The Effectivity of *Centella asiatica* Extract on Salivary Neutrophils Proliferation in Severe Early Childhood Caries

Mieke Sylvia AR[1], Muhammad Luthfi[2]*, Aqsa Sjuhada Oki[2], Yuliati[2], Darmawan Setijanto[3], Adioro[4]

1. Department of Forensic Odontology, Faculty of Dental Medicine Universitas Airlangga, Surabaya 60115;
2. Department of Oral Biology, Faculty of Dental Medicine Universitas Airlangga, Surabaya 60115;
3. Department of Public Health, Faculty of Dental Medicine Universitas Airlangga, Surabaya 60115
4. Department of Dental Conservation, Faculty of Dental Medicine Universitas Airlangga, Surabaya 60115

Abstract

Dental caries, defined as multifactorial chronic disease, begins with the biofilm microbial complex change that is affected by sugar intake, salivary flow, and behavior. Neutrophils are the first line of defense against microbes. Besides kill microbes by phagocytosis, releases reactive oxygen species (ROS) and contains antimicrobial peptide, neutrophils activation also sets up the immune response. Saponin triterpenoids contained in *Centella asiatica* demonstrated immune-modulator effects, while pectin isolated from *Centella asiatica* showed immune-stimulatory activity.

To analyze the effectivity of *Centella asiatica* extract as an immune-modulator in salivary neutrophil proliferation as innate immunity effector cells in severe early childhood caries.

Preparation of extract of *Centella asiatica* were done using and isolation of neutrophils from the saliva of severe early childhood caries (S-ECC) using the magnitude of beads and marker label CD177 were analyzed using flow cytometry. Immune-modulator activity of *Centella asiatica* extract on salivary neutrophils proliferation was analyzed using MTT assay. Results: Salivary neutrophil proliferation increased after centella extract administration started from 50 µg/ ml.

The extract of *Centella asiatica* effectively increase salivary neutrophil proliferation on administration 50 µg/ ml.

Clinical Article (J Int Dent Med Res 2018; 11(2): pp. 551-555)

Keywords: Salivary neutrophils, *Centella asiatica*, Severe early childhood caries.

Received date: 29 November 2017 **Accept date:** 06 February 2018

Introduction

Dental cavity, is demineralization process as a result of oral environment imbalance that involve bacterial plaque and saliva[1]. Early childhood caries (ECC) is a major oral health problem that affect children. Based on American Dental Association (ADA), ECC presents multi-factorial enamel disease in early childhood[2], it is characterized by the presence of one or more decay teeth, missing teeth due to caries, or filling in deciduous teeth[2]. ECC is a multifactorial disease involving the interaction between host susceptibility, cariogenic bacteria, cariogenic diet and host behavior[3].

Corresponding author:
Muhammad Luthfi, Department of Oral Biology, Faculty of Dental Medicine Universitas Airlangga. Jl. Mayjend. Prof.Dr. Moestopo 47 Surabaya 60132, Indonesia.
E-mail: m.luthfi@fkg.unair.ac.id

According to Filstrup et al (2003), ECC prevalence is still considered high. Studies found that ECC prevalence reached 80% in developing countries, that five times higher than asthma, seven times higher than allergy, and fourteen times higher than chronic bronchitis[4]. A study by Fitriani (2007) in Semarang, stated that 90.5% children in city and 95.9% children in suburb area suffered from caries, while a study stated that 30.8% children in Gunung Anyar, Surabaya suffered from caries; and 29.2% suffered from severe early childhood caries[5,6]. ECC with decay-exfoliation-filling teeth (def-t) index score more than 6 denotes destructive state since involving several teeth, including upper anterior teeth; and referred as severe early childhood caries (S-ECC)[7].

One of contributing factor that plays important role in ECC is an immunologic aspect in the oral mucosa that requires secretory immunoglobulin A (SIgA)[8] and *Streptococcus*

mutans[9]. *Streptococcus mutans* is considered as cariogenic bacteria due to its acidogenic and aciduric nature. Acidogenic is the ability to create acid environment, while aciduric is the ability to survive in acid environment[7]. *Streptococcus mutans* is predominant cariogenic bacteria. Bacterial elimination occurs through activating alternative pathway, leading complement C3 activation that leads to membrane lysis, pathogen opsonization and neutrophil recruitment. These processes can trigger specific immune response[10].

Neutrophils are innate immunity effector cell that are recruited in inflammation and act as first line defense towards pathogenic microbes through phagocytosis. Recent studies have focused on neutrophils as major component in host defense against bacteria[11]. Besides eliminating bacteria through phagocytosis, releasing reactive oxygen species (ROS) and antimicrobial peptides, neutrophils also control immune response activation[12].

In Indonesia, *Centela asiatica* (*C. asiatica*) has been widely used as traditional medicine. *C. asiatica* contains triterpenoid saponin, pectin. The phenolic and methanolic extract compound that contained in *C. asiatica* have different potency, triterpenoid saponin shows immunomodulator ability[13], pectin act as immune-stimulating agent[14], and methanolic extract has immunomodulator activity and stimulate cellular mediated immune system that increase neutrophil phagocytic function[15,16].

Against the background of foregoing theory, thus this research was conducted to find the effectivity of *C. asiatica* extract towards salivary neutrophil proliferation in S-ECC patients.

Methodology

Sample

This was a cross-sectional observational study with kindergarten student children aged 4-6 years old as sample.

Ethical clearance was approved by ethical committee of Faculty of Dental Medicine, Universitas Airlangga (No. 255/KKEPK/FKG/XI/2016). All patients' parents that participated in this study were given explanation about the study prior signing consent form. Samples were taken from kindergarten student in Surabaya that have undergone dental examination and scored based on def-t index. Saliva was taken from children with def-t score more than 6 which is known as severe early childhood caries.

Centella asiatica Extract Preparation

Extract were prepared from *C. asiatica* leaves using methanol as solvent agent. 50grams of *C. asiatica* leaves were immersed in 200ml methanol 80% in a tube, then vibrated on 156 rpm for 24 hours in 37°C. Thereafter, the mixture was filtrated using filter disk 0.22 µm, and methanol was separated using rotary evaporator (Heidolph Laborota 4000) in 65°C subsequently. The remaining filtrate was freeze dried using freeze-dryer until become powder. The filtrate then stored in biofreezer in -70°C for 24 – 48 hours. The freeze-dried extract (1 mg) was diluted in 1ml Dimethyl sulfoxide (DMSO), resulting extract with 1000 mg/ml concentration. The extract was diluted until reach 1 µg/ml – 1000 µg/ml. 100 µl extract was added to neutrophil cell culture that isolated from patients' saliva.

Salivary Neutrophil Isolation

Saliva was taken by instructing kindergarten student children with S-ECC to gargle 10 ml NaCl 1.5% for 30 seconds, then spitted in sterile glass. This procedure was repeated for four times. Hereafter, saliva was centrifuged with 450g for 15 minutes in 4°C. Sample then mixed with 2 ml Roswell Park Memorial Institute Media 1640 (RPMI 1640)[17].

Salivary Neutrophil Identification

Salivary neutrophil identification was conducted using cell sorting Human Neutrophils Enrichment kit (Easy Sep) with CD177 marker using flow cytometry.

Salivary Neutrophil Proliferation Assessment

MTT assay is standard method for analyzing cell viability. This is colorimetric test that measure cell proliferation, based on reducing tetrazolium yellow, 3-(4,5-dimethythiazol-2)-2,5-difenil tetrazolium bromide (MTT) by mitochondria succinate dehydrogenase. MTT goes inside the cell toward mitochondria, then reduced and solved become purple formazan crystal. Those cells then solved using organic solvent (formazan) and released, then measured using spectrophotometer[18].

Salivary neutrophil was isolated from kindergarten student's saliva and devided into two groups, namely control group (without *C. asiatica* extract administration) and treatment group (with *C. asiatica* extract administration on 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml concentration).

C. asiatica extract on 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml concentration were diluted in DMSO and 100 ml isolated neutrophil from S-ECC children was added (6x105 cell/ml) in flat microtiter plate 96 wells (Cellstar, Greiner, German), then incubated for 24 hours on 37°C with 5% CO2. Cell culture on RPMI-1640 media that did not treated with the extract was considered as negative control. After 24 hours incubation, 10 µl MTT (5mg/ml) was added to each well and incubated for 4 hours with temperature 37°C. Precipitated purple formazan that formed was diluted in 100 ml DMSO then incubated for 30 minutes. Hereafter, absorbance rate was evaluated on wave length 650 nm using ELISA. The percentage of viable cell was measured using the following formula:

$$\% \text{ Proliferating cell} = \frac{\text{Mean treatment group absorbance level}}{\text{Mean control group absorbance level}} \times 100$$

The mean value and standard deviation of cell viability was used to measure the percentage of cell concentration changes between control and treatment group. All procedures were repeated 6 times and the results are shown as mean ± SD.

Results

Salivary neutrophil proliferation assessment using MTT assay from ECC patient showed that group treated with *C. asiatica* showed higher proliferating rate compared to control group. The highest proliferating rate was recorded by group treated with *C. asiatica* at 100 µg/ml concentration. Neutrophil proliferating rate was increasing along with the increasing extract concentration, and decreased at 250 µg/ml concentration.

(S-ECC)	N	Mean	Standard Deviation	95%CI
Control	6	5124.8415	984.59049	4091.5772 - 6158.1059
25 µg/ ml	6	6585.4018	3553.77964	2855.9388 - 10314.8648
50 µg/ ml	6	13436.9874	1201.75153	12175.8265 - 14698.1483
100 µg/ ml	6	15180.0623	1509.99727	13595.4173 - 16764.7072
250 µg/ ml	6	13466.2035	2281.30512	11072.1206 - 15860.2864
500 µg/ ml	6	25683.9574	3690.54048	21810.9727 - 29556.9421
1000 µg/ ml	6	22146.8049	8225.30054	13514.8814 - 30778.7285

Table 1. Mean and Standard Deviation of Salivary Proliferating Rate in Control Group and Treatment Group.

Independent t-test analysis showed there was no significant difference between control group and group treated with *C.asiatica* extract with 25µg/ml concentration. The significant differences were recorded between control group and group treated with 50 µg/ml concentration and above. Those results showed that the effectivity of *C. asiatica* extract on proliferating salivary neutrophil that was isolated from S-ECC patients started at 50 µg/ml concentration.

Discussion

Severe early childhood caries (S-ECC) is infection disease involving teeth that is caused by *S. mutans*, thus trigger immune specific response[10]. In saliva, neutrophil is considered as the first line defence against pathogenic microbes[19]. Neutrophils are potential in eliminating microbes since containing antimicrobial substances, namely kationic peptides, proteases, and lactoferin; besides, also have the ability to secrete reactive oxygen species (ROS) and nitrogen[20]. that can be directly triggered by cytokine and chemokine through pattern-recognition receptor (PRRs) and cytokine receptors[21].

Neutrophil proliferation in infected area is an important aspect of innate immune response against pathogen invasion to eliminate pathogenic bacteria[11]. This statement is compatible with previous studies stated that the level of circulating neutrophil in infection state increase up to 40 times. This explained that infected area sent signal to mobilize neutrophil rapidly from bone marrow towards infected area[22].

	Control	25	50	100	500	1000
Control	**-**	**0.355**	**0.000***	**0.000***	**0.000***	**0.004***
25		**-**	**0.001***	**0.003***	**0.000***	**0.004***
50			**-**	**0.051**	**0.000***	**0.048***
100				**-**	**0.000***	**0.093**
250					**0.000***	**0.049***
500					**-**	**0.369**
1000						**-**

Table 2. The significant difference of proliferating salivary neutrophil of E-SCC patients after treated with extract with various concentration.

Based on Table 2, shows increasing proliferating salivary neutrophil after treated with *C. asiatica* extract on 25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml compared to control group. Those results indicate that *C. asiatica* extract has the ability as immunomodulator and immune-stimulator, that increasing innate immune response by promoting salivary neutrophil proliferation on S-ECC patient. The significant difference from control group was recorded from treatment group that treated with *C. asiatica* extract started from 50µg/ml concentration. These results gave the same results with the previous studies, which stated that saponin and triterpenoid contained in *C. asiatica* can act as immunomodulator[13]. Whereas pectin contained in *C. asiatica* extract can stimulate immune system*14* and act as immunomodulator*23*. Besides that, methanolic extract of C. asiatica has the ability to stimulate immune system by increasing phagocytosis function of neutrophil[15].

C. asiatica extract contains four major triterpenoids, namely asiatic acid, madecassic acid, asiaticoside and madecassoside, that act as anti-microbial agent, anti-oxidant, and anti-cancer, and also therapeutic agent in wound healing, that can stimulate cell migration and proliferation24. With such functions, triterpenoids are thought to increase neutrophil proliferation, that leads to increase phagocytosis activity in infected area, thus may reduce infection in S-ECC. Neutrophil proliferation in infected area can increase neutrophil amount so that produced cytokine will also increase, that promotes innate immune response[14,23].

Within the limitations in this study, further study that evaluate the effect of incubation *C. asiatica* extract time towards salivary neutrophil proliferation is required.

Conclusion

Based on the result of this study, it is concluded that C. asiatica extract can effectively promotes salivary neutrophil proliferation on 50 □g/ml concentration.

Acknowledgement

The authors would like to thank the Ministry of Research, Technology, and Higher Education of Indonesia and the Head of Research and Innovation Department Universitas Airlangga to give a grant funding.

References

1. Alazmah A. Early Childhood Caries: A Review. Early Child Caries A Rev J Contemp Dent Pract J Contemp Dent Pr. 2017;1818(88):1-61.
2. Kokoceva Ivanovska Olga, Jankulovska Mira, Mijoska Aneta, Efka Zabokova Bilbilova, Meri Pavlevska, Gordana Todorovska Ultrastructural Changes of the Initial Lesion at Early Childhood Caries Clinical Article J Int Dent Med Res 2017; 10(1): 36-41
3. Begzati A, Berisha M, Mrasori S, et al. Early Childhood Caries (ECC) — Etiology, Clinical Consequences and Prevention. In: Virdi PM, ed. Emerging Trends in Oral Health Sciences and Dentistry. 2015. book edited by Mandeep Singh Virdi, ISBN 978-953-51-2024-7
4. Filstrup SL, Briskie D, Da Fonseca M, Lawrence L, Wandera A, Inglehart MR. Early Childhood Caries and Quality of Life: Child and Parent Perspectives. Pediatr Dent. 2003;25(5):431-440.
5. Sutjipto RW, Satiti D, Departemen K, Kedokteran I, Anak G. Prevalensi early childhood caries dan severe early childhood caries pada anak prasekolah di Gunung Anyar Surabaya. Dent J (Majalah Kedokt Gigi). 2014; 4: 47
6. Fitriani. Faktor Risiko Karies Gigi Sulung Anak (Studi Kasus Anak TK Islam Pangeran Diponegoro Semarang. 2007. Undergraduate thesis, Diponegoro University.
7. Tinanoff N, Reisine S. Update on early childhood caries since the Surgeon General's Report. Acad Pediatr. 2009;9(6):396-403.
8. Setiawati F, Sutadi H, Rahardjo A. Relationship between Breastfeeding Status and Early Childhood Caries Prevalence in 6-24 months old children in Jakarta. J Int Dent Med Res 2017; 10(2): 308-312
9. Fejerskov O, Kidd E, Nyvad B, Baelum V. Dental Caries: The Disease and Its Clinical Management.; 2008.
10. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-435.
11. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. Annu Rev Pathol. 2014; 9:181-218.
12. Vatansever F, de Melo WCMA, Avci P, et al. Antimicrobial strategies centered around reactive oxygen species--bactericidal antibiotics, photodynamic therapy, and beyond. FEMS Microbiol Rev. 2013;37(6):955-989.
13. Ayu NP, Suryani L, Studi P, et al. Effect of Centella asiatica (L.) Urban Leaves Extract toward Leukocyte Count and Lymphocyte Percentage in Balb/c Mice Infected with Salmonella typhimurium. Mutiara Med. 2014;14(1):15-24.
14. Agrawal M, Tyagi T. Therapeutic efficacy of Centella asiatica (L.) and Momordica charantia: As traditional medicinal plant. J Plant Sci J Plant Sci Spec Issue Med Plants. 2015;3(1):1-9.
15. Mali RG, Hatapakki BC. Ravindra G. Mali et al: An in vitro Study of Effect of Centella asiatica on Phagocytosis by Human Neutophils 297 An in vitro study of Effect of Centella asiatica on Phagocytosis by Human Neutrophils. Int J Pharm Sci Nanotechnol. 2008;1(3):297-301.
16. Seevaratnam V, Banumathi P, Premalatha MR, Sundaram S, Arumugam AT. FUNCTIONAL PROPERTIES OF CENTELLA ASIATICA (L.): A REVIEW. Int J Pharm Pharm Sci. 2012;5(suppl 5):1-11.
17. Gasparoto TH, Oliveira CE, Vieira NA, et al. Activation pattern of neutrophils from blood of elderly individuals with Candida-related denture stomatitis. Eur J Clin Microbiol Infect Dis. 2012;31(6):1271-1277.
18. Patel S, Onkarappa R, Gurumurthy S. Pretreatment studies on wheat straw and rice straw. Internet J Microbiol. 2008;7(1).
19. Fábián TK, Hermann P, Beck A, Fejérdy P, Fábián G. Salivary defense proteins: their network and role in innate and acquired oral immunity. Int J Mol Sci. 2012;13(4):4295-4320.
20. Kobayashi SD, Malachowa N, DeLeo FR. Influence of Microbes on Neutrophil Life and Death. Front Cell Infect Microbiol. 2017; 7:159.
21. Theilgaard-Mönch K, Porse BT, Borregaard N. Systems biology of neutrophil differentiation and immune response. Curr Opin Immunol. 2006;18(1):54-60.
22. Strydom N, Rankin SM. Regulation of circulating neutrophil numbers under homeostasis and in disease. J Innate Immun. 2013;5(4):304-314.
23. Sushen U, Chouhan A, Ali K, Ranjesh V. Medicinal Properties of Centella Asiatica (L.): A Review. Eur J Pharm Med Res. 2017;4(9):261-268.
24. Gohil KJ, Patel JA, Gajjar AK. Pharmacological Review on Centella asiatica: A Potential Herbal Cure-all. Indian J Pharm Sci. 2010;72(5):546-556.