

Original Research Paper

Immunologic Aspect of Mice (*Mus musculus*) Dental Caries Induced by *Streptococcus mutans*

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Article history

Received: 22-07-2016

Revised: 19-01-2017

Accepted: 16-03-2017

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Abstract: Dental caries (tooth decay), cavities or caries is a breakdown of teeth due to activities of bacteria acid producing in the presence of fermentable carbohydrates. The most responsible bacteria for dental cavities are *Streptococcus mutans*. Dental shape, saliva pH, normal flora, diets were suggested as predisposition factors for dental plaque and caries. The aim of this research was to study immunologic response in dental caries induced by *S. mutans*. This research was experimental study, using post-test control and complete random design. Eighteen mice (*Mus musculus*) with 18 weeks age, 20-30 gr. body weight, were divided into 2 groups: Infected group and negative control. In the infected group, the mice were given cariogenic food (plus 5% sucrose) and infected by *S. mutans* (3×10^5 cfu/mL). *S. mutans* was inoculated onto the enamel and between the teeth. On the 4th day after infection, the mice were euthanized and parotid glands were removed for immunologic examinations (TNF- α , TLR3, TLR4, IL-10, IgA, IFN- γ) and measured by flow cytometry. The data were analysed using one-way ANOVA, $\alpha = 0.05$. The results showed that infection of *S. mutans* caused dental caries marked by changes of the enamel teeth colour, turn to yellow and accompanied by the emergence of black dots. The TNF- α , TLR3, TLR4, IgA, IFN- γ of parotid glands decreased, but the IL-10 increased significantly. Infection of *S. mutans* onto the enamel and between the teeth of mice caused dental caries and immunologically *S. mutans* suppress the mice immune system, both cellular and humoral, both innate and adaptive immunity.

Keywords: Dental Caries, *S. mutans* and Immunology

Introduction

Dental caries or tooth decay, cavities, caries, is a breakdown of teeth due to activities of bacteria. The cavities may be a number of different colours from yellow to black. The earliest sign of a new carious lesion is the appearance of a chalky white spot on the surface of the tooth, indicating an area of demineralization of enamel. It can turn brown but will eventually turn into a cavitation. Once a cavity forms, the lost tooth structure cannot be regenerated (Wikipedia, 2015).

The cause of caries is bacterial breakdown of the hard tissues of the teeth (enamel, dentin and cementum). This occurs due to acid made from food debris or sugar on the tooth surface. Simple sugars in food are these bacteria's primary energy source and thus a diet high in simple sugar is a risk factor. The acid is produced when sugars (mainly sucrose) react with bacteria present in the dental

biofilm (plaque) on the tooth surface. The acid produced leads to a loss of calcium and phosphate from the enamel and creates holes in the tooth (cavities); this process is called demineralization (DHF, 2015).

Bacteria, acid, food pieces and saliva combine in the mouth to form a sticky substance called plaque. Plaque adheres to the teeth, commonly on the back molars, just above the gum line on all teeth and at the edges of fillings. If mineral breakdown is greater than build up from sources such as saliva, caries results, plaque begins to build up on teeth within 20 min after eating. If it is not removed, tooth decay will begin, the teeth turns into a substance called tartar or calculus. Plaque and tartar irritate the gums, resulting in gingivitis and periodontitis (Fotek, 2015).

Cavities usually do not hurt, unless they grow very large and affect nerves or cause a tooth fracture. An untreated cavity can lead to a tooth abscess. Untreated

tooth decay also destroys the inside of the tooth (pulp) (Fotek, 2015). The destruction spreads into the softer, sensitive part of the tooth beneath the enamel (dentine). The weakened enamel then collapses to form a cavity and the tooth is progressively destroyed. Caries can also attack the roots of teeth should they become exposed by gum recession (DHF, 2015).

The mouth contains a wide variety of oral bacteria, but only a few specific species of them are believed to cause dental caries: *Streptococcus mutans* and *Lactobacillus*. *S. mutans*, is cariogenic and is a major cause of dental caries. One characteristic of this bacteria is being able to stick to all surfaces location of its habitat within the oral cavity. The organisms can produce high levels of lactic acid following fermentation of dietary sugars and are resistant to the adverse effects of low pH, properties essential for cariogenic bacteria (Wikipedia, 2015; Peres *et al.*, 2002).

The *S. mutans* produces biofilm in order to adhere on to the host surface through its receptors in salivary pellicle (Tanzer, 1992). Streptococcus infection will induce immune responses, both innate and adaptive immunity, also both cellular and humoral immunity. Glands parotis is the biggest of salivary gland and have primary function in oral infection (Wikipedia, 2015).

Procedures

Animal Models (Mus musculus)

This research is experimental study, using post test control and completely randomized designs. Male mice (16 weeks age, 20-30 gr. body weight) as animal model. The mice were divided into 2 groups. The minimal number of mice (n) for every group was 9, which calculated using Kusnirningrum (2008), equation $t(n-1) \geq 15$:

t = Number of treatment

n = Number of sample

The mice were injected ampicilline for 3 days before treatment in order to killed and inhibited the growth of the oral bacteria (Ghasempour *et al.*, 2014) and were given cariogenic diet by adding 5% sucrose (Michalek *et al.*, 1977; Peres *et al.*, 2002).

Streptococcus mutans Preparation

Streptococcus mutans was obtained from the Laboratory of Microbiology, Medical Faculty of Brawijaya University. The bacteria was propagated in Tryptone Yeast Cystine Agar (Lab M TYC Medium, Cat-No. LAB035-A), incubated at 37°C, for 48 h. Five colonies on TYCA were selected and propagated into Brain Heart Infusion (BHI, Oxoid CM1135) medium, incubated at 37°C, for 24 h. Infection dose of *S. mutans* was 3.10^5 cfu/mL. As much as 0.1 mL bacteria were

inoculated onto the enamel (surface) and sulcus between the mice teeth (Anggraeni *et al.*, 2005).

Isolation of Parotis Gland Cells

The mice were euthanized by cervical dislocation method and the blood were collected from cardiac puncture.

Parotid glands were removed and placed in sterile Phosphat Buffer Saline (PBS). The gland was homogenized and then filtered using sterile gauge. The filtrate was centrifugated at 2500 rpm, 4°C, for 5 min. The sediment was gland parotis cells.

Parameters

Immunologic study determined based on the innate and adaptive immunity from glands parotis. Parameters were measured to streng then the presence of dental caries are TNF α , TLR3, TLR4, IL-10, IgA, IFN γ . All these parameters were measured by flow cytometry method in relative values (%).

Analysis

The datas were analysed statistically by one-way ANOVA, with a Confidence Interval (CI) 95%.

Results and Discussion

The mice were infected by 0.1 mL of infection dose *S. mutans* (3×10^5 cfu/mL) onto the surface and insulcus between the teeth. *S. mutans* produced biofilm in order to adhere onto the host surface through its receptors in salivary pellicle. The salivary pellicle has several kinds of receptors, as mediator attachment of the bacteria of the oral cavity on the tooth surface (Tanzer, 1992). *S. mutans* has the ability to ferment carbohydrates and the acid produced leads to demineralisation, a loss of calcium and phosphate from the enamel, is important in the carious process (Peres *et al.*, 2002).

In this research, to accelerate the occurrence of dental caries, mice were given a cariogenic foods by adding 5% sucrose. Sucrose is one of the highest cariogenic potential. Enhancement of cariogenic potential was associated with foods containing approximately 1% or more hydrolysable starch in combination with sucrose or other sugars (Mundorff *et al.*, 1990). Dental shape, saliva pH, normal flora and diet can be predisposition factor for dental caries (Peak, 2015). In this research, on the 4th of day after infection, anatomic changes of the teeth were examined. The teeth colour turned into yellow with the presence of black dots (Fig. 1). It was compared to normal teeth, looking white, clean, with no colour dots (Fig. 2). Yellow colour of teeth caused by the formation of dental plaques is an early signs of dental caries and followed by the formation of yellow, brown and black dots. Black dots is an progressive form of dental plaques because it happens to tooth decay (Wikipedia, 2015).

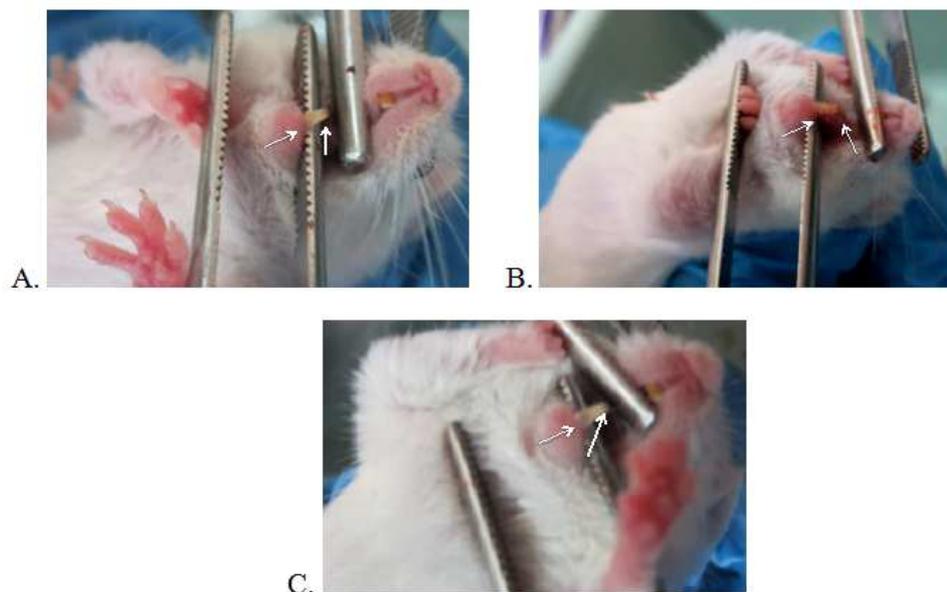


Fig. 1. A, B and C. The teeth of mice infected by *S. mutans*. The enamel turned into yellow colour accompanied by the presence of black dots (arrows)

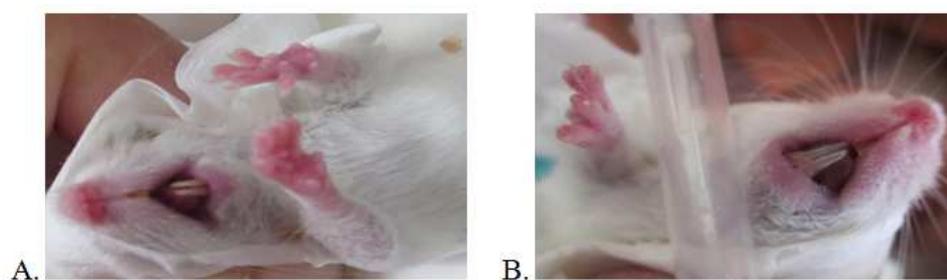


Fig. 2. A and B. Normal mice, with white colour of teeth, clear without the presence of yellow or brown area, or black dots

Table 1. Tukey Test for TNF α , IFN γ , TLR3, TLR4, IL-10 and IgA ($\alpha = 0.05$)

Group	Mean \pm SD (%)					
	TNF α	IFN γ	TLR3	TLR4	IL-10	IgA
Negative control	2.58 \pm 0.12 ^b	3.49 \pm 0.03 ^b	1.12 \pm 0.12 ^b	1.22 \pm 0.03 ^b	0.31 \pm 0.31 ^a	4.37 \pm 0.03 ^b
Infected mice	0.67 \pm 0.12 ^a	1.10 \pm 0.22 ^a	0.89 \pm 0.09 ^a	0.70 \pm 0.11 ^a	2.02 \pm 0.21 ^b	1.32 \pm 0.02 ^a

Santarem *et al.* (2013), reported about an immunosuppressor crude extracellular products of *Streptococcus mutans* (CEP-Sm) secreted by *S. mutans* that plays a role in the protection of the bacteria in the host. In his research, CEP-Sm suppressed the proliferative response to phytohaemagglutinin of human peripheral blood mononuclear cells and the primary immune response of C57BL/6 mice to sheep erythrocytes. It is a good input to the continuation of our research on the process of immunosuppression by *S. mutans*.

The use of parotid glands in this study was to detect immune responses, because the glands is the biggest of

salivary glands, is one of peripheral lymphoid organs having a primary function in host defenses to oral infection. These glands secrete saliva and *salivary Alpha-Amylase* (sAA), that possess function to break down amylose, amylopectin, inhibit bacteria attachment on oral surface and eliminate bacteria from the oral cavum (Wikipedia, 2015). Saliva also acts to dilute and neutralise the acid, which causes demineralisation and is an important natural defence against caries. Aside from buffering plaque acids and halting the demineralisation of enamel, saliva provides a reservoir of minerals adjacent to the enamel from which it can remineralisation and “heal” once the acids have been neutralised (DHF, 2015).

In this study, both of innate and adaptive immunity, cellular and humoral immunity were examined.

By one-way ANOVA and post hoc test (Tukey), with CI. 95%, showed: TNF- α ; TLR3, TLR4; IgA and IFN- γ decreased significantly in infected mice compared to the normal group (Table 1); but contrary to IL-10, increased significantly in infected mice (Table 1). From the results of this study it can be summarized that *S. mutans* has ability to suppress host immune system.

TNF- α is particularly produced by activated mononuclear phagocytes. The principals physiologic function of TNF- α is to stimulate the recruitment of neutrophils and monocytes to sites of infection and to activate these cells to eradicate microbes (Abbas and Lichtman, 2006). Decrease of this cytokine, may reduce the number and function of mononuclear phagocytes, antigen-stimulated T cells, NK cells and will have effect decreasing of the number and function innate and adaptive immunity. Whereas the mononuclear phagocytes and NK cells play role in cellular immunity and T cells play role both cellular and humoral immunity in TCD₄ and TCD₈ activation.

Toll-Like Receptor (TLR) is a membrane signal receptor play very important role activation of innate immunity to microbes. TLR is protein class, single form, in cell membrane expressed by macrophages and dendritic cells. TLR recognise pattern molecular structure of microbes. Once the microbes induce physical barrier damages, such as skin or membrane mucous, will be recognized by TLR and activate immunity system. Some TLRs are TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9, TLR10, TLR11, TLR12 and TLR13 (Abbas and Lichtman, 2006). Infection of *S. mutans* causing dental caries may through decrease of TLR-3 and TLR-4, leading to decreasing of number and quality of innate immunity cells such as macrophages, NK cell and decreasing associated cytokines such as TNF α , IL-1, IFN γ and IL-12.

Interferon- γ (IFN- γ) is a cytokine produced by Th₁, TCD₈ and NK cells, important in innate and adaptive immunity, particularly in Cell-Mediated Immunity (CMI). IFN- γ activates macrophages to destroy phagocytosed microbes. Different function shown by IL-10, is an inhibitor of activated macrophages and dendritic cells and is thus involved in the control of innate immune reactions and CMI. While the macrophages respond to microbes by secreting cytokines ANND by expressing co-stimulator that enhance T cells activation and CMI. Macrophages responses are terminated by IL-10 (Abbas and Lichtman, 2006). From this research IL-10 elevated and caused reduction of the macrophages function.

The innate and adaptive immune responses are two fundamental aspects of the immune system that respond to infections such as dental caries. The mucosal tissue associated with the exocrine glands and saliva

contributes to the production of immunity in the oral cavity. These regions contain cells responsible for antigen internalization and antibodies specific to oral bacteria. The secretory antibodies that protect against dental caries are IgA and IgG. In this research IgA decreased. It can be associated with immunosuppressive effects which cause reduction of TNF- α , TLR3, TLR4, IFN- γ and increasing of IL-10. In immunosuppression condition, *S. mutans* growth well and causing dental caries. Supporting the immunosuppressive condition, *S. mutans* produces biofilm in order to adheres, colonize on the tooth surface and protects the microbe from drugs, antibodies and phagocytosis.

Taken together it can be concluded that the host immunity did not undergo an effective immune response, both the innate and adaptive immunity may play a small role in preventing colonization and subsequent plaque development. Some possibilities that can lead to decreased host immunity against *S. mutans*. *S. mutans* may have strategies to disrupt the immune system, such as increasing production of biofilm, increases acid production, or cause apoptosis of immune cells. It requires further research to detect possible occurrence of immune cells apoptosis, increase of biofilm production or increasing the ability to ferment carbohydrates in dental caries patients.

Conclusion

An infection dose (3×10^5 cfu/mL) of *S. mutans* causes dental caries, due to the nature of cariogenic *S. mutans* and also due to the ability *S. mutans* to suppress host innate and adaptive immunity, suppress host cellular and humoral immunity and influenced by cariogenic diet.

Acknowledgement

Thank you for Prof. Dr. Muhaimin, SSi. and the students of Veterinary Medicine Faculty of Brawijaya University (Intan, Yessie, Nisa) given to the over all support.

Ethics

This article contains material that is original and unpublished. The animal models certified by the Ethical Committee Medical Research of Medical Faculty, Brawijaya University (No. 423-KEP-UB, 20 October 2015).

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Streptococcus mutans caused dental caries by many roles, such as production of biofilm that protects bacteria from host immunity (phagocytes and antibodies) and attaching to the dental surfaces, their ability to carry out fermentation producing acids and their ability to suppress the cellular and humoral host immunities. This study showed that TNF α , TLR3, TLR4, IFN γ and IgA decreased but IL-10 increased significantly (Table 1). TNF α , IFN γ are important cytokine for cellular and humoral immunity, TLR3, TLR4 important for innate immunity activation only. IL-10 is one of regulator cytokine that control innate immunity and cellular immunity. In this study, both of the cellular and humoral host responses were activated in response to dental caries and *S. mutans* infection. There was suppression of the immune responses when compare to normal mice.

The harm of *S. mutans* is its ability to destroy hard tissue of tooth: Enamel, dentin and cementum. By fermentation, the bacteria produce lactic acid, creating acidic oral environment. The effects of this acid include the demineralization of crystals in the enamel, over time until the bacteria physically penetrate the dentin. Demineralization is an early lesion on the dental enamel and characterized by white spots lesion preceded the formation of microcavity (smooth surface caries) (Fig. 1 A-C). Demineralised (softened) enamel of teeth surface, in the fissures (grooves) of teeth or in between the teeth. It may progress, then creates holes in the tooth (cavities) and looks as a number of different colours from yellow to black dots. These kinds of lesions are strongly associated with heavy infection by *S. mutans* on tooth surface (Wikipedia, 2015; Fotek, 2015; Peres *et al.*, 2002). Normal teeth look clear, white with no white, brown, yellow or black spot (Fig. 2A and B).