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Peroxidase Activity in Saliva and Caries Susceptibility

Aktywność peroksydazowa w ślinie a podatność na próchnicę

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Abstract

Background. Total peroxidase activity in whole human saliva is due to salivary peroxidase and myeloperoxidase. These enzymes catalyse thiocyanate oxidation by H_2O_2 generating hypothiocyanate, acting as an antibacterial agent. Therefore, the increase in the enzymatic activity could modify the dental caries development.

Objectives. Comparison of peroxidase activity in saliva (SP) in relation to caries susceptibility.

Material and Methods. 66 12–14-year-old caries-free (DMFT = 0; group CF, N = 22) and susceptible (DMFT \geq 4, n = 44) subjects were examined. In supernatants of unstimulated mixed saliva (SM), activities of salivary peroxidase (SP) and myeloperoxidase (MP), as well as total protein (P) and thiocyanate (T) concentration and salivary flow rate (V) were measured. In submandibular/sublingual saliva (SS), levels of SP, P and T were assayed as well as salivary flow rate (V). Level of SP activity was measured by two methods using ABTS (SP-1) and NbsSCN as a substrate. The latter method was performed with use of non-dialyzed (SP-2) and dialyzed (SP-3) salivary samples. MP activity was measured by NbsCl method. Content of P was assayed by Lowry et al. method and T by nitrate ferric method.

Results. The obtained mean values were as follows: in CF group in SM: V – 0.19 ± 0.09 ml/min, P – 1.71 ± 0.85 mg/ml, T – 1.66 ± 0.70 mmol/l, SP-3 – 1.90 ± 0.48 mIU/ml, MP – 0.63 ± 0.25 mIU/ml; in SS: V – 0.16 ± 0.11 ml/min, P – 0.84 ± 0.46 mg/ml, T – 1.28 ± 0.65 mmol/l, SP-3 – 0.19 ± 0.07 mIU/ml and in CS group in SM: 0.19 ± 0.11 , 1.22 ± 0.51 , 1.32 ± 0.58 , 1.93 ± 0.66 , 0.78 ± 0.22 ; in SS: 0.15 ± 0.11 , 0.60 ± 0.23 mg/ml, 0.99 ± 0.51 , 0.17 ± 0.07 , respectively.

Conclusion. The obtained data may suggest some differences of salivary components in caries-free and -susceptible subjects (Dent. Med. Probl. 2005, 42, 3, 405–411).

Key words: saliva, peroxidase, myeloperoxidase, caries susceptibility.

Streszczenie

Wprowadzenie. Aktywność peroksydazową w ludzkiej ślinie całkowitej stanowi aktywność ślinowej peroksydazy i mieloperoksydazy. Enzymy te katalizują utlenianie przez H_2O_2 tiocyanków tworząc hipotiocjany, które działają antybakteryjnie. Zatem wzrost aktywności enzymatycznej mógłby modyfikować rozwój próchnicy zębów.

Cel pracy. Porównanie aktywności peroksydazowej w ślinie (SP) w odniesieniu do podatności na próchnicę.

Material i metody. Zbadano 66 dzieci 12–14-letnich, w tym wolnych od próchnicy (PUW/Z = 0; grupa CF, n = 22) i podatnych na próchnicę (PUW/Z \geq 4, n = 44). W supernatantach niestymulowanej śliny mieszanej (SM) oznaczano aktywności ślinowej peroksydazy (SP) i mieloperoksydazy (MP) oraz stężenia białka całkowitego (P) i tiocyanków (T), a także szybkość wydzielania śliny (V). W wydzielinie z gruczołów podżuchwowych i podjęzykowych (SS) oceniano SP, P i T oraz szybkość wydzielania (V). Poziom aktywności SP oceniano dwiema metodami, stosując ABTS (SP-1) i Nbs-SCN jako substrat. Tą drugą metodą przeprowadzano w niedializowanych (SP-2) i dializowanych (SP-3) próbkach śliny. Aktywność MP mierzono metodą Nbs-Cl. Zawartość P oceniano metodą Lowry'ego et al., a T metodą z zastosowaniem azotanu żelaza.

Wyniki. Uzyskano następujące średnie wartości wynoszące odpowiednio: w grupie CF w ślinie SM: V – 0.19 ± 0.09 ml/min, P – 1.71 ± 0.85 mg/ml, T – 1.66 ± 0.70 mmol/l, SP-3 – 1.90 ± 0.48 mIU/ml, MP – 0.63 ± 0.25 mIU/ml; w ślinie SS: V – 0.16 ± 0.11 ml/min, P – 0.84 ± 0.46 mg/ml, T – 1.28 ± 0.65 mmol/l, SP – 0.19 ± 0.07 mIU/ml, a w grupie CS w ślinie SM: 0.19 ± 0.11 , 1.22 ± 0.51 , 1.32 ± 0.58 , 1.93 ± 0.66 , 0.78 ± 0.22 , a w ślinie SS: 0.15 ± 0.11 , 0.60 ± 0.23 mg/ml, 0.99 ± 0.51 , 0.17 ± 0.07 .

Wniosek. Uzyskane dane mogą sugerować pewne różnice w stężeniu składników śliny u osób bez próchnicy i podatnych na próchnicę (Dent. Med. Probl. 2005, 42, 3, 405–411).

Słowa kluczowe: ślina, peroksydaza, mieloperoksydaza, podatność na próchnicę.

Whole human saliva contains a great number of antibacterial agents which are usually divided into two main categories, non-immunoglobulin (innate) and immunoglobulin (acquired) factors. The major components of the non-immunoglobulin group are peroxidase and myeloperoxidase systems, lysozyme, lactoferrin, agglutinins, histidine-rich protein, anionic protein and phagocytic cells [1, 2]. Peroxidase activity in saliva is due to salivary peroxidase (SP) and myeloperoxidase (MP). The enzymes catalyse the oxidation of thiocyanate ions (SCN^-) by hydrogen peroxide (H_2O_2), produced mainly by oral bacteria, to generate the oxidized forms of hypothiocyanous acid (HOSCN) or hypothiocyanite anion (OSCN^-). The reaction final product – hypothiocyanite (OSCN^-) is a strong oxidising agent. It plays an important role in the control of bacteria. The enzymes also prevent the oral accumulation of potential cytotoxic levels of the hydrogen peroxide (H_2O_2). Peroxidase is secreted by the acinar cells of salivary glands. Myeloperoxidase is produced by polymorphonuclear leukocytes (PMNs) and its concentration increases in the gingival fluid during inflammation and this increase may be reflected in whole saliva as well. Peroxidase and myeloperoxidase differ in their substrate specificity so that MP can oxidize chloride to OCl^- , whereas SP cannot [1–4]. Thiocyanites (SCN^-) are naturally occurring salivary components. The salivary glands concentrate SCN^- from the blood serum and secrete them into saliva. The SCN^- content in saliva is in the range from 0.1 to 5 mmol/l [5, 6].

The goal of this study was to compare peroxidase activity in saliva in relation to caries susceptibility.

Material and Methods

Sixty-six 12–14-year-old schoolchildren, out of whom 22 were caries-free (DMTF = 0; group CF) and 44 caries-susceptible (DMFT \geq 4; group CS) were examined. The mean number of caries affected teeth was DMFT 5.77 and DMFS 8.87. The subjects had clinically healthy periodontal tissues and oral mucosa.

Samples of resting mixed (SM) and submandibular/sublingual (SS) saliva were collected by drooling from the floor of the mouth into ice-chilled test tubes. Salivary flow rate (V) was calculated (ml/min). Chemical assays were performed in the salivary supernatant obtained after centrifugation of saliva samples (18 000 g, 15 min). The total protein content (P) was measured by Lowry et al. method [7] and thiocyanite (T) by nitrate ferric method [8]. Peroxidase activity (SP)

was measured using two different methods. The first assay was done using ABTS [2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid] as a donor – SP-1. [9]. This method is interfered to a significant extent by salivary SCN^- ions leading to an underestimation of the true peroxidase activity. The second assay was based on the oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to 5,5-dithiobis-2-nitrobenzoic acid (Nbs_2) by OSCN^- ions generated during the oxidation of SCN^- [10]. Although it is believed that this method is not interfered with by SCN^- ions peroxidase activity was performed in two ways, i.e. in non-dialyzed – SP-2 and in dialyzed salivary samples (against quartz-distilled water using Spectrapor membrane tubing) – SP-3. The replacement of SCN^- by Cl^- in the assay mixture makes the method suitable for determination of myeloperoxidase activity since Cl^- is oxidized to OCl^- by MP, but not by SP [10].

The data from the measurements of salivary components were expressed as crude concentrations (concentrative-volumetric). Moreover, levels of enzymes were expressed as relative concentration (amount/secreted protein – specific activity and amount/flow rate). The obtained data were analysed by ANOVA.

Results

Mean values of analysed salivary parameters are presented in Table 1. Significantly higher total protein and thiocyanate contents in mixed and submandibular/sublingual saliva of caries-free subjects (group CF) in comparison to caries-susceptible ones (group CS) were observed.

Thiocyanate occurring in blood and mainly saliva are of extrinsic origin. Some vegetables contain SCN^- precursors (broccoli, cabbage, cauliflower, brussels sprout). Moreover, they result from detoxification of thiocyanate performed in liver, kidney and intestines, where hydrocyanide is transformed to SCN^- by thiosulphate sulphotransferase. Substantial source of thiocyanate is smoking and inconsiderable some nutritional products – almonds, manioc [11]. Significantly higher salivary SCN^- concentrations were found in smokers in comparison to non-smokers subjects [5, 12]. Moreover, Tenovuo et al. [5] observed the increase in SCN^- concentration along with the number of daily cigarettes. According to them, the only known SCN^- function in saliva is acting as an oxidizable cofactor in peroxidase system. Lamberts et al. [12] found significant negative correlation between SCN^- level and peroxidase activity in whole saliva. Reznick et al. [13] observed a sharp drop of peroxidase activity after smoking a single

Table 1. Mean (SD) crude concentration of analyzed salivary parameters

Tabela 1. Wartości średnie i odchylenia standardowe analizowanych wskaźników śliny wyrażonych jednostkach stężeniowo-objętościowych

Mixed saliva (SM) (Ślina mieszana)				
Group (Grupa)	CF (osoby bez próchnicy)	CS (osoby podatne na próchnicę)	Significant difference (Istotność różnic)	
	mean (SD) (średnia (SD))	mean (SD) (średnia (SD))		
Salivary flow rate (Szybkość wydzielania śliny) ml/min	0.19 (0.08)	0.19 (0.11)	–	
Protein (Białko) mg/ml	1.71 (0.85)	1.22 (0.51)	0.05	
Thiocyanate (Tiocyjanki) mmol/l	1.66 (0.70)	1.32 (0.58)	0.02	
Peroxidase activity – SP-1 by ABTS method (Aktywność peroksydazy SP-1, oznaczanej metodą ABTS) mIU/ml	1017.68 (397.92)	964.42 (497.02)	–	
Peroxidase activity SP-2 by NbsSCN method in non-dialyzed saliva (Aktywność peroksydazy SP-2, oznaczanej metodą NbsSCN w niedializowanej ślinie) mIU/ml	1.63 (0.40)	1.79 (0.60)	–	
Peroxidase activity – SP-3 by NbsSCN method in dialyzed saliva (Aktywność peroksydazy SP-3, oznaczanej metodą NbsSCN w dializowanej ślinie) mIU/ml	1.90 (0.48)	1.93 (0.66)	–	
Myeloperoxidase activity (Aktywność mieloperoksydazy) mIU/ml	0.63 (0.25)	0.78 (0.22)	0.05	
Submandibular/sublingual saliva (Ślina podżuchowo-podjęzykowa)				
Salivary flow rate (Szybkość wydzielania śliny) ml/min	0.16 (0.11)	0.15 (0.11)	–	
Protein (Białko) mg/ml	0.84 (0.46)	0.60 (0.23)	0.02	
Thiocyanate (Tiocyjanki) mmol/l	1.28 (0.65)	0.99 (0.51)	0.05	
Peroxidase activity – SP-1 by ABTS method (Aktywność peroksydazy SP-1, oznaczanej metodą ABTS) mIU/ml	155.90 (95.65)	164.87 (97.90)	–	
Peroxidase activity SP-2 by NbsSCN method in non-dialyzed saliva (Aktywność peroksydazy SP-2, oznaczanej metodą NbsSCN w niedializowanej ślinie) mIU/ml	0.18 (0.06)	0.16 (0.07)	–	
Peroxidase activity – SP-3 by NbsSCN method in dialyzed saliva (Aktywność peroksydazy SP-3, oznaczanej metodą NbsSCN w dializowanej ślinie) mIU/ml	0.19 (0.07)	0.17 (0.07)	–	

Table 2. Values of Pearson's correlation coefficient of salivary peroxidase levels measured by different methods**Tabela 2.** Wartości współczynnika korelacji Pearsona poziomów ślinowej peroksydazy oznaczanej różnymi metodami

Mixed saliva (MS) (Ślina mieszana)			
Group CF (Grupa osób bez próchnicy)			
	SP-1	SP-2	SP-3
SP-1		0.499	0.429
SP-2	0.533		0.756
SP-3	0.623	0.936	
Group CS (Grupa osób podatnych na próchnicę)			
Submandibular/sublingual saliva (Ślina podżuchowo-podjęzykowa)			
Group CF (Grupa osób bez próchnicy)			
	SP-1	SP-2	SP-3
SP-1		0.655	0.598
SP-2	0.746		0.965
SP-3	0.663	0.910	
Group CS (Grupa osób podatnych na próchnicę)			

cigarette in smokers and non-smokers, which after 30 min returned to the presmoking level, presumably due to the secretion of new saliva. In present study, the difference in salivary thiocyanate concentration between caries-free and caries-susceptible subjects could be elucidated by various intake of their precursors with diet. However, Mandel [14] evaluating hypothiocyanate in whole saliva obtained by expectoration and in stimulated submandibular saliva did not find statistically significant difference between caries-resistant and -susceptible adults. Grahn et al. [1] displayed no correlation between thiocyanate concentration in stimulated mixed saliva and caries prevalence expressed by DMFS and DS scores.

The level of peroxidase activity (crude concentrations) determined by three ways in both salivary secretions did not reveal any significant differences between groups. Similarly, Lamberts et al. [12] evaluating flow rate, thiocyanate and salivary peroxidase (by ABTS method) in unstimulated whole saliva did not notice significant differences between caries-free and -active subjects neither. Although all examined subjects had clinically healthy periodontal tissues, the myeloperoxidase activity was higher in caries-susceptible than in caries-free ones. Schiott et al. [15] observed that even in subjects with healthy periodontal tissue, leukocytes are found in the saliva and become the source of enzyme. Their findings were confirmed

in the present study as in the resting mixed saliva of all subjects with healthy periodontal tissue the MP activity was detected.

According to previously published results [1, 14], present data did not display significant difference in SP level (crude concentration) of mixed and submandibular/sublingual saliva between caries-free and -susceptible subjects, and in correlation with the number of caries affected teeth. Jentsch et al. [15], in a prospective study lasting over 4 years, studied caries increment and some salivary constituents but did not find any link of peroxidase activity in unstimulated and stimulated whole mixed saliva with caries lesions development. In recently published paper, Hanning et al. [16] described the occurrence of peroxidase in experimental pellicles.

In presented results, the total protein concentration and peroxidase activity (irrespective of measurements) in mixed saliva were significantly higher ($p < 0.001$) than in submandibular/sublingual saliva. The analysis of correlation did not display any significant correlation between analysed salivary components. The findings agree with the data obtained by Grahn et al. [1] who did not noticed any significant correlation between flow rate of stimulated mixed saliva and levels of SP, MP and protein. However, they revealed negative correlation of flow rate with thiocyanate concentration.

The values of Pearson's correlation coefficient of the three ways of salivary peroxidase measurements are presented in Table 2. Proportion of SP activity obtained in non-dialysed to dialysed samples of mixed saliva was 85% in CF group and 92% in CS group. Values of correlation coefficient were 0.756 and 0.936, respectively. Percentage of SP activity in non-dialysed to dialysed samples of submandibular/sublingual saliva was in both groups 94% and correlation coefficient was 0.965 in CF group and 0.910 in CS group. Activity level of SP determined by ABTS method was significantly positive correlated with SP evaluated by Nbs-SCN method in non-dialysed and dialysed salivary samples. The data confirm the results obtained by Mansson-Rahemtulla et al. [10] which were performed with use of purified salivary peroxidase enzyme that at pH 5.6 of assay mixture the rate of oxidation was high and independent of SCN^- concentration when it was 1–3 mmol/l (range of the level observed in saliva). However, in a mixture of enzymes salivary peroxidase and myeloperoxidase (condition occurring in human saliva) the presence of SCN^- results in a false Cl⁻ activity. Salivary peroxidase will catalyse the peroxidation of SCN^- to OSCN^- , and OSCN^- will oxidize NbsH to (Nbs)₂ but when myeloperoxidase is present in the same

Table 3. Mean (SD) relative concentration of salivary peroxidase and myeloperoxidase activities in dialyzed saliva**Tabela 3.** Wartości średnie i odchylenie standardowe pochodnych koncentracji ślinowej peroksydazy i mieloperoxydazy w dializowanej ślinie

Mixed saliva (Ślina mieszana)			
Group (Grupa)	CF (osoby bez próchnicy)	CS (osoby podatne na próchnicę)	Significant difference (Istotność różnic)
	mean (SD) średnia (SD)	mean SD średnia (SD)	
SP-3/mg protein (SP-3/mg białka) mIU/mgP	1.37 (0.72)	1.76 (0.80)	0.01
SP-3/flow rate (SP-3/szybkość wydzielania) mIU·mg/ml ²	14.30 (14.77)	12.02 (6.45)	–
MP/mg protein (MP/mg białka) mIU/mg P	0.50 (0.36)	0.73 (0.36)	0.01
MP/flow rate (MP/szybkość wydzielania) mIU/mg P	5.37 (3.96)	5.17 (2.80)	–
Submandibular/sublingual saliva (Ślina podżuchwowo-podjęzykowa)			
SP-3/mg protein (SP-3/mg białka) mIU/mg P	0.35 (0.33)	0.37 (0.24)	–
SP-3/flow rate (SP-3/szybkość wydzielania) mIU·mg/ml ²	1.94 (1.48)	1.70 (1.13)	–

solution, it will oxidize the Cl^- to OCl^- which also will oxidize NbsH. Therefore, the assay system did not allow to differentiate between the OSCN^- and OCl^- formed because both anions will oxidize the NbsH and in order to determine true MP activity, SCN^- should be removed.

Mean level of peroxidase activity (mIU/ml) obtained in present study is slightly lower than values for stimulated mixed saliva presented by Grahn et al. [1] – 1.00 ± 0.31 mIU/ml and Kirstila et al. [18] – 1.37 ± 0.69 mIU/ml using the NbsSCN assay method. Moreover, present findings can confirm the data obtained by Tenovuo et al. [19] and suggest that salivary peroxidase system is significantly lower in stimulated than in resting saliva.

In present study, the obtained level of myeloperoxidase activity (mIU/ml) is close to values presented by Grahn et al. [1] for non-dialysed stimulated mixed saliva (0.66 ± 0.21 mIU/ml). However, Kirstila et al. [18] reduced by half the level of MP in the supernatant of stimulated mixed saliva than the one described in data.

Similarly to Grahn et al. [1] the authors observed no significant correlation between the level of enzyme activity and salivary flow rate.

However, Smith et al. [20] suggested some correlation of MP activity with flow rate as they found lower activity in mixed and parotid saliva at higher rate of stimulation. Nevertheless, at higher flow rate of both secretions they did not detect the enzyme activity at greater numbers of examined subjects.

Mean relative concentrations of SP in dialysed salivary samples are presented in Table 3. Specific SP and MP activities (i.e. activity calculated per 1 mg protein) in mixed saliva, but not in submandibular/sublingual, turned out to be significantly higher in CS group in comparison to CF one. However, SP activity in both secretions calculated per salivary flow rate did not display any significant differences between groups.

Discussion

Biological importance of myeloperoxidase in the saliva is not fully clarified. It can indirectly influence the OSCN^- formation using hydrogen peroxide necessary for the oxidation of SCN^- during formation of OCl^- from chloride ions, but this

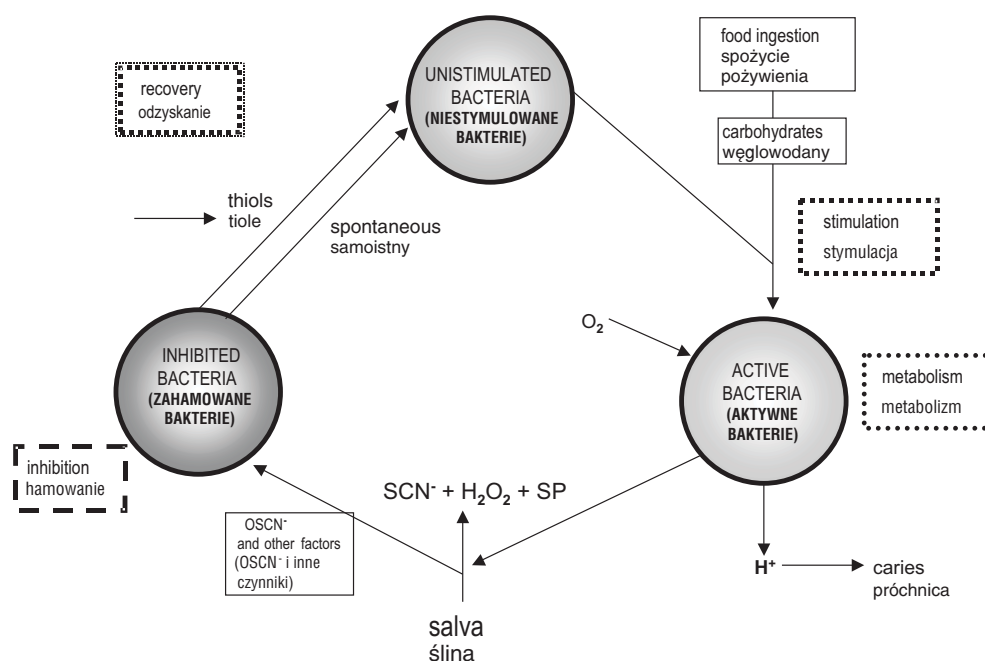


Fig. 1. Regulation of plaque bacteria by salivary peroxidase system proposed by Tenovuo et al. [19]

Ryc. 1. Regulacja bakterii płytki przez system ślinowej peroksydazy zaproponowana przez Tenovuo et al. [19]

reaction has probably minor importance because of greater affinity of the enzyme to SCN^- than to Cl^- in salivary pH conditions. Nevertheless, the presence of myeloperoxidase in saliva has to be taken into consideration during the study of salivary peroxidase system [20].

Tenovuo et al. [21] explained the role of salivary peroxidase system *in vivo* conditions (Fig. 1). After eating dietetic carbohydrates, they stimulate the metabolism of some species of aerobic bacteria, which produce H_2O_2 . Further, this production is stimulated by bacterial metabolism of the glycoproteins and mono-disaccharides occurring in saliva. The hydrogen peroxide reacts with SCN^- and peroxidase present in saliva and plaque yielding OSCN^- and probably other short-lived oxidation products. They can inhibit the bacterial metabolism by declining sugar transport and by inhibiting key glycolytic enzymes and diminish the production of acids. Inhibition of metabolism causes the drop of peroxide secretion and decrease of OSCN^- generation. At lower OSCN^- concentrations, spontaneous or thiol-induced recovery of inhibited bacteria may take place. As peroxidase system does not inhibit the growth of all oral bacteria, and besides *in vivo* conditions OSCN^- concentrations are low, the rate of acid production depends main-

ly on individual bacterial flora and saliva composition. Moreover, it should be taken into consideration that the other salivary defensive factors can contribute to the control of oral microorganism as well.

It could be suggested that levels of SP system constituents alone are not direct determinants of susceptibility or resistance to dental caries. Probably, the whole system acts more efficiently than each of its components. It should not skip its cooperation with other salivary defensive factors. It can be suggested that the results of studies on functional aspect of all antibacterial salivary factors evaluating their bactericidal potential, ability to agglutination and prevention of bacterial adherence to hydroxyapatite and capability of buffering would be the clue of defence mechanisms against caries forming factors. Moreover, the concentration of the peroxidase systems components in the oral cavity are in a state of dynamic flux regulated by bacterial metabolism, salivary flow rate, food intake and others factors.

As saliva is a complex system of quantitative and qualitative relationships of its constituents, the complete elucidation of the relationship between the salivary peroxidase system and dental caries will require further studies which go beyond the single point type measurements of the present study.

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