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Review article

The antioxidant capacity of saliva

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Abstract

Background/aims: Saliva, a heterogeneous fluid comprising proteins, glycoproteins, electrolytes, small organic molecules and compounds transported from the blood, constantly bathes the teeth and oral mucosa. It acts as a cleansing solution, an ion reservoir, a lubricant and a buffer. In addition to its other host-protective properties, saliva could constitute a first line of defence against free radical-mediated oxidative stress, since the process of mastication and digestion of ingested foods promotes a variety of reactions, including lipid peroxidation. Moreover, during gingival inflammation, gingival crevicular fluid flow increases the change of saliva composition with products from the inflammatory response; this, in turn, could have some rôle in controlling and/or modulating oxidative damages in the oral cavity. This is the reason why the antioxidant capacity of saliva has led to increasing interest, and the development of techniques suitable for saliva antioxidant evaluation.

Materials and Methods: Here, we review the current peer-reviewed literature concerning the nature and characteristics of free radicals, reactive oxygen species, oxidants, pro-oxidants and antioxidants in saliva, especially pro-oxidant and antioxidant features, as well as current methods for assessing the antioxidant capacity of saliva.

Results and Conclusions: In the last decade, several methods have been developed for assaying the antioxidant activity of saliva, indicating an increasing interest of researchers and clinicians. Unfortunately, systematic studies of saliva are still lacking, even in healthy populations.

Key words: saliva; free radicals; reactive oxygen species; antioxidants; total antioxidant capacity

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Whole saliva represents a mixture of the secretions of the major (submandibular, sublingual, parotid) and minor (accessory) salivary glands, together with the gingival fluid. The secretions from the different glands have been shown to differ considerably, to be complex in composition and to be affected by different forms of stimulation, time of day, diet, age, gender, a variety of disease states, and several pharmacological agents (Mandel 1974). Part of the volume of saliva is produced in response to stimulation accompanying chewing, about 60% being produced under resting conditions (Wei et al. 1986, Moore et al. 1994). During sleep, flow from the major glands virtually ceases (Wei et al. 1986). Saliva may be described as a heterogeneous fluid composed of proteins, glycoproteins, electrolytes, and small organic molecules, as well as compounds transported from blood (FDI Working Group 1992). The proteins in saliva have been found in concentrations of approximately 3% of plasma, and most have antibacterial properties (Edgar 1992), as they include both nonimmunoglobulins and secretory antibodies, especially secretory IgA, lysozyme, lactoferrin and peroxidase (Tenovuo et al. 1986). This fluid constantly bathes the teeth and oral mucosa; it acts as a cleansing solution, an ion reservoir, a lubricant and a buffer. Once saliva is present at the tooth surface, the buffering action of saliva may help to prevent further demineralisation by the plaque acid (Wei et al. 1986, Whelton, 1996, Edgar & Higham 1996). In addition, saliva forms the acquired pellicle, a protective layer on the tooth surface.

Recently, it has been claimed that the imbalances in levels of free radicals and

reactive oxygen species with antioxidants may play an important rôle in the onset and development of several inflammatory oral pathologies (Chapple 1997, Battino et al. 1999). Recently, we extensively reviewed current evidence for oxidative damage in the chronic inflammatory periodontal diseases (CIPD), and the possible therapeutic effects of antioxidants (Battino et al. 1999). Physiologically free radical/reactive oxygen species in the mouth are derived mainly from polymorphonuclear neutrophils (PMN), which may also help to control bacterial growth by the well-known "respiratory burst" (RB). Such physiological processes are usually efficiently counteracted by intrinsic antioxidant systems: if such systems fail, tissue damage can result.

Saliva may constitute a first line of defence against free radical-mediated oxidative stress, since the process of mastication promotes a variety of such reactions, including lipid peroxidation (Terao & Nagao 1991). Moreover, during gingival inflammation, gingival crevicular fluid (GCF) flow increases, adding to saliva with products from the inflammatory response. This is why the antioxidant capacity of saliva is of increasing interest, and why several laboratories have devised methods for its evaluation.

Free Radicals, Reactive Oxygen Species, Oxidants, Pro-Oxidants and Antioxidants: What are they?

Oxygen may be considered a gaseous nutrient (Forster & Estabrook 1993) that cannot effectively be replaced by any other element (George 1964). It is required for all mammalian energy needs. The evolution of efficient aerobic respiration allowed the development of complex multicellular organisms (aerobes) that use oxygen to oxidise (i.e., burn) fuels rich in carbon and hydrogen (i.e., nutrients) to produce the different forms of energy needed for life. However, the reduction of molecular oxygen to water is accompanied by a large free energy release that results in a great variety of chemical species, such as intermediates, depending upon environmental conditions. Biologically, either 1-, 2-, 3- or 4-electron reduction may occur, giving rise to free radicals (FR) and/or reactive oxygen species (ROS). The reactivity and associated toxicity of ROS (otherwise called "partially reduced oxygen products") may be major contributors to the pathogenesis of several chronic degenerative diseases (Pryor 1986, Halliwell & Gutteridge 1990, Cheeseman & Slater 1993, Rice-Evans & Burdon 1993, Halliwell 1994, Sies 1997). In the last 2 decades, over 80 clinical conditions have been identified in which involvement of FR and ROS has been suggested, and over 6500 and 4500 papers, respectively, have appeared on these topics in the last 5 years, including some dealing with ROS- and/or FR-mediated inflammatory disease.

An FR is commonly defined as an atomic or molecular species with 1 or more unpaired electrons in its structure, and can be positively or negatively charged or electrically neutral. The most important FRs in biological systems are radical derivatives of oxygen (e.g., $O_2\cdot^-$, OH·, OOH·, RO·, ROO·, RCOO·, RCOOO·, ArO·, ArOO·, etc.). Other highly reactive compounds are known as ROS. ROS include not only oxygen FR but also non-radical oxygen derivatives involved in oxygen radical production (e.g., $^1O_2$, O_3, H_2O_2, HOCl, etc.). Oxygen FR are not uniquely important, although they are often the initial species formed. Many other FR/ROS exist: nitric oxide and nitric dioxide, thiyl radicals, carbon-centred radicals that result from the attack of an oxidising radical on amino-acids; carbohydrates, fatty acids or DNA bases are only the most widely diffused examples (Chapple 1997, Battino et al. 1999).

From a chemical point of view, oxidation is defined as a loss of electrons and, therefore, an oxidant or an oxidizing agent is a substance that accepts electrons and causes another reactant to be oxidized (Prior & Cao 1999). Pro-oxidant is a synonym for ROS, indicating a toxic substance that can cause oxidative damage to biological targets. From a biomedical point of view, an antioxidant may be defined (Halliwell 1997) as a substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly prevents or delays a pro-oxidant-initiated oxidation of the substrate. Among the different classifications of antioxidant defences proposed, it appears that a functional classification of antioxidants based on the way they act (Niki 1996) could be more useful. According to this suggestion, antioxidant defence systems in vivo are mainly of three kinds.

(a) *Preventive antioxidants*, that suppress the formation of FR (e.g., superoxide dismutase, catalase, glutathione peroxidase and −S-transferase, carotenoids, transferrin, albumin, haptoglobin, caeruloplasmin).
(b) *Radical-scavenging antioxidants*, that scavenge radicals to inhibit chain initiation and break chain propagation (e.g., albumin, bilirubin, carotenoids, ubiquinol, uric acid, vit. A, vit. C, vit. E).
(c) *Repair and "de novo" enzymes* that repair the damage and reconstitute membranes (DNA repair enzymes, lipase, protease, transferase).

Pro-oxidant and Antioxidant Features of Saliva

Saliva contains many biochemical systems known to be involved in soft-tissue repair, and many antibacterial components including lysozyme, lactoferrin and salivary peroxidase. Human whole saliva contains a complex peroxidase system, the major components of which include different forms of lactoperoxidase secreted by the salivary glands and myeloperoxidases from PMN.

One of the most important functions of salivary peroxidase is the control of oral bacteria that form dental plaque, to imbalances in the ecology and which lead and to dental caries and to CIPD. Salivary peroxidase catalyses the peroxidation of the thiocyanate ion (SCN^-) to generate oxidation products (O_2SCN^-, O_3SCN^-, $(SCN)_2$, HOSCN and the more stable $OSCN^-$) that inhibit the growth and metabolism of many micro-organisms (Tenovuo et al. 1986, Pruitt et al. 1986). It seems that this enzyme can also function as a catalase. H_2O_2 may reach significant levels in human saliva. Since H_2O_2 is highly toxic for human cells and $OSCN^-$ is not, the peroxidation of SCN^- in vivo may serve the dual purpose of limiting the accumulation of toxic levels of H_2O_2, which are produced by commensal bacteria and by the salivary glands while, at the same time, providing $OSCN^-$ and HOSCN.

It should also be taken into account that the GCF is constantly mixed with saliva and its flow increases with gingival inflammation; increased GCF flow relates to increased PMN levels which, in turn, contribute to overall peroxidase enhancement by myeloperoxidase activity. Myeloperoxidase is a chlorine-con-

taining enzyme in the azurophil granules of neutrophils and blood monocytes that catalyzes the oxidation of chloride and reduction of H_2O_2 to form hypochlorous acid (HOCl), a reactive oxygen species that can induce peptide bond scission and the formation of low molecular weight chloramines with bactericidal potential (Miyasaki 1991). The amount of O_2 and H_2O_2 (the latter produced during respiratory burst), consumed in order to oxidize chloride, can account for up to 40% or more of the total available in these cells (Foote et al. 1983). Myeloperoxidase may accumulate during sleep, when salivary flow is very low, with consequent slow removal of PMN products. It has been suggested that higher myeloperoxidase levels are present in low flow rate whole saliva supernatants of subjects with severe gingival inflammation, probably owing to the enhanced numbers of PMN which enter the oral cavity (Smith & Yang 1984).

On the other hand, saliva is also rich in antioxidants, mainly uric acid, with lesser contributions from albumin, ascorbate and glutathione (Moore et al. 1994, Lynch et al. 1997, Meucci et al. 1998, Zappacosta et al. 1999), and it has been demonstrated that saliva has a rôle in suppressing the lipid peroxidation of ingested foods (Terao & Nagao 1991). Salivary urate concentration greatly varies (between 40 and 240 μM) depending on experimental conditions (Moore et al. 1994, Lynch et al. 1997, Meucci et al. 1998, Zappacosta et al. 1999). It has been reported that uric acid is the major antioxidant in saliva, accounting for more than 85% of the total antioxidant activity of resting and stimulated saliva from both healthy and periodontally-compromised subjects (Moore et al. 1994). Albumin concentration is comparatively low, about 10 μM, apparently in the same range of that of ascorbic acid (Moore et al. 1994). Ascorbic acid appears to be particularly concentrated in GCF where its level it has been reported to be 3× higher than in plasma (Meyle & Kapitza 1990). Glutathione concentration is about 2 μM (Zappacosta et al. 1997), but it is not clear whether it is the same thiol with antioxidant activity found in gingival crevicular fluid and saliva. Responsibility for such activity has been identified: a thiol of low molecular weight (<10 kd), the activity of which has been reproduced from the cytosol of neutrophils and reproduced using L-cysteine (Chapple et al. 1997). Finally, traces of other antioxidants (transferrin, lactoferrin and caeruloplasmin) capable of binding metal ions are found in both saliva and GCF (Chapple 1997) and probably account for the 5–10% of antioxidant activity in saliva that other authors ascribed to unknown antioxidants (Moore et al. 1994). In fact, in healthy humans, these compounds enable iron and copper (the two elements involved in the FR production via the "metal-catalysed Haber-Weiss reaction") to be safely bound: the former is transported by the proteins transferrin and lactoferrin, and the latter is inactivated mainly by binding to caeruloplasmin and by the activity of albumin-SH groups.

Total Antioxidant Capacity: What does it Mean and how to Measure it

FR/ROS production and actions are rather complex and their interaction is frequent (Battino et al. 1999). The antioxidant defence systems are also highly complex (Chapple et al. 1997, Battino et al. 1999), constituting an effective network capable of counteracting FR/ROS effects. It is essential to evaluate the amounts and/or the activities of the different antioxidants when assessing antioxidant status in vivo. However, since FR/ROS and antioxidant systems appear to act in concert rather than alone, investigations of individual antioxidant activity may be misleading, and the measurement of any individual antioxidant may be less representative of the whole antioxidant status. Moreover, the number of different antioxidants makes it difficult, and also expensive, to measure each antioxidant separately, especially during daily clinical treatments. For these reasons, research is now being directed towards assays that evaluate the so-called "Total antioxidant capacity" (TAC) of biological fluids, including saliva. Recently, Prior & Cao (1999) exhaustively reviewed the methods employed in determining TAC, mainly of serum and plasma. However, a critical literature analysis reveals that the TAC of saliva has been measured by only 3 methods using 3 different biochemical techniques: (a) a spectrophotometric assay; (b) an enhanced chemiluminescence assay; (c) a cyclic voltammetry assay.

As far as (a) is concerned, several techniques have been developed for the measurement of the TAC of fluids and all are essentially inhibition methods: an FR is generated, there is an end point by which the presence of the radical is detected, and the TAC of the added fluid inhibits the end point by scavenging the free radical. The spectrophotometric assay, also called "Trolox equivalent antioxidant capacity" (TEAC), is an adaptation of the ABTS assay (Moore et al. 1994, Rice-Evans & Miller 1994) based on the inhibition by antioxidants of the absorbance of the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) ($ABTS^{\cdot+}$). This blue/green chromogen produces characteristic absorption maxima in the near UV region and at 660 nm, 734 nm and 820 nm. $ABTS^{\cdot+}$ is formed by the interaction of ABTS with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin with H_2O_2. In the presence of antioxidants, the absorbance of $ABTS^{\cdot+}$ is inhibited to an extent and on a timescale dependent on the TAC of the substance investigated. In order to standardize the assay, Trolox, a vitamin E analogue, is usually employed. Thus, solutions can be compared with Trolox and with one another expressing their antioxidant activity as TEAC. The TEAC method was first used (Moore et al. 1994) to compare the antioxidant activity of saliva in healthy individuals with that of patients with CIPD. The data obtained confirmed the pivotal rôle played by uric acid, which was shown to be the main antioxidant of saliva. An increased production of antioxidants was also associated with stimulation of salivary flow. Finally, the TEAC of saliva was not found to be compromised in patients affected by CIPD and the authors suggested that this may be due to an increased local production of antioxidants due to increased GCF flow. Such a situation would result in no net local depletion of antioxidants. More recently, the salivary TEACs of patients with fluctuating urate leves (Meucci et al. 1998) and of smokers (Zappacosta et al. 1999, Kondakova et al. 1999) have been investigated. In the former study, TEAC was assessed in whole as well as parotid and submandibular/sublingual saliva of controls and haemodialyzed patients (Meucci et al. 1998). In controls, the highest TEAC values were in parotid saliva, while in haemodialyzed patients, the highest values were typical of whole saliva. In these subjects, predialysis samples for each kind of saliva had TEAC values higher than the corre-

sponding levels in normal individuals. At the end of a dialysis session, a remarkable decrease of TEAC was found for all 3 kinds of saliva. Therefore, elevated plasma levels of uric acid are accompanied by higher TEAC values. A similar observation was made in hyperuricaemic patients affected by remarkably higher plasma uric acid levels, and who displayed concomitantly very high TEAC values. Finally, both total protein concentration and uric acid level showed a good positive correlation with salivary TEAC. The current data did not enable any definitive conclusion about a possible protection of saliva against CIPD in haemodialyzed patients, because none of the 25 subjects examined showed any periodontitis. The authors wondered whether high uric acid dependent-TEAC could reflect a periodontal protection factor. However, some methodological features make this data less reliable. For instance, only an average probing depth for the 25 subject patients was presented, and disease severity varies from site to site around individual teeth and around the mouth. Other investigations concerned the evaluation of TEAC in human saliva of smokers and non-smokers (Zappacosta et al. 1999, Kondakova et al. 1999). In the 1st study, it was suggested that salivary TEAC and uric acid are not affected by smoking one cigarette, and also that no differences in these variables existed between smokers and non-smokers (Zappacosta et al. 1999). Only glutathione concentration fell after smoking a single cigarette. Unfortunately, it was not explained why non-smokers were characterized by very low glutathione levels, identical to those of smokers after smoking a single cigarette. In the 2nd study, the rôle of uric acid as the major salivary antioxidant was confirmed, as well as the positive correlation between TEAC and uric acid (Kondakova et al. 1999). It was also confirmed that there are no differences in salivary uric acid and TEAC between smokers and non-smokers, as was the fact that smoking a single cigarette has no demonstrable effect on TEAC or uric acid.

The 2nd method for measuring salivary antioxidant activity (b) is based on chemiluminescence assays (Chapple et al. 1997, Hirayama et al. 1997, Hirayama & Yida 1997). One enhanced chemiluminescence assay (ECL), is based on the horseradish peroxidase (HRP)-catalysed oxidation of luminol by H_2O_2 (Chapple et al. 1997). The light produced from the reaction is enhanced by p-iodophenol which prolongs and intensifies the light signal. The resulting signal can be temporarily suppressed by antioxidants. Such suppression lasts until the antioxidants are exhausted. The antioxidant capacity of the solution under test can be calculated from a standard curve run with a calibrant. This rapid, simple and reproducible method has provided evidence that salivary TEAC of patients with chronic periodontitis was lower than for a periodontitis-free group. The 2nd chemiluminescence method is based on antioxidant-dependent quenching of chemiluminescence generated from lipid hydroperoxide and isoluminol/microperoxidase reagent. When an antioxidant is present in the assay mixture, it scavenges the lipid oxyradical and quenches the production of light (Hirayama et al. 1997). This method was used to evaluate antioxidants by measuring the half-inhibition concentration (I_{50}) of biological fluids, including saliva. A further chemiluminescence method is based on the generation of OH· by the Fenton reaction and its subsequent determination by chemiluminescence. This simple, sensitive and useful method evaluates the OH·-scavenging ability of biological fluids such as saliva (Hirayama & Yida 1997).

The 3rd method for measuring salivary antioxidant activity (c) seems the least diffused and requires the use of the cyclic voltammetry technique (Kohen et al. 1999). The method is designed to evaluate salivary TAC, taking into account that the major FR scavengers in saliva are molecules with reducing properties. The cyclic voltammetry procedure reported recently (Chevion et al. 1997, Kohen et al. 1999) evaluated the overall reducing power of low molecular weight antioxidants in saliva. Following preparation, the sample is placed into a well with 3 electrodes: the working (e.g., glassy carbon), the reference (Ag/AgCl) and the auxiliary (platinum wire). The application of a constant rate potential to the working electrode, either toward the positive potential (to evaluate reducting equivalents) or toward the negative electrode (to evaluate the oxidizing species), allows the recording of a potential current curve or "cyclic voltammogram". In the cyclic voltammogramm of saliva, one anodic wave was found, indicating one group of reducing low molecular weight antioxidants, and it was demonstrated that the compounds comprising the wave correlated with the TAC of saliva. However, not all the common antioxidants donate their electrons to the working electrode at a sufficient rate. This is why thiol compounds, such as glutathione, should be detected using different electrodes (e.g., Au/Hg). Finally, the sensitivity of this procedure is relatively low: it is possible to determine reducing equivalents to as low as 1–10 μM.

Conclusions

It has been widely reported (Chapple 1997, Battino et al. 1999) that FR/ROS are often essential for biological processes and that tissue damage can easily take place when antioxidant systems do not efficiently counteract their action. In this sense, the mouth is a critical site because there is evidence that something of this nature may occur in inflammatory diseases, including CIPDs. In the last decade, several methods have been developed for assaying the antioxidant activity of saliva, indicating an increasing interest of researchers and clinicians. Since saliva has found application as a diagnostic aid in an increasing number of clinical situations (Mandel 1990), and a systematic study of its antioxidant capacity is still lacking, it is hoped that the technologies reviewed may find applications in the near future.

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Zusammenfassung

Die antioxidative Kapazität des Speichels

Hintergrund/Zielsetzung: Der Speichel, eine heterogene Flüssigkeit bestehend aus Proteinen, Glykoproteinen, Elektrolyten, kleinen organischen Molekülen und Bestandteilen aus dem Blut, umspült andauernd Zähne und Mundschleimhäute. Er wirkt als Reinigungslösung, Reservoir für Ionen, als Schmiermittel und als Puffer. Zusätzlich zu seinen anderen Abwehreigenschaften könnte der Speichel eine erste Verteidigungslinie gegen durch freie Radikal verursachten oxidativen Stress sein, da der Prozess der Nahrungs-

zerkleinerung und -verdauung eine Vielzahl von Reaktionen auslöst einschließlich der Lipidperoxidation. Darüber hinaus erhöht sich während gingivaler Entzündung der Sulkusflüssigkeitsfluss und verändert die Zusammensetzung des Speichels durch Produkte der Entzündungsreaktion. Dies könnte eine Rolle bei der Kontrolle und/oder Beeinflussung oxidativer Schäden in der Mundhöhle spielen. Dies sind die Gründe dafür, warum die antioxidative Kapazität des Speichels zu einem wachsenden Interesse und zur Entwicklung von Techniken geführt hat, die die Bestimmung der antioxidativen Kapazität des Speichels erlauben.

Material und Methoden: In diesem Übersichtsartikel wird die akutelle Literatur hinsichtlich der Natur und Charakteristika freier Radikale, reaktiver Sauerstoffarten, Oxidantien, Prooxidantien und Antioxidantien im Speichel, insbesondere Eigenschaften der Pro- und Antioxidantien sowie aktuelle Methoden zur Bestimmung der antioxidative Kapazität des Speichels, dargestellt.

Ergebnisse/Schlussfolgerungen: Während des vergangenen Jahrzehnts wurden mehrere Methoden für die Bestimmung der antioxidativen Kapazität des Speichels entwickelt, was für ein wachsendes wissenschaftliches und klinisches Interesse spricht. Unglücklicherweise fehlen noch systematische Studien zum Speichel selbst für gesunde Kollektive.

Résumé

La capacité antioxydante de la salive

Origine/but: La salive, fluide hétérogène constitué de protéines, de glycoprotéines, d'électrolytes, de petites molécules organiques et de composés transportés du sang, baigne constamment les dents et les muqueuses buccales. Elle agit comme une solution nettoyante, comme réservoir d'ions, comme lubrifiant et comme tampon. En plus de ces propriétés protectrices pour l'hôte, la salive pourrait constituer une première ligne de défense contre le stress oxydatif dû aux radicaux libres puisque le processus de mastication et de digestion des nourritures ingérées induit une variété de réactions, telle la peroxidation des lipides. De plus, pendant l'inflammation gingivale, le flux gingival sulculaire augmente et altère la compositon de la salive par les produits de la réponse inflammatoire. Cela, à son tour, pourrait avoir un rôle dans le contrôle ou la modulation des dommages oxydatifs dans la cavité buccale. C'est la raison pour laquelle la capacité antioxydant de la salive a connu un intérêt croissant et le développement de techniques fiables pour l'évaluation des antioxydants salivaires.

Matériaux et méthodes: Ici, nous passons en revue de façon concise la littérature actuelle concernant la nature et les caractéristiques des radicaux libres, des espèces réactives à l'oxygène, des oxydants, des pro-oxydants et des antioxydants dans la salive, particulièrement les caractéristiques pro-oxydante et antioxydante et les méthodes actuelles de mise en évidence des capacités antioxydantes de la salive.

Résultats et conclusions: Lors de la dernière décade, plusieurs méthodes ont été développées pour tester l'activité antioxydante de la salive, ce qui prouve un intérêt grandissant des chercheurs et des cliniciens. Malheureusement, des études systématiques sur la salive manquent même pour les populations saines.

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