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Research paper

Quantitative detection of epidermal growth factor and interleukin-8 in whole saliva of healthy individuals

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ABSTRACT

Objectives: This study aims to create consensus concerning the use of a methodology by which the handling of saliva is standardized and quantitative detection of IL-8 and EGF in whole saliva is achieved. Our study involves evaluating the extent to which the pre-treatment of saliva samples with an anionic detergent – sodium dodecyl sulphate (SDS) – improved detection levels for IL-8 and EGF.

Methods: Whole saliva samples ($n = 28$) were collected from healthy individuals and a protease inhibitor cocktail was added immediately. They were treated with either SDS or PBS for 20 min and were then applied to a sandwich ELISA.

Results and conclusions: Saliva is a complex viscous fluid that requires degrading before the analysis of salivary biomarkers. We found that pre-treatment of samples with SDS significantly increased the detection levels for both EGF (293%) and IL-8 (346%) when compared with PBS-treated pairs ($^{***}P < 0.001$). According to the results we recommend: (i) pre-treatment of whole saliva samples with SDS for quantitative analysis (ii) using secretory output instead of concentration in the presentation of results to avoid individual variations and (iii) taking into consideration gender, age and meal intake since these have an impact on the secretory output of salivary proteins.

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1. Introduction

Saliva is a complex and dynamic biological fluid that has numerous functions in the oral cavity. Recent technological developments have enabled an exponential increase in the use of saliva as a diagnostic fluid. Saliva is now being used as a diagnostic tool for monitoring the overall systemic health of an individual. The possibilities for using saliva in the diagnosis of conditions such as cardiovascular disease, abnormal endocrine function, the presence of infection (viral or bacterial), renal disease and cancer are now attracting attention (Kaufman and Lamster, 2002). *How can saliva serve as a diagnostic tool?* Saliva is a "real-time" fluid, which means that its content changes continuously according to the physiological status of the individual (Schipper et al., 2007). Although saliva secretion is controlled largely by the nervous system, hormones and blood-borne substances originating from remote organs may also affect its composition (Aras and Ekstrom, 2006, 2008). This means that the proteins found in saliva may be of diagnostic value in seeking biomarkers for the diagnosis and progression of disease. Several studies have been conducted to try to identify protein biomarkers in whole saliva that could be associated with the pathogenesis of oral diseases (St John et al., 2004; Rhodus et al., 2005; Adisen et al., 2008). However, thus far the lack of standardized laboratory protocols in the analysis of highly complex and viscous saliva specimens has meant that accurate and reliable detection of protein biomarkers has yet to be

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achieved (Schipper et al., 2007; Messana et al., 2008). In whole saliva, the main source of proteins is from major (parotid, submandibular, sublingual) and minor salivary glands, but serum-derived gingival fluid, epithelial cells, bacteria and bacterial products also contribute to its great complexity. Microscopically, saliva is composed of clusters of particles called salivary micelles, which are formed by the intermolecular interactions of proteins (e.g. mucins) and ions (e.g. calcium) (Schipper et al., 2007). Mucins are a family of heavily glycosylated proteins and they are responsible for the viscoelastic character of saliva. Mucins are relatively abundant but in widely varying concentrations in human saliva. Because of its highly complex character, using saliva as a laboratory specimen has been problematic since the micelle formation masks the counts of small proteins and this results in inaccurate readings (Kelly et al., 2002). It would therefore be of immense value if a common, standardized assay protocol could be developed that allowed the sensitive detection of small proteins in saliva. For this purpose, we have tested a method of pre-handling saliva that reduces protein clusters (micelles) and creates a homogenous fluid prior to analysis. We found that pre-treatment of saliva with the anionic detergent sodium dodecyl sulphate (SDS) was effective in yielding accurate and reproducible readings in ELISA.

2. Materials and methods

2.1. Saliva collection and handling

All participants in the study donated saliva in accordance with the ethical approval received from the Central Ethical Review Board in Gothenburg, Sweden. All samples were taken with the understanding and written consent of each subject and according to the above-mentioned principles.

Un-stimulated whole saliva was collected from 28 healthy, non-smoking individuals aged between 20–39 (n = 14) and 40–65 (n = 14) with an almost equal gender balance: females (n = 15) and males (n = 13). To obtain diurnal changes for EGF and IL-8 in saliva, the collection was performed at regular intervals three times per the day 1) in the morning (n = 28), between 8:00 a.m. and 10:00 a.m. 2) just after lunch, between 12:00 p.m. and 1:00 p.m. (n = 7) and 3) in the late afternoon, between 3:00 p.m. and 4:00 p.m. (n = 7). All subjects were told to refrain from eating, drinking or carrying out any oral hygiene procedures for at least 1 h before the collection of morning and late afternoon saliva. The subjects were asked to expectorate once a minute for ten minutes into a pre-weighed 50-ml tube that was kept on ice. For each individual the volume of the saliva was measured and calculated as secretory rate (ml/min). Protease inhibitor cocktail tablets (Sigma-Aldrich, S8830; one tablet diluted in 4 ml distilled water and used 25 μl/ml), and EDTA (Sigma-Aldrich, 2 mM) were immediately added to minimize protein degradation. The samples were then divided into 250 μl aliquots to reduce the risk of the fluid entering cycles of freezing and thawing and it was immediately stored at −80 °C to await analysis. The samples were prevented from undergoing any form of centrifugation, which would have led to loss of proteins. This is presented in the result section. The exceptions to this were 5 samples (n = 5) that were subjected to centrifugation in order to evaluate its effect. After collection and the addition of protease inhibitors, these samples were divided into two pairs and one of these pairs was subjected to cold centrifugation at 1000 *g* for 10 min.

2.2. Sandwich ELISA

An enzyme-linked immunosorbent assay was performed to determine the IL-8 and EGF levels in the saliva of healthy individuals (n = 28). Sandwich ELISA was then performed according to the manufacturers' instructions (R&D systems, USA), except in the case of the pre-treatment saliva samples. For these samples, the anionic detergent sodium dodecyl sulphate (SDS, Sigma-Aldrich) was used to dissociate the mucin particles. A concentration gradient (0.1–1%) of SDS was applied to the saliva samples, which were then incubated for 20 min either in SDS (50 μl for 200 μl saliva) or in PBS (50 μl for 200 μl saliva). They were then placed onto a 96-microwell plate in a duplicate dilution series (from 1/2 to 1/16). We observed that the concentration of SDS higher than 0.8% inhibited protein detection. The ideal concentration was found to be 0.4% (1.38 mM) for both IL-8 and EGF. This was determined by analysing the linearity of IL-8 and EGF concentrations obtained through a double dilution series (n = 4). The final SDS concentrations after dilution of the samples were 0.05% and 0.012% respectively. The dilution factor for calculations used for IL-8 and EGF was 2 and 8 respectively. The same pre-treatment protocol was also applied on recombinant human IL-8 and recombinant human EGF, which were supplied by the manufacturer and served as standards in ELISA measurements.

In order to analyse intra-assay variation, two replicates of seven paired samples were pre-treated with SDS or PBS. These were then put through a single assay in a dilution series (1/2 to 1/16). To analyse inter-assay variation, five paired samples that had been pre-treated with SDS or PBS were put through two independent assays in a dilution series (1/2 to 1/16). Intra- and inter-assay variations (%) were calculated for each sample using the following formula: (standard deviation of concentration) / (mean of concentration) × 100.

3. Statistics

The statistical significance of differences was calculated using paired or unpaired Student's *t*-test (GraphPad Prism). Probabilities of <5% were considered significant. Values are the means ± s.e.m.

4. Results

4.1. Pre-treatment with SDS increased the detection levels for IL-8 and EGF in a sandwich-ELISA

Table 1 shows the effect of SDS or PBS pre-treatment on the detection of EGF and IL-8 (n = 14). Pre-treatment of saliva samples with SDS (0.4%, working concentration) significantly increased the detection level for EGF by 293% and for IL-8 by 346% compared to the pairs that had been pre-treated with PBS ($^{***}P < 0.001$, see Table 1). The concentration of PBS-treated samples was 373 ± 91.6 pg/ml (EGF) and 394 ± 47 pg/ml (IL-8), while the concentration of SDS-treated samples was significantly higher at 1003 ± 179.6 pg/ml and 1662 ± 160 pg/ml (n = 14, $^{***}P < 0.001$) respectively. Pre-treatment

Table 1
Shows pre-treatment of saliva samples with SDS significantly increased the detection levels (pg/ml) for both EGF and IL-8 compared to untreated pairs (n = 14, ***P < 0.001).

Sample no	Pre-treatment with PBS		Pre-treatment with SDS		% Increase with SDS	
	EGF pg/ml	IL-8 pg/ml	EGF pg/ml	IL-8 pg/ml	EGF	IL-8
1	130	601	444	2171	241	261
2	41	518	463	2430	1029	369
3	199	666	682	2776	242	317
4	61	268	421	1605	590	499
5	169	132	343	778	157	489
6	357	231	907	1100	154	376
7	266	348	1973	1380	641	267
8	808	228	1891	1402	134	514
9	373	330	1130	1251	203	281
10	147	288	519	1215	253	319
11	289	501	798	1623	176	237
12	351	307	809	1508	131	390
13	1244	702	2520	2596	102	264
14	787	396	1145	1436	46	263
Mean ± SEM	373 ± 91.6	394 ± 47	***1003 ± 179.6	***1662 ± 160	293 ± 73	346 ± 26

of recombinant human EGF and IL-8 (used as standards) with SDS had no effect on concentration levels when compared to untreated pairs.

4.2. The effect of SDS pre-treatment on reproducibility of the analysis

Intra-assay variations were significantly reduced following pre-treatment with SDS to 2.2 ± 0.5% for EGF and 2.7 ± 0.9% for IL-8. This compares with 16.5 ± 6.5% (P < 0.001) and 25 ± 5.5% (P < 0.001) respectively among the samples that had been pre-treated with PBS. Pre-treatment with SDS also resulted in significantly reduced inter-assay variation: 7.1 ± 1.2% for EGF and 16.7 ± 3% for IL-8. This compares with 43.7 ± 9.6% (P < 0.05) and 49.9 ± 10.2% (P < 0.05) respectively among the samples that had been pre-treated with PBS.

4.3. Reduced secretory output for EGF and IL-8 after meal intake

The secretory activity of the salivary glands varies according to the time of day and meal intake. For this reason, we measured secretory output on three occasions during the day (morning, just after lunch and late afternoon) for 7 individuals. Fig. 1 shows that the secretory output of EGF (488.6 ± 44 pg/min) and IL-8 (793.8 ± 159 pg/min) at lunchtime was significantly lower than the morning output levels, which were 672.7 ± 53 pg/min (P < 0.05) and 1117.2 ± 133.5 pg/min (P < 0.05) respectively. However, the afternoon output of EGF (595.8 ± 87 pg/min) and IL-8 (1171.8 ± 288 pg/min) showed no changes from the morning values (P > 0.05).

4.4. Secretory output of EGF and IL-8 varies according to the age and gender of the individual

Saliva secretion capacity varied greatly between individuals (n = 28), with a mean of 0.65 ± 0.06 ml/min. The highest secretion level was 1.5 ml/min and the lowest, 0.31 ml/min. Because of this variation the total secretory output (pg/min) of EGF and IL-8 was calculated for each individual by multiplying the concentration of EGF or IL-8 (pg/ml) by the secretion rate for the respective individual (ml/min).

The mean concentration of salivary samples that were collected in the morning (n = 28) was 1533 ± 121 pg/ml for EGF and 1905 ± 229 pg/ml for IL-8. The secretory output was 898 ± 107 pg/min and 1064 ± 120 pg/min respectively. Females (n = 15) had a significantly lower secretory output for EGF (745.3 ± 100.8 pg/min, Fig. 2) and IL-8 (842.8 ± 75.1 pg/min, Fig. 2B) than males (n = 13); the levels were 1074 ± 192.1 (P < 0.05) and 1136 ± 217.4 pg/min (P < 0.001) respectively.

In the age group 20–39 of mixed gender (n = 14) a significantly lower total secretory output for EGF at 744.5 ± 66.3 pg/min was found than in the age group 40–65 of mixed gender (n = 14), which had an output of 1075 ± 211 pg/min (P < 0.001, Fig. 3). However, comparison of IL-8 output in these groups showed no significant difference, measuring 1064 ± 120 pg/min (n = 14) and 880.6 ± 188 pg/min (n = 14) respectively (P > 0.05, Fig. 3).

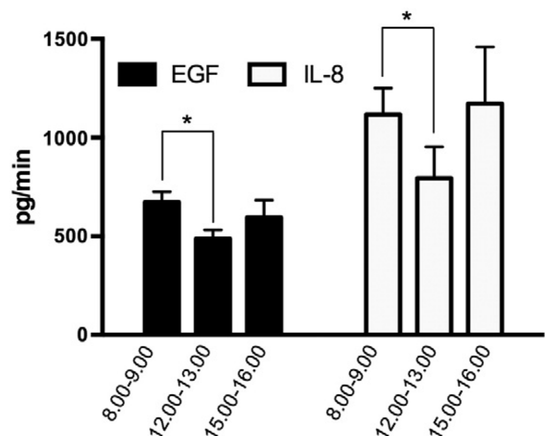

Fig. 1. Columns represent secretory output (pg/min) of EGF or IL-8 (mean ± SEM, n = 7) at three different time periods of the day. Comparisons were made between morning and lunch saliva samples of same individuals (n = 7). *P < 0.05 (paired *t*-test).

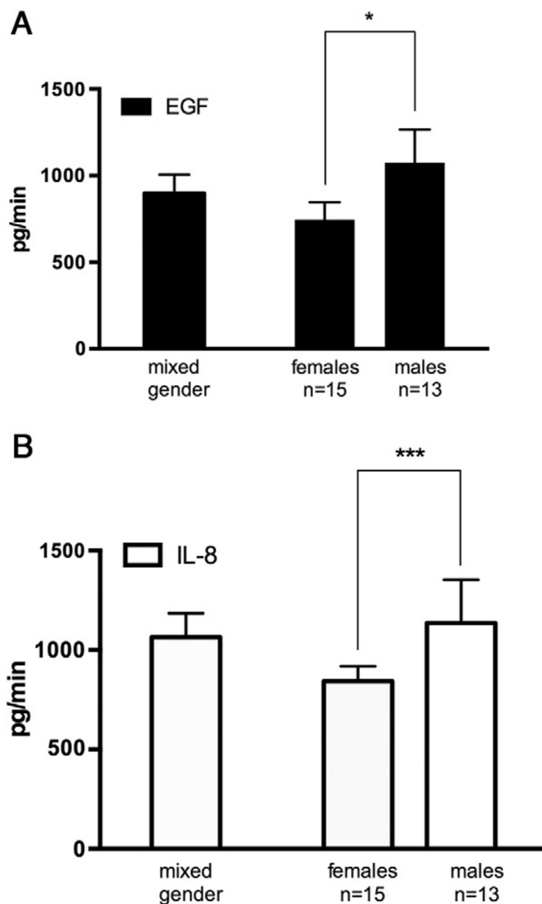

Fig. 2. A. Comparison of EGF secretion between males (n = 13) and females (n = 15), Columns represent secretory output (pg/min) of EGF (mean ± SEM). *P < 0.05 (unpaired *t*-test). B. Comparison of IL-8 secretion between males (n = 13) and females (n = 15), columns represent secretory output (pg/min) of EGF (mean ± SEM). ***P < 0.001 (unpaired *t*-test).

Females in the age group 40–65 (n = 7) showed significantly higher secretory output for EGF (916.5 ± 201 pg/min) than females in the age group 20–39 (595.4 ± 29.4 pg/min n = 8), (P < 0.001). However, when these groups were compared for IL-8 output the older age group showed slightly lower secretory output at 712.8 ± 69 than the younger, at 932.6 ± 125.6 pg/min though this difference was statistically insignificant (P > 0.05). Males in the age group 40–65 (n = 6) had significantly higher secretory output for EGF at 1260 ± 403 pg/min than males in the age group 20–39 (n = 7); the output was 914.8 ± 108.3 pg/min (P < 0.01). However, the output of IL-8 for males in the older group (n = 6) was slightly lower 1048 ± 372.7 pg/min than that for the males in the younger group (1239 ± 218, n = 7), though this again lacked statistical significance (P > 0.05).

4.5. Effect of centrifugation on detection levels of EGF and IL-8 before and after SDS pre-treatment

We have also analysed the effects of centrifugation on the concentration levels of EGF and IL-8 in saliva samples taken from 5 individuals. We found that the mean concentration of EGF (771.6 ± 235 pg/ml) and IL-8 (609 ± 59 pg/ml) following centrifugation before SDS pre-treatment (0.4%) was significantly lower than that found in the corresponding samples that had not been subjected to centrifugation but SDS pre-treatment (0.4%): 1078 ± 270 (P < 0.001) and 954 ± 107 pg/ml (P < 0.001) respectively. The mean percentage protein loss was 50 ± 9% for EGF and 56.5 ± 11% for IL-8 and there was considerable variation between individuals.

However the concentration of EGF and IL-8 showed no significant difference for the saliva samples (n = 5) that were first pre-treated with SDS (0.4%) and later subjected to centrifugation than the corresponding pairs that were not subjected to centrifugation but pre-treated with SDS (0.4%). The mean percentage difference was 2.1 ± 0.1% (P > 0.05) for EGF and 1 ± 0.1% for IL-8 (P > 0.05) as compared to un-centrifuged pairs. Centrifugation of the samples after SDS pre-treatment neither caused any significant loss of proteins nor seemed to be advantageous in the assay methodology.

5. Discussion

Salivary proteins interact with each other under physiological conditions to initiate biological activities that are responsible for bacterial agglutination and for the immune response in the oral cavity (Young et al., 1997). Interactions between the proteins result in multi-component particles of various sizes known as salivary micelles (Schipper et al., 2007; Young et al., 1999). The formation of micelles is a selective process that involves specific salivary proteins including mucins, lactoferrin, amylase, sIgA and proline rich proteins (Soares et al., 2004). Calcium ions are also important for the maintenance of the structure of the micelles. Small soluble proteins like EGF and IL-8 are often bound to various molecules that are present in biological fluids such as glycoproteins (Kelly et al., 2002). It is therefore often problematic to recognize them in saliva since the relevant epitopes are packed within a complex structure.

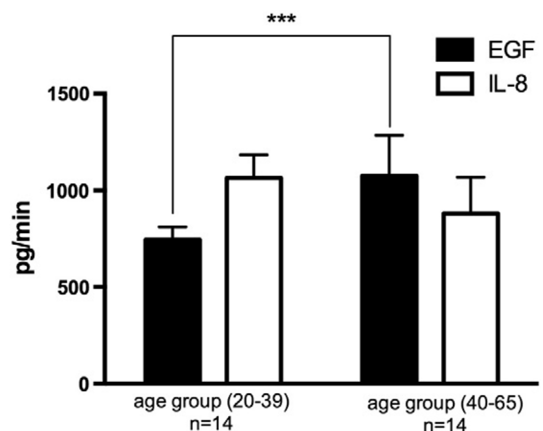

Fig. 3. The effect of age on the secretory output of EGF and IL-8. Columns represent secretory output (pg/min) of EGF (mean ± SEM) and IL-8 (mean ± SEM) compared for individuals aged between 20 to 39 (n = 14) and 40 to 65 (n = 14). ***P < 0.001 (unpaired *t*-test) for age group comparison as regards to EGF. There was no statistically significant difference between age groups for IL-8 (unpaired *t*-test).

The handling and preservation of saliva can have dramatic effects on its biochemical and physiochemical properties. This study shows the importance of how saliva is handled when it is to be used as a diagnostic tool. It also provides a well-studied methodology for the quantitative detection of EGF and IL-8 in whole saliva. The viscosity of our samples varied considerably from light to heavily viscous presumable depending on various quantities of large glycoproteins known as mucins. Thus, the variation in viscosity is a significant problem in ELISA-based readings. However, the major problem with mucins is not only that they make the saliva more viscous, but that also they bind to other proteins and thereby make these unrecognisable in quantitative analysis (Kelly et al., 2002; Young et al., 1999). In this study we therefore tested several surfactants (Triton X-100, and SDS; sodium dodecyl sulphate) and a reducing agent called dithiothreitol (DTT) that is known to reduce viscosity (Schipper et al., 2007; Raynal et al., 2003). Although pre-treating of saliva samples with all of these agents considerably reduced the viscosity, reproducible readings for EGF and IL-8 were still lacking in our analysis. However, pre-treatment of samples with SDS greatly decreased the viscosity and increased the level of detection of EGF by 293% and of IL-8 by 346%. Sodium dodecyl sulphate (SDS) is an anionic surfactant but has an unknown ability to break down mucin aggregates into individual molecules (14). In solutions, SDS associates with mucin molecules and breaks the physical bonds that create aggregates. SDS has effectively been used as a detergent in ELISA for detection of soluble proteins (Lechtzier et al., 2002) and blood group substances (McCabe et al., 1988). An earlier study reported a reduction in the size of mucin molecules in a concentration range from 0.75 mM to 2 mM (14) from bovine saliva. In our analysis the best results were achieved at 1.38 mM (used as a working concentration in the pre-treatment of saliva) for quantitative detection of EGF and IL-8. However, we recommend that the concentration of SDS should be carefully tested specifically for each biomarker since the detection of CCL-22 and IL-1 beta in whole saliva required 10 times weaker concentration (data not shown). We did not study why other surfactants but SDS has been effective in saliva analysis. However, SDS has a significant effect by associating with mucin molecules in hydrophobic interactions (14) and other agents that have been tested lack this capacity.

The secretory activities of salivary glands are highly individual and change throughout the day according to diurnal rhythm, meal intake and the health status of the individual. The amount of secreted proteins then varies accordingly (Aras and Ekstrom, 2006, 2008). A previous radioimmunoassay study showed that EGF secretion capacity varied greatly according to the rate of saliva secretion. The same study showed that it would be misleading to use concentration as a measurement of EGF secretion because EGF concentration is affected by the dilution of saliva (McGurk et al., 1990). This is in harmony with our results and we therefore strongly recommend using the rate of EGF secretion (secretory output) instead of concentration. The effects of EGF and IL-8 on secretion have yet to be studied in this way and thus far there is no well-tested method for detecting the quantities of growth factors and cytokines. Using our newly developed methodology we therefore studied the diurnal effects of EGF and IL-8 and the impact of meal intake on secretory output. We demonstrated that the secretory output of EGF diminished by 27% and of IL-8, by 29% following the consumption of a midday meal. However, we found no such changes in the saliva samples taken in the morning and in the afternoon without food intake. It is well recognised that eating exerts a powerful stimulus upon salivary glands and because of long-acting reflex mechanisms, proteins that are packed in the secretory granules are released following a meal (Ekstrom et al., 1998). Since EGF is produced and stored in the salivary glands and secreted reflexively (Ino et al., 1993), it is likely to be secreted in lower concentration following a meal. This correlates with our results. However, the extent to which IL-8 is present in secretory granules has not been investigated and the impact of meal intake on this cytokine is therefore unknown.

Animal studies have shown that the synthesis of growth factors in the salivary glands is androgen-dependent and that males secrete much higher levels of growth factor into their saliva than do females (Byyny et al., 1974). Although this phenomenon has not been demonstrated in human salivary glands, we observed a 30% higher secretory output of EGF and 26% higher output of IL-8 in males than in females. In the older age groups, our results showed 54% higher levels of secretion of EGF in females and 38% higher in males. Other studies have found no significant differences in the composition, salivary flow and buffering capacity of saliva among different age groups. However, mucins have been found to decrease (Denny et al., 1991), while transforming growth factor-α has been found to increase with age (Humphreys-Beher et al., 1994) in whole saliva. In line with our results, a recent proteomics study reported increased amounts of immune-related proteins in elderly healthy individuals (Ambatipudi et al., 2009). Although we have not observed any changes for IL-8 secretion according to age, the changes for EGF secretion in the elderly may play an important role in maintaining homeostasis in the oral cavity.

Although the standardisation of methods of preparing saliva is essential if saliva is to be used as a laboratory material, consensus on collection and assay methods is still lacking. The methods used for preparing samples have a direct impact on the accuracy, reproducibility, and quantification limits used in analysis. Moreover, saliva contains high levels of proteases and the use of protease inhibitors therefore has a significant impact on the level of detection of protein biomarkers. Many biological fluids must be processed prior to analysis in order to remove proteins that may interfere with the properties of the sample (Kelly et al., 2002). Our results demonstrated that an anionic surfactant is effective in enhancing the level of detection and the reproducibility of analysis of whole saliva. We also refrained from centrifuging samples since our results showed that considerable quantities of EGF and IL-8 were lost in this process unless pre-treated with SDS before centrifugation. Previous studies have also noted this problem (Young et al., 1999) and have recommended freezing samples instead of centrifuging them and this allows the cellular components of the saliva to break down (Francis et al., 2000).

In conclusion, saliva has great potential for use as a research material in studying the pathogenesis of oral diseases. However, this requires a well-designed methodology. This study therefore proposes a standard method for sampling and handling saliva and for presenting the results. Based on our results we recommend: (i) pre-treatment of whole saliva samples with SDS for quantitative analysis (ii) using secretory output instead of concentration in the presentation of results so that the problem

of individual variations in secretory activity is avoided and (iii) taking into consideration gender, age and meal intake since these have an impact on the secretory output of salivary proteins.

Saliva is the main source of EGF in the oral cavity and has an important role in cell mitosis and wound healing (Ohshima et al., 2002). Besides IL-8 is a pro-inflammatory cytokine and mainly responsible for chemotaxis of phagocytes into tissues and increased level of IL-8 is an indication of inflammation (Graves and Jiang, 1995). In the future, it would be interesting to study the association of EGF and IL-8 levels with the pathogenesis of oral mucosal disorders such as recurrent aphthous ulcerations, lichen planus, leukoplakia and oral cancer.

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Author Contributions

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