Blood contamination, a problem or a lucky chance to analyze non-invasively Myokines in mouth fluids?

Barbara Ravara (1,2,3), Sandra Zampieri (1,4,5,6), Helmut Kern (4,5), Ugo Carraro (1,2,3)

(1) CIR-Myo, Interdepartmental Research Center of Myology, University of Padova, Italy; (2) Department of Biomedical Sciences (DSB), University of Padova, Italy; (3) A&C M-C Foundation for Translational Myology, Padova, Italy; (4) Ludwig Boltzmann Institute of Rehabilitation Research, St Pölten, Austria; (5) Institute of Physical Medicine and Rehabilitation, Prim. Dr. H Kern GmbH, Amstetten, Austria; (6) Department of Surgery, Oncology and Gastroenterology (DISCOG), University of Padova, Italy

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Abstract

Use of saliva in clinical studies are increasing to identify methods less invasive than blood sampling in search for systemic changes of biomarkers related to physical activity, aging, late aging and rehabilitation. The consensus is that the diagnostic value of whole saliva is compromised by the presence of blood, but we are looking at the contamination as a major opportunity for non-invasive analyses of serological biomarkers. The aim of this preliminary study was to evaluate the presence of serum in mouth fluids of healthy seniors and the eventual changes after a modest trauma, i.e., tooth brushing. Seven heathy persons, aged more than 65 years, drooling saliva in a test tube provided the fluids for the analyses. After low speed centrifugation, small aliquots of supernatants were frozen in liquid nitrogen and stored at -80° until use. Aliquots were thawed and used for quantification by the Lowry method of total proteins and by colorimetric ELISA of serum albumin, fibrinogen and lysozyme. Hemoglobin content was quantified by Spectrophotometry. Adjustment of saliva dilution, after a preliminary test, increased the homogeneity of the analytes' content determined by colorimetric ELISA. The control reference to judge the quantity of serum in saliva was a pool of sera from age-matched healthy persons. Saliva collected from the seven healthy elderly person before and after tooth-and-gum, brushing presented measurable amount of the analytes, including fibrinogen, a minor component of the pooled sera. Tooth brushing did not induced statistically significant difference in analytes' contents, suggesting that a measurable blood contamination is a frequent event in elderly persons. In conclusion, fibrinogen analysis in saliva is a promising approach to quantify serological biomarkers by a non-invasive procedure that will increase acceptability and frequency of analyses during follow-up in aging and rehabilitation.

Key Words: Saliva, blood contamination, fibrinogen, non-invasive analyses of plasm proteins.
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In response to contraction and physical activity, skeletal muscle cells produce and release Myokines, some of the several hundred cytokines and other small proteins or proteoglycan peptides that cells may release. They have autocrine, paracrine and endocrine effects at picomolar concentrations.[1] Their systemic evaluation is generally performed by tissue, plasma or serum analysis at protein/peptide or nucleic acid levels.[2,3] These invasive approaches limit sampling frequency and the persons' acceptability of the analyses.[4-8] Blood and urine are the most important diagnostic fluids, but saliva is becoming collected because of its non-invasive procedure.[9] Saliva is considered as an alternative to blood for detecting viral antibodies and for monitoring of hormones and lipid-soluble drugs. Several commercial kits to detect antibodies of human immunodeficiency virus (HIV), human papillomavirus, and hepatitis C virus using oral fluids are in use.[10] Many commercial kits, using saliva to detect hormones and drugs are available for research and diagnostic purposes.[11-14] To improve the its clinical use, it is mandatory to standardize collection, storage, and processing to determine contents of serum proteins in

Table 1. *Total protein of PRE-POST tooth-brushing saliva by Lowry method.*[15]

Test	Standard dilutions 1		Standard dilutions 2	
	µg/µl		µg/µl	
	PRE	POST	PRE	POST
Mean	4.49	4.72	2.41	2.47
SD	2.27	3.21	1.71	3.24
p by student test	0.7605		0.9492	

Table 2. *Serum Albumine in PRE-POST tooth-brushing saliva by Colorimetric ELISA*[3]

Test	Standard dilutions 1		Standard dilutions 2	
	ng/ml		ng/ml	
	PRE	POST	PRE	POST
Mean	120292.81	61479.79	116510.1	171598.84
SD	142445.34	47910.70	133152.95	93690.04
p by student test	0.3209		0.1580	

Table 3. *Lysozyme in PRE-POST tooth-brushing saliva by Colorimetric ELISA*[3]

Test	Standard dilutions 1		Standard dilutions 2		
	ng/ml		ng/ml		
	PRE	POST	PRE		POST
Mean	12074.21		5193.02	2230.65	2104.24
SD	17943.09		5123.17	1193.28	1744.10
p by student test	0.3485		0.8768		

saliva samples.[10] Considering that many devices used to collect saliva influence level of serum contamination, a rational approach is to take to a minimum the impact of saliva collection. Indeed, we started our reevaluation process for a consistent user-friendly clinical approach of the saliva from the less invasive procedure that is, by passive drooling and spitting in a test tube, before and after submitting tooth and gum to a gentle trauma, i.e., tooth brushing. Our preliminary results support our hope that a generally considered limitation of saliva approach (i.e., serum contamination) may became a major clinical opportunity: to evaluate the responses of aged persons to physical and pharmacological treatments to delay counteract the unavoidable process of elderly decay, as gently and as much frequrntly during the follow-up.

Materials and Methods

From seven healthy volunteers, aged over 65 years, saliva was collected by dribbling in test tubes. Mouth fluids were collected before and after tooth brushing. After low speed centrifugation, small aliquots of supernatants were frozen in liquid nitrogen and stored at -80° until use. Aliquots were thawed to quantify total proteins by the Lowry method,[15] Hemoglobin by spectrophotometry,[16] and, by colorimetric ELISA,[3] Serum albumin, Fibrinogen, and Lysozyme. Control was a pool of sera from age-matched volunteers. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad software, La Jolla, CA, USA). Linear regression analysis was applied to evaluate correlation between parameters and their contents in serum and saliva. Significance was set at $p<0.05$ and data are presented as mean ± standard deviation.

Results

The purpose of this study was to test presence of plasma proteins contamination in saliva of aged healthy persons before and after thoot brushing. Table 1 shows the results of total protein analysis of the pre vs post tooth-brushing saliva. In the duplicate tests, there are not significant differences in both samples. Similar results are presented in Tables 2 and 3, for Serum Albumin and Lysozyme

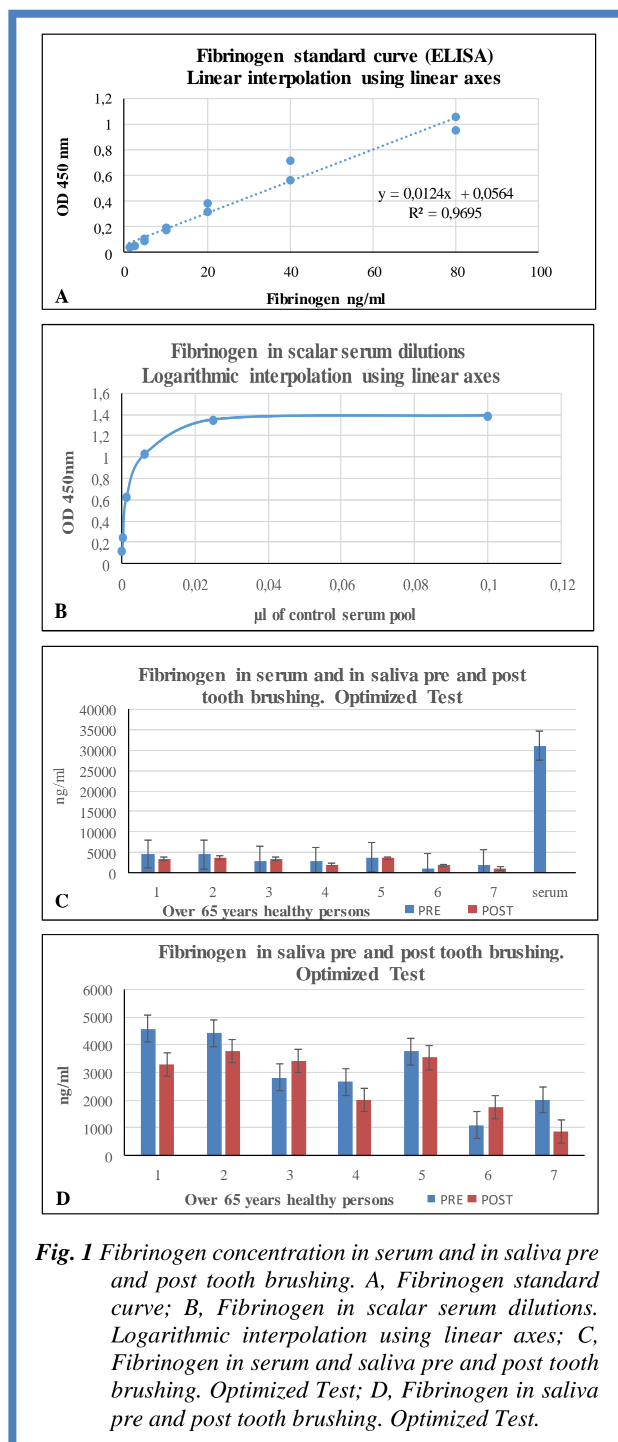

***Fig. 1** Fibrinogen concentration in serum and in saliva pre and post tooth brushing. A, Fibrinogen standard curve; B, Fibrinogen in scalar serum dilutions. Logarithmic interpolation using linear axes; C, Fibrinogen in serum and saliva pre and post tooth brushing. Optimized Test; D, Fibrinogen in saliva pre and post tooth brushing. Optimized Test.*

analyses, respectively. Figure 1. shows the results of the Fibrinogen analysis in the pooled sera and the saliva pre and post tooth brushing. Panel A is the standard curve that allows quantitating by Colorimetric ELISA the Fibrinogen in the samples. Since there were not published data on the content of Fibrinogen in the human serum, that is after Fibrinogen was converted to Fibrin during blood coagulation, we quantitated content of Fibrinogen in a scalar series of dilutions of the control pooled sera (Figure 1, B). This analysis provided the information to establish the residual content of free Fibrinogen in control serum (around 30.000 ng/ml) and its content in the samples of human saliva pre and post tooth brushing, by allowing to identifying an approximate dilution factor for the saliva samples (Figure 1, C and D and Table 4, Table 5). After a preliminary standard test at the same dilution factor for all saliva samples (Table 4), optimization of the dilution factors resulted in improved results (Panels B and D of Figure 1 and Table 5). Indeed, optimized dilution increased Fibrinogen values and decreases variability among the 14 saliva samples (seven pre- and seven post- tooth brushing) tested by Colorimetric ELISA. Table 5 confirms that optimization of dilution results in statistically significant increased values and decreased variability of Fibrinogen content in saliva. All saliva samples collected from the seven healthy elderly presented measurable amount of the analytes (Tables 1 to 3), including Fibrinogen, a minor component of the control pooled-sera (Figure 1, C). Furthermore, tooth brushing did not induced significant differences in contents of analytes, suggesting that a measurable blood contamination is a very frequent event, at least in this group of healthy elderly persons (Tables 4 and 5), wheter all data were analyzed (20 pre tooth brushing vs 28 post tooth brushing) or the saliva samples from the five elderly persons were compared (20 pre tooth brushing vs 20 post tooth brushing)

Discussion

Saliva collection

To improve the clinical use of saliva, it is essential to standardize the procedures for collection, processing, and storage of saliva samples.[10] Several saliva collecting methods and devices have been developed. Literature reports three types of collection methods for saliva sampling. The first type is a simple tube, which facilitates saliva collection by passive drooling and spitting. Its main advantage is that it provides adequate volume of mouth fluids. [10] Cotton roll and other absorbing materials including inert polymers have been used as media in the devices for collecting saliva, leading to Salivette® and Intercept®. The absorbing materials are soaked into saliva and then inserted into a container that is centrifuged to obtain saliva. However, several studies have demonstrated the low recovery of several substances including steroid hormones and peptides when using this method.[17,18,19] Considering that many proposed methods for measuring the level of blood contamination in saliva samples mainly adopted the method of measuring plasma protein levels in saliva, saliva collection using these tubes may also affect the blood contamination levels in samples. Furthermore, some methods adopted the procedures of chewing the absorbent materials, which may lead to increasing the gingival exudates. The increased gingival exudates could increase the likelihood of blood contamination in the collected samples. Several reports have mentioned the increased concentrations of oxidative stress markers and

Table 4. *Fibrinogen analyses, Standard vs Optimized dilutions in PRE vs POST tooth-brushing saliva by Colorimetric ELISA*[3]

Test	Standard dilutions		Optimized dilutions	
	ng/ml		ng/ml	
	PRE	POST	PRE	POST
Mean	1258.79	1591.21	3057.01	2666.22
SD	937.96	496.73	1281.22	1111.97
p by student t test	0.216		0.554	

Table 5. *Fibrinogen analyses, Standard vs Optimized dilutions in PRE+POST tooth-brushing saliva by Colorimetric ELISA*[3]

Test	Standard dilutions		Optimized dilutions
	ng/ml		ng/ml
20 vs 28 Data	PRE+POST		PRE+POST
Mean	1425.00		2861.61
SD	714.74		1152.73
p by student t test		< 0.001	
20 vs 20 Data	PRE+POST		PRE+POST
Mean	1425.00		3090.32
SD	714.74		1144.54
p by student t test		< 0.001	

C-reactive protein in saliva samples collected by the chewing procedure using Salivette® with cotton medium compared to those collected by swab or drooling method.[20,21] The third collector type, such as SalivaBio Oral Swab® and ORAcollect DNA® adopted a swab method that captures cells and saliva in the mouth. They are easy-to-use, but they collect very limited quantity of saliva and cells.

In our preliminary re-evaluation of the mouth fluids, we decide to take at the minimum the risk of modify the saliva and its analytes contents, adopting the simple method to collect saliva by drooling and spit procedure.

Serum contamination of saliva

The present study has important implications for aged patients that seldom accept to volunteer for invasive collection of samples, even blood, when not strictly related to diagnosis, management and rehabilitation of diseases. Saliva and sweat could be an interesting alternative, in particular for studies including healthy seniors. It is a generally shared opinion that the diagnostic value of body fluids, in particular saliva, is affected by the level of blood contamination, originating from either gingival inflammation or loss of oral mucosal integrity or other factors. Several methods, including visual inspection, use of strips for urinalysis, and measurement of plasma proteins levels in saliva, were used to quantitate the level of blood contamination, but each method has limitations.[10] Although transferrin has has been regarded as one of the most reliable markers of blood/serum contamination of saliva, several factors, including age, gonadal hormones, salivary flow rate, chewing performance, oral microorganisms, and pathologies, might affect the salivary levels of transferrin. On the other hand, in our opinion, whatever the co-origins of contaminating serum, the fact *per se* has a value, because the serum will carry in the mouth fluids all its relatively small molecules, hopefully, Myokines included.[1-8] In blood, the serum is the fluid and solute components of blood after clotting. Anti-coagulated blood yields plasma containing fibrinogen and clotting factors. Coagulated blood yields serum without fibrinogen, although some clotting factors remain.[22-24] Our present results confirm the presence of measurable amount of plasma protein in mouth fluids, including traces of fibrinogen. The main advantage of fibrinogen is its very limited presence in mouth fluids, becuase it would be used as a more reliable index of serum contamination in comparison to other plasma proteins. Its concentration in serum will allow to identifying dilution factors for more realistic comparison of actual contents of Cytokines and Myokines in saliva. Having measured

its content in the control serum, the amount of blood contamination of each saliva sample could be determined. Indeed, after optimizing dilution factors the contents of fibrinogen of the seven volunteers were precisely determined and very similar in concentration. An added advantage is savings of time and costs to analyze circulating Cytokines and Myokines, specifically those muscle secreted, during physical activity and rehabilitation protocols in aging. Indeed, next steps of our research program will be to quantitate anti- and pro-inflammatory Cytokines and then skeletal-muscle-specific Myokines in the saliva of elderly persons. Whether those measurements will correlate or not with eventual managements to delay/counteract aging decay, is open to future trials, but our positive preliminary results warrants that those aims are worth to be tested.
In conclusion, analysis of Fibrinogen is a promising approach to quantify serum contamination of mouth and skin fluids,[25-33] and thus of circulating biomarkers (Cytokines and Myokines) by non-invasive methods.

List of acronyms

ELISA - enzyme-linked immuno-sorbent assay

Authors contributions

UC, BR, research conception and implementation, BR SZ, data collection and analysis, BR, SZ, HK and UC, manuscript conception and writing.

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Conflict of Interest

The authors have no conflicts to disclose.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Corresponding Author

Barbara Ravara, Department of Biomedical Sciences (DSB), University of Padova, Italy
Via Ugo Bassi, 58/B 35131 Padova, Italy
Phone +39 049 8276030
Email: barbara.ravara@unipd.it

E-mails of co-authors

Sandra Zampieri: sanzamp@unipd.it
Helmut Kern: helmut@kern-reha.at
Ugo Carraro: ugo.carraro@unipd.it

ORCID iD

Barbara Ravara: 0000-0002-0159-3245
Sandra Zampieri: 0000-0001-6970-0011
Helmut Kern: 0000-0001-9661-8814
Ugo Carraro: 0000-0002-0924-4998

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