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Influence of a hypoiodite mouth-wash on dental plaque formation *in vivo*

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SUMMARY

This study describes an *in vivo* inhibition of dental plaque growth after peroxidase-generated hypoiodite (OI^-) mouth-washes. After giving up all other usual hygiene procedures nine healthy volunteers washed their mouth using 10 ml of the mouth-wash [H_2O_2 (0.005%), KI (50 mM) and lactoperoxidase (0.04%)] three times a day for 1 minute for 3 days. The initial oxidation power of this mixture represented 430 ± 11 μM oxidised cysteine (n=6), dropping down to 87 ± 6 μM after the solution was spat out (n=5). A saline solution served as a negative control, and a 0.2% chlorhexidine digluconate solution as a positive control. Proximal dental plaque between mandibular canine and lateral incisor (left and right) was collected after 3 days using standardized sterile toothpicks, then analysed for ATP and protein content. ATP concentrations dropped to 49% of the control values after OI^- rinsing, and to 9% after chlorhexidine rinsing while the protein content dropped to 48% for OI^- versus 31% for chlorhexidine. However, when considering the ATP content per protein μg, only the decrease to 6% of the initial value in the chlorhexidine testing was significant while the drop to 81% for the OI^- testings was not significant. This study points out a negative effect of OI^- on plaque growth *in vivo*.

KEY WORDS:

Peroxidase - Hypoiodite - Dental plaque - ATP.

RÉSUMÉ

Cette étude décrit l'inhibition de la croissance *in vivo* de la plaque dentaire après traitement à l'aide d'un bain de bouche contenant de la peroxydase et produisant de l'hypoiodite (OI^-). Neuf personnes ont utilisé ce rinçage pendant une minute, 3 fois par jour pendant 3 jours, cependant qu'elles cessaient toute autre pratique d'hygiène bucco-dentaire; une solution saline servant de contrôle négatif et une solution de chlorhexidine de contrôle positif. Des échantillons de plaque interproximale furent prélevés de manière standardisée à l'aide de cure-dents stériles et leur contenu en ATP et en protéines furent mesurés. Les concentrations en ATP après traitement à l'OI^- ne représentaient plus que 49% des valeurs des contrôles négatifs; le traitement à la chlorhexidine 31%. Le rapport ATP/masse protéique est fortement abaissé après traitement à la chlorhexidine (6%) mais se maintient à 85% de la valeur témoin après traitement à l'OI^-.

MOTS CLÉS:

Peroxydase - Hypoiodite - Plaque dentaire - ATP.

INTRODUCTION

Periodontitis as well as carious tooth decay is mostly the result of uncontrolled proliferation and metabolism of oral bacteria. In normal conditions, continuous plaque growth is limited by both immune factors, such as salivary antibodies, and non-immune defence factors, such as lysozyme, lactoferrin, and peroxidase systems (Tenovuo, 1991). Human whole saliva contains both salivary peroxidase (SP), originating from the major salivary glands, and leukocyte myeloperoxidase (MP). Bovine milk lactoperoxidase is also used in toothpastes for its possible antiseptic properties. All peroxidase enzymes catalyse the oxidation of thiocyanate (SCN^-) by hydrogen peroxide (H_2O_2), generating the corresponding oxidized forms: hypothiocyanous acid and hypothiocyanite ($HOSCN/OSCN^-$) (Thomas, 1981). Iodide (I^-) is also a possible substrate for SP, MP and LP, but is a thousand times less concentrated than SCN^- in human saliva (Tenovuo and Makinen, 1976). Antibacterial properties of $HOSCN/OSCN^-$ are demonstrated *in vitro* against *Streptococcus mutans, S. sobrinus* and *Lactobacillus casei* (Lumikari *et al.*, 1991) and against anaerobes such as *Eikenella corrodens, Eubacterium yurii,...* (Courtois *et al.*, 1992). $HOSCN/OSCN^-$ is also an inhibitor of the growth and replication of viruses such as HSV_1 and HIV (Courtois *et al.*, 1990; Pourtois *et al.*, 1990).

However, all these observations were made *in vitro,* and could not, so far, be repeated *in vivo,* calling in question the capability of $HOSCN/OSCN^-$ to exert any bacterial inhibition in the presence of a normal salivary flow (Midda and Cooksey, 1986; Lenander-Lumikari *et al.*, 1993). The only *in vivo* effect of a lactoperoxidase-SCN^- containing toothpaste has indeed been claimed for supra gingival plaque formation and gingival inflammation in radiation-induced xerostomic patients (Van Steenberghe *et al.*, 1994).

In presence of I^-, SP and LP produce hypoiodite (OI^-), a specific growth inhibitor when tested against *Candida albicans in vitro* (Majerus and Courtois, 1992), even if less efficient in presence of saliva. *In vitro,* OI^- also inhibits the growth and metabolism of *Streptococcus sanguis,* one of the first colonizers of dental surfaces (Courtois *et al.,* 1995).

The present study attempts to measure the efficiency of a OI^--generating mouth rinse against plaque forming bacteria *in vivo,* both on the inhibition of *de novo* dental plaque formation (protein content) and on the modulation of the adenosine triphosphate (ATP) content of bacterial plaques.

MATERIAL AND METHODS

Chemicals: All chemicals are of reagent grade (H_2O_2, KI, citric acid monohydrate, D^+ glucose anhydrous and soluble starch are purchased from Merck, Darmstädt, Germany; 5,5'-dithio-bis(2-nitrobenzoic acid) ($(NbS)_2$) and cysteine are from Sigma chemical Co, St Louis, USA; BCA protein assay reagents from Pierce, Rockford, Illinois, USA; Tris and the ATP bioluminescence CLS kit from Boëhringer Mannheim, Mannheim, Germany and I_2 from Union Chimique Belge, Brussels, Belgium).

The enzyme preparation was a bovine lactoperoxidase powder with a purity index (A_{412}/A_{280}) of 0.6 and a specific activity of 600 ABTS units/mg (Biopole, Brussels, Belgium).

Chemical Assays: Bacterial ATP content is determined by chemoluminescence on a LKB Wallac 250 Luminometer (LKB-Pharmacia, Uppsala, Sweden).

Protein contents are determined using the BCA method (Smith *et al.,* 1985).

$HOSCN/OSCN^-$ and HOI/OI^- concentrations (or total oxidation power) are evaluated using the oxidation of cysteine as an indicator (Aune and Thomas, 1978) and expressed as μM of oxidized -SH groups.

Iodine concentrations in the mouthwash preparation are determined by means of a colorimetric assay based on the reaction of iodine with starch. A 0.1% starch solution is mixed with defined concentrations of I_2 in 0.1 M citrate buffer at pH 5.5. The optical densities are read at 640 nm, in order to establish a standard curve, on which the I_2 concentration of the unknown sample is determined.

Subjects: Nine adults ranging in age from 22 to 62 years volunteered for this study. All of them had a healthy gingival status, and were free of any medications for a period of at least one month before the experimentation.

Experimental procedure: Ten ml of an extemporaneous mixture of H_2O_2 (0.005%), KI (50 mM) and LP (0.4 mg/ml) suspended in a 100 mM citrate buffer at pH 5.5 are tested as a mouthwash (LP+KI powder; H_2O_2 solution).

After professional tooth-cleaning, the volunteers replaced all usual hygiene procedures by mouth rinses (1 min 3 times a day for 3 days), respectively after breakfast, after lunch and before bedtime. For all subjects, 10 ml of a saline solution served as a negative control, followed after 2 weeks by the OI^- solution, and after 2 more weeks by a positive control, using 10 ml of a 0.2% chlorhexidine (CHX) digluconate solution (Hibident®).

This study was approved by the Ethical Committee of the Medical Faculty of the Free University of Brussels.

Dental plaque sampling: Both ATP content and plaque growth are depending on [1] the ratio alive/death bacterial cells and [2] the level of metabolic activity (D'Eustachio and Levin, 1967). Meal times and fasting intervals do also regulate bacterial growth in dental plaques (Quirynen and Van Steenberghe, 1989). For the reasons, all test periods begin and end between 10 and 11 AM, at least 3 hours after breakfast, but a standard energy supply is provided 5 minutes before plaque sampling under the form of 10 ml of a 0.1 M glucose mouthwash for 1 minute. In all instances the same dentist collects the deposit between mandibular canine and lateral incisor on both sides using sterile standardized toothpicks (Sanex®) introduced into the interproximal space just above the papilla tip until contact is obtained with both teeth. Moving the toothpick upward in close contact with the teeth allows plaque collection on defined surfaces, repeated from week to week. The tips of the toothpicks covered with adherent plaque are immediately cut off with sterile scissors; immersed in 1 ml sterile distilled water; dispersed by a 10 seconds ultrasonication; divided in 2 parts (1 for ATP determination and 1 for protein assessment) and refrigerated in dry ice to preserve the ATP content.

Repeated sampling of the same site on a healthy volunteer refraining from oral hygiene procedures for 3 days every week over 6 weeks yields variation coefficients of 19% for ATP concentration and 13% for protein content.

Statistical analysis: The results are given as mean ±SEM (n=number of subjects). Statistical analysis is performed on «Graphpad Instat» and consists in Student's paired t tests, using two-tailed p values.

RESULTS

After mixing for one minute, the oxidation power of the H_2O_2-KI-peroxidase mixture is equivalent to the oxidation of 430±11 μM cysteine (n=6), reflecting the level of OI^-/HOI concentration. After rinsing for 1 minute, the cysteine oxidation potential in the saliva mixed peroxidase solution (this time reflecting both OI^-/HOI and endogenous $OSCN^-$/HOSCN) falls down to 87±6 μM (n=5).

After 3 days without hygiene procedures, the mean bacterial ATP content is 116.6±13.4 nM (n=18) in the placebo samplings; this mean falls down to 56.6±16.8 nM (n=18) in the samplings after OI^- treatment; and it falls even lower to 10±6.4 nM (n=18) following CHX application. These results are shown in Fig. 1. The drop in ATP content to 49% of its initial value after OI^- rinsing is significant at p=0.0043 level; and the drop to 9% after CHX rinsing significant at p<0.0001.

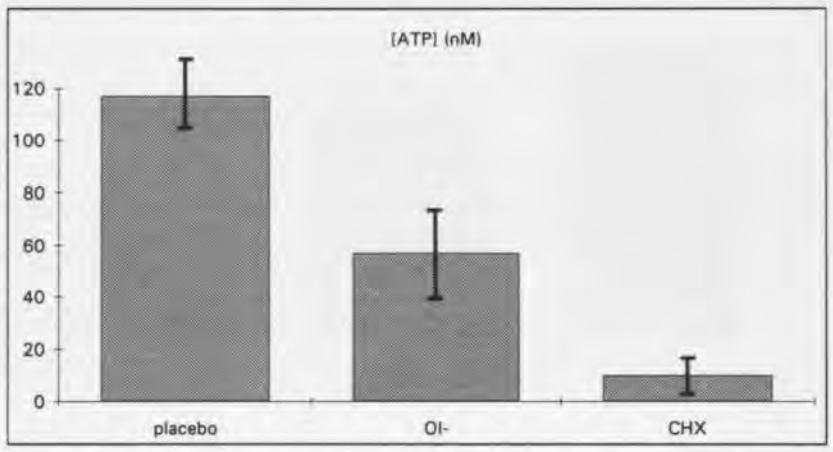

Fig. 1: ATP content in 3 days old dental plaque after placebo, OI^- or CHX treatments.
Fig. 1: Contenu de la plaque en ATP après 3 jours de rinçage placebo, CHX ou OI^-.

The mean protein content in the samples, falls from 45.2±6.4 μg (n=18) after placebo rinsing down to 21.7±5.4 μg (n=18) after OI^- rinsing; that is to 48% of the value in the controls or unrepressed bacterial colonies. This reduction is considered as significant (p=0.007). After CHX rinsing the deficit in protein synthesis is even larger since the mean protein content then falls down to 13.9±4.7 μg (n=18) (31% of the value in placebos), which is a significant difference since p=0.001. These results are shown in Fig. 2.

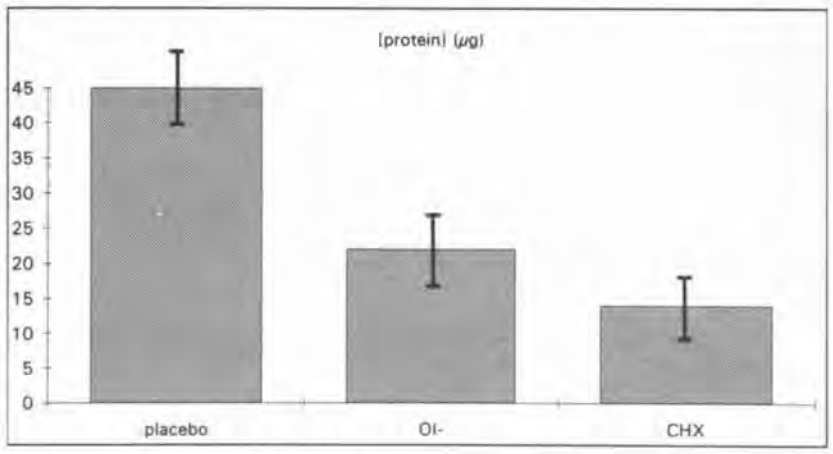

Fig. 2: Protein content in 3 days old dental plaque after placebo, OI^- or CHX treatments.
Fig. 2: Concentration en protéines dans la plaque après 3 jours de rinçage placebo, CHX ou OI^-.

When considering the mean arithmetical ratios between the ATP and protein concentrations (nM/μg) for each sample; 3.2±0.2 (n=18) in the control group for 2.5±0.5 (n=18) in the OI^- group and 0.2±0.1 (n=18) in the CHX group (Fig. 3), which represents 81% of the placebo testings after OI^- washings versus 6% after CHX rinsings, only the latter value is significant (p=0.0001).

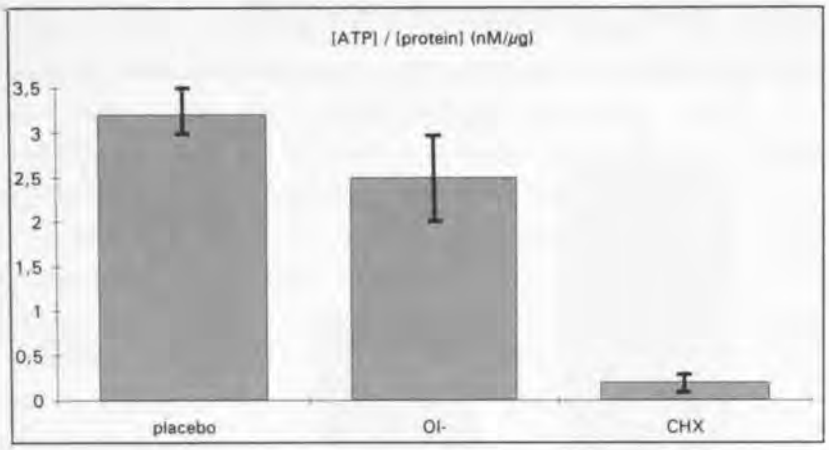

Fig. 3: Different arithmetical ratios between ATP versus protein concentrations after placebo, OI^- or CHX treatments.
Fig. 3: Concentration en ATP par µg de protéines après 3 jours de rinçage placebo, CHX ou OI^-.

Although the OI^- generating mouthwash contains 0.64% I^-, only 0.015±0.002% of this element is found under the form of I_2 in the mouthwash after addition of H_2O_2 (n=6).

DISCUSSION

Inhibition by chlorhexidine mouth rinses of bacterial ATP synthesis was already investigated in dental plaques developing on polyester patches (Vanden Abbeele and Pourtois, 1993). In the present work, plaque samples are collected by proceeding tooth picks interproximally along adjacent tooth surfaces, at selected sites. The operator is unaware of which type of rinse was being tested. Precise localization of the scraping allows to uniformly repeat the same sampling pattern, so that the volume and content of two samples from a same site would be influenced mainly by environmental variables, here the different types of rinsing. The use of toothpicks for evaluating the prevalence of *mutans streptococci* on individual tooth surfaces compared favourably with other sampling methods such as amalgam carvers, needles, or dental floss (Wennerholm *et al.*, 1995).

The glucose rinse five minutes before sampling was made to optimize the conditions for ATP production. Any drop in ATP content could thus be ascribed to either: [1] reduced bacterial growth (and accordingly reduced biomass); [2] reduced mean anabolic potential in bacteria; and [3] combination of [1] and [2].

After rinsing with the peroxidase system, the deficiencies in ATP (±48%) as well as in proteins (±48%), expressed in percent of the values found in placebos, are much alike; the plaques accordingly bear witness of growth inhibition but this treatment had but little influence on the mean energy potential volume unit in the plaque mass. This points out the inability for HOI/OI^- to penetrate inside the plaque mass, even for concentration up to 430 μM.

On the contrary, after CHX treatment, ATP concentration dropped more readily than the proteins in the samples. As already well documented (Netuschil *et al.*, 1988), beside dental plaque growth inhibition, CHX treatment appears to leave a high number of death cells among a depleted population of bacteria.

The plaque growth inhibition due to the H_2O_2-iodide-peroxidase mixture cannot be assigned to hydrogen peroxide since no H_2O_2 is detected as long as peroxidase is present in the solution (Courtois *et al.*, 1995).

The inhibitory agent appears to be one of the products of the lactoperoxidase catalyzed reaction: that is the hypoiodous acid (IOH) and its OI^- cation:

$$OI^- + H^+ \Leftrightarrow IOH$$

However, in solution, this molecule coexists with iodhydric acid and iodine:

$$IOH + I^- + H^+ \Leftrightarrow H_2O + I_2$$

One can ask therefore whether iodine could be the main bacteriostatic species. Practically, we measured an I_2 concentration of 0.015%. However, at a 0.1 concentration, iodine alone could not demonstrate any anti-plaque efficiency in experiments based on polyester patches as colonizing areas (results not shown).

The obvious anti-plaque effect obtained using the H_2O_2-iodide-lactoperoxidase mixture should therefore be attributed to hypoiodite. The concentration of this anion was measured in the rinsing solution before and after mixing with saliva. Results showed that 343 μM of cysteine are oxidized within 1 min of rinsing and that 82% of the oxidizing potential was

consumed during mouth washing, by -SH (or $^{-}C=C^{-}$) radicals either in saliva, on mucosal surfaces or in dental plaques.

After mixing saliva with a glucose-glucoseoxidase/thiocyanate/peroxidase system (e.g. Biotène® tooth paste), Lenander-Lumikari *et al.* (1993) measured a [$OSCN^{-}$] peak as high as 200 μM, but this concentration was not followed by any inhibition of commensals such as *S. mutans* and *Lactobacillus*, provided the testing was made in saliva. So the two different anions $OSCN^{-}$ and OI^{-} display very dissimilar antiseptic properties.

This could be explained in part by the presence in some bacterial species of a specific enzyme – a NADH dependent hypothiocyanite oxido-reductase (NHOR) – that reduces $OSCN^{-}$ into SCN^{-} (Oram and Reiter, 1966; Courtois *et al.*, 1992) which may shield them from $OSCN^{-}$ though not from OI^{-}. A bacterium like *S. sanguis* for instance, a first generation colonizer of enamel, can resist $OSCN^{-}$ thought it is susceptible to OI^{-} (Courtois *et al.*, 1995). The same statement does not apply to *S. mutans*, a bacterium that is devoid of NHOR and, if susceptible to $OSCN^{-}$ *in vitro* (Lumikari *et al.*, 1991) is resistant to this anion in the presence of saliva (Lenander-Lumikari *et al.*, 1993). The lack of inhibition *in vivo* must be attributed to some fostering agent(s) or deceiving target(s) in saliva. More research should be aimed at defining these protective mechanisms.

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