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Increased expression and levels of human β defensins (hBD2 and hBD4) in adults with dental caries

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ABSTRACT

Introduction: Defensins are small anti-microbial peptides produced by epithelial cells. These peptides have a broad range of actions against microorganisms, including Gram-positive and Gram-negative bacteria. Human defensins are classified into two subfamilies, the α-, and β- defensins, which differ in their distribution of disulphide bonds between the six conserved cysteine residues. Defensins are found in saliva and others compartments of the body. Human β defensins 2 (hBD2), beta defensins 4 (hBD4) and alpha defensins 4 (hNP4) in saliva may contributes to vulnerability or resistance to caries. This study aimed to determine a possible correlation between caries and levels of defensins measuring the expression in gingival tissue and concentrations in saliva samples.

Methods: Oral examinations were performed on 100 adults of both genders (18-30 years old), and unstimulated whole saliva was collected for immunoassays of the three peptides and for the salivary pH, buffer capacity, protein, and peroxidase activity. mRNA levels of defensins in gingival sample were assessed by semi-quantitative RT-PCR technique.

Results: The median salivary levels of hBD2 and hBD4 were 1.88 μg/ml and 0.86 μg/ml respectively for the caries-free group (n=44) and 7.26 μ/ml (hBD2) and 4.25 μg/ml (hBD4) for all subjects with evidence of caries (n=56). There was no difference in the levels of hNP4, salivary pH, and proteins between groups, however the peroxidase activity and buffer capacity (interval 6.0-5.0) were reduced in caries group. Transcriptional levels of hBD2 and hBD4 did correlate with caries experience, the mRNA expression of hBD2 and hBD4 were significantly higher in patients with caries than in patients with no-caries ($p < 0.01$).

Conclusion: We conclude that high salivary levels and expression of beta defensins, low peroxidase activity and buffer capacity may represent a biological response of oral tissue to caries. Our observation could lead to new ways to prevent caries and a new tool for caries risk assessment.

Keywords: Saliva, Antimicrobial Peptides, Defensins, Caries.

INTRODUCTION

Saliva plays a central role in oral health (1,2). Saliva is produced and secreted from salivary glands. The basic secretory units of salivary glands are clusters of cells called acini. These cells secrete a fluid that contains water, electrolytes, mucus, enzymes and

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antimicrobial peptides, all of which flow out of the acinus into collecting ducts. Antimicrobial peptides are important members of the host defense system. They have a broad ability to kill microbes (3). In human the two main antimicrobial peptide families are defensins and cathelicidins. Defensins are small anti-microbial peptides act by disrupting the structure or function of microbial cell membranes, and are found in saliva and others compartments of the body (3). Evidence is accumulating that defensins play an important role in defense against pathogens and they are considered as part of innate immune response (3). They have generally been considered to contribute to mucosal health; however, it is possible that these peptides can be considered biological factors that influence the appearance of caries. Defensins are cysteine-rich, cationic peptides with β-pleated sheet structures. Mammalian defensins are classified into three subfamilies, the α-, β- and θ-defensins, which differ in their distribution of and disulphide bonds between the six conserved cysteine residues (4). Defensins have activity against a wide variety of bacteria, fungal, and viral targets. Under optimal conditions, antimicrobial activity of defensins is observed at concentrations as low as 1-10 μg/mL. The major mechanism of antimicrobial activity of all defensins is thought to occur through interaction with the membrane of the invading microbe resulting in a release of the cell contents (5). Model bacteria (*Escherichia coli* ML-35) that were treated by defensins became permeable to small molecules. In bacteria, permeabilization coincided with the inhibition of RNA, DNA and protein synthesis and decreased bacterial viability as assessed by the colony forming assay. Conditions that interfered with permeabilization also prevented the loss of bacterial viability, indicating that permeabilization is essential for bacterial killing (6).

The human β defensins are widely expressed in oral tissues including gingival epithelium, salivary glands, ducts and saliva (3,4). It is known that these peptides are involved in defense against bacteria that can colonize the oral mucosa. The presence of defensins in saliva implies their potential role in protecting tooth structure from bacterially-induced caries. The hBDs have broad antimicrobial activity against oral microorganisms such as *Streptococcus mutans, Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (3,5). The amount of hBDs expressed in saliva varies between individuals. This has been previously demonstrated for α- and β -defensins, histatin, and proline-rich proteins (7,8). This study shows that the levels of these peptides in unstimulated saliva vary greatly between individuals, even when differences in total salivary protein are considered. To date there are no reports of normal values of defensins in human saliva. It has been proposed that the variation in concentration of defensins in saliva could be attributed to the genetic factors. The genes for hBDs lie in a cluster on human chromosome 8. Several genes in this region can occur as multiple repeated copies (9). It is not well known if human with several copies of hBD2, for example, produce more defensins than other peoples. In the same way, individual differences in the quantity of α- and β -defensins may be genetically determined. There is also data for genetically determined factors in susceptibility to caries. Some evidence suggests individual differences in caries experience in patients within the same family (9). These individual differences suggest that genetic factors may play an important role in caries resistance or susceptibility. The purpose of this study was to determine a possible correlation between hBDs levels in saliva and caries experience in adults. We show high levels and high expression of hBDs in adults with caries experience. Our findings suggest that high salivary levels of hBD2 and hBD4 may contribute to caries response.

METHODS

Patients

This study was approved by the Institutional Review Board of the Applied Biotechnology Laboratory. Written informed consent was obtained from each participants. One hundred subjects participated in the study. Oral examinations were performed by trained calibrated clinicians using standardized procedures. All samples were obtained with informed consent. Examiners were instructed to rank subjects separately for active caries as follows: 0, caries-free group; 1, mild (one to three caries); 2, moderate (four to six caries); 3, severe (more than six caries). All patients were apparently healthy and were excluded from the study patients with a history of systemic disease or taking medications likely to influence periodontal health.

Collection of gingival samples

Tissue samples from caries group (n=4) were taken from interproximal sites showing redness and/or bleeding on probing, but no clinical attachment loss. The control specimens (caries-free group) (n=4) were collected during impacted third molar extraction surgery. All tissue samples were placed in Trizol solution and stored at -80ºC until the analysis.

Collection of saliva

Saliva samples were collected (4 to 6 ml) in a tube containing Nonidet P-40 to a final concentration of 0.2% v/v. Saliva was cleared by centrifugation twice at 3000 x g for 20 min at 4°C. Total protein concentration was evaluated in the supernatant by bradford assay (10). Cleared unfractionated saliva was used for pH and buffer capacity determinations. Aliquots (200 µl) of supernatant were acid extracted by the addition of an equal volume of 1 M HCl/1% trifluoroacetic acid overnight at 4ºC (11). The sample was centrifuged, and the supernatant was concentrated by vacuum evaporation and resuspended in distilled water equal to the starting sample volume (11). Acid-extracted saliva was used for immunoassay (ELISA).

pH and buffer capacity determinations

Cleared unfractionated saliva was used for pH determination with a portable pH-meter (Cole Parmer ACCUMET AB15). The buffer capacity was determined by titration using 1 mL of saliva, with 0.01 M HCl and after each addition of acid the change in pH was monitored up to pH 5.0. The buffer capacity was analyzed by ranges of pH. The volume of acid added to the saliva was calculated for each interval considered: initial pH-7.0, pH 7.0-6.0, and pH 6.0-5.0. The buffer capacity was expressed in volume (mL) of the acid added to 1 mL of saliva in the pH range considered, instead of equivalents of H.

Activity of peroxidase

Peroxidase activity was measured in the patients' saliva according to the 2-nitrobenzoic acid-thiocyanate (NBS-SCN) assay as previously described (12). Briefly, the calorimetric change induced by the reaction between the enzyme and the substrate, Dithiobis 2-Nitrobensoic Acid (DTNB) in the presence of mercapto-ethanol, was read at a wavelength of 412 nm for 20 s. One unit of enzyme activity was defined as the level of enzyme activity needed to cleave 1 µmol of NBS/min at 22°C, using a molar extinction coefficient of 12,800.

ELISA

We coated 96-well immunoplates (MaxiSorp™; Nunc) with 100 µL of Acid-extracted saliva diluted in 0.05 mol/L carbonate buffer, pH 9.6, 4 °C, for 12 h. Subsequently, we blocked the wells with 200 µL of 1% bovine serum albumin (BSA) in PBS at room temperature for 2 h. After washing 5 times with 200 µL PBS containing 1 mL/L Tween 20, we incubated 100 µL/well with PBS containing 1% BSA and a 1:1000 dilution of anti-human BD2, BD4 or HNP4 (Santa Cruz Biotechnology) at room temperature for 2 h. The plates were washed 5 times with PBS containing 1 mL/L Tween 20, and wells were incubated at room temperature with 100 µL of peroxidase-coupled secondary antibody (Santa Cruz Biotechnology, cat. No. sc-2350 (for BD2 and HNP4) or cat. No. sc-2370 (for BD4) diluted to 1:5000 in PBS plus 1 mL/L Tween 20 for 30 min. Plates were washed 5 times as described above, and incubated with 100 µL of substrate (0.2M Na2HPO4, 0.1M citric acid, 0.1% H_2O_2, 15mg O-phenylenediamine dihydrochloride) to each well in the dark at room temperature for 10 min. Stop solution (100 µl, 0.5M H_2SO_4) was added to each well. Absorbance was measured at 405nm using a microtiter plate spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA).We quantified hBDs by simultaneous ELISA runs using recombinant hBDs as calibrators.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from all tissue samples by Trizol™ (Invitrogen) as previously descried (12, 13). RNA concentration and purity were measured using a spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA). Total RNA (1 µg) was reverse transcribed into cDNA using a commercial kit (Invitrogen ThermoScript™ RT-PCR System), according to the manufacturer's instructions. Control reactions to check for DNA contamination were run in parallel with samples processed without reverse transcriptase. PCR was performed in a final vol-

ume of 25 µl containing 1 µl of the reverse transcription reaction, 50 µM deoxynucleotide triphosphates, 1.5mM MgCl2, 50mM Tris–HCl (pH 8.0), 1 IU Taq DNA polymerase and 0.2 µM each of sense and antisense hBD2 and hBD4 primers (see sequences below). PCR was performed in an Eppendorf Mastercycler STM thermocycler for 35 cycles consisting of denaturation at 94°C (1min), annealing at 60°C (1min) and extension at 72°C (1min). Amplification was terminated by a final extension step at 72°C for 5min. A negative control without the cDNA template was run with every assay to evaluate the overall specificity. The integrity of the template RNA was checked by confirming expression of β-actin mRNA. The primer sequences were: β-actin sense, CACGCCATCCTGCGTCGGAC; β-actin antisense, CATGCCATCCTGCGTCTGGAC; hBD2 sense, TTCCTGATGCCTCTTCCA; and hBD2 antisense, ATGTCGCACGTCTCTGA; hBD4 sense, GGCAGTCCCATAACCACATATTC; and hBD4 antisense, TGCTGCTATTAGCCGTTTCTCTT, hNP4 sense, TGCCGGCGAACAGAACTTCGT; and hNP4 antisense, ACCGATGATGGCGTTCCCAGC, Aliquots (10 µl) of the polymerase chain reaction products were electrophoresed on 1.5% agarose gels and stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen™). Densitometric analyses were performed using the image analysis software Quantity One (Bio-Rad laboratories, Hercules, CA, USA). Briefly, the digital image was analyzed to determine the pixel intensity of each band. Relative quantities of hBD2 and hBD4 mRNA among different preparations were calculated as the ratio of the hBD2: β-actin and hBD4: β-actin pixel intensities from three independent RT-PCR experiments. Positive results were based on the presence of DNA bands of the expected size.

RESULTS

Caries experience

Fifty-four females and 46 males participated in the study. All subjects were between 18 and 32 years of age. Overall, the adults were healthy, with 90% having no history of disease. Oral examination showed that 15% had loose teeth. Sixty-one percent of the population reported having regular dental care. Gin-

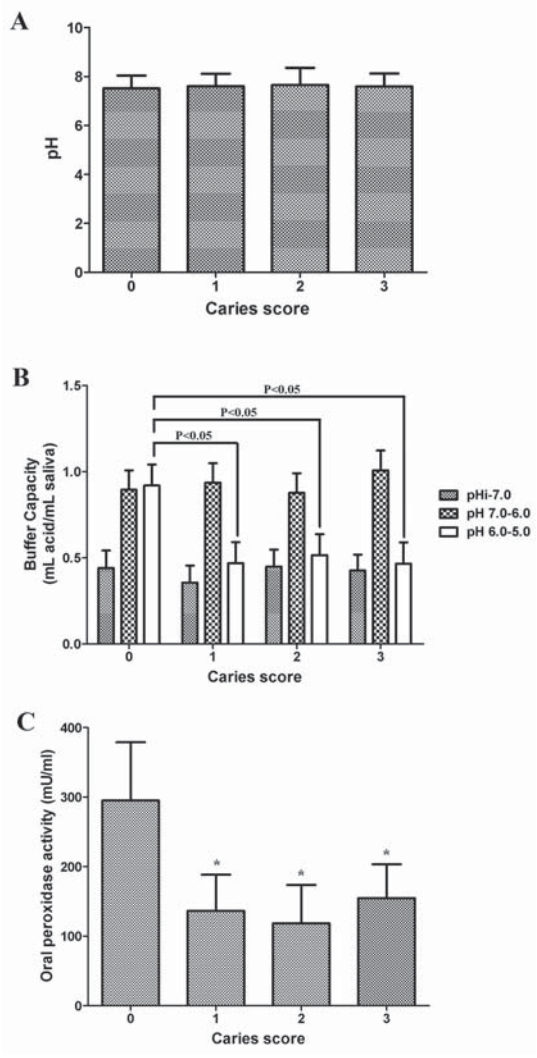

FIGURE 1. pH, Buffer capacity and oral peroxidase activity in saliva. Cleared unfractionated saliva was used for pH determination. The buffer capacity was determined by titration using 1 mL of saliva, with 0.01 M HCl and after each addition of acid the change in pH was monitored up to pH 5.0. The buffer capacity was analyzed by ranges of pH. The volume of acid added to the saliva was calculated for each interval considered: initial pH-7.0, pH 7.0-6.0, and pH 6.0-5.0. No difference in the saliva pH between the groups was noted (A). Considering the pH intervals analyzed, the buffer capacity showed no difference between the groups either in the initial interval pH - 7.0 or pH 7.0-6.0. In the interval pH 6.0-5.0 the caries-free group showed a higher value than the control group (P<0.05) (B). Peroxidase activity was measured as previously described (12). The caries-free group showed significantly higher oral peroxidase activity than each of the groups with caries (*, P<0.05).

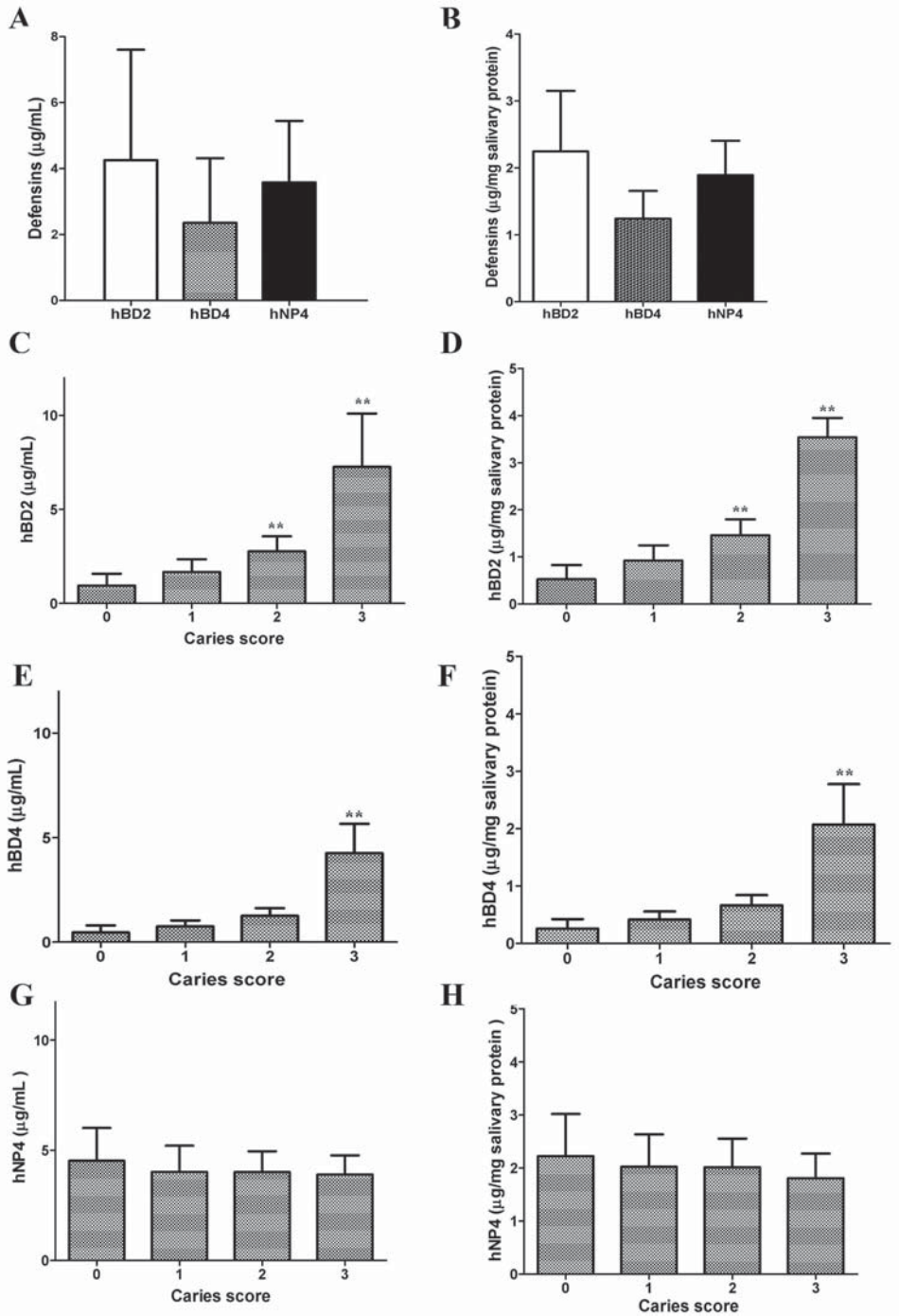

FIGURE 2. Defensins levels in saliva as a function of caries score. Saliva was thawed and cleared by centrifugation twice at 3000 x g for 20 min at 4°C, proteins were precipitated with HCl-TCA. Defensins concentrations were determined by ELISA using anti-hBD2, anti-hBD4, or anti-hNP4 as primary antibody, as indicated in Materials and methods (12). The figure shows the measured concentrations of defensins expressed as µg/ml (A), and relative to salivary protein in µg/mg protein (B). hBD2, hBD4 and hNP4 concentrations in saliva, expressed as µg/ml; (C, E and G) and relative to salivary protein in µg/mg protein (D, F and H). The caries group showed significantly higher hBD2 and hBD4 concentration (A and B) than each of the groups with no caries (**, P<0.01). Each assay was carried out in three independent experiments, and results are reported as mean±S.D.

givitis was noted in only a small number of subjects (10%). forty-four subjects (44%) had no dental caries; 19 (19%), 25 (25%), and 12 (12%) had caries scores of 1, 2, and 3 or greater, respectively.

Salivary analysis

The median protein concentration of unstimulated saliva samples (n=44) were 1.35 mg/ml (range from 0.63 to 2.67 mg/ml) for caries-free group and 1.29

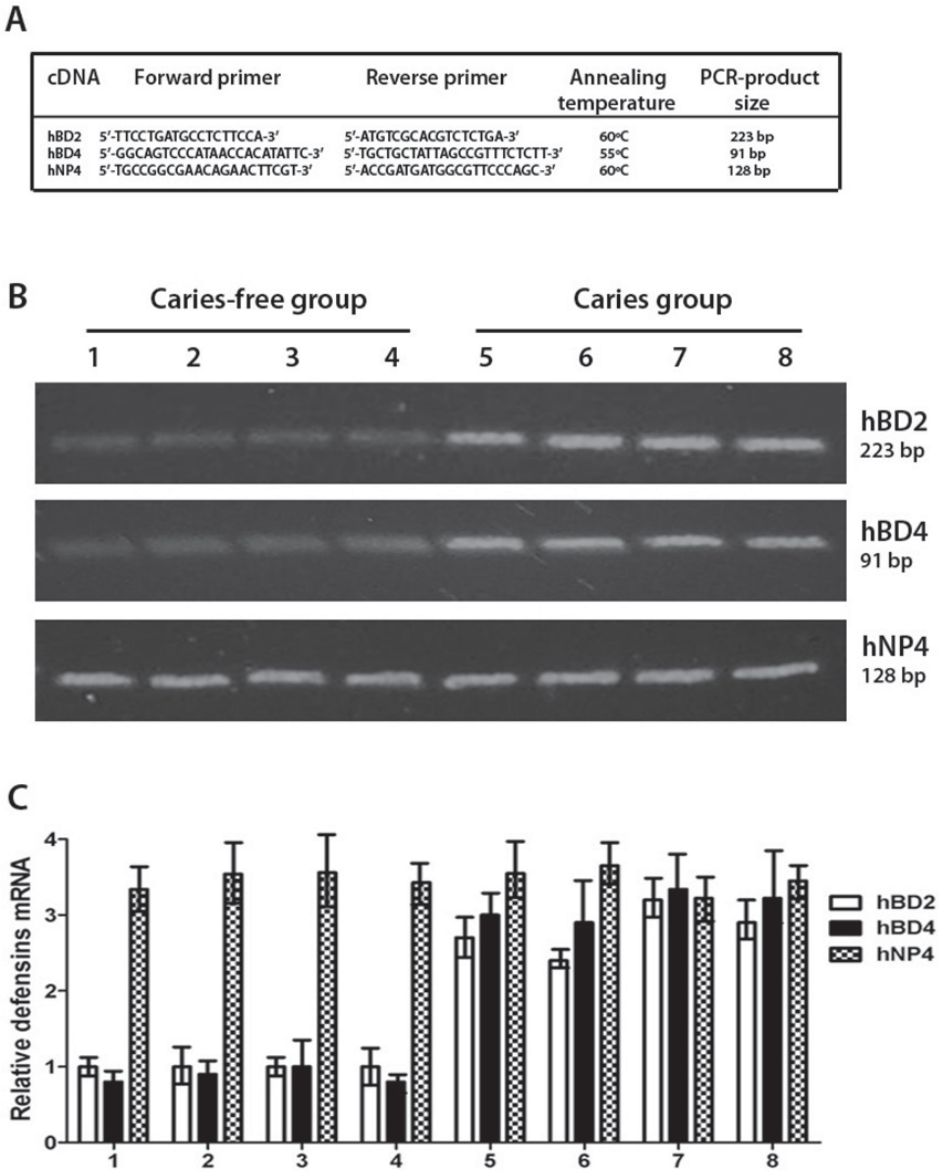

FIGURE 3. Quantification of differentially-expressed defensins mRNAs by RT-PCR. (A) Specific primers and annealing temperatures employed. (B) RT-PCRs for hBD2, hBD4 and hNP4 were carried out from gingival samples divided in two main groups: caries-free (1-4) and caries (5-8). The PCR-products were run onto 2% agarose gel electrophoresis. Control reactions without reverse transcriptase were carried out. PCR was performed in a final volume of 25µl containing 1µl of the reverse transcription reaction, 50µM of dNTPs, 1.5mM MgCl2, 50mM Tris–HCl (pH 8.0), 1 IU Taq polymerase and 0.2µM each of sense and antisense primers. Specific PCR for a constitutively expressed gene (β-actin) was carried out as a positive control (data no shown). The relative amount of product was quantified by densitometric analysis of DNA bands (C). Defensins-mRNA expression levels are shownnormalized to β-actin. Results are mean ± SEM of three independent experiments.

mg/mL (range from 0.79 to 2.89 mg/mL) for all subjects with evidence of caries (n=56). The salivary protein concentration showed no correlation with age, gender, or caries score (data not shown). No difference in the saliva pH between the groups was noted (Figure 1A). Considering the pH intervals analyzed, the buffer capacity showed no difference between the groups either in the initial interval pH - 7.0 or pH 7.0-6.0. In the interval pH 6.0-5.0 the caries-free group showed a higher value than the control group (P<0.05) (Figure 1B). The peroxidase activity was reduced for all subjects with evidence of caries when compared with the control group (P< 0.05) (Figure 1C). Finally, the levels of salivary defensins hBD2, hBD4 and hNP4 were in the µg/ml range (Figure 2A). hBD levels were also normalized to the protein concentration in whole saliva for each sample (Figure 2B).

Association between defensins and caries experience

In order to evaluate the relationship of defensins levels and caries experience in the population, we used the Kruskal-Wallis nonparametric test based on rank as previously reported (11,14). We found a significant difference in the level of hBD2 and hBD4 among different caries groups (P < 0.01). Differences were observed for both the median level of salivary defensins concentration (µg/ml) and salivary defensins relative to salivary protein (µg/mg) (Figs. 2C-2F). On the other hand, there was not difference in the level of hNP4 among different group (Figs. 2G and 2H). The median salivary levels of hBD2, hBD4 and hNP4 were 7.26 µg/ml, 4.25 µg/ml and 4.52 µg/ml respectively for the caries group (n=44) and 1.88 µ/ml (hBD2), 0.86 µg/ml (hBD4) and 3.91 µg/ml (hNP4) for all subjects with no evidence of caries (n=56). The defensins value relative to total salivary protein was 3.53 µg/mg (hBD2), 2.07 µg/mg (hBD4) and 2.22 µg/mg (hNP4) protein in the caries group and 0.96 µg/mg (hBD2), 0.44 µg/mg (hBD4) and 1.98 µg/mg (hNP4) protein in the caries-free group (P < 0.01). hBD2 and hBD4 concentration was positively correlated with caries score (r = 0.7525 and r = 0,7201 respectively), and the correlation is significant at the 0.0001 level (P < 0.0001). No correlation was found between caries level and hNP4 concentration. Additionally, semi-quantitative RT-PCR was used to determine whether the caries group present an increase in oral epithelial cell expression of hBD2 and hBD4 mRNA. As shown in Figure 3, in the caries group there was a significantly higher expression of hBD2 and hBD4 compared with defensin levels in caries-free group. On the other hand, there was no difference in the expression of alpha defensin 4 (hNP4). Together, the results demonstrate that hBD2 and

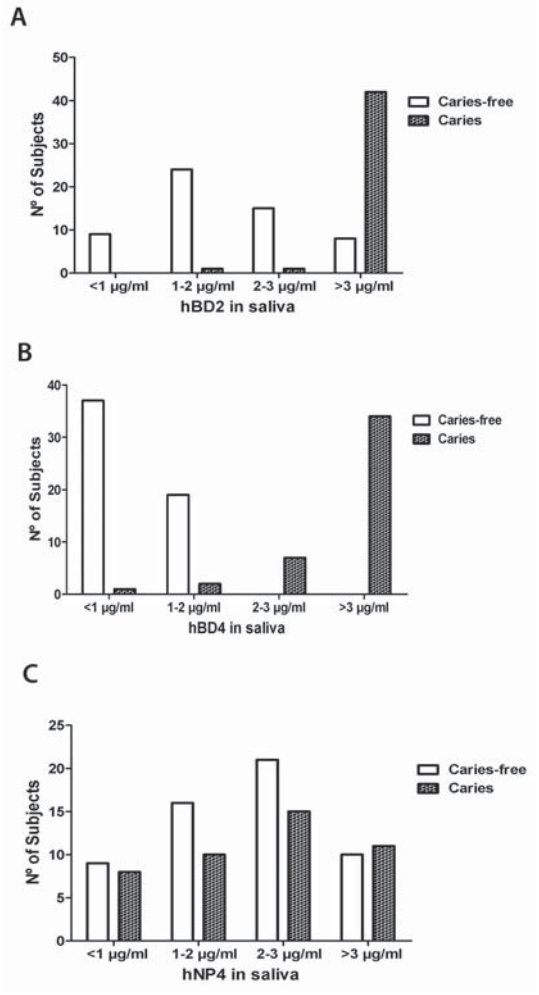

FIGURE 4. Defensins values and caries in the population. The number of subjects with no caries (open bars) compared to those with caries (filled bars) with hBD2 (A), hBD4 (B) and hNP4 (C) concentrations (µg/ml saliva) in the ranges indicated. Note that an increasing proportion of subjects had caries as the defensins (hBD2 and hBD4) concentration increased. hNP4 analysis showed no significant differences among the population, with the same levels of hNP4 in caries and caries free group.

hBD4 were upregulated in caries group. To further examine the relationship of defensins with caries, the hBD2, hBD4 and hNP4 concentration range was evaluated in subjects with no caries compared to those with caries (Figure 4A-C). hBD2 analysis showed an increasing proportion of subjects had caries as the defensins concentration increased; 86% of the subjects with defensins levels lower than 3.0 μg/ml (n = 48) had no caries, but only 15% of the subjects with hBD2 levels greater than 3.0 μg/ml (n = 8) had no caries (Figure 4A). Similar analysis for hBD4 is shown in Figure 4B. The results showed the same trend with higher levels of hBD4 in the caries group than in those with no caries. On the other hand, hNP4 analysis showed no significant differences among the population with the same levels of hNP4 in caries and caries free group (Figure 4C).

DISCUSSION

Salivary constituents are potential candidates as biological factors influencing caries risk. Many salivary protein components, such as glycoprotein, immunoglobulins, agglutinin, lactoferrin, and defensins are thought to have a role in defense in the oral cavity (15). The salivary protein concentration showed no correlation with age, gender, or caries score. According to Rudney *et al*. (16) a high protein concentration in the saliva contributes to greater adherence of *S. mutans*, the first resident of dental plaque, however, in this work there was no difference in protein levels between caries and no caries groups. The mean saliva pH values of the 2 groups were similar. In literature results are conflicting with respect to saliva pH. Factors such as collection methods (sites in the oral cavity), the ages, and diet can influence results (17). In the range of pH 7.0-6.0 the buffer capacity of saliva of the two groups was no different. In fact, the range pH 7.0-6.0 constitutes the most important pH interval related to dental cavity formation, since in this range two pKs of two buffer systems are found, namely, the bicarbonate/carbonate system with a pK around 6.1 and the phosphate buffer system with a pK around 6.8. The presence of these two buffer systems in this range is the cause of the higher acid consumption in this pH interval. However, in the interval pH 6.0-5.0 the caries-free group showed a higher value than the control group ($P<0.05$). It is recommended to continue doing investigations in order to assess the levels of sodium bicarbonate in plasma because there are evidence that the bicarbonate is the most important buffer system of the saliva (18). In the same way, as Smith suggests (19), when the concentrations of bicarbonate of sodium in plasma are high, this excess can be excreted by the salivary glands, probably by the acinar cells. On the other hand, Lamanda (20) demonstrated that salivary buffering between pH 3.4 and 5 was not based on hydrogencarbonate and dihydrogenphosphate but rather on proteins. However, further studies have to be undertaken to identify the protein buffer components in the human salivary proteome. The presence of the low capacity buffer in caries group evaluated could be related to high dental caries risk, which might cause modifications in the acid-base physiologic homeostasis, causing a decrease of the systemic buffer system and so, of the capacity salivary buffer. The peroxidase activity is significantly greater in caries-free adults than in those with caries. Oral Peroxidase (OPO) is composed of two peroxidase enzymes, salivary peroxidase (SPO) and myeloperoxidase (MPO). The SPO secreted from the major salivary glands, mainly the parotid gland (21), contributes 80% of OPO activity, while MPO, produced by leukocytes in inflammatory regions of the oral cavity (22). Oral peroxidase is an enzyme with antimicrobial properties, and in the mouth, it is secreted by salivary glands and catalyzes the oxidation of thiocyanate by hydrogen peroxide to produce on oxidized form of thiocyanate. The product of the reaction catalyzed by peroxidase inhibits bacterial growth (23). In this investigation, the decrease of peroxidase activity observed in caries group may be linked to the increase of dental caries risk. Numerous studies have investigated the correlation among these salivary proteins and caries experience, but no studies have shown reliable association between a single salivary component and caries experience.

The expression of defensins in saliva and throughout the oral cavity suggests that they may have a central role in protecting tooth structure from dental caries as well as protecting oral mucosa. Several reasons for this proposal are 1) Defensins have broad antimicrobial activity; 2) they stimulate the acquired immune system and could function to enhance IgA production as well as IgG production (24); 3) these defensins may function to keep overall bacteria in check and to help prevent biofilm formation. Thus, oral defensins may provide a natural antibi-

otic barrier. There are several new findings of this study. First, hBD2, hBD4 and hNP4 are detectable in saliva but show extensive variation in concentration between subjects (Figure 2A-B). The concentration of defensins (BD2 and BD4) in unstimulated saliva of adults has not been previously reported, although healthy adults had a mean value of 0.8 μg/ml for other antimicrobial peptide such as human defensins-1(25). Second, salivary defensins (hBD2 and hBD4) are significantly greater in caries adults than in those with caries-free, however there are no difference in hNP4 levels between groups (Figure 2G-H). Third, the defensins levels found in saliva in this study are in the range of effective antimicrobial function, especially considering the low salt concentration in saliva and the synergistic action of the peptides. Fourth, the correlation of a salivary cationic defensins with caries experience suggests the possible protective effect of hBD2 and hBD4. Conversely, low levels of defensins may result in increased susceptibility to caries. In this work, salivary defensins concentrations showed large variation between individuals, with a significantly higher level of salivary defensins in adults with caries. Finally, this study shows the simultaneous expression of human hBD2 and hBD4 in caries and caries free gingival tissue samples detected by semi-quantitative RT-PCR. Previous studies demonstrated the constitutive expression of hBD2 in oral tissues (26). Our analysis of gene expression in caries and caries free group showed differential transcriptional levels for the defensins. In samples isolated from caries group, hBD2 and hBD4 expression was at a higher level than caries-free group. The lower expression of hBD2 and hBD4 in caries-free group could explain the lower concentration of these antimicrobial peptides in saliva. The salivary levels of hBD2 and hBD4 may represent a genetically determined factor that contributes to caries susceptibility. The large variation in the concentration of defensins in saliva could be due to previously demonstrated polymorphisms in sequence and copy number in the genes encoding these peptides (27).

Saliva is an easily available sample which can be collected noninvasively and used to measure and monitor the risk for caries (28). The oral cavity, which is colonized by numerous microorganisms, contains a wide selection of antibacterial peptides that play an important role in maintaining its complex ecological homeostasis. We have shown that adults with caries have a significantly higher expression and levels of defensins (hBD2 and hBD4) based on both the RT-PCR and ELISA (Figure 2-3). Future studies could lead to development of means to enhance endogenous oral peptide expression, utilization of these peptides as therapeutics, and to a simple test for clinical evaluation of caries risk.

CONCLUSION

Salivary defensins are potential candidates as biological factors influencing caries response. The higher expression of defensins in saliva suggests that they may have a central role in protecting tooth structure from dental caries as well as protecting oral mucosa We conclude that high salivary levels and expression of beta defensins may represent a biological response of oral tissue to caries. However, these suggestions deserve further investigation.

CONFLICT OF INTEREST

The authors declare no competing interests.

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