SALIVARY PROTEIN CHARACTERISTICS FROM SALIVA OF CARIOUS LESION-FREE AND HIGH CARIES ADULTS

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ABSTRACT

It has been argued that specific salivary proteins could have a protective effect against caries, but data from the many available studies are rather contradictory. The purpose of this study was to analyze whether there is a relationship between protein concentration, electrophoretic profile and concentration of salivary IgA and the presence or absence of caries in adults. Adults with high caries activity (HC) and without caries lesions (CF), assessed by ICDAS criteria, were asked to provide unstimulated saliva samples. Protein concentration (µg/mL) was determined using the Bradford method. Western blotting was used to detect IgA. Data were compared using Student's t test at p<0.05. Total protein concentration in CF was higher (50.65±7.5 µg/mL) than in HC individuals (26.80±2.5 µg/mL) (p=0.001). More protein bands were visualized in the gels from CF than the HC group (p=0.001). CF subjects showed higher salivary IgA concentration (11.27±0.5 µg) than HC individuals (1.71±0.2µg) (p=0.001).Salivary composition in high caries experience and caries-free young adults seems to differ in terms of the type and amount of proteins. Further research is needed to expand these findings.

Key words: *Dental caries, saliva, salivary proteins and peptides, Immunoglobulin A, Blotting, Western, electrophoresis.*

CARACTERÍSTICAS DE PROTEÍNAS SALIVALES DE ADULTOS LIBRES Y CON ALTO NÚMERO DE LESIONES DE CARIES

RESUMEN

Se ha descrito que proteínas salivales específicas podrían tener un efecto protector sobre la caries, sin embargo, los datos de los-numerosos estudios disponibles son contradictorios. El propósito de este trabajo fue analizar si existe una relación entre la concentración total de proteínas, perfil electroforético y la concentración de IgA salival y la presencia o ausencia de lesiones de caries en adultos. Se obtuvieron muestras de flujo salival no estimulado de adultos con alta actividad de caries (HC) y sin lesiones de caries (CF), evaluados según criterios ICDAS. La concentración total de proteínas (mg / ml) se determinó utilizando el método de Bradford. Para detección de IgA se empleó Western Blot. Los datos se compararon mediante la prueba t student, estableciendo diferencias significativas si p<0,05. La concentración total de proteínas en CF fue mayor (50,65 ± 7,5 mg / ml) que en individuos HC (26,80 ± 2,5 mg / ml) (p=0,001). En los geles, se visualizó un mayor número de bandas de proteínas en CF que en el grupo HC (p=0,001). Los Sujetos CF mostraron mayor concentración de IgA salival (11,27 ± 0,5 µg) que los individuos HC (1,71 ± 0,2 µg) (p=0,001). La composición salival de sujetos adultos jóvenes con alta experiencia y libres de caries, parece ser diferente en función del tipo y la cantidad de proteínas. Se requiere de más investigación para profundizar estos resultados.

Palabras clave: *Caries dental, saliva, proteínas y péptidos salivales, Inmunoglobulina A, Western blot, electroforesis.*

INTRODUCTION

Saliva contains several types of proteins, which constitute less than 1% of its total composition[1]. Despite the low proportion, this organic fraction actively participates in the defense of the oral cavity against deleterious agents[2]. Among the approximately one thousand salivary proteins currently described, only some have been related with protective activity against oral diseases. For example, differences in the amount and type of salivary proteins have been reported in subjects with and without systemic diseases, including diabetes[3], cystic fibrosis[4] and oral cancer[5]. Similarly, different protein patterns have been suggested in dental caries. Peroxidase, lysozyme, lactoferrin and histatin have been described as providing protective activity against the disease.

Although the mechanisms involved in the putative anticariogenic activity of salivary proteins have not been fully identified, it has been proposed that some

proteins, such as those from the peroxidase systems, lysozyme, lactoferrin and histatins, may interfere with sucrose metabolism or promote bacterial aggregation with the subsequent removal of cariogenic bacteria[6]. Another caries-protective mechanism involves mucins and proline-rich proteins. These proteins are adsorbed onto the surface of the dental hard tissues to form the acquired pellicle, which actively regulates the demineralization and remineralization process[7]. The interplay between specific proteins and certain bacterial species conditions the bacterial composition of the oral biofilm[8,9]. Due to these specific functions, salivary proteins could be one of the most important factors in protecting the host against caries[10]. Hence, it would be reasonable to assume that a specific protein composition of saliva could have an effect on caries onset. The many available studies regarding the role of salivary proteins in caries are rather contradictory[11]. In fact, it has been proposed that proline-rich proteins and salivary IgA may participate in the opsonization and impairment of bacterial adherence to the tooth surface in caries-free subjects [8,12]. Also, IgA may have an anti-caries effect due to inhibition of bacterial adherence, neutralization of some enzymes and bacterial toxins and to the synergistic activity with other salivary proteins such as lactoferrin or lysozyme[13]. Conversely, one study showed that higher lysozyme concentration from the parotid or submandibular saliva was unrelated to differential caries susceptibility[14]. Furthermore, other studies indicate that subjects with higher protein concentration[15] or IgA levels[13] in saliva had more carious lesions than those people with lower content, suggesting a protein-concentration effect.

The analysis of salivary protein composition can provide insight into the role played by these factors on the caries process. Due to the lack of certainty on this issue and the contradictory results reported in the literature, our aim was to analyze whether protein concentration, electrophoretic profile and salivary IgA concentration are related to the presence or absence of caries lesions in adult subjects.

MATERIALS AND METHODS

Subjects

From the patients attending to the Dental Clinics of the University of Talca, twenty young adults without clinical or radiographic caries lesions, missing teeth and restorations (DMFT = 0) (CF) were recruited. A second group of 20 subjects with high caries activity (HC), defined as having at least 3 lesions ICDAS codes 5 or 6 (mean 3.9 ± 0.7 lesions) was also invited to participate. Participants read and signed an informed consent. The study protocol and the consent form were approved by the Bioethics Committee of the University of Talca and the study was performed in accordance with the Declaration of Helsinki. Twelve women and 8 men (mean 24 ± 2 years old) were included in the CF group, while 11 men and 9 women 25 ± 3 years old were included in the HC group. Clinical procedures included general health questions and an intraoral examination. An experienced clinician, calibrated in the ICDAS criteria for carious lesion detection, performed all the examinations. Bitewing radiographs were obtained for each subject to determine the presence of proximal caries. All clinical examinations were conducted at a dental clinic (C8, Sirona Dental Systems GmbH, Bensheim, Germany). Participants received oral prophylaxis before the examination. Data from the exam were recorded in a specially-designed record. Subjects with oral or systemic conditions that could affect saliva flow or composition, subjects taking medication that decreases salivary flow and smokers were not included in the study[16].

Saliva samples

In a separate session, each participant was requested to provide unstimulated saliva. The subject was comfortably seated, with their head bent forward to gently pour saliva secreted for 15 minutes into a sterile 15 mL tube (Kima, Arzergrande, Italy)[16,17]. Each volunteer was instructed to refrain from rinsing or eating at least 2 hours prior saliva collection. To minimize the influence of the circadian rhythms on salivary flow, all samples were collected in a single session under the same conditions and by the same operator between 9:00 and 11:00 AM[18].

To minimize protein loss, each tube contained 60 µL of a protease inhibitor (Protease Inhibitor Cocktail Set I, Merck, Darmstadt, Germany) samples were kept at -20°C in a container with dry ice for a maximum of two hours during transportation to the laboratory[2]. Each sample was centrifuged at 29,000 *g* for 20 min at 4°C (Eppendorf 5810, Eppendorf AG, Hamburg,

Germany). Using 1 mL acetone and 40 µL DL-Dithiothreitol (DTT) (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA), a 1 mL aliquot from the supernatant was precipitated. Samples were stored 2 h at -80°C and then centrifuged at 4°C at 16,000 g (Eppendorf 5415 C, Eppendorf AG Hamburg, Germany), washed 3 times with 1 mL 90% acetone and 4 mL DTT. The supernatant was discarded and the resulting pellet was re-suspended in 100 µL of a solution of 1M Tris-HCl pH 8 + DTT 10 mM and 10% PMSF (v/v). The resulting suspension was used for the quantification of total protein concentration, saliva electrophoresis and immunological identification of IgA.

Salivary protein concentration

Protein concentration (µg/mL) was assessed with 10 µL of the suspension from the previous step by the Bradford method[19] with spectrophotometer (Victor ™ X - Multilabel Plate Reader, Perkin Elmer, MA, USA) at 595 nm. Using a calibration curve with albumin (Merck, Darmstadt, Germany), the optical density (OD) was converted to µg/mL using the Gen5 Data Analysis Software (Biotek, WA, USA).

Salivary protein electrophoresis

After determining the protein concentration in each sample, a standardized amount of sample, equivalent to 30 µg of protein was loaded in each well of a10% denaturing polyacrylamide gel with a molecular weight marker in the first lane (Spectra Multicolor Broad Range Protein Ladder, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). The procedure was performed in electrophoresis chambers (Mini-PROTEAN, Bio-Rad, California, USA) at 90V for 30 min and then at120V until completion. Gels were stained with Coomassie Blue for 30 min and washed for 2 h in a de-staining solution based on acetic acid and methanol. The number of bands in each gel was obtained by a digital scanner (Perfection V300 Photo Scanner, Epson America Inc., USA) and analyzed with the Gel-Pro Analyzer software (Version 4.0.00.001 for Windows, Media Cybernetics Inc., Rockville, MD, USA).

Western blotting for IgA

To identify IgA, a specific polyclonal antibody (Rabbit Anti-IgA, Santa Cruz, Dallas, USA) was used in western blot membranes (Nitrocellulose membrane for hybridization 11,327 - 41BL, Sartorius, Madrid, Spain). Membranes were blocked for 1 h with 8% skim milk and washed three times with TBS (Tris-Buffered Saline). Membranes were incubated with the primary antibody (IgA 0.2 µg/mL) under overnight stirring at 4°C. Subsequently, the membrane was washed with TBS-Tween (TBST) for 4 min and incubated with a mouse anti-rabbit IgG secondary antibody conjugated with HRP (2 mL + 10 mL TBS) under agitation for 1 h. Each membrane was washed four times with TBST for 3 min and bands were evidenced by immersion in 10 mL of a developing solution for 3 min (Super Signal, West Pico Chemiluminescent Substrate, Thermo Fisher Scientific Inc., MA, USA). Images of the bands were obtained through a digital camera (Fine Pix S2980, Fuji Film, Tokyo, Japan) and analyzed using the Gel-Pro Analyzer software (Media Cybernetics Inc.). To assess intensity, resulting bands were quantified and compared with those obtained in a standard curve with known concentrations of IgA.

Statistical Analysis

Normal distribution of all data was tested with Kolmogorov-Smirnov ($p>0.05$). Student's *t*-test was used to compare the differences between the study groups regarding total protein concentration, number of electrophoretic bands and IgA concentration. The differences were considered statistically significant if $p<0.05$. All analyses were performed using SPSS v15.0 software for Windows (IBM Corporation, NY, USA).

RESULTS

CF subjects showed higher ($p=0.001$) protein concentration in saliva (50.65 ± 7.5 µg / mL) than HC individuals (26.80 ± 2.5 µg/mL) (Fig.1). When the number of bands was evaluated, CF participants had an average of 12 ± 0.7 electrophoretic bands (Fig. 2A), which was higher ($p=0.001$) than HC subjects, who only had a mean of 7 ± 0.4 (Fig. 2B). The most distinctive difference in the electrophoretic pattern between groups occurred between 40 and 70 kDa. In that molecular weight range, bands in the CF group were more numerous and more intense than in the HC group. Likewise, a18 kDa band was observed in both groups, but with notoriously higher intensity in the CF subjects (Fig. 2).

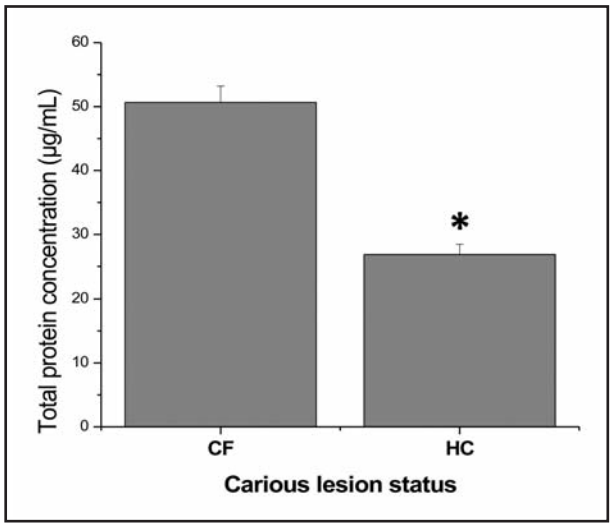

*Fig. 1: Total salivary protein concentration. Salivary protein concentration was assessed in CF and HC individuals. Bars show mean values (µg/mL) and error bars indicate standard deviation. * : p< 0.05.*

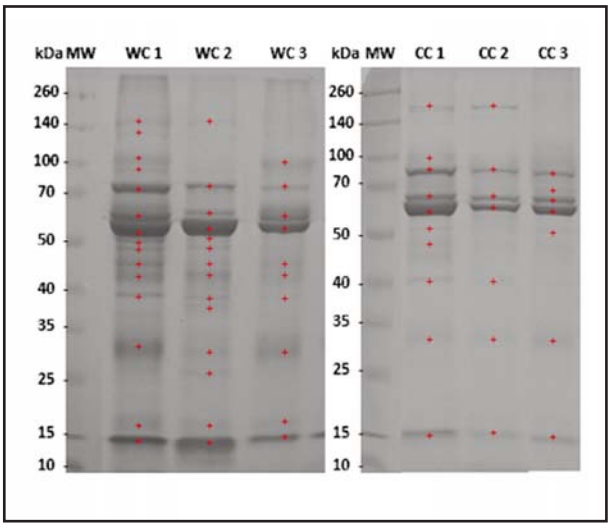

Fig. 2: Number of electrophoretic bands in CF and HC subjects. Denaturing polyacrylamide gels were made for CF and HC participants and the resulting number of bands were counted and compared between the groups. Panel A shows the number of bars obtained in three CF individuals, while panel B depicts the number of protein bands from saliva from three HC subjects. Black marks highlight the area of the gel zone (between 40 and 70 KDa) where most of the variability took place. Black arrow points to the area of the gel where a 18 KDa band was visualized only in CF subjects.

When IgA expression was compared between study groups by Western blotting, densitometry showed greater intensity (p=0.001) in the IgA bands (50 – 55 kDa: IgA heavy chain [20]) obtained in CF subjects than in HC subjects (Fig. 3A). When the concentration of salivary IgA was quantified by densitometric digital analysis, CF subjects showed higher concentration (11.27 ± 0.5 µg) than HC individuals (1.71 ± 0.2 µg) (p=0.001) (Fig. 3B).

DISCUSSION

The chief finding in our study was that protein concentration, electrophoretic profile and concentration of salivary IgA differed between young adults with no caries experience and those with a high number of lesions. Total salivary protein concentration was higher in subjects without lesions. Very few studies have reported on this issue and their results are as yet inconclusive[11]. Although some studies have reported a difference in total concentration of salivary proteins between individuals with different caries experience[21-24], the methodologies used are heterogeneous and the results cannot easily be compared with ours. Given the lack of a standard protocol that could have been used as a gold standard, we tested several methods to optimize protein yield from saliva. After the pilot studies testing the final methods presented herein, we created a working protocol that may be used in further studies on the subject.

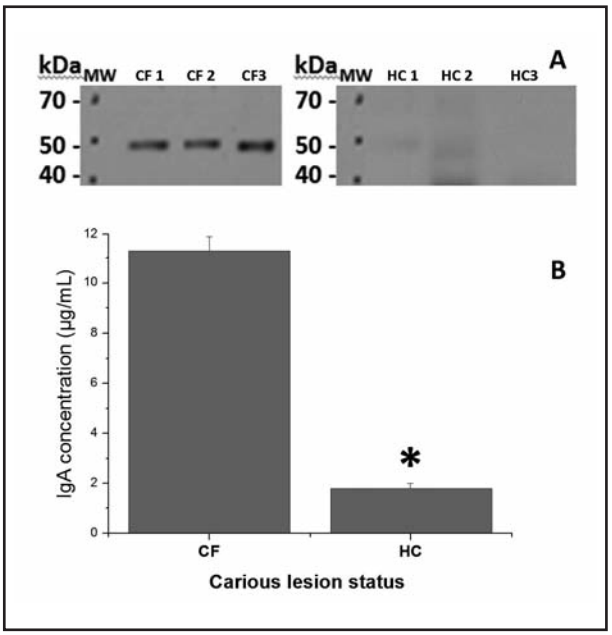

*Fig. 3: IgA identification by Western Blot in CF and HC participants. Panel A shows the resulting WB bands at about 50 KDa in CF and HC individuals. Band intensity was quantified using software and normalized against a standard curve. Panel B shows the resulting mean IgA concentrations in both CF and HC subjects. Error bars show standard deviation. *: p< 0.05.*

When the electrophoretic pattern was compared between study groups, CF subjects had a higher number and variety of proteins in saliva. These differences are expressed most clearly in the bands between 40 and 70 kDa. Salivary proteins corresponding to this molecular weight include Amylase (46-60 kDa), IgA (70 kDa), Albumin (67.5kDa), Haptocorrin (60-80 kDa), α2 glycoprotein (41 kDa), α–Amylase (58 kDa), Catalase (55 kDa), Enolase 1 (47 kDa) and Transferrin (52 kDa)[25, 26]. Although electrophoresis is a rather basic analysis, this preliminary approach to a more detailed study of the protein component of saliva and its relation to caries confirms the hypothesis that there are differences in protein content in the saliva of sound individuals compared to those severely affected by caries. These encouraging results call for further research aimed at specific protein identification. In fact, the antibody Anti-IgA provides information on monomeric, dimeric and secretory IgA. We have not individualized the different origin of the proteins, as they can all be present in total unstimulated saliva. Further studies should be conducted to establish the specific characteristics of the antibody. Hence, molecular sequencing techniques may be used to identify and characterize the variants of the proteins between both groups. Precise identification of one or more proteins exclusively present incaries- free individuals opens up the field to novel strategies for caries prevention or treatment. In fact, new salivary peptides with high bactericidal power have been described, with very low potential for bacterial resistance[27].

Regarding IgA, our results are consistent with others that have shown that IgA is more abundant in caries-free subjects than in people with caries[28-31]. In this study, IgA concentration was nine times higher in CF subjects than in the HC group. IgA is the main immunoglobulin involved in the defensive response of saliva[28]. IgA may interfere with adhesion and neutralizes bacterial products alone or in association with other proteins such as lysozyme and lactoferrin[32]. Although a clear association between IgA concentration and presence of caries remains elusive[33], our findings are comparable to other studies reporting low incidence of carious lesions in subjects with higher IgA concentrations[34-36]. In this sense, IgA deficiency may represent an interesting novel risk factor for caries[31]. Furthermore, most of these studies have been conducted on children, so this report is one of the few that shows this relationship in adults.

It is important to mention that all the participants in this study had normal unstimulated salivary flow, as recommended[37]. This methodological approach avoids the potential source of bias as a result of taking samples from individuals with low salivary flow. Indeed, a previous study showed that a marked increase or decrease in salivary flow volume was related to a proportional change in salivary protein concentration due to a dilution effect on the salivary molecules[22]. Furthermore, some authors suggest the use of stimulated saliva for the analysis of its biochemical characteristics because it is easy to obtain and to standardize the procedures[38]. Nevertheless, in this study we used unstimulated saliva. While unstimulated saliva is in contact with the dental tissues most of the time in the mouth, stimulated saliva mainly occurs during food consumption[39]. Thus, it may be speculated that unstimulated salivary flow has a more permanent effect on caries. It is reasonable to assume that continuous contact with the hard tissues of the tooth implies a greater protective effect of unstimulated saliva than of stimulated secretion. Thus, the acquired pellicle, which allows bacterial adhesion to the tooth, may be formed mainly from unstimulated saliva. Other researchers have used saliva directly obtained from one or more salivary glands[40-42]. In this study, we decided to analyze whole saliva, as it better represents the actual clinical situation. The etiological dental biofilm is exposed to whole saliva and not only to the secretion that comes from the gland[25, 43]. Importantly, IgA may have been derived from the crevicular fluid and may have been pooled with that from the salivary glands. More precise techniques should explore this possibility.

In contrast to the results of a study conducted in a Colombian population[44] reporting that men with high number of caries have a differential electrophoretic band located at 17 kDa, we found a band at 18 kDa, which is consistently observed in the gels from CF subjects. Proteins described with that molecular weight include Cofilin (18 kDa), Cystatin (17 kDa), IgG–J chain (16 kDa), prolactin-induced protein (17 kDa) and extra-parotid glycoprotein (EP-GP) (18 kDa)[25, 26]. The differences between the studies are not surprising. As mentioned, protocols designed to obtain salivary proteins differ greatly among researchers. To

develop a reliable protocol, we made several attempts and used alternative methods. The main difficulty to overcome was the very small protein concentration in saliva. Human saliva contains only 1% organic matter, of which only a small fraction corresponds to proteins. Procedures therefore clearly require optimization. For example, once obtained from saliva, proteins were precipitated with acetone and all procedures were performed immediately and under cold conditions to prevent degradation. Additionally, samples were treated at all times with protease inhibitors, also to prevent loss and peptide denaturation. Besides these technical factors during sample processing, the variability in the molecular weight of the bands between studies may derive from the fact that proteins can form complexes with each other or with polysaccharides[45]. Degradation may also explain differences reported in the literature[46]. As a result, bands with larger or smaller molecular size may arise. Undoubtedly, more sophisticated and precise techniques are required to clearly identify proteins that may be present in higher or lower amount in the groups studied. Despite the differences in protein concentration, electrophoretic profile and salivary IgA concentration between the two groups under testing, current state-of-the-art precludes using these variables as biomarkers for dental caries[11]. If more precise protein characterization develops in the future to allow a clear distinction between some proteins that may be only present in caries patients, saliva may well serve as an easily obtainable target for caries risk assessment. Proteomic analysis with mass spectrometry should be used in future research. Since saliva is a readily accessible secretion that can be obtained from the mouth without any surgical or invasive procedure, sampling is usually completely pain-free. Hence, samples may be obtained from small children without the complications inherent to other sampling sites of the body, such as the gut or the bloodstream. Furthermore, the cost for obtaining large samples is minimal, allowing for massive widespread application[47]. Although saliva is already being used for the detection of specific antibodies in some diseases (HIV, Hepatitis C), hormones or drugs, its use for diagnostic purposes is still limited due to the variability and polymorphism in the salivary proteome. Given the potential for accurate caries risk assessment from salivary proteins, further research is strongly encouraged.

Despite the limitations of the experimental setting, total protein concentration, number of electrophoretic bands and concentration of salivary IgA appear to be higher in adults with no caries experience than those who have a high number of carious lesions. Salivary composition, particularly IgA, may be a potential target for caries risk assessment.

ACKNOWLEDGEMENTS

The authors thank Dr. Luis Morales-Quintana, Dr. María Cecilia Opazo and Dr. Patricio Ramos Campos for helping with the experimental settings to standardize the techniques for protein quantification. Funded by a Grant from the Interdisciplinary Excellence Research Program on Healthy Aging (PIEI-ES), University of Talca, Talca, Chile to Ramiro J. Castro and a Chilean Government Grant Fondecyt number 1140623 to Rodrigo A. Giacaman.

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