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ANTIOXIDANT ACTIVITY OF SALIVA AND PERIODONTAL DISEASE

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The antioxidant activity of saliva has been investigated in 28 apparently healthy individuals and seven dental patients with periodontal disease. The results show that the major aqueous antioxidant component of whole saliva is uric acid, with lesser contributions from ascorbic acid and albumin. All are present at lower concentrations than those found in the plasma water. The total antioxidant activity (TAA) of saliva correlates ($r^2 = 0.972$) with the concentration of uric acid, which contributes more than 70% of the TAA. Stimulation of salivary flow is associated with increased production of antioxidants. The antioxidant potential of saliva does not appear to be compromised in patients with periodontal disease but this may relate to the antioxidant flow from the gingival crevicular fluid.

KEY WORDS: Antioxidant activity, uric acid, albumin, ascorbic acid, saliva, periodontal disease.

INTRODUCTION

In spite of the current interest in the significance of free radical involvement in disease processes, there has been minimal research into free radicals and antioxidants in the oral cavity and their potential significance in dentistry.

Whole saliva represents the secretions of the major (parotid, submandibular, sublingual) and minor accessory salivary glands, together with the gingival crevicular fluid.[1] The normal daily secretion of saliva is about 500 mls, about 60% being produced under resting conditions. This resting saliva is present in the mouth most of the time. Stimulated saliva (by mastication and gustation) tends to be less viscous than its resting counterpart with the increased proportion of water acting to dilute the various gland-derived constituents of the saliva.

The proteins in saliva are found in concentrations approximately 35 times lower than in plasma[1] and several of these have antibacterial properties[2] including the salivary peroxidase system which produces hypothiocyanous acid (via hydrogen peroxide and the thiocyanate ion).[3–5]

Gingival crevicular fluid (GCF) is a constituent of saliva with a composition similar to serum and is regarded as an inflammatory transudate. GCF flow rate increases in gingival inflammation because the epithelium lining the gingival crevice becomes more permeable in these areas.[6–8]

No conclusive information is as yet available concerning which factors in saliva might be predictive of susceptibility or resistance to periodontal disease,[9] which is

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of multifactorial aetiology. Some studies have addressed the assessment of th level of inflammation by measuring the numerous serum proteins that travers the crevicular epithelium and become part of the gingival crevicular fluid. Othe have concentrated on monitoring the large array of enzymes derived from bacteri and the gingival crevicular fluid.

The aim of this investigation was to analyse the total antioxidant activity (TAA of whole saliva by a method based on the quenching of the ABTS radical cation, to assess the contribution of the various antioxidants to this activity and to identif differences between the saliva of individuals with apparently healthy gingivae an those with periodontal disease, examining whether the whole saliva of those wit periodontal conditions might have a lower TAA than that of healthy individual secondary to increased free radical production in inflamed gingivae.

MATERIALS AND METHODS

Saliva collections were made at the standardised time of 10.30 am each day sinc resting saliva secretion displays a circadian rhythm, with its highest rate at abou 5 pm. The subjects were instructed not to eat, drink, smoke or take heavy physic exercise for two hours prior to collection. Resting saliva samples were collecte into plain universal containers for 15 minutes. Salivas were then collected aft masticatory stimulation: each subject chewed 1 gram of paraffin wax and expe torated intermittently for two minutes (to allow the salivary glands to reach the maximum rate of secretion) and then saliva was collected during a further minutes of mastication. Collection tubes remained on ice during collection t minimise enzymatic degradation of saliva. Samples were centrifuged immediate after collection for 15 minutes at 3000 r.p.m. at 4°C and the supernatant remove for immediate analysis. Twenty-eight subjects (12 males, 16 females – age range 2 to 50 years) with apparently healthy gingivae were chosen. While these subjects ma not all have had perfectly healthy gingivae, they reflected the periodontal conditic of the general population. In contrast, the subjects with periodontal disease ha conditions that were sufficiently severe to necessitate dental treatment. Saliv samples were taken from seven patients of UMDS Dental School, each of who had been referred from their own general dental surgeon for the treatment periodontal disease. Samples were collected from these patients before they we clinically assessed as this process tends to result in bleeding gingivae (blood prese in the saliva would interfere with the subsequent analyses) and also changes gingival crevicular fluid (GCF) production due to irritation of the tissues. Saliv collection from patients was performed in an identical manner to that of healtl subjects. Access to the patients' notes, once clinical assessment had been complete gave an indication of the type and severity of the periodontal disease present.

Salivary total antioxidant activity (TAA) was estimated by an adaptation the ABTS assay,[10] which involves the interaction of the ferrylmyoglobin radica produced from activation of metmyoglobin, with the phenothiazine compour ABTS [2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] forming the ABT radical cation. This blue/green chromogen produces characteristic absorptic maxima in the near u.v. region, but also at 660 nm, 734 nm and 820 nm. In tl presence of antioxidants, the absorbance of the ABTS radical cation is inhibit to an extent and on a timescale dependent on the total antioxidant capacity. Tl assay was standardised using the vitamin E analogue, Trolox (Hoffman-La Roche

Pure antioxidant substances can be compared with Trolox and with one another by means of this assay and this is expressed as the Trolox Equivalent Antioxidant Capacity or TEAC. The TEAC is defined as the mM concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. Ascorbic acid, uric acid and α-tocopherol have a TEAC value of 1.0.[10] Human albumin (Sigma) has a TEAC of 0.69.

The assay was calibrated using dilutions of Trolox in phosphate buffered saline. The temperature of the reaction was controlled at 30°C and absorbance readings were taken at 734 nm on a Cobas Bio centrifugal analyser (Roche Diagnostic Systems). The sample was added to mixed ABTS/myoglobin reagent and then hydrogen peroxide was added to start the reaction (final concentrations being 2.5 μM metmyoglobin, 150 μM ABTS, 375 μM H_2O_2, and 0.84% sample fraction, with a measuring time 60 seconds). A logit/log 4 plot was made of absorbance change versus concentration for the Trolox standards and from this the total antioxidant activity of the saliva samples was derived.

Uric acid was measured by uricase/4-aminophenazone method, using reagents from Randox Laboratories (Diamond Road, Crumlin, Co. Antrim, N. Ireland BT29 4QY). Uric acid present in the sample is converted by the action of uricase to allantoin and hydrogen peroxide. The latter, catalysed by peroxidase present in the reagent mixture, oxidises the 4-aminophenazone to a red quinoneimine compound which absorbs at 520 nm.

The concentration of human albumin in each saliva sample was measured by the bromocresol purple method, using reagents from Randox Laboratories. Albumin binds quantitatively to the indicator bromocresol purple (5,5-dibromo-o-cresolsulphonphthalein) and the albumin-BCP complex absorbs maximally at 600 nm. A human albumin standard was used to calibrate the assay.

The concentration of ascorbic acid in each saliva sample was estimated by the ferrozine method.[11] This is a ferrous chromogenic method which depends on the reduction of ferric to ferrous ions by ascorbate, which is simultaneously converted to dehydroascorbate. The ferrous ions formed are subsequently reacted with ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulphonic acid)1,2,4-triazine]. The chromogenic complex absorbs maximally at 550 nm. Freshly prepared ascorbic acid standards were used to calibrate the assay.

The Student's paired t-test was used to evaluate the significance of differences between resting and stimulated saliva samples; the Student's unpaired t-test was used to evaluate the significance of differences between saliva samples from healthy subjects and from patients. P values of less than 0.05 were accepted as statistically significant.

RESULTS

For each subject, the salivary flow rate was noted and mean salivary flow rates calculated for healthy, resting (R) and stimulated (S) saliva (Table 1).

The rate of stimulated salivary flow was characteristically variable with a mean rate of 1.93 ml/min ± 1.0 and was approximately six times higher than that of resting saliva (0.33 ml/min ± 0.16), close to the mean rates quoted previously[1] (1.7 ml/min and 0.32 ml/min, respectively). There were no significant differences between the values for males and females.

The total antioxidant activity of resting and stimulated saliva was assessed

TABLE 1
The mean salivary flow rates for healthy, resting (R) and stimulated (S) saliva

	n	Resting (R) Mean (ml/min) ± s.d.	Stimulated (S) Mean (ml/min) ± s.d.
Total	28	0.33 ± 0.17	1.93 ± 1.07
Male	12	0.40 ± 0.2	2.36 ± 1.25
Female	16	0.28 ± 0.13	1.60 ± 0.81

applying the ABTS assay. The total salivary antioxidant status is shown in Table 2, revealing a significantly lower activity in stimulated as compared with resting saliva ($p < 0.005$). The measurement of individual antioxidants (Table 3) in the saliva samples demonstrated that urate is the major antioxidant in saliva, with minor contributions from albumin and ascorbate. Calculation of antioxidant production rates (μM/minute - Table 3) showed that when the salivary flow is taken into account significantly more urate, albumin and ascorbate is, in fact, secreted during salivary stimulation ($p < 0.005$) than during the resting state. The antioxidant capacity and composition of the saliva from patients with periodontal disease was measured and the details of the subjects investigated are summarised in Table 4. The results (Tables 5 and 6) showed no significant differences between resting and stimulated normal saliva and that from the patients, respectively, for total antioxidant activity or individual antioxidant levels, although the uric acid concentrations in patients' saliva samples were widely spread. The contribution of the measured antioxidants to the total antioxidant activity was determined. The calculation is based on the Trolox equivalent antioxidant capacity for the urate, albumin and ascorbate in relation to their concentrations. Taking the mean concentrations for both resting and stimulated saliva in healthy subjects and that of patients with periodontal disease (Table 7), the data show that the water-soluble antioxidants in saliva measured here, especially urate, account for the majority of the antioxidant activity.

DISCUSSION

Whereas in plasma the major contributors to the antioxidant content are albumin[12,13] and urate,[14,15] the major antioxidant in saliva is urate, with lesser contributions from albumin and ascorbate. In addition, antioxidant production in stimulated saliva is enhanced compared with the resting state if the flow rate is taken into account. One possible explanation for increased antioxidant production in stimulated saliva is that the act of mastication could expel gingival crevicular fluid (GCF) into the saliva via the muscular actions of the tongue, lips, and cheeks. Although calculation of antioxidant production rates in individual subjects suggests that stimulated saliva is a potentially more effective antioxidant medium than resting saliva (perhaps indicating that the antioxidants might be secreted with ductal saliva) this is contradicted by a lack of correlation between flow rate and the salivary TAA (data not shown).

The findings here also indicate that there is no significant change in the antioxidant status of saliva in periodontal disease. This pertains even after correction for the salivary flow rate (data not shown). Correlating the uric acid content with

TABLE 2
The Total Antioxidant Activity (TAA) of resting (R) and stimulated (S) saliva samples from healthy subjects

	Saliva type	n	TAA (μM) ± s.d.
Total	R	28	246 ± 64
Total	S	28	144 ± 40
Male	R	12	271 ± 74
Male	S	12	165 ± 41
Female	R	16	229 ± 50
Female	S	16	126 ± 30

TABLE 3
The urate, albumin and ascorbate concentrations (μM/L) and production rates (μM/min) in resting (R) and stimulated (S) saliva samples from healthy subjects

	Saliva type	n	Urate (μM) mean ± s.d.	Urate (μM/min) mean	Albumin (μM) mean ± s.d.	Albumin (μM/min) mean	Ascorbate (μM) mean ± s.d.	Ascorbate (μM/min) mean
Total	R	28	219 ± 64	0.070	12 ± 7	0.0038	9 ± 6	0.0028
Total	S	28	104 ± 36	0.200	8 ± 2	0.0161	9 ± 4	0.0160
M	R	12	233 ± 74	0.092	12 ● 7	0.0040	8 ± 7	0.0032
M	S	12	127 ± 40	0.281	9 ± 3	0.0203	8 ± 4	0.0212
F	R	16	206 ± 56	0.055	13 ± 6	0.0036	9 ± 5	0.0026
F	S	16	87 ± 19	0.137	8 ± 1	0.0130	9 ± 5	0.0121

TABLE 4
Details of patients with periodontal disease from whom saliva was collected

Patient number	Sex	Age	Clinical assessment
1	female	45	moderate chronic periodontitis, chronic gingivitis, poor oral hygiene
2	male	36	advanced chronic periodontitis, mild chronic gingivitis, good oral hygiene
3	female	25	chronic gingivitis, poor oral hygiene
4	male	36	advanced chronic periodontitis, moderate chronic gingivitis, poor oral hygiene
5	female	35	moderate chronic periodontitis, moderate chronic gingivitis, poor oral hygiene
6	female	59	moderate chronic periodontitis, moderate chronic gingivitis, poor oral hygiene
7	male	29	advanced chronic periodontitis, moderate chronic gingivitis, poor oral hygiene

the TAA in all types of saliva (Figure 1) shows that urate is the extracellular antioxidant that contributes most to the TAA of whole saliva in all conditions studied ($r^2 = 0.972$). The contribution of albumin, ascorbic acid and other antioxidants to the salivary TAA are represented by the intercept. The albumin levels in fresh normal saliva samples reported here are 2–3 times those reported by others.[16] Previously reported albumin levels in the resting saliva of normal subjects, stored

TABLE 5
The total antioxidant activity and antioxidant composition of resting saliva samples from patients

Patient no.	Salivary flow rate (ml/min.)	TAA (μM)	Urate (μM)	Albumin (μM)	Ascorbate (μM)
1	0.13	459	344	13	8
2	0.27	278	202	7	5
3	0.40	419	345	8	4
4	0.53	245	195	10	8
5	0.50	152	116	10	5
6	0.43	315	330	7	2
7	0.10	253	252	27	13
Mean ± s.d.	0.34 ± 0.17	303 ± 106	255 ± 89	11.7 ± 7.0	6.4 ± 3.6

TABLE 6
The total antioxidant activity and antioxidant composition of stimulated saliva samples from patients

Patient no.	Salivary flow rate (ml/min)	TAA (μM)	Urate (μM)	Albumin (μM)	Ascorbate (μM)
1	1.40	180	144	8	8
2	2.00	167	126	8	7
3	0.80	315	257	8	8
4	2.20	155	112	8	5
5	1.00	76	23	11	11
6	1.25	137	115	7	2
7	0.67	172	140	15	5
Mean ± s.d.	1.33 ± 0.58	172 ± 72	131 ± 69	9.3 ± 2.8	6.6 ± 2.9

TABLE 7
The contribution of urate, ascorbate, albumin and unknown antioxidants to the total antioxidant activity of resting and stimulated saliva from healthy and diseased subjects

	Individual Antioxidants (μM)			Total antioxidant Activity		
Sample	Urate	Ascorbate	Albumin	Calculated	Measured	Unknown antioxidants %
TEAC value	1.02	0.99	0.69	-	-	-
Saliva:						
Healthy *Resting*	223	9	6	238	246	4.0
Healthy *Stimulated*	106	9	8	123	144	8.0
Saliva:						
Diseased *Resting*	260	6	8	274	303	9.5
Diseased *Stimulated*	134	7	6	147	172	14.5

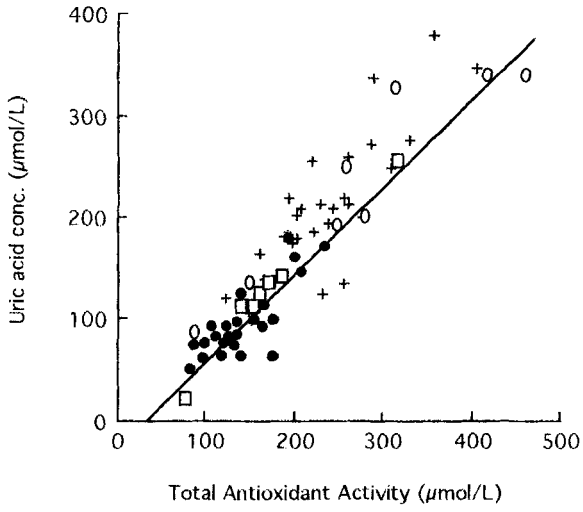

FIGURE 1 The relationship between total antioxidant activity and uric acid concentration in saliva samples (resting and stimulated) from 28 healthy volunteers and 7 patients with periodontal disease. $R^2 = 0.972$, + healthy, resting saliva, ● healthy, stimulated saliva, ○ diseased, resting saliva, □ diseased, stimulated saliva.

frozen at −20°C prior to use, are in the range of up to 3.5 μM. While the ELISA method used by these workers is a more sensitive procedure than that applied here, this low figure was derived from analysis of samples that had been frozen and stored, which may have affected the immunoreactivity of the salivary albumin.

The results obtained demonstrate that whole saliva from patients with periodontal conditions does not have a lower TAA than that from healthy individuals. Thus the inflammatory processes associated with periodontal disease leading to the increased production of free radicals at these sites are not detectable through changes in antioxidant status or levels. There may be several explanations for this (Figure 2). Firstly, some of the extracellular antioxidants may be derived from gingival crevicular fluid (GCF) rather than from pure ductal saliva. The increased production of GCF associated with gingivitis and periodontitis may then balance the local decrease of antioxidants. Secondly, any oxidative stress arising from the inflammatory state in periodontal disease may play only a minor role; there is no documented evidence that reactive oxygen species are produced in large enough amounts to be an important factor in the destruction of the periodontium and hence other bacterial and host-derived factors (e.g. enzymes, such as the collagenases) may have a greater effect.

Thus although salivary antioxidants may have a local effect scavenging oxidants produced in inflammatory processes associated with periodontal disease, any resulting decrease in the concentration of antioxidants may be balanced by increased production of gingival crevicular fluid, and the latter may be a source of some of these antioxidants rather than the pure glandular salivary secretions. It is likely that other components of saliva (e.g. salivary peroxidase and lactoferrin) are more significant in the protection of the oral tissues from the toxic species generated in inflammatory conditions than are the water-soluble antioxidants in saliva.

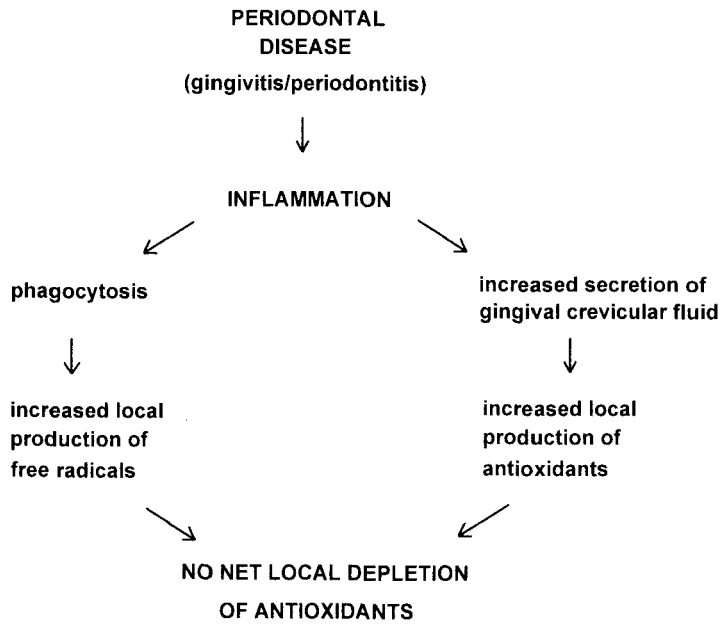

FIGURE 2 Hypothesised relationship between free radical production and salivary antioxidants in periodontal disease.

The major antioxidant in saliva is thus concluded to be urate; however, the relatively low concentrations of ascorbate present may also make an essential contribution to the salivary antioxidant activity since free radical scavenging by urate in the absence of ascorbate can lead to further macromolecular damage if the urate radicals formed are not re-cycled.[17,18]

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