic pH was found to repress the phagocytic capacity of neutrophils. This study provides a basis for future strategies to prevent dental caries by promoting phagocytic activity and maintaining a neutral pH in the oral cavity.

INTRODUCTION

Dental caries results from tooth decay due to the activity of pathogenic microbes in the oral cavity, and most cases occur in children aged between 4–6 years. *Streptococcus mutans* is the primary microorganism that causes dental caries (Fekrazad *et al.*, 2017; Peterson *et al.*, 2013; Jiang *et al.*, 2017). *S. mutans* has both acidogenic and acidophilic characteristics, which allow it to survive and dominate in the oral cavity (Lemos *et al.*, 2008; Nekoofar *et al.*, 2009). The bacteria initiate biofilm formation and stimulate the migration of neutrophil cells and an inflammatory response (Kwiatkowska *et al.*, 1999). Neutrophils eliminate the bacteria by phagocytosis via the Fc receptor, which recognizes

opsonized antigens by either the immunoglobulin (CD89) or complement (CD35) receptors (Garcia *et al.*, 2002; Manolea *et al.*, 2009; Robinson *et al.*, 1995). The CD89⁺ and CD35⁺ cells have many roles in phagocytosis. However, the effect of extracellular pH on phagocytosis remains unclear.

S. mutans triggers an adaptive immune response including CD4⁺ and CD8⁺ T lymphocytes (Hurlbutt *et al.*, 2010). The adaptive immune response begins when dendritic or neutrophil cells present *S. mutans*-derived peptide fragments to naïve T cells, which are then activated to become effector T cells (CD4⁺ or CD8⁺). Furthermore, *S. mutans* produce metabolites that increased the acidity of the oral cavity to evade the immune system. As the homeostasis of immune cells depends on pH, an acidic environment in the oral cavity may inhibit the ability of neutrophil and lymphocyte cells to eliminate *S. mutans*. Therefore, the aim of this study was to investigate the role of oral cavity pH on the phagocytic capacity of neutrophils and the activation of T cells, and their ability to eliminate *S. mutans*.

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MATERIALS AND METHODS

Sample collection

A total of 30 saliva samples were obtained from preschool children aged 6 years in Surabaya, Indonesia. Samples were separated into two groups, 15 from children with severe caries and 15 from caries-free children, as identified by a dentist based on the def-t index. One hour before saliva sampling, subjects were not allowed to eat, drink, chew gum or brush their teeth. Salivary samples were taken between 9:00 to 11:00 A.M., with consideration for circadian rhythm. This study was approved by the Ethics Committee of Brawijaya University.

Streptococcus mutans isolation

Streptococcus mutans were obtained from the Laboratory of Microbiology in the Faculty of Medicine at Brawijaya University. The *S. mutans* were grown in brain heart infusion broth for 48 hours in an anaerobic jar, then harvested by centrifugation at 12000 rpm for 10 minutes at 4°C. The pellet was washed with phosphate buffered saline (PBS) and stained with 50 µL carboxyfluorescein diacetate succinimidyl ester in 1000 µL PBS, then incubated at 37°C for 60 minutes in the dark. After incubation, the suspension was washed twice with PBS. The *S. mutans* pellet was resuspended in PBS and then killed by heating at 60°C for 60 minutes. The bacteria was stained using the fluorescent marker carboxyfluorescein diacetate succinimidyl ester (CFSE), and the stained bacteria were then used to examine the phagocytic capacity of neutrophils.

Neutrophil isolation

Neutrophil cells were collected from the saliva by centrifugation at 450×g for 15 minutes at 4°C. The cell pellet was mixed with 2 mL RPMI complete medium (supplemented with 10% Fetal Calf Serum) and filtered using a Millipore filter. The number of viable cells was determined using trypan blue staining, after which cells were counted using a hemocytometer. Three million cells were grown in RPMI medium on culture plates until the assay.

Determination of the phagocytic capacity of neutrophils

CFSE-stained bacteria (*S. mutans*) were added to wells containing neutrophils and incubated for 90 minutes at 37°C in 5% CO₂. The cells were then harvested by centrifugation at 2500 rpm for 5 minutes at 4°C. The pellet was stained with PE anti-human CD89 antibody (BioLegend, San Diego, CA, USA). The stained cells were pipetted into a cuvette for flow cytometry and 300 µL PBS was added. The phagocytosis of neutrophils (CFSE⁺ CD89⁺) was measured using a flow cytometer using a standard method of BD FACS Calibur.

Analysis of neutrophil surface molecules

The expression of CD66b, CD89 and CD35 molecules on the neutrophil surface, related to their phagocytosis activity, were detected by staining cells with FITC anti-human CD66b, PE anti-

human CD89 and PE anti-human CD35 antibodies (BioLegend). The stained cells were measured using flow cytometry following standard methods of BD FACS Calibur.

Isolation and profile of lymphocyte cells

Lymphocytes were obtained from the saliva samples. Lymphocyte cells were detected using FITC anti-human CD4, PE anti-human CD62L and PE anti-human CD8 antibodies (BioLegend). The stained cells were measured using flow cytometry following the standard method of BD FACS Calibur manufacturer's instructions.

Data analysis

Neutrophil and lymphocyte cell populations were identified using standard flow cytometry methods from BD FACS Calibur. The relative numbers of all parameters were analyzed using BD CellQuest software. All data were statistically analyzed using an independent t-test in SPSS version 16.

RESULTS

This study showed that the phagocytosis ability of neutrophils (CFSE⁺CD89⁺) in children with dental caries was significantly lower than in children without caries (Fig. 1). The decreased phagocytosis observed in the dental caries group may have been caused by an acid pH in the oral cavity due to metabolites derived from *S. mutans*. Therefore, we evaluated the effect of pH on neutrophil activity. We found that the relative phagocytic activity of neutrophil cells (CFSE⁺CD89⁺) was lower at an acidic pH compared to an alkaline pH. A decrease in extracellular pH reduces oxygen consumption by 80–90%, which is implicated in superoxide production and neutrophil activity (Lardner, 2001; Simchowitz, 1985; Brekke *et al.*, 2007). Furthermore, we investigated the number of activated neutrophils by measuring the number of CD11b⁺CD35⁺ and CD66b⁺CD89⁺ cells. The relative number of neutrophils that expressed the CD11b⁺CD35⁺ complement receptor was significantly higher in the caries group compared to the caries-free group at an acid pH but did not significantly differ at an alkaline pH. The greatest number of activated neutrophils were found in the caries group at an acidic pH. However, the relative number of neutrophils that expressed the immunoglobulin G (IgG) Fc receptor II (CD66b⁺CD89⁺) was lower in children with caries than caries-free children at both an alkaline and acidic pH (Fig. 2).

In children with caries, the relative number of naïve CD4⁺CD62L⁺ cells was significantly lower than that of free caries children in both acidic and alkaline saliva (Fig.3 A). It is fascinating that CD4⁺ T cells isolated from alkaline saliva reach to 60% of total cells, whereas in children with caries reach to 30% of the total cell. This shows the importance role of CD4⁺ T cells protecting children from caries in alkaline saliva. In children with acidic saliva showed that the relative number of CD4⁺ T cells did not show any difference. In contrast to CD4 T-cells, CD8 T cells showed no difference in both caries and caries-free children. It is interesting that CD4 T-cells can enter saliva 600 times greater in caries free children with alkaline saliva (Fig. 3 B and C).

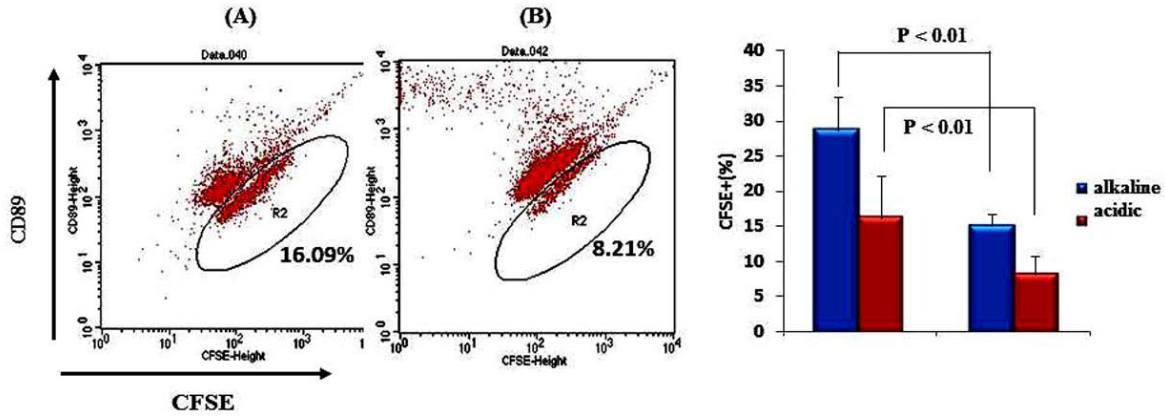


Fig. 1: The phagocytic activity of neutrophils was disrupted by an acidic saliva pH. (A) Neutrophil of caries free children can engulf *S. mutans* (CFSE⁺CD89⁺) (16.09%). (B) Neutrophil of children with dental caries can engulf *S. mutans* (CFSE⁺CD89⁺) (8.21%). The number of neutrophil that engulf *S. mutans* (CFSE⁺CD89⁺) in children with dental caries was significantly lower compare to that of caries free children. The lowest phagocyte activity of neutrophils was observed in children with dental caries with an acidic saliva pH (right panel).

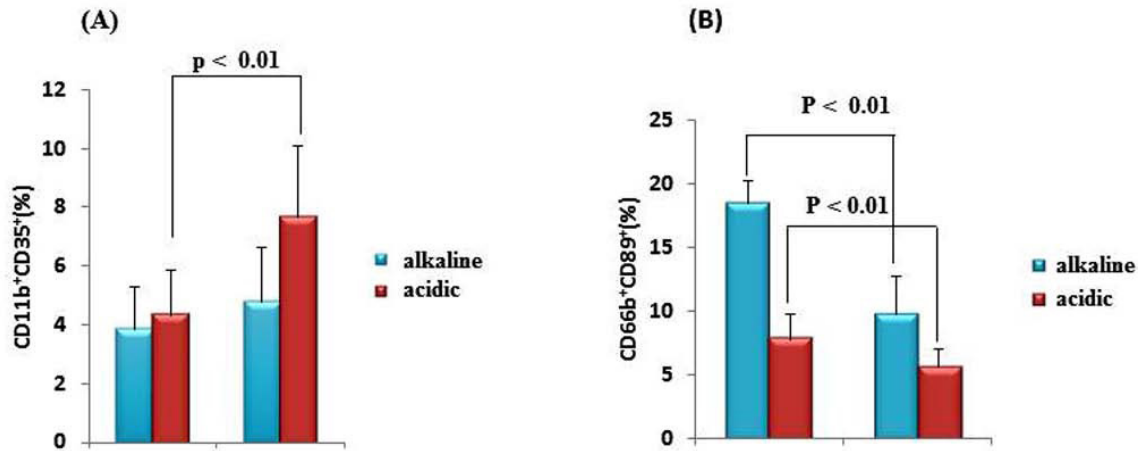


Fig. 2: An acidic oral cavity increased the number of activated neutrophils and reduced the number of neutrophils that expressed immunoglobulin. (A) Children with dental caries with acidic saliva had the highest relative number of neutrophils that expressed the complement receptor (CD11b⁺CD35⁺) as activated neutrophils. (B) The number of neutrophils that expressed the immunoglobulin G (IgG) Fc receptors II (CD66b⁺CD89⁺) was lower in children with caries than in children without caries.

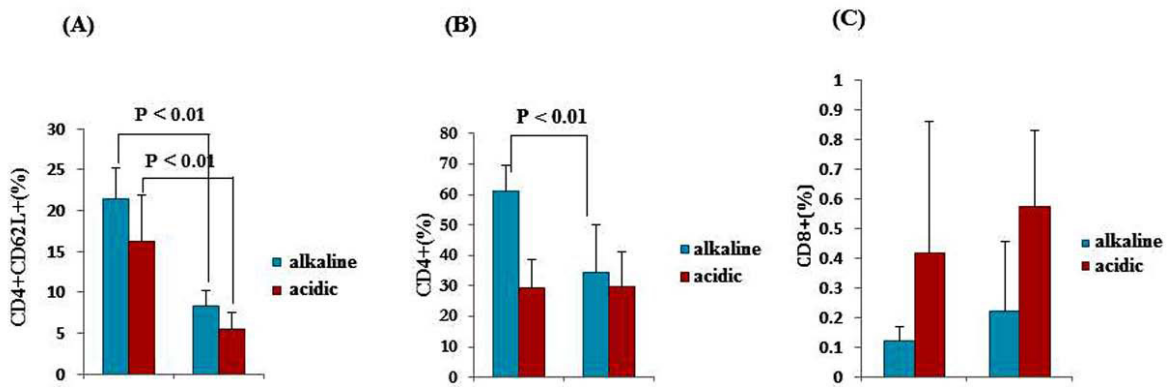


Fig. 3: (A) The relative number of naïve T cells (CD4⁺CD62L⁺) was decreased in children with caries in either acidic or alkaline saliva. (B) The number of activated T cells increased in alkaline saliva of caries free children. (C) Percentage of cytotoxic T cells (CD8⁺) at an acidic and alkaline saliva.

DISCUSSION

The results obtained in this study suggest that the neutrophils were activated by complement proteins rather than immunoglobulin activity. The decreased number of CD66b⁺CD89⁺ cells may have been caused by the abundance of immunoglobulin in the oral cavity (Omar *et al.*, 2011). The interaction of CD89 with immunoglobulin results in phagocytosis (Simchowitz *et al.*, 1985; Yin *et al.*, 2007; Rogers *et al.*, 2004; Stockmeyer *et al.*, 2000). Phagocytosis plays a significant role in the host-dependent mechanisms to eliminate infectious pathogens via the process of engulfment (Jiang *et al.*, 2017). In addition, acidic conditions activate the complement C3b protein which opsonizes microbes. These can then be recognized by neutrophil cells via the complement receptor (CD35⁺), thereby mediating the ingestion of C3b-coated particles by phagocytes (Yin *et al.*, 2007; Rogers *et al.*, 2004). Therefore, although the number of cells expressing the complement receptor (CD11b⁺CD35⁺) was increased, there was no corresponding increase in phagocytic activity of the neutrophils. This phenomenon indicates that an acidic pH disrupts the phagocytosis mechanism of neutrophils, even though these cells highly expressed complement receptors.

Neutrophils may present the *S. mutans* peptide which activates naïve T cells to become effector or memory T cells. The data indicated that children with dental caries had a decreased number of naïve T cells (CD4⁺CD62L⁺) at both an alkaline and acidic pH. However, the lowest number of naïve T cells was observed in the acidic pH group. Furthermore, we also observed the status of activated T cells and found that the number of CD4⁺ (Th) cells in children with dental caries were significantly decreased in alkaline conditions when compared to caries-free children (Fig. 3B). Nevertheless, the number of CD4⁺ cells in children with and without dental caries were similar at an acidic pH. In addition, the number of CD8⁺ (Tc) cells was not significantly affected by the presence of caries and the pH. However, the relative number of CD8⁺ T cells were lower than CD4⁺ T cells. Immunohistologic studies of dental caries are known that CD8 cells increase but CD4 cells and B cells appear in substantial numbers. This result is consistent with a previous study which reported that the number of CD8⁺ cells was less than CD4⁺ cells in human dental pulp, while T and B lymphocytes occasionally formed some clusters (Castellano *et al.*, 2000; Angelova *et al.*, 2004; Simsek *et al.*, 2005; Hahn *et al.*, 2000).

This study found that the presence of dental caries controls the activation of naïve T cells to become effector T cells (CD4⁺), which is associated with the pH of the environment. Impaired development of effector T cells from naïve T cells may be due to high amount of regulatory T cells, CD4⁺CD25⁺. In general, regulatory T cells have the power of suppressing and preventing the immunocompetent cells from activation. There is evidence that the balance of Th1 and Th2 in children with caries did not differ significantly with healthy children. This indicates that the T cell cytokines did not predominantly involve (Roa *et al.*, 2008; Rifa'i *et al.*, 2013; Lee *et al.*, 2011, Hahn *et al.*, 2000). There were reports that children with caries have IgA titers higher than a healthy child, this will be evident that B cells are involved in order to prevent children from caries (Saito *et al.*, 2001). The composition of fimbria on *S. mutans* in children with caries differs

from children with caries. This shows that the adhesion of bacteria to the host determines the occurrence of caries (Perrone *et al.*, 1997).

The decrease in the number of naïve T cells is generally caused by a change of status into activated or memory cells (Rifa'i, 2013; Lee *et al.*, 2011). In alkaline saliva of children who are free from caries, the amount of CD4 T-cell is known more than children with caries. We suppose that CD4 T cells have better survival than CD8 T cells against alkaline conditions. Thus CD4 T cells that are homing in the area around the salivary gland have a higher chance to penetrate the salivary fluid (Fig. 3B).

CONCLUSIONS

An acidic pH, associated with the presence of dental caries, increased the expression of complement receptors (CD11b⁺CD35⁺) on neutrophils, in addition to activating naïve T cells to become effector T cells (CD4⁺). Therefore, elevated CD11b⁺CD35⁺ cells were unable to enhance phagocytic activity. The phagocytic activity was found to be influenced by saliva pH, rather than an elevated expression of complement receptors.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

ETHICAL APPROVAL

This study involved human participants and was approved by the Ethics Committee of Brawijaya University.

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