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Evaluation of lysozyme concentration and *S.mutans* colonies on children with early childhood caries and caries free after using 0.1% lysozyme toothpaste

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Abstract. The prevalence of Early Childhood Caries (ECC) in various countries is quite high, even at the age of 2 years the incidence is quite apprehensive. One simple and effective ECC alternative prevention is tooth brushing by using lysozyme toothpaste. Lysozyme can kill S.mutans bacteria by hydrolizing bacterial cell walls. This study aims to compare the salivary lysozyme concentration and the number of S.mutans colonies between pre-treatment and after 1 month tooth brushing by using 0.1% lysozyme toothpaste. This research is single masked clinical trial. The sample size of this study is 73 under 3 years old children, consisting of 31 caries-free children and 42 ECC children. The research subjects are obtained from Posyandu in Medan Selayang and Medan Johor District by using purposive sampling. Criteria for ECC is based on the American Association of Pediatric Dentistry (AAPD). Data analysis use Mann-Whitney and Wilcoxon tests because the data are not normally distributed, $p<0.05$. The result shows that after tooth brushing by using lysozyme toothpaste, reduction of lysozyme concentration was detected on both groups of caries-free and ECC children compared to the state before tooth brushing, but it is not statistically significant ($p>0.05$). Similiarly, reduction in number of S.mutans was detected after tooth brushing by using lysozyme toothpaste, although it is not statistically significant ($p>0.05$). As conclusion, lysozyme toothpaste can reduce the growth of S.mutans bacteria, but is only inhibitory or bacteriostatic. Therefore, lysozyme toothpaste can be used as an alternative of caries prevention for early childhood.

1. Introduction

Early Childhood Caries (ECC) is a condition that often be found in children from various countries[1]. According to American Academy of Pediatric Dentistry (AAPD) the definition of ECC is stated as the presence of carious lesions on the surface of primary teeth (with or without cavity), missing teeth due to caries or filled teeth in under 6 years old children [2].

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The prevalence of ECC in various countries is quite high, even at an early age of 2 years old, the incidence is quite apprehensive.The prevalence of ECC in children of 2 years old and under is 27,66% with dmft of 3,65+3,12 [3]. In Srilanka, the prevalence of ECC in children aged 1-2 years has increased to 32,19% with mean dmft 2,01 and dmfs 3,83 [1].The prevalence of ECC in children of 3 years old and under in Medan City (Indonesia) is higher, reaching 57,7% with dmft 2,17+4,49 [4].

One of easy way to prevent caries which is done by parents and quite effective is brushing their children's teeth. But in reality, some 2 years old children never have their teeth been brushed by their parents. Research show that the proportion of children whose teeth are brushed ≥1 time a day by mothers is only 29%, while the rests are 30,1% whose teeth are brushed <1 times a day and 25,3% whose teeth have never been brushed. While the proportion of children who use toothpaste every brushing their teeth is only 30%; the rests 27,66% is under category sometimes, and 27,9% never use it [3].

The usage of fluoride toothpaste for under 2 years old children must be with carefulness, considering the possibility of baby swallowing the toothpaste may cause fluorosis in the teeth if it occurs frequently. AAPD recommends using fluoride toothpaste for children under 2 years of age if they suffer moderate or high caries, and it is recommended that the amount of the given toothpaste is only smear. Children under 1 year old of age can clean their teeth by using only a soft toothbrush without toothpaste or with non-fluoride toothpaste [5].

Recently, fluoride toothpaste has been developed with additional several active substances such as lysozyme, lactoferrin and lactoperoxidase to increase effectiveness in caries prevention, but its usage is still not familiar for public. Experimental studies of tooth brushing using fluoride toothpaste containing lysozyme, lactoferrin and laktoperoxidase after 1 week has showed significant reduction in the number of *S. mutans* and *L.acidophilus* ($p < 0.001$) in children suffering from Severe-ECC[6].

The toothpaste used in this study is lysozyme toothpaste without the addition of fluoride. This toothpaste is intended for children under the age of 3 years. There is addition of the active ingredient lysozyme because of its antibacterial ability. The activity of lysozyme as an antimicrobial is related to its ability in lytic actionon bacteria[7] by hydrolyzing β (1-4) bonds between N- *acetylmuramic* and N-*acetylglucosamine acids* in the peptidoglycan layer of bacterial cell walls[8]. The result of glycosidic bonds hydrolysis will cause small pores inside the bacterial cell walls so that the bacteria die. Lysozyme mainly hydrolyzes the cell walls of Gram-positive microorganisms, such as *S. mutans* [9] .

Lysozyme as a strong cationic protein can also mediate bacterial aggregation and inhibit bacterial attachment and also activate autolisin of bacteria by destroying bacterial cell walls [7,8]. The activity of lysozyme will survive when the enzyme is absorbed in the pellicle [10]. Cationic molecular absorption of bacterial walls is highly dependent on pH and ionic strength [7].

This study aims to compare salivary lysozyme concentrations and the number of colonies of *S.mutans* between pre-treatment and after 1 month tooth brushing by using tooth paste without fluoride in under 3 years old children.

2. Methods

2.1. Subjects

The research is single masked clinical trial. The sample size of this study is 73 under 3 years old children, consisting of 31 caries-free children and 42 ECC children. The research subjects are obtained from Posyandu in Medan Selayang and Medan Johor District by using purposive sampling.

The subject criteria including having at least 2 primary teeth incisors that have erupted, a good general health state, not taking drugs that affect saliva at least 1 month before, the parents permission by giving informed consent. Based on American Association of Pediatric Dentistry (AAPD) criteria of ECC, caries-free child is defined as not having caries or filled or extracted teeth caused by caries. This research has received approval from the Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara.

2.2 Caries examinations and saliva sampling
All children were examined visually by sunlight. The caries examination (dmft) used a mouth mirror and a half moon explorer. The results of dmft were recorded on questionnaire.

Time of collecting saliva was between 09.00-11.00 WIB. Children were prohibited from drinking and eating 1 hour before collecting the saliva. Saliva were obtained unstimulated with 2ml disposable pipette. Saliva was inserted into a sealed plastic tube stored in an ice box to prevent hydrolysis of salivary protein, and quickly taken to Terpadu laboratory Faculty of Medicine Universitas Sumatera Utara and Microbiology laboratory Faculty of Medicine Universitas Sumatera Utara.

2.3 Microbial examination
Culture of *S.mutans* used *trypticase soy-yeast* -20 per cen *sucrose-bacitracin* (TYS20B) media. The media was put into anaerobic jar (anaerobic conditions, 10% H_2 , 10% CO_2 and 80% N_2) then stored in an incubator at 37^0C temperature for 2x 24 hours. The number of *Colony Forming Units* (CFU / ml saliva) was calculated, colony counting was done twice. The result of the calculation of the number of *S. mutans* colonies was the mean value of the calculation results 1 and 2.

2.4 Chemical saliva examination
The chemical examination that would be carried out was an examination of salivary lysozyme concentration. Examination of lysozyme concentrations used Human LZMc ELISA *Kit* (LZM) 96 *wells* (® Fine Test). Examination of lysozyme quantity used ELISA sandwich technique. Lysozyme concentration in the sample was assessed by comparing optical density (OD) of the sample with a standard curve. The sample reading used a 450 nm wave micro reader.

2.5 Tooth brushing with tooth paste
The child who has his/her teeth been examined and saliva collected is then given a lysozyme toothpaste. Lysozyme toothpaste contains lysozyme active ingredient (@ Sigma) of 0.1% concentration and acetic acid as lysozyme stabilization ingredient. Other basic ingredients of toothpaste are calcium carbonate, Na benzoate, Na CMC, Tragacanth, Glycerol, Sorbitol, flavoring agent (Vanilla) and water. Control toothpastes only contain basic ingredients of toothpaste without active substances. The choice of lysozyme toothpaste or control toothpaste by the subject of the study was done randomly. Each groups were divided into 2, one group was given lysozym toothpaste and the rest was given control toothpaste. After 1 month usage, children's teeth were examined again to see observe their dmft and saliva was retaken for examination of *S. mutans* colonies and lysozyme concentrations. The data was then compared with the initial data before treatment.

2.6 Data analysis
Analysis test to determine mean difference of lysozyme concentration and *S.mutans* colonies between caries-free and ECC children groups used Mann-Whitney test because the data are not normally distributed. Analysis test to determine mean difference of lysozyme concentration and *S.mutans* colonies between before and after tooth brushing used Wilcoxon test. The significance value used is $p < 0.05$.

3. Results

3.1 Subject characteristics and base data
Thirty-one caries-free children consist of 45.16% male and 54.84% female; while 42 ECC children consist of 57.14% male and 42.86% female. Mean dmft and dmfs for ECC children group are 4.83± 2.17 and 5.74±3.01 (Table 1).

Table 1. Characteristics of research subject

Children category	n (%)	Gender		Mean age	Mean dmft	Mean dmfs
		M (%)	F (%)			
Caries free	31 (42.47)	14 (45.16)	17 (54.84)	22.26±4.28	0	0
ECC	42 (57.53)	24 (57.14)	18 (42.86)	24.74±5.18	4.83±2.17	5.74±3.01
Total	73 (100)	31 (42.47)	42 (57.53)	23.68±4.94		

Based on the children groups, no significant mean difference of lysozyme concentration is found between caries-free and ECC children (p> 0, 05). Difference in the number of *S. mutans* colonies is found between two groups. In ECC children, *S. mutans* colonies are found more than in caries-free children (p = 0.001) (Table 2).

Table 2. Mean comparison of lysozyme concentration and *S.mutans* colonies based on groups of children

Children Category	n	Mean lysozyme concentration (ng/ml)	p	Mean *S.mutans* colony (CFU/ml)	p
Caries free	31	1160.28±1129.63	0.938	21.68±54.22	0.001
ECC	42	1450.81±1634.03		688.50±1038.39	

3.2 After tooth brushing result

It's found substantially that lysozyme concentration is reduced after tooth brushing using both lysozyme toothpaste and control toothpaste, compared with the state before tooth brushing. But the reduction is not statistically significant (p>0.05) (Table 3).

Table 3. Mean comparison of lysozyme before and after tooth brushing in each group of children

Children Category	n	Lysozyme toothpaste		p	Control toothpaste		p
		Initial data of lysozyme concentration (ng/ml)	Lysozyme concentration after 1 month (ng/ml)		Initial data of lysozyme concentration (ng/ml)	Lysozyme concentration after 1 month (ng/ml)	
Caries free	31	1088,32 ±1037,98	670.96 ± 749.55	0.170	1259.92 ±1282.71	716.08 ±625.69	0.075
ECC	42	1433.43 ±1546.30	1343.45 ±1192.24	0.855	1471.85 ±1777.23	737.09 ±775.41	0.227

There is no value difference (Δ) of lysozyme before and after tooth brushing using lysozyme toothpaste between group of caries-free children and group of ECC children (p = 508), similar with the result after tooth brushing using control toothpaste which show no difference between caries-free and ECC children group (Table 4). There is reduction found in the number of *S. mutans* colonies after tooth brushing using lysozyme toothpaste compared with the state before tooth brushing, but it's not statistically significant (p = 0.543). Likewise the other groups which show no difference in the number of colonies after tooth brushing using both lysozyme toothpaste or control toothpaste (Table 5). There is no value difference (Δ) of *S.mutans* colonies before and after tooth brushing using both lysozyme toothpaste or control toothpaste between group of caries-free children and group of ECC children (p >0.05) (Table 6).

Table 4. Comparison of mean difference of lysozyme before and after tooth brushing between two different groups

Children Category	**n**	Lysozyme toothpaste	p	Control toothpaste	p
		Δ Lysozyme concentration (ng / ml)		Δ Lysozyme concentration (ng / ml)	
Caries free	31	417.37 ± 1278.61	0.508*	543.85 ± 1096.48	0.715**
ECC	42	89.98 ± 1739.99		734.76 ± 1684.78	

*Independent t-test
** Mann-Whitney test

Table 5. Comparison of the number of *S. mutans* colonies before and after tooth brushing in each group of children

Children Category	**n**	Lysozyme toothpaste		p	Control toothpaste		p
		Initial data of *S. mutans* colonies (CFU / ml)	*S. mutans* colonies after 1 month (CFU / ml)		Initial data of *S. mutans* colonies (CFU / ml)	*S. mutans* colonies after 1 month (CFU / ml)	
Caries free	31	23.11 ± 63.05	25.56 ± 34.27	0.070	19.69 ± 41.38	47.69 ± 58.06	0.147
ECC	42	630.04 ±1098.99	273.26 ±567.17	0.543	759.26 ±984.98	193.63 ±348.85	0.070

Table 6. Comparison of mean difference of *S. mutans* colonies before and after tooth brushing between two different groups

Children Category	**n**	Lysozyme toothpaste	p	Control toothpaste	p
		Δ *S.mutans* Colony (CFU / ml)		Δ *S. mutans* colonies (CFU / ml)	
Caries free	31	2.44 ± 44.52	0.895	28 ± 77.33	0.055
ECC	42	356.78 ± 1035.68		565.63 ± 893.44	

4. Discussion

At the beginning of the study, the ratio of lysozyme concentration between ECC and caries-free children showed no significant difference status (p> 0.05) (Table 2). The results of this study correspond to Felizardo *et.al* who showed that lysozyme was not related to dmft [11]. Hao and Lin in their research also revealed no difference of lysozyme between caries-free children and children with caries (dmft> 5)[12]

This study results are different from Moeslemi *et.al.* who found significantly higher levels of lysozyme concentrations in saliva without stimulation in the group of caries-free compared to the ECC (p = 0.04)[8]. Other researchers also reported differences in concentration between ECC and caries-free, but the concentration of salivary lysozyme without stimulation on ECC was higher than caries-free[13]. Likewise, Letsirivorakul *et.al* got the same results. The sample of unstimulating saliva taken from 32 caries-free children and 32 ECC children showed that the concentration of lysozyme in ECC was higher than caries-free (p = 0.008)[14].

In substance, this study shows that children with ECC prone to have higher concentrations of lysozyme than caries-free, but the difference in concentration is not statistically significant (Table 2). The high lysozyme concentration in ECC compared with caries-free may be due to compensation

mechanisms. The presence of caries will increase the number of *S.mutans*, so lysozyme which acts as a defense mechanism will increase its secretion due to stimulation of these bacteria [15].

Before treatment, the number of *S. mutans* colonies in ECC was higher than caries-free ($p < 0.05$) (Table 2). The results correspond to other researchers who found that ECC had more *S. mutans* than caries-free [16,17].The number of *S. mutans* in saliva is a microbial parameter for determining caries risk. Children who have more *S.mutans* in number increase caries to higher number after 1 year observation compared with the children who have lesser *S. mutans* [18]. Children with more *S. mutans* than 10^5 (CFU/ml) suffered more caries by 34.2%, compared to children with lesser *S. mutans* than 10^5 (CFU / ml) of 27% [19].

After tooth brushing treatment using lysozyme and control toothpaste, it's as shown in Table 3 the reduction of mean lysozyme concentration on each caries-free children group and ECC children group, but the reduction is not statistically significant ($p > 0.05$) (Table 3). Reduction of lysozyme concentration occured after regular tooth brushing, may be due to reduction of *S.mutans* number in children (Table 5). Pre-treatment questionnaire result shows that more children do not brush their teeth regularly everyday and even few of them have never had their teeth brushed. Mass *et al.* found an association between lysozyme values and the number of *S. mutans* [20]. The amount of *S. mutans* decreases due to lysozyme contained in toothpaste. This lysozyme acts as an antibacterial. Therefore the production of immune *innate* lysozyme formed in the saliva of children will decrease.

In the group of caries-free, there is reduction found in lysozyme concentration after tooth brushing using lysozyme toothpaste more than the reduction in lysozyme concentration in children with ECC (Table 4). This maybe due to lesser *S.mutans* colonies in number in caries-free children compared with children with ECC (Table 5). Besides, additional lysozyme source from outside (from toothpaste in this case) can quickly help inhibiting *S.mutans* growth.

Based on the difference (Δ) in lysozyme decreases between caries-free and ECC children after tooth brushing using both lysozyme and control toothpaste, there is no significant difference found (Table 4). The same result can be found in the difference of the number of *S. mutans* colonies, which is also not significant between the two groups (Table 6). The results of this study are different from Gudipaneni *et.al* research, who revealed that usage of toothpaste containing lysozyme, lactoferrin and lactoperoxidase after 1 week showed a significant reduction in the number of *S. mutans* ($p < 0.001$) in children suffering from Severe-ECC [6].

Although no significant differences are found in the difference of lysozyme concentration and *S. mutans* colonies between the two groups, but it does not mean that lysozyme toothpaste do not play role in the reduction of the number of *S. mutans*. Lysozyme toothpaste in this study is inhibitory or bacteriostatic to the growth of *S. mutans* bacteria.

5. Conclusion

The 0.1% lysozyme toothpaste can reduce the growth of *S. mutans* bacteria but is only inhibitory or bacteriostatic, so that it can be used as an alternative preventive way of dental caries occurrence in early childhood.

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