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In vitro antioxidant activities of antioxidant-enriched toothpastes

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Abstract

Several forms of periodontal diseases (PD) are often associated with modified phagocytosing leukocytes and contemporary free radical production. Host antioxidant defenses could benefit from toothpastes used as adjuncts to counteract plaque-associated bacteria. The aim of the present study was to determine possible antioxidant activity (AA) of 12 differently antioxidant-enriched toothpastes, regardless of their efficacy as antimicrobial agents. Toothpastes were enriched alternatively with sodium ascorbyl phosphate, α-tocopherol acetate, pycnogenol, allantoin and methyl salycilate or a mixture of these. AA was tested in a cell-free system with a ABTS-decolorization assay improved by means of a flow injection analysis device. Comet assay, using NCTC 2544 keratinocytes, was performed to test if it was possible to identify any protection against *in vitro* DNA fragmentation provoked by a challenge with H_2O_2 in cultures pre-incubated with toothpaste extracts.

Only toothpastes containing sodium ascorbyl phosphate displayed clear AA with I_{50} values ranging between 50 and 80 mg of toothpaste/ml water. COMET analysis of cells challenged with H_2O_2 in presence of toothpaste extracts revealed a limited protection exerted by sodium ascorbyl phosphate. The results described herein indicate that toothpastes containing sodium ascorbyl phosphate possess AA. All the data were obtained in systems *in vitro* and the demonstration of *in vivo* AA is desirable. These findings could be useful in the treatment and maintenance of some forms of PD and should be considered when arranging new toothpaste formulations.

Keywords: *Antioxidants, toothpaste, periodontitis, comet assay, total antioxidant activity*

Introduction

Periodontal diseases (PD) affect 10 to 15% of any population[1,2] thus resulting among the most widespread chronic conditions worldwide with evident severe consequences also from a socio-economic and public health point of view.

PD show both inflammation and destructive lesions in periodontal tissues: in fact, PD are chronic inflammatory conditions featured by loss of connective tissue, alveolar bone resorption and formation of periodontal pockets as a result of the complex interaction between pathogenic bacteria and the host's immune response.[3] Among the host's responses, polymorphonuclear neutrophils (PMN) act as the initial host defence against the periodontal pathogens. After stimulation by bacterial antigens a series of different activities is activated including phagocytosis and the production of large amount of reactive oxygen species (ROS) as a result of

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the so-called "respiratory burst". In addition to being released into the phagosome, ROS are released into the extracellular environment. These ROS, together with more ROS produced by myeloperoxidase during phagocytic degranulation, have the capacity to destroy the pathogen but contribute also to the host tissue destruction in the same area.[4,5]

The damage mediated by ROS can be mitigated by antioxidants, thus products for oral hygiene and care (e.g. mouthrinses, toothpastes) able to deliver antioxidants in, or close to, the site of ROS production, may be valuable adjuvants in the PD treatment and maintenance.

The aim of the present study was to determine possible antioxidant activity (AA) of 12 toothpastes regardless of their efficacy as polishing or antimicrobial agents. For this, we used the very fast, simple and reproducible FIA-enhanced ABTS method which we considerably improved recently.[6]

Moreover, comet assay was also used to evaluate prevention of *in vitro* DNA fragmentation.

Materials and methods

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic) diammonium salt (ABTS), as well as all other reagents and solvents used for experimental assays were of biochemical or analytical grade from Fluka (Fluka Chemie, Buchs, Switzer land) and Sigma (St. Louis, MO, USA).

The basic materials [glycerin (Henkel, Dusseldorf, Germany), sorbitol (Roquette, Lestreme, France), titanium dioxide (Kemira Pigments Oy, Pori, Finland), sodium saccharin (Productos Aditivos, Barcelona, Spain), sodium methylparaben (Nipa Laboratories Ltd, Deeside, UK), xanthan gum (Rhodia, Aubervilliers, France), sodium fluoride (Merck, Whitehous NJ, USA), D-panthenol (Basf, Germany)] used for assembling the toothpastes had a high level of purity and were apt for oral use. The antioxidants tested, alone or in combination, were sodium ascorbyl phosphate and α-tocopherol acetate (Roche, Basel, Switzerland), pycnogenol® (Biolandes, Le Sen, France), allantoin (Merck, Whitehous NJ, USA) and methyl-salicylate (Symrise, Holzminden, Germany). Reagents for cell cultures were obtained from Bio-Whittaker (Walkersville, USA). The Live/Dead® Viability/Cytotoxicity Kit (L-3224) was supplied by Molecular Probes (Eugene OR, USA).

Toothpaste preparations

The 12 experimental toothpastes used in the study were prepared using the same base, to which different antioxidants were added. The base was completely conventional and consisted of a humectant system (glycerin and sorbitol), an abrasivity system with silicas that give the product a medium degree of abrasivity, titanium dioxide acting as a dye, an alkyl sulfate as surfactant and sodium saccharine as sweetener. Sodium methylparaben was used for preservation, and xanthan gum (a natural polymer) was used for thickening. Additional active ingredients, lacking antioxidant properties, were 0.332% sodium fluoride (equivalent to 1500 ppm fluoride ion) and 1% D-panthenol. The aroma, when used, was the same for all tested products.

The antioxidants were added, alone or in combination, in the experimental toothpastes, according to the scheme in Table I and in the following concentrations: 0.525% sodium ascorbyl phosphate, 0.5% α-tocopherol acetate, 0.04% pycnogenol®, 0.1% allantoin and 0.1% methyl salicylate. Toothpastes were formulated and prepared in the Galenic Department of Dentaid, S.L., using a Berents model Becomix-Mini-Lab 2.5 brand mixer/homogenizer. Toothpastes were stored in 150 ml storing tubes.

Sample treatment for AA assays

Toothpaste extracts were obtained as follows: increasing amounts of toothpaste (10,25,50,70,100 mg)

Table I. Scheme of antioxidant contents in the experimental toothpastes used.

Toothpastes (Batch no.)	Aroma	Vitamin C phosphate	Vitamin E acetate	Pycnogenol®	Allantoin	Methyl salicylate
P/AGN-03-00/01	No	No	No	No	No	No
P/AGN-04-00/01	Yes	No	No	No	No	No
P/AGN-05-00/01	Yes	No	No	Yes	No	No
P/AGN-06-00/01	Yes	No	No	No	Yes	No
P/AGN-07-00/01	Yes	Yes	No	No	No	No
P/AGN-08-00/01	Yes	No	Yes	No	No	No
P/AGN-09-00/01	Yes	Yes	Yes	No	No	No
P/AGN-10-00/01	Yes	Yes	Yes	Yes	No	No
P/AGN-11-00/01	Yes	Yes	Yes	Yes	Yes	No
P/AGN-12-00/01	Yes	No	No	No	No	Yes
P/AGN-13-00/01	Yes	Yes	Yes	Yes	No	Yes
P/AGN-14-00/01	Yes	Yes	Yes	Yes	Yes	Yes

completely dissolved in 1 ml of HPLC-grade H_2O were centrifuged at 5000*g* for 10 min in a ALC model PK121R multispeed refrigerated centrifuge. The supernatants were immediately assayed by the FIA-ABTS method.

The supernatants obtained by the extraction of 50 mg toothpastes/ml H_2O were filtered on a 0.2 μm filter (Schleicher & Schuell Gmbh, Dassel, Germany) and added to the cell cultivation media (10% of the final volume).

FIA-ABTS

The so-called ABTS-decolorization assay consists in the production of the ABTS radical cation ($ABTS^{\cdot+}$) by reacting ABTS stock solution with potassium persulfate in the dark for 12–16 h before use.[7] $ABTS^{\cdot+}$, which possesses absorption maxima in the visible range, results stable in this form for more than two days if cautiously stored in the dark at 25°C. When biological or chemical samples, containing antioxidant molecules, react with $ABTS^{\cdot+}$ they scavenge the radical, in this way producing an inhibition of absorbance. The percentage inhibition is calculated and plotted as a function of concentrations of antioxidants and of Trolox for the standard reference data. The solution of ABTS radical cation was produced by reaction of a 7 mM aqueous solution with 2.45 mM potassium persulfate, and maintained in the dark for 12 h before use. This solution was stable for 2 days. The working solution was obtained diluting 1:50 with PBS buffer pH 7.4 the stock solution. The flow injection system[6] consisted of a Beckman 126 HPLC pump, a Beckman model 166 UV/Vis detector, a Rheodyne Model 7125 manual injection valve equipped with a 5 μl sample loop, a reaction coil (1.0 mm i.d., 1.56 mm o.d., 100 cm long) (Sigma-Aldrich). The reaction coil was folded to obtain a serpentine-knotted coil in order to achieve better performance in mixing sample and reagent. The oven was a BEER 1000P (Eurisco Diagnostic, Cornedo Vicentino, Italy). The results are expressed as mM trolox equivalent antioxidant capacity (TEAC), according to the validated methods[6,7] and also the corresponding I_{50} were calculated. I_{50} is defined as the substance amount (in mg of toothpaste/ml H_2O of the toothpaste extract) with the capacity to inhibit half of the $ABTS^{\cdot+}$ formation.

Cell culture

The immortalized human keratinocyte cell line NCTC2544 (purchased from ICLC-Interlab Cell Line Collection, Genova, Italy) used throughout this study was maintained at 37°C and 5% CO_2/95% air in RPMI supplemented with non-essential amino-acids (1%), 2 mM L-glutamine, 1 U/ml gentamycin, 100 U/ml penicillin/streptomycin and 10% (v/v) fetal bovine serum. The cells were cultured as adherent monolayers, routinely split 1:4 every 3–4 days and used for each experiment at the third passage. Seventy-two hours prior to the experiments, the cells were placed into 6-well plates (diameter of each well = 3.5 cm) and 48 h prior to the experiment toothpaste extracts (100 μl) were added to the culture medium.

Cell treatments

When monolayers of cells reached approximately 5×10^5 cells/well they were washed twice with phosphate buffered saline (PBS). Cells were challenged with H_2O_2, diluted at a concentration of 150 μM in PBS, added directly to the wells. The control monolayer was mock-treated with PBS alone. After incubation for 30 min the medium was discarded and cells were washed with PBS.

Cell viability/cytotoxicity was determined and finally the comet assay was performed.

Viability/cytotoxicity

The Live/Dead® Viability/Cytotoxicity Kit (L-3224) was supplied by Molecular Probes (Eugene OR). This kit provides a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells with two probes that measure two recognized parameters of cell viability: calcein-AM for intracellular esterase activity and ethidium homodimer (EthD-1) for plasma membrane integrity. The polyanionic dye calcein is retained within live cells,

Table II. Total antioxidant activity of toothpaste extracts (expressed as TEAC values) and I_{50} values.

Toothpastes (Batch no.)	TEAC (mM Trolox/100 mg toothpaste × ml H_2O^{-1})	I_{50} (mg toothpaste /ml H_2O)
P/AGN-03-00/01	0.504	n.d.
P/AGN-04-00/01	0.532	n.d.
P/AGN-05-00/01	0.224	n.d.
P/AGN-06-00/01	0.222	n.d.
P/AGN-07-00/01	1.896	75.0
P/AGN-08-00/01	0.370	n.d.
P/AGN-09-00/01	2.379	49.7
P/AGN-10-00/01	1.915	73.8
P/AGN-11-00/01	1.755	75.9
P/AGN-12-00/01	0.478	n.d.
P/AGN-13-00/01	2.236	63.5
P/AGN-14-00/01	1.922	76.1

n.d. = not detectable in the range of toothpaste's extract concentrations used. Absent (n.d.) values indicate the absence of I_{50} values in the range of extract concentrations tested (10–100 mg toothpaste/ml H_2O). I_{50} values were obtained directly by the respective inhibition curves (see "Materials and Methods" section for details). Each curve was obtained by means of five separate extract concentrations, and each concentration was performed in triplicate.

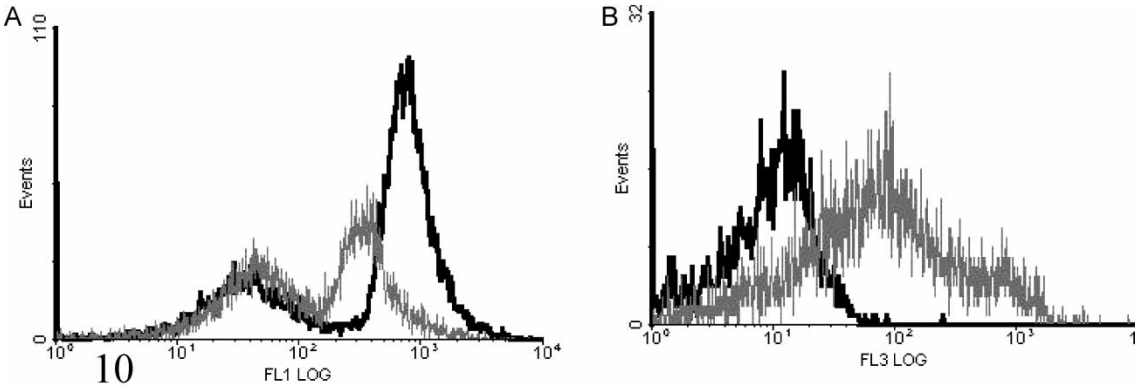

Figure 1. Representative flow cytometric graphics of Live/Dead® Viability/Cytotoxicity assay in control and H_2O_2-treated cells: esterase activity of the control cells (black) and the H_2O_2-treated cells (grey) using Calcein-AM (Figure 1A) and damage of membranes of the control cells (black) and the H_2O_2-treated cells (grey) using EthD-1 (Figure 1B). The experiment shown is representative of three others performed.

producing an intense green fluorescence in live cells (ex/em ~495 nm/~515 nm; FL1). EthD-1 enters cells with damaged membranes, producing a red fluorescence in dead cells (ex/em ~495 nm/~635 nm; FL3). The samples were analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Miami, FL, USA) interfaced to System II software (Beckman Coulter). An average of 10,000 cells from each sample were collected. Green fluorescences from Calcein-AM were measured in the FL1 channel through a 525 nm band-pass filter (BP) in combination with a 550 nm dichroic long pass (DL) mirror, red fluorescences from EthD-1 were measured in the FL3 channel, using a 620 nm BP and a 645 nm DL. Cell viability was assessed using fluorescein diacetate (1 μg/ml) and propidium iodide (5 μg/ml) as probes for detecting, respectively viable and non-viable cells.[8] Preparations were examined under a fluorescent microscope (Nikon Eclipse E800). Cell viability was calculated on each slide, by randomly selecting 50 cells and evaluating the percent of live or dead cells. For each sample three different slides were prepared.

Table III. Cell viability assessed using fluorescein diacetate and propidium iodide.

	% live cells
Controls	83.5 ± 10.2
H_2O_2-treated	60.8 ± 12.3
P/AGN-03-00/01	80.6 ± 14.7
P/AGN-03-00/01 + H_2O_2	58.9 ± 11.5
P/AGN-04-00/01	78.8 ± 15.1
P/AGN-04-00/01 + H_2O_2	62.3 ± 10.8
P/AGN-05-00/01	81.8 ± 12.7
P/AGN-05-00/01 + H_2O_2	63.0 ± 14.1
P/AGN-06-00/01	79.3 ± 14.2
P/AGN-06-00/01 + H_2O_2	57.4 ± 13.7
P/AGN-07-00/01	82. 5 ± 11.1
P/AGN-07-00/01 + H_2O_2	61.3 ± 14.5
P/AGN-08-00/01	81.1 ± 12.3
P/AGN-08-00/01 + H_2O_2	60.9 ± 15.6
P/AGN-09-00/01	80.9 ± 14.8
P/AGN-09-00/01 + H_2O_2	58.7 ± 11.4
P/AGN-10-00/01	84.5 ± 12.6
P/AGN-10-00/01 + H_2O_2	59.8 ± 13.5
P/AGN-11-00/01	82.3 ± 15.1
P/AGN-11-00/01 + H_2O_2	63.4 ± 12.7
P/AGN-12-00/01	80.6 ± 12.4
P/AGN-12-00/01 + H_2O_2	60.1 ± 14.6
P/AGN-13-00/01	77.9 ± 13.8
P/AGN-13-00/01 + H_2O_2	62.3 ± 11.9
P/AGN-14-00/01	83.4 ± 12.7
P/AGN-14-00/01 + H_2O_2	60.3 ± 10.9

In the table are indicated the percent of live cells found for NCTC 2544 keratinocytes in the different situations tested: Controls, H_2O_2-treated cells, toothpaste extracts-treated cells and toothpaste extracts/H_2O_2-treated cells. The data showed the mean ± SD from three different slides.

Comet assay

DNA breaks were measured using the alkaline Comet assay as described previously.[9] Treated and control keratinocytes were suspended in 100 μl of 1% (w/v) low melting point agarose in PBS, pH 7.4, at 37°C and immediately pipetted onto a frosted glass microscope slide precoated with a layer of 1% (w/v) normal melting point agarose (NMPA) similarly prepared in PBS. Finally, the slide was coated with 100 μl NMPA. The agarose was allowed to set at 4°C for 10 min and the slide was immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, NaOH to pH 10.0 and 1% (v/v) Triton X-100) at 4°C for 1 h to remove cell proteins. Slides were then placed for 40 min in a double row in a 260 mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA before electrophoresis at 25 V for 30 min at temperature of 4°C. Slides were washed three times for 5 min with 0.4 M Tris-HCl, pH 7.5, at 4°C. After neutralization, they were stained with 10 μl of 50 μg/ml SYBR GREEN and examined, using a B-2A filter, on a Nikon Eclipse E600 fluorescence microscope equipped with a COHU High Performance CCD Camera. For each slide, 100 randomly selected cells were scored visually according to the relative intensity of fluorescence of

Table IV. The effect of toothpaste extracts on the formation of H_2O_2-induced DNA breaks in NCTC 2544 keratinocytes was evaluated by the formation of DNA breaks with the comet assay.

	Controls a	H_2O_2 b	P/ AGN03+ H_2O_2 c	P/ AGN04+ H_2O_2 d	P/ AGN05+ H_2O_2 e	P/ AGN06+ H_2O_2 f	P/ AGN07+ H_2O_2 g	P/ AGN08+ H_2O_2 h	P/ AGN09+ H_2O_2 i	P/ AGN10+ H_2O_2 l	P/ AGN11+ H_2O_2 m	P/ AGN12+ H_2O_2 n	P/ AGN13+ H_2O_2 o	P/ AGN14+ H_2O_2 p	Statistical analysis ($P < 0.01$)
Class 1	85.5 ± 3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	a vs. all
Class 2	10.3 ± 1.1	1.8 ± 0.7	2.9 ± 0.6	2.1 ± 0.5	3.2 ± 0.8	2.1 ± 0.9	4.1 ± 2.3	3.0 ± 1.1	4.2 ± 1.6	3.9 ± 1.7	3.5 ± 1.9	3.9 ± 1.6	4.1 ± 1.9	3.9 ± 1.7	a vs. all
Class 3	3.1 ± 1.5	25.6 ± 3.8	27.6 ± 3.6	34.0 ± 4.1	31.0 ± 5.9	33.7 ± 5.6	53.3 ± 6.9	36.1 ± 5.5	50.7 ± 5.6	49.7 ± 7.2	50.4 ± 6.5	34.2 ± 5.8	48.3 ± 6.3	47.1 ± 5.9	a vs. all g,i,l,m,o,p vs. b,c,d,e,f,h,n
Class 4	1.1 ± 0.6	66.4 ± 4.5	63.5 ± 7.1	58.3 ± 7.9	60.7 ± 6.1	57.9 ± 6.3	38.9 ± 4.5	55.6 ± 6.2	41.2 ± 6.3	42.1 ± 5.9	41.0 ± 6.3	56.3 ± 6.1	42.7 ± 5.9	44.2 ± 6.4	a vs. all g,i,l,m,o,p vs. b,c,d,e,f,h,n
Class 5	0.0	6.2 ± 2.5	6.0 ± 2.1	5.6 ± 1.9	5.1 ± 1.5	6.3 ± 1.2	3.7 ± 1.9	5.3 ± 1.2	3.9 ± 1.7	4.3 ± 2.1	5.1 ± 1.9	5.6 ± 1.5	4.9 ± 1.6	4.8 ± 1.8	a vs. all
Total Damage	119.8 ± 6.3	377.0 ± 16.5	372.6 ± 15.6	367.4 ± 19.7	367.7 ± 17.4	368.4 ± 18.1	342.2 ± 20.1	363.2 ± 19.6	344.8 ± 15.6	346.8 ± 21.2	347.7 ± 22.5	363.6 ± 19.1	348.4 ± 17.9	349.9 ± 18.8	a vs. all

The results are presented both by averaging the percentage of each comet class from 100 counted cells in one slide and total score for each slide. The data showed the mean ± SD from three samples for each treatment. Statistical analysis was performed as indicated in the text.

the head and tail and assigned a value of 1 to 5: type 1, undamaged cells without tail; type 2, cells with a tiny tail; type 3, cells with a dim tail; type 4, cells with a clear tail; and type 5, tail alone (head of comet very small, most of DNA in tail). The results are presented as the average percentage of each score from 100 counted cells and total score from 100 counted cells in one slide. The total score is calculated as follows: Total score = (number of cells with score 1 × 1) + (number of cells with score 2 × 2) + ⋯ + (number of cells with score 5 × 5).

The total score for 100 cells/comets can thus range from 100 (all undamaged) to 500 (all maximally damaged) for each slide.[10] Visual scoring is the only feasible method when dealing with very large numbers of slides.[11] The results were validated by comparison with computerized image analysis of selected comets using Komet II (Perceptive Ltd., Liverpool, UK). The data are expressed as the mean ± SD of three different slides for each toothpaste extract tested.

The ANOVA test was used to compare groups. Homogeneity of variance was checked by Leven's test. When significant differences were detected ($P < 0.01$), Scheffe's test was used for a *post hoc* comparison. All tests were performed using a PC Statistical Package (SPSS for Windows, 6.1, SPSS Inc Chicago, IL, USA).

Results

Data regarding TEAC values of the toothpastes investigated are shown in Table II. Two groups of toothpastes have been identified: one in which members exhibited a negligible TEAC value (about 0.5 mM Trolox/100 mg toothpaste × ml H_2O^{-1} or less) and the other in which members had TEAC values higher than 1.7 mM Trolox/100 mg toothpaste × ml H_2O^{-1}. For the members of the former group it was possible, experimentally, to identify any of the I_{50} values in the range of the toothpaste's extract concentration used (10–100 mg toothpaste/ml H_2O). On the other hand, for the members of the latter group, it was also easy to determine the corresponding I_{50}, that were between 49.7 and 76.1 mg toothpaste/ml H_2O).

Figure 1 for the live/dead assay shows the results obtained with control cells and H_2O_2-treated cells.

Figure 1A shows the intensity of green fluorescence that indicate esterase activity. H_2O_2 treatment induced significant decreases of fluorescence mean (6.63) with respect to the fluorescence mean present in the control cells (35.2) indicating that a H_2O_2 treatment induced a lesser activity of esterase in the cells. Figure 1B shows the intensity of red fluorescence which indicates cell mortality since EthD-1 binds to nucleic acids when the membrane of the cells are damaged. H_2O_2 treatment induced significant increases of fluorescence mean (7.19) with respect to

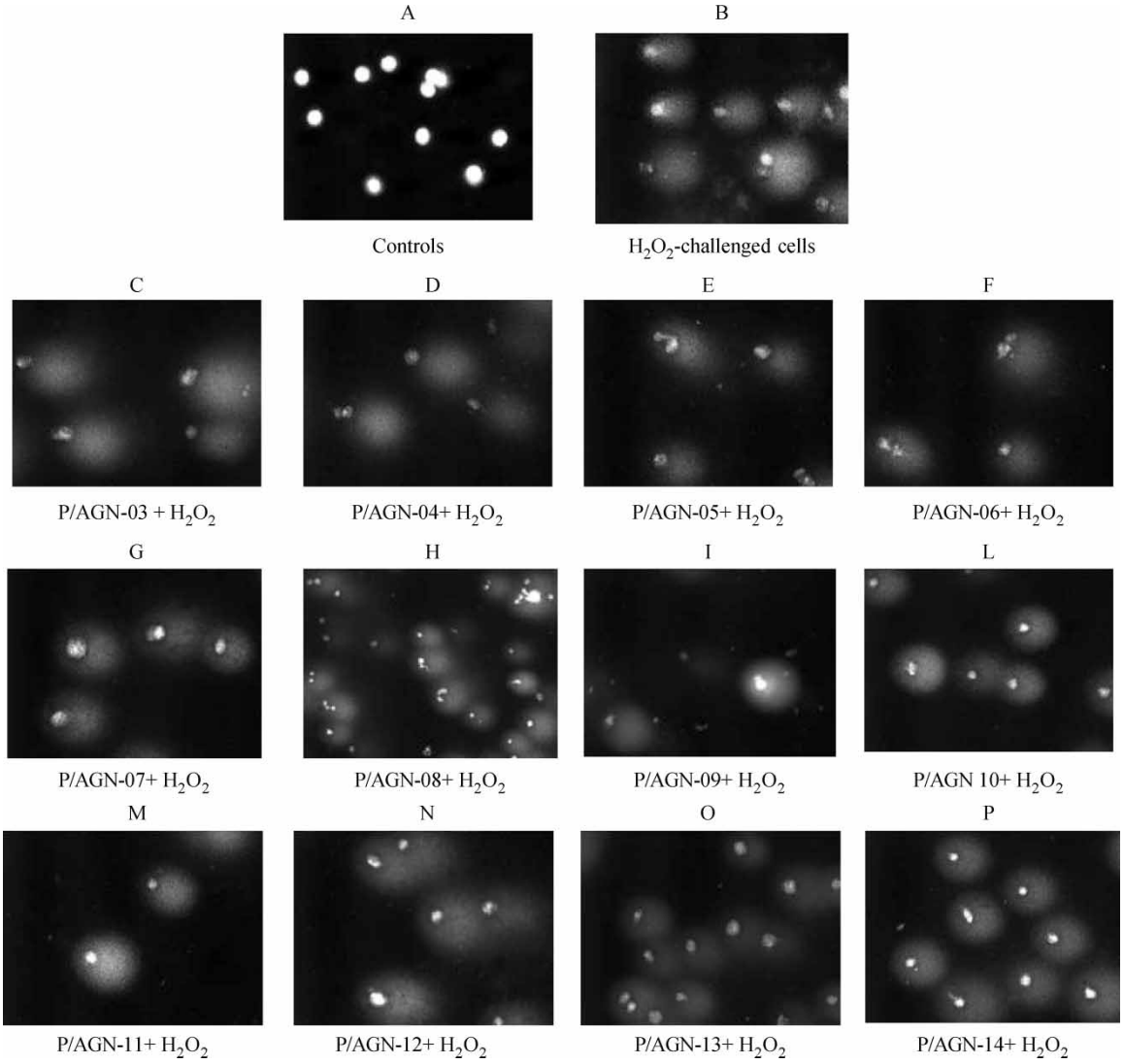

Figure 2. Representative images for each cell population after having undergone comet assay for evaluating the possible prevention of *in vitro* DNA fragmentation by means of extracts from antioxidant-enriched toothpastes: Control NCTC 2254 keratinocytes (A); Cells challenged with 150 μM H_2O_2 for 30 min (B); Cells pre-treated throughout 48 h with P/AGN-03 (C), P/AGN-04 (D), P/AGN-05 (E), P/AGN-06 (F), P/AGN-07 (G), P/AGN-08 (H), P/AGN-09 (I), P/AGN-10 (L), P/AGN-11 (M), P/AGN-12 (N), P/AGN-13 (O) and P/AGN-14 (P) extracts before having been challenged with 150 μM H_2O_2 for 30 min. See Table I for toothpaste abbreviations and contents.

the control cells (0.16) indicating that H_2O_2 treatment enhances cell mortality. Table III shows the percent of the living cells, respectively in the control cells, in the H_2O_2 treated cells and in the corresponding preparations treated throughout 48 h with each of the toothpaste extract. When the cells (both controls and H_2O_2-challenged) were previously treated with toothpaste the data were unmodified, indicating the absence of any influence (both toxicity and protection) of the toothpaste extracts on immediate cell vitality: this piece of information was valuable for avoiding any artifactual response of the comet assay used later on.

The results of comet assays are summarized in Table IV. Control cells, i.e. toothpaste-untreated and H_2O_2-unchallenged cells, were more than 85% in class 1, about 10, 3 and 1% in classes 2, 3 and 4, respectively. An image of this kind of cell population is shown in Figure 2A. DNA of H_2O_2-challenged cells (Figure 2B) was highly dispersed and the images obtained were of perfect comet-shapes. In this case the distribution in classes of damage was therefore different from controls with class 4 being the most numerous (66.4%); classes 3, 5 and 2 with respectively 25.6, 6.2 and 1.8 completed the cells' distribution. Among toothpaste extract-treated cells it is possible to identify two main groups, according to the number of cells distributed in the different class of damage: on one side the group of toothpastes P/AGN03, P/AGN04, P/AGN05, P/AGN06, P/AGN08 and P/AGN12 (Figure 2C–F,H and N) and, on the other, the group of toothpastes P/AGN07, P/AGN09, P/AGN10, P/AGN11, P/AGN13 and P/AGN14 (Figure 2G,I,L,M,O and P). The former group had class 3 values between

27.6 and 36.1% and class 4 values between 55.6 and 63.5%; the latter group in the ranges 47.1–53.3% and 38.9–44.2% for the respective classes 3 and 4. Both groups displayed very similar values both for class 2 and class 5.

Discussion

The number of toothpastes available as OTC-products is continuously increasing. Many of them are used to provide adjunctive benefits in addition to the merely abrasive aid provided by the mechanical role of toothbrushes and, in some cases, it is claimed that their content in antioxidants may have an important role in scavenging part of the ROS involved in the onset and development of PD. The very reliable and sensitive FIA-ABTS method used for assaying AA of antioxidant-enriched toothpastes suggests that some formulations may actually display a considerable AA. It seems clear that only sodium ascorbyl phosphate, among all the antioxidant molecules used, still acts as antioxidant once it is released from the experimental toothpaste. It also seems that this feature is maintained by sodium ascorbyl phosphate both when it is present alone in the toothpaste and when it is used in combination with other antioxidants.

The results indicate that the AA reasonably depends upon the water solubility of the compounds employed. In fact, while sodium ascorbyl phosphate, featured by a high solubility, really carries out its antioxidant properties, other molecules with lower hydrophilicity are probably partially retained in the paste and for this reason it is impossible to find considerable AA in the corresponding extracts. In this sense, two different examples may be represented by methyl-salicylate and Vitamin E. The former, being a derivative of salicylic acid is a strong antioxidant[12,13] but the mild solubilization of the toothpaste in water was probably not sufficient to allow its release from the matrix. The latter is a substance whose well-known antioxidant properties[14,15] reside mainly in its OH phenolic group. Acetylation of this group, while increasing its solubility, simultaneously reduces its AA.

Another, and more vigorous, kind of extraction from the toothpastes using hydrophobic solvents was, on the other hand, not possible because such procedure would have been too different from, and absolutely not comparable with, the conditions of usage of the products. These data agree and confirm previous findings recently obtained evaluating the AA of mouthrinses[13] and may explain why some toothpastes containing allantoin and even α-tocopherol failed to obtain any clinical influence on gingival inflammation.[16]

The treatment of cells with toothpaste extracts over 48 h indicate that they were unable to affect cells' vitality. This evidence allowed to perform the evaluation on DNA strand breaks, after a H_2O_2 insult, on a constant number of living cell populations. In fact, the same viability/cytotoxicity results obtained with controls and H_2O_2-treated cells were also found for toothpaste extracts-treated cells and toothpaste extracts/H_2O_2-treated cells, respectively.

The comet assay allows sensitive detection of ROS-induced DNA strand breaks[9] and represents an extremely important tool for assessing the protective effects of antioxidants.[17] In order to evaluate the actual toothpaste-extract efficacy in cell protection from ROS insult we chose to challenge all the extracts previously tested with the FIA-ABTS method.

Comet assay, performed on comparable living cell populations, provided data which were not completely unambiguous. The statistical analysis of the classes of damage revealed that differences were found only in classes 3 and 4. The data seem to confirm that sodium ascorbyl phosphate may act as antioxidant *in vitro* also protecting cell DNA after an H_2O_2-induced damage. In fact, the cells treated with toothpaste-extracts obtained from preparations containing sodium ascorbyl phosphate shifted the distribution of the majority of cell population to class 3. However, the overall pattern of classes of damage did not completely change, as had occurred in other investigations.[13,18] The outcome can be found in the total score results which did not display differences among any of the H_2O_2-treated cells: this was due to minimal differences which had occurred in classes 2 and 5. These last results indicate that, at least at the pseudo-physiological concentration used, a tendency exists for toothpastes-containing sodium ascorbyl phosphate to protect cellular DNA.

Ascorbate has long been a candidate for modulating PD: the effects of ascorbate on extracellular matrix and immunologic and inflammatory responses provide a rationale for hypothesizing it as a risk factor for PD.[19] Consequently, several studies have attempted to study the degree of association between vitamin C and periodontal status and response to therapy.[20–22] Investigations carried on in humans demonstrated that (i) ascorbate depletion due to a 4-day ascorbate lacking diet produces increase in gingival bleeding on probing[20], (ii) a relationship exists between reduced dietary ascorbate and increased risk for periodontal disease for the overall population utilizing the third national health and nutrition examination survey (NHANES III) which is representative of the US civilian, non-instituzionalized population[19], (iii) ascorbate consumption is lower in edentate subjects and plasma ascorbate levels correlate with edentulism.[21] On the other hand, the AA of saliva drastically changes during some pathological states[23] and several researchers have largely demonstrated that decreased AA of saliva and gingival crevicular fluid are associated with PD.[24–26] There is evident that any device

delivering ascorbate but mainly capable of actually releasing it and increasing its levels, especially at the sites of interest (i.e. periodontal pockets) may represent a valuable tool for increasing the oral AA of PD patients and consequently a valuable aid during the classical therapy.

The results described here indicate that specific and suitable toothpaste preparations may possess and exert AA *in vitro*; therefore, a demonstration of *in vivo* AA is necessary and desirable in order to produce worthwhile information for the preparation of new toothpaste formulations.

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