

Age-Related Changes in Salivary Antioxidant Profile: Possible Implications for Oral Cancer

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Oral cancer's much higher prevalence among older people may be due to an age-related reduction in protective salivary antioxidant mechanisms and/or an age-related increase in the magnitude of oral carcinogen attack, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing DNA aberrations. This study found a significantly reduced total value of salivary antioxidant capacity in elderly persons (as measured by overall antioxidant capacity [ImAnOx] assay), (46% of healthy individuals, $p = .004$), increased oxidative stress (86% increase in carbonyl concentrations—indicators of enhanced ROS attack, $p = .001$), and increased salivary concentrations and total values of RNS (7-fold and 3-fold higher respectively, $p = .001$), all contributing to increased DNA oxidation of oral epithelial cells. Salivary oxidative stress-related changes in the intimately related saliva and oral epithelium compounded with higher viscosity of saliva may explain the higher prevalence of oral cancer in the elderly population. Administration of local therapeutic agents (i.e., antioxidants) to the oral cavity should be considered.

ORAL cancer, the most common malignancy of the head and neck and having a high rate of morbidity and mortality (>50%) (1–5), has a much higher prevalence among older people as compared with the younger population. This high prevalence among the elderly population may result from an age-related increase in the magnitude of the attack of the oral carcinogens (as free radicals, which include reactive oxygen and nitrogen species—ROS and RNS—causing various DNA mutations and/or aberrations). It may also result from an age-related reduction in oral protective mechanisms based on the salivary antioxidant system, or from both. Ma and colleagues (6) recently demonstrated that oxidative and nitritative stress contributes to the development of oral cancer from premalignant lesion leukoplakia.

RNS are produced by the reaction of ROS or other free radicals with nitric oxide (NO) (7). NO[•] is a free radical which plays an important role in host defense and homeostasis when generated at a low level and for a brief period of time, but becomes genotoxic and mutagenic when generated at higher concentrations for prolonged periods. NO[•] may mediate DNA damage through the generation of RNS, the inhibition of DNA damage-repair mechanisms, or the formation of carcinogenic nitrosamines (8). Accordingly, it is not surprising that human saliva also contains antinitrosamine inhibitory agents (9) which are part of its antioxidant system. Moreover, salivary nitrosamine production and RNS metabolism are also based on dietary nitrates (NO₃). They are absorbed from the upper gastrointestinal (GI) tract and are actively concentrated from the plasma into the saliva by the salivary glands through a transport system similar to that for iodide, thiocyanate and perchlorate (10). In the oral cavity the salivary nitrates

are turned into nitrites (NO₂) which are of special importance as carcinogenesis promoters, because they react with amines and amides to form the carcinogenic nitrosamines (11,12). Both NO₃ and NO₂ also originate from breakdown of the NO molecule and thus are in equilibrium with it.

The salivary antioxidant system, which has a fundamental anticarcinogenic role in the oral cavity, is aimed at fighting ROS and RNS, which originate from smoking, alcohol, food, drink, and/or various other volatile sources, entering freely into the oral cavity through the body's largest open gate—the mouth. The salivary main antioxidant enzyme is peroxidase, and uric acid is the main salivary antioxidant molecule (13). The anticarcinogenic capability of saliva was demonstrated in a study published in 1997 in which saliva was shown to significantly inhibit the initiation and progression of oral cancer in an animal model (14). Further credence to this salivary anticarcinogenic capacity against oral cancer was demonstrated by Nishioka and colleagues (15), who, using the Ames test, found that saliva inhibited the mutagenicity of oral cancer inducers as cigarette smoke and 4-nitroquinoline-1-oxide (4NQO). Moreover, Wu and colleagues (16) reported recently that saliva plays an important role in cigarette-induced DNA damage; their study is supported also by *in vitro* studies (17).

Based on the above accumulative data one may conclude that an age-related reduction in the salivary antioxidant capacity and/or increase in the salivary content of RNS and/or ROS that concomitantly results in enhanced oxidative stress in the oral cavity may explain the increased prevalence of oral cancer in the elderly population. The purpose of the current study was to evaluate various salivary antioxidant parameters and nitrogen species (and the free radical-related damage they cause to the proteins) in elderly persons.

MATERIALS AND METHODS

Patients and Study Design

For this analysis, 80 participants (45 men and 35 women, 20–80 years old) were enrolled and identified according to age. Participants analyzed in the current study were all healthy, functioning individuals, and none used any saliva-affecting drugs (such as anticholinergics or antidepressants). Sjogren's syndrome or other connective tissue diseases, previous radiotherapy and/or chemotherapy, or any oral mucosal and/or gingival disease were considered exclusion criteria. An analysis of age-related changes in salivary antioxidant profile was performed across a broad age range of these participants (20–80 year old), using linear regression. This profile included peroxidase—the most important salivary antioxidant enzyme, uric acid—the most important salivary antioxidant molecule, total antioxidant status (TAS), and the overall antioxidant capacity (ImAnOx).

Two age groups composed of the youngest (20- to 25-year-old) and the oldest (70- to 80-year-old) participant, 22 individuals in each, were further analyzed. The parameters were presented not only as specific concentrations but also as total salivary values (i.e., concentration multiplied by flow rate), to evaluate the total amount of the antioxidant secreted into the oral cavity. The salivary oxidative stress level was assessed both by measuring nitrogen species (NO, NO₃, NO₂) and by examining the protein carbonyl level (both are indicators of nitrate and oxidative stress inflicted on the saliva).

Sialometric Analysis

Saliva was collected using the widely accepted procedure often described (13), controlled to avoid saliva loss through drooling or swallowing. Whole saliva was collected in resting conditions in a quiet room during the morning, between 9 AM and 12 PM, at least 1 hour following food intake. Participants were asked to collect saliva in their mouths and to spit it into a wide-mouthed test tube for 5 minutes. Saliva flow rates were estimated by measuring the volume of saliva thus collected, which was then centrifuged and kept at 4°C until analysis (within 1 week of collection, because previous studies have shown that during this period of time, saliva remains stable with respect to the parameters analyzed in such conditions). However, for each examination, new standards were prepared and calibration tools were used.

Salivary Antioxidant and Protein Analysis

The salivary antioxidants and total protein measurements were performed as previously described (18).

Peroxidase Activity

Peroxidase activity was measured both in the patients' serum and saliva according to the thionitrobenzoic acid (NBS) assay as previously described (19). Briefly, the calorimetric change induced by the reaction between the enzyme and the substrate, dithiobis 2-nitrobenzoic acid (DTNB) in the presence of mercaptoethanol, was read at a wavelength of 412 nm for 20 seconds.

Uric Acid

Uric acid concentration was measured both in the serum and saliva of the participants, as previously described (13), using a kit supplied by Sentinel CH (Milan, Italy). Uric acid is transformed by uricase into allantoin and hydrogen peroxide which, under the catalytic influence of peroxidase, oxidizes the chromogen (4-aminophenazone/*N*-ethyl-methylanilin propane-sulfonate sodium) to form a red compound the intensity of color of which is proportional to the amount of uric acid present in the sample and which is read at a wavelength of 546 nm.

TAS

TAS was assessed both in the saliva and serum samples as previously described (18). Briefly, this assay is based on a commercial kit supplied by Randox (Oceanside, CA) in which metmyoglobin in the presence of iron is turned into ferryl myoglobin. Incubation of the latter with the Randox reagent 2,2'-azido-bis-[3-ethylbenzothiazolino-6-sulfonate] (ABTS) results in the formation of a radical colored blue-green which can be detected at 600 nm.

Salivary Antioxidant Capacity

An enzyme-linked immunosorbent assay (ELISA) colorimetric test system (Immundiagnostik AG, Bensheim, Germany) for the determination of antioxidative capacity was performed by the reaction of antioxidants in the saliva with a defined amount of exogenously provided hydrogen peroxide (H₂O₂). The antioxidants in the saliva sample eliminated a certain amount of the provided hydrogen peroxide. The residual H₂O₂ was determined colorimetrically by an enzymatic reaction that involves the conversion of tetramethylbenzidine (TMB) to a colored product. After addition of stop solution, the samples were measured at 450 nm in a microtiter plate reader. The quantification was performed with a calibrator. The difference between applied and measured concentration in a defined time is proportional to the reactivity of the antioxidants of the sample (antioxidant capacity).

Salivary Nitrogen Species Analysis

Salivary NO, NO₃, and NO₂ concentrations were measured by the method of Griess modified by Fiddler (20) using the NO and the Total Nitric Oxide assays kits (catalog no 907-010/020) of Assay Designs Inc. (Ann Arbor, MI). This method is based on a two-step process. The first step is the conversion of NO₃ to NO₂ using tin metal powder, and the second is the addition of sulphanilamide and *N* (-naphthyl) ethylenediamine (Griess reagent). The latter converts NO₂ into a deep purple azo-compound, which was measured colorimetrically at 540 nm. NO products were expressed as mmol/dL saliva.

Salivary Carbonyls

Salivary carbonyls were analyzed for both the old and young groups by Western blot performed with an Oxyblot Kit S-71250 (Intergen, Purchase, NY) using specific anti-dinitrophenylhydrazine (DNPH) antibodies. Between 25 and 30 mL of saliva supernatant was applied to each well,

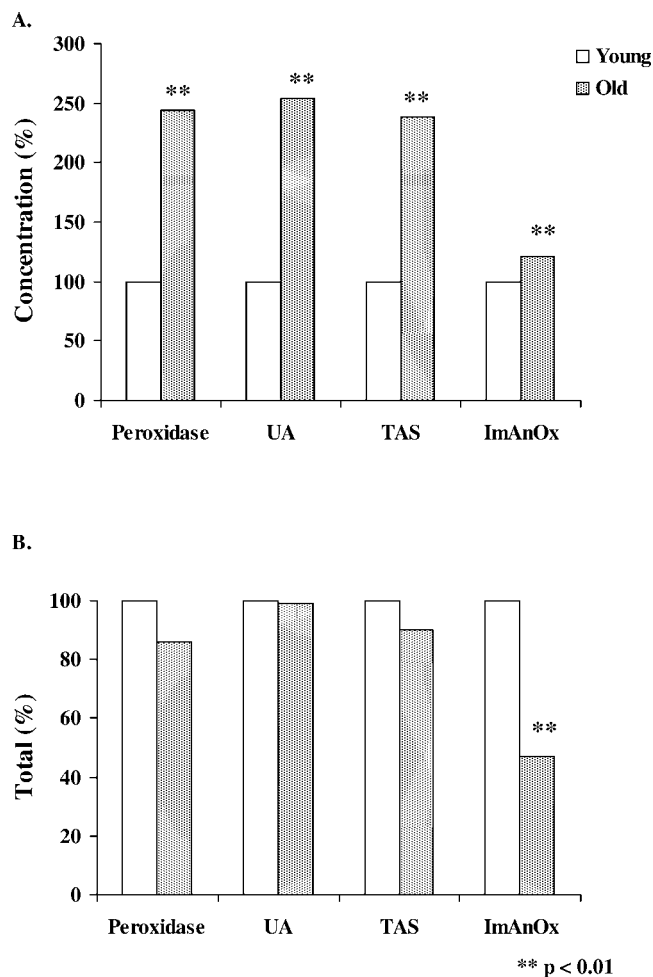


Figure 1. Salivary concentrations (A) and total output amounts (B) of peroxidase, uric acid (UA), total antioxidant status (TAS), and overall antioxidant capacity of the oral cavity (ImAnOx) in young (white bars, $n = 22$) versus old (gray bars, $n = 22$) individuals.

corresponding to 60 μg of protein. Finally, saliva proteins were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as was described previously (21). In addition to using the Western blot, which is a qualitative examination, we also measured the salivary carbonyls quantitatively using the NWK-PCK01 protein carbonyl ELISA kit (Northwest Life Science Specialties, LLC, Vancouver, WA).

Statistical Analysis

For categorical variables, frequencies, percentages, and distribution were calculated. For continuous variables, ranges, medians, means, and standard errors were calculated. Due to the large inborn variability of parameters in saliva, median values were calculated. Nonparametric statistical tests were used, as is acceptable for small-sample-size groups (<30). Distributions of categorical variables were compared and analyzed by the Fisher–Irwin exact test. The medians between subgroups of participants were compared by the Wilcoxon

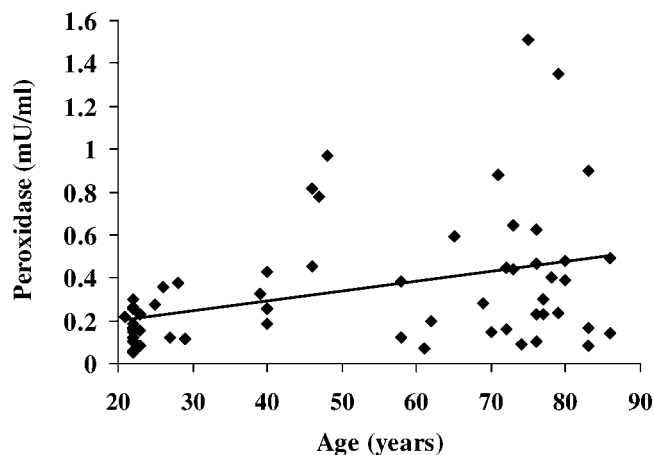


Figure 2. Correlation (Pearson analysis) between age and concentration of salivary peroxidase ($r = 0.48$).

rank–sum test (pairs of subgroups). Correlation between pairs of variables was calculated by the Pearson correlation.

RESULTS

Sialometric Analysis

An age-related decrease was demonstrated for the salivary flow rates. The mean flow rate of the young group was 0.46 ± 0.05 mL/min, and that of the elderly group was significantly lower by 60% (0.19 ± 0.04 mL/min), ($p = .002$).

Salivary Antioxidant and Protein Analysis

The overall oral antioxidant capacity as expressed by the median salivary total level of ImAnOx was lower by 54% ($p = .004$) in the elderly participants as compared with the young participants, whereas the peroxidase, uric acid, and TAS were unaltered by age (Figure 1).

In contrast to these total values, which represent the total amount of the antioxidant in the oral cavity, the salivary concentrations of these antioxidants were significantly higher with age, as salivary flow rates were significantly decreased and became more concentrated. The salivary total protein concentration in the elderly group was 63.5 mg/dL, significantly higher by 32% than in the young group ($p = .05$). The median salivary concentrations of peroxidase, uric acid, TAS, and ImAnOx in the young group were 0.16 mU/mL, 2.07 mg/dL, 0.24 mmol/L, and 264 mmol/L, respectively, and they increased significantly in the elderly group by 144% ($p = .004$), 154% ($p = .0001$), 138% ($p = .0001$), and 21% ($p = .0023$), respectively. The Pearson correlation coefficients between age and peroxidase, age and uric acid, and age and TAS levels were 0.48 ($p = .0023$), 0.51 ($p = .0001$), and 0.52 ($p = .0001$), respectively (Figures 2–4). The Pearson correlation coefficient between age and ImAnOx levels was 0.52 ($p = .0001$).

Salivary Nitrogen Species Analysis

The median salivary concentrations of the NO, NO₃, and NO₂ in the young group were 9.5 $\mu\text{mol/L}$, 93.7 $\mu\text{mol/L}$, and

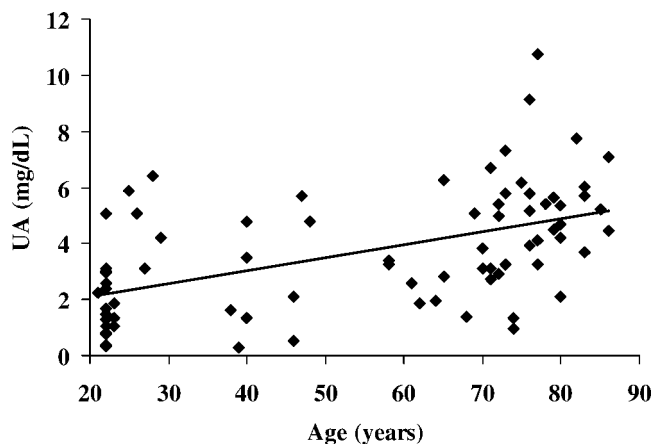


Figure 3. Correlation (Pearson analysis) between age and concentration of salivary uric acid (UA) ($r = 51$).

99.2 $\mu\text{mol/L}$, respectively, whereas in the elderly group these concentrations were significantly higher by 777%, 635%, and 786%, respectively ($p = .001$) (Figure 5). The total values of these three RNS were increased by 310%, 254%, and 314%, respectively ($p = .001$).

Salivary Carbonyls

The salivary carbonyl concentration in the elderly group was 0.54 nmol/mg, significantly higher than that of the young group by 86% ($p = .001$). In Figure 6, the Western blot analysis of the saliva secreted by elderly (columns 1–7) and by young participants (columns 9–14), shows the protein carbonyl levels as measured by positive anti-DNPH antibodies (see Material and Methods section). It is apparent that the proteins originated in the saliva of the young group are significantly less oxidized.

DISCUSSION

The most important and novel finding of the current study is that elderly people have significantly reduced total salivary antioxidant capacity (only 46% of healthy individuals; $p = .004$) and increased oxidative stress (85% increase in carbonyls, which are the products of ROS oxidation; $p = .001$). This oxidative stress was demonstrated also by the profoundly increased salivary nitrogen species (salivary RNS were 7-fold higher in the elderly group; $p = .001$). This increased oxidative stress might contribute also to an increased level of DNA oxidation of the oral epithelial cells, which in turn may result in the development of cancer. In fact, it was previously found that, although ROS and RNS are involved in the initiation and promotion of multistep carcinogenesis, both are inhibited (neutralized) by antioxidants (22,23). When the equilibrium is broken either by reduced levels of antioxidants or enhanced levels of ROS and RNS, an oxidation process of the DNA occurs and cancer evolves. Whether the increase in RNS was the event that led to the consumption and reduction of the salivary antioxidants remains an open question. The opposite might be true. In any case, it explains the increased oxidative stress inflicted in the oral cavity. Such a salivary antioxidant

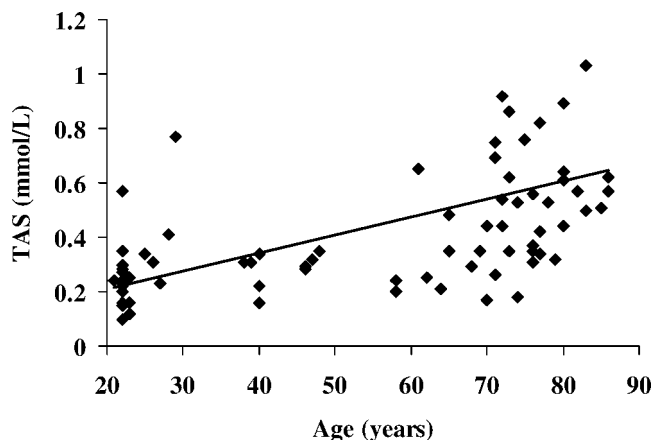


Figure 4. Correlation (Pearson analysis) between age and concentration of salivary total antioxidant status (TAS), ($r = 52$).

reduction, simultaneously accompanied by an increase in salivary carbonyls, was previously reported in other conditions also (13). As for the decrease in salivary secretion, which results in increased saliva concentration, it is noteworthy to appreciate that this is also accompanied by a reduced salivary rheological capacity, i.e., a reduced ability to flow and arrive at the sites where the salivary protective capacity is required (24). Thus the concentrated saliva in the elderly group (demonstrated by the increased concentrations of the proteins, peroxidase, uric acid, and TAS) adds to the already reduced antioxidant-protective capacity mentioned earlier and is in accord with the decrease in salivary secretion previously reported in the elderly population (25–29). This reduction might be induced by age-related changes in the salivary gland parenchyma (30,31) and/or by the prevalent administered therapy for medical problems in elderly persons, as suggested by others (32–34).

Our results are in accord with the study published by Salvolini and colleagues (35), who found a significant

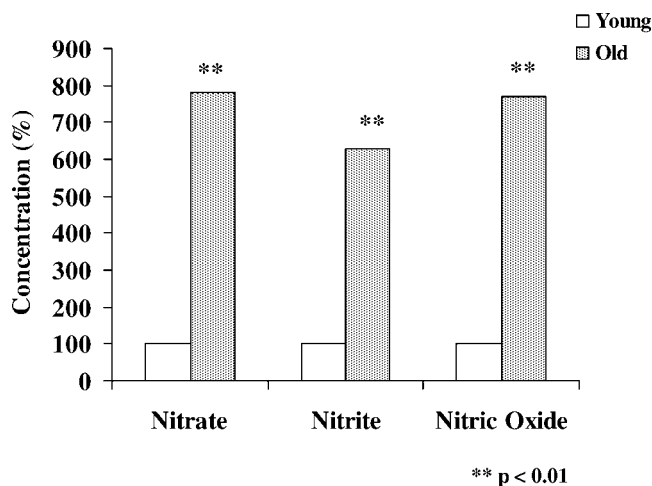


Figure 5. Salivary concentrations of salivary nitrates (NO_3), nitrites (NO_2), and nitric oxide (NO) in elderly (gray bars, $n = 22$) versus young (white bars, $n = 22$) individuals.

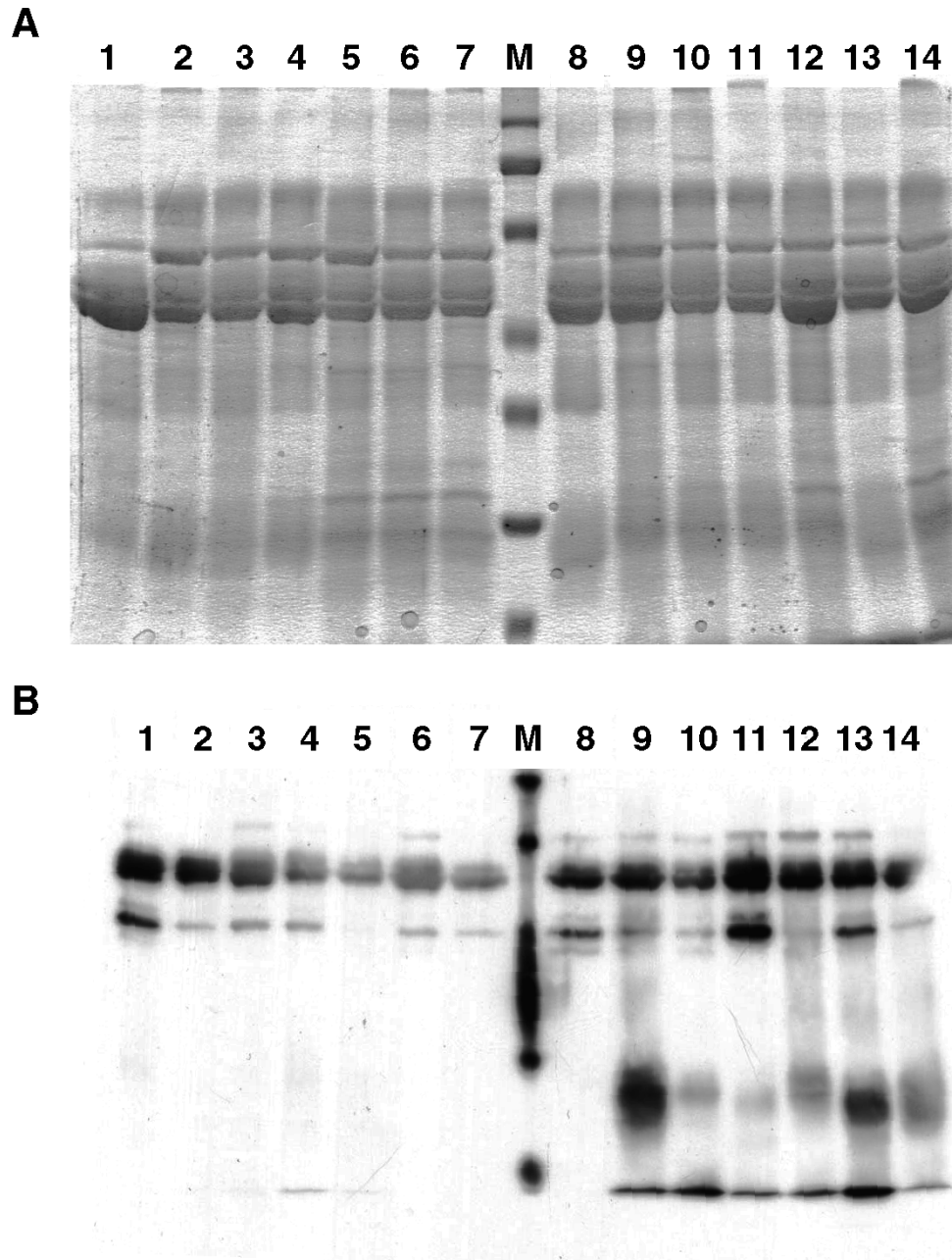


Figure 6. Western blot analysis with anti-dinitrophenylhydrazine (DNPH) antibody for salivary protein carbonylation pattern (oxidation level). (A) Coomassie staining. (B) protein carbonyls. Lanes 9–14: increased level of protein carbonyls in the elderly individuals as compared with the saliva secreted in the young individuals (lanes 1–7). M, molecular weight marker.

age-related decrease in the activity of the peroxidase, the salivary pivotal antioxidant enzyme, accompanied by a significant increase in saliva lipid peroxide levels, indicating enhanced free-radical production that may contribute to tissue damage. Moreover, whole-saliva NO content showed a significant increase with age, and accordingly, Salvolini and colleagues (35) concluded that during the aging process, the oral tissues may become more susceptible to environmental factors due to a modification in the balance between various antimicrobial agents. Our results are also in accord with those reported by Kohen and

colleagues (36), who found that the total saliva-reducing ability decreases with age as indicated by cyclic voltammetry measurement (without determination of the specific reducing compound within the saliva). Further credence for our results may be found in the report of Yeh colleagues (37), who reported an age-related increase in the salivary protein concentration.

Presenting the data both as salivary concentrations and as salivary total values is helpful to better understand the biological significance of the data; the total values represent the overall amount of antioxidant in the oral cavity

(concurrently taking into consideration the increased salivary concentrations and the reduced flow rates in the elderly population).

Summary

The demonstrated salivary oxidative stress-related changes, the intimate contact between this saliva and the oral mucosa, and the increase in saliva concentration (higher viscosity) may explain the higher prevalence of oral cancer in the elderly population. It also presents a unique opportunity to evaluate the oral status and perhaps even to intervene, as saliva can be easily harvested and monitored (for its antioxidants, RNS, carbonyls, e.g.). Furthermore, one should consider administering local therapeutic agents as antioxidants to the oral cavity of elderly persons.

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