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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.

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