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Notes

Impact of Saliva Collection and Processing Methods on Aspartate Aminotransferase, Creatin Kinase and Lactate Dehydrogenase Activities

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We aimed to investigate the impact of saliva collection and processing methods on AST, CK and LDH. Saliva was collected from 17 healthy participants by a passive drool. Each saliva sample was distributed into 3 aliquots: not treated, centrifuged, and passed through cotton. Centrifugation improved the precision of assays and produced lower values of AST and CK. The use of cotton resulted in decreased levels of LDH. This data stress the importance of the standardization of sample processing to measure enzymes in saliva.

Keywords Saliva, collection, enzyme

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Introduction

In recent years there has been increasing interest in saliva as a biological fluid for clinical and experimental studies.[1,2] Saliva sampling in comparison with blood it is painless, non-invasive, rapid and can be carried out by patients or careers to facilitate the self-management of disease monitoring at home or at a care/clinical setting.[1] However, the variability concerning the methods of collection and processing could influence the final results.[3-7]

Aspartate aminotransferase (AST), creatine kinase (CK) and lactate dehydrogenase (LDH) in saliva were shown to be useful to test the activity of gingivitis and periodontal disease, and to monitor the effects of therapy.[8,9] In addition these enzymes in saliva can change in situations of intensive exercise,[10] peptic ulcers,[11] myocardial infarction[12] and muscle damage.[13] To the authors' knowledge, no data about any possible influence of pre-analytical factors on measurements of these three enzymes in saliva have been reported. Thus, the aim of this study was to investigate the impact of saliva collection and processing methods on their measurements.

Material and Methods

Participants

The study was approved by The University of Murcia Ethical Institutional Board, and all participants signed an informed consent.

We recruited 17 healthy participants: 11 females (28 – 58 years) and 6 males (28 – 62 years). Participants received extensive verbal instructions that addressed procedures for saliva sample collection, avoiding coughing into the collection tube, abstaining from eating, brushing teeth, using a mouthwash, chewing gum or smoking for the hour prior to saliva collection. Exclusion criteria included the existence of any co-morbid and/or oral disease (*e.g.* periodontal disease and gingivitis), autoimmune, infectious, musculoskeletal, or malignant disease, and recent operation or trauma, identified with a questionnaire. Additionally, participants had to be symptom free of fever and/or a cold, and had maintained to achieve good oral hygiene.

Saliva collection and processing methods

Saliva samples were obtained by passive drool into sterile 10 mL centrifuge tubes over a 2-min period.

To evaluate any possible variation in the precision of the enzymatic assays due to centrifugation, intra- and inter-assay coefficients of variation (CV) were calculated using not-treated and centrifuged saliva samples. For this, three saliva samples were each subdivided into two aliquots, one of which was not-treated, and the other one was homogenized and centrifuged ($10000 \times g$ for 10 min at 4°C). The intra-assay CV was calculated after the analysis of saliva samples five times in a single assay run. The inter-assay CV was determined by analyzing the same samples in five separate runs carried out on five consecutive days. All samples used were frozen in aliquots at –80°C and only the vials needed for each run were used.

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All samples were homogenized before analysis.

In order to evaluate saliva collection and processing methods, saliva samples from 17 participants were aliquoted into 3 aliquots. One aliquot [Not treated] (300 µL) was used for direct measurements of selected analytes. The second aliquot [Centrifuged] (300 µL) was centrifuged (10000 × *g* for 10 min at 4°C) as previously described.[1,9] In the remaining saliva (third aliquot, cotton), a piece of cotton from the collection device Salivette (Salivette, Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) was introduced and left for 5 min in order to let the cotton absorb saliva. Then sodden cotton was centrifuged (10000 × *g* for 10 min at 4°C). After centrifugation, samples were transferred into polypropylene vials (microcentrifuge tube, 1.5 mL; Daslab; Barcelona, Spain) and measurements of the selected analytes were performed.

As an effect of centrifugation that was observed for AST and CK, different centrifugation conditions were also evaluated. Saliva samples obtained by passive drool from 5 of 17 participants were subdivided into 5 aliquots each. One aliquot was not centrifuged (not treated), the second (C1) was centrifuged 10000 × *g* for 10 min at 4°C, the third aliquot (C2) was centrifuged 10000 × *g* for 5 min at 4°C, the forth aliquot (C3) was centrifuged 5000 × *g* for 10 min at 4°C, and the fifth aliquot (C4) was centrifuged 5000 × *g* for 5 min at 4°C.

To evaluate the enzymatic activity of the saliva sediment, samples obtained from 5 of 17 participants by passive drool were analyzed both prior and after centrifugation (following those conditions set as the most appropriate in prior approaches). The sediments were re-suspended by the addition of 1 mL saline and mixed in a vortex for 30 s; thus, the enzymatic activities in those re-suspended pellets were analyzed. In order to correct for a possible dilution effect of the sediment after saline addition, as well as a different amount of sediment observed between samples, all results in this approach were expressed as IU/L and IU/mg protein.

Analysis

AST and CK were measured by commercial kits (Beckman Coulter, Brea, USA) based on the International Federation of Clinical Chemistry (IFCC) recommendations. LDH was measured by a commercial kit (BioSystems S.A., Barcelona, Spain) and total protein concentration was determined using a commercial colorimetric method (Spinreact, Barcelona, Spain). Calibrators were provided by the manufacturers of the commercial kits. All of the assays were validated for use with saliva samples, and were performed in an automated biochemistry analyzer (Olympus A400, Hamburg, Germany) at 37°C.

Statistical analysis

A statistical analysis was performed using routine descriptive statistical procedures and software (Graph Pad Prism, Ver. 5; San Diego, CA, USA). The results were evaluated for normality by using the D'Agostino & Pearson omnibus normality test; given that the majority of datasets were not normally distributed, differences in the concentrations of analytes in different aliquots were evaluated using the Friedman test, followed by Dunn's multiple comparison test. Correlations between the variables were estimated using the Spearman correlation coefficient. The CVs of the assays were calculated as the standard deviations divided by the mean value of the analyzed replicates × 100%. Values of $P < 0.05$ were considered to be significant for two-sided analysis.

Results

Intra-assay CVs for AST, CK, and LDH were below 20, 23, and 8%, respectively, for not-treated saliva samples, and below 6, 8, and 1%, respectively, for centrifuged samples. Inter-assay CVs for AST, CK, and LDH were below 35, 32, and 15, respectively, for not-treated saliva samples, and below 11, 9, and 3, respectively, for centrifuged samples.

Median (range) values of salivary AST, CK, and LDH in the differently treated aliquots are presented in Table 1. Median salivary AST and CK activities were 3.7 and 9.3-fold, respectively, lower in centrifuged aliquots when compared with not-treated aliquots ($P < 0.001$ and $P < 0.01$, respectively). No significant effect was observed between different centrifugation conditions on these analytes (Table 2).

The use of cotton significantly affected the concentrations of the three analytes studied when compared with not-treated aliquots (Table 1). However, when the activities were compared between the centrifuged and cotton aliquots, statistically significant changes were observed only in LDH (Table 1).

Saliva sediment showed high enzymatic activities when the results were expressed per mg of protein (Table 3). This activity seemed to be higher for AST and CK in the sediments than in the supernatants, although the results were only statistically significant for AST. Regarding LDH, the supernatant showed higher activity than the sediment, but without statistical significance.

Table 1 Median (range) data of salivary muscle enzymes in fresh saliva samples (results are in IU/L)

Aliquot	AST	CK	LDH
Not treated	53.6 (10.7 – 420.9)	256.7 (41.4 – 1276.8)	313.5 (46.9 – 847.8)
Centrifuged	14.3 (1.9 – 45.3)[b]	27.7 (3.8 – 86.5)[a]	378.9 (28.2 – 867.7)
Cotton	13.6 (1.8 – 48.2)[b]	18.6 (3.6 – 50.9)[b]	168.2 (3.2 – 578.5)[b,c]

a. $P < 0.01$.
b. $P < 0.001$ *vs.* values obtained in aliquot 1 (not treated sample).
c. $P < 0.01$ *vs.* values obtained in aliquot 2 (centrifuged).

Table 2 Median (range) AST and CK values in 5 aliquots submitted to different centrifugation conditions

	Not treated	C1	C2	C3	C4
AST	18.1 (15.0 – 104.6)	8.0 (4.8 – 39.4)	7.2 (5.3 – 40.6)	7.5 (5.0 – 40.7)	8 (5.2 – 41.7)
CK	61.8 (50.3 – 262.7)	17.8 (5.7 – 37.4)	15.8 (6.5 – 73.3)	16.7 (5.3 – 30.1)	16.6 (5.7 – 92.1)

One aliquot was not centrifuged (not treated), second (C1) was centrifuged 10000 × *g* for 10 min at 4°C, third aliquot (C2) was centrifuged 10000 × *g* for 5 min at 4°C, forth aliquot (C3) was centrifuged 5000 × *g* for 10 min at 4°C, and fifth aliquot (C4) was centrifuged 5000 × *g* for 5 min at 4°C (results are in IU/L).

Table 3 Median (range) AST, CK and LDH values in 5 saliva samples before centrifugation (A), in supernatants after centrifugation at 10000 × *g* for 10 min at 4°C (B) and in pellets resuspended in 1 mL saline (C) (results are in IU/L and IU/mg of protein)

		A	B	C
AST	IU/L	28.5 (10.7 – 60.2)	17.4 (6.9 – 23.6)	7.2 (3.9 – 10.2)[a]
	IU/mg	63.0 (20.4 – 106.5)	41.8 (16.6 – 43.3)	105.9 (25.2 – 145.8)[a]
CK	IU/L	95.7 (41.4 – 424.4)	22.7 (6.8 - 76.9)	25.1 (3.3 – 48.8)
	IU/mg	125.6 (111.7 – 751.3)	47.8 (24.5 – 131.5)	173.1 (21.2 – 854.9)
LDH	IU/L	487.4 (105.1 – 660.6)	437.6 (84.8 – 585.5)	50.5 (22.0 – 67.1)[a]
	IU/mg	1169.4 (209.2 – 1178.0)	1065.4 (203.6 – 1077.0)	870.0 (143.5 – 1096.0)

a. $P < 0.05$ with A (non treated sample).

Table 4 Spearman correlation coefficients between values of enzymes measured in not-treated saliva, saliva after centrifugation, and after use of cotton

Analyte	Centrifuged *vs.* Not treated		Cotton *vs.* Not treated		Centrifuged *vs.* Cotton	
	r	*P*	*r*	*P*	*r*	*P*
AST	0.825	<0.001	0.8412	<0.001	0.957	<0.001
CK	0.389	0.01	0.678	0.01	0.880	<0.001
LDH	0.956	<0.001	0.9455	<0.001	0.926	<0.001

The Spearman test revealed a statistically significant correlation between the not-treated aliquots, centrifuged, and cotton aliquots for the three evaluated enzymes (Table 4).

Discussion

Centrifugation significantly improved the precision of the evaluated assays. Saliva samples can contain cellular debris, mucus and remainders of food, resulting in a non-homogeneous sample and possible interferences that can increase the assay imprecision. For this reason centrifugation would be preferred in order to obtain cleaner specimens and to improve the assay performance. However, a negative impact of centrifugation was observed on smaller size enzymes, such as AST and CK (90 and 80 kDa, respectively), but not LDH (140 kDa). Similarly, Mohamed *et al.*[1] observed a decrease in the salivary C-reactive protein (CRP) (115 kDa) and myoglobin (16.7 kDa), but not immunoglobulin E (160 kDa) after centrifugation. This finding is unexpected, since it appears that the centrifugation force applied tends to pull the larger proteins down,[1] and thus decrease should have been noticed in larger size analytes.

The AST and CK activities in the sediments would be sufficiently high to explain the decrease in these activities in the supernatants. In contrast, the LDH activity in the sediment was not higher than that in the supernatant, which would also explain the lack of an effect of centrifugation in this enzyme.

When cotton was used, decreases were found for LDH. The use of cotton has been shown to interfere with measurements of a number of analytes in saliva in humans, increasing testosterone, progesterone, or dehydroepiandrosterone scores,[14,15] while resulted in decreased levels of CRP and myoglobin.[2] Therefore, it could be suggested that LDH can adhere to the cotton fibers, while decreasing its activity.[15]

A positive correlation was observed between differently treated aliquots for each of the enzymes measured. Although these data could indicate that each of the three systems could produce comparable values, centrifugation (with or without use of cotton) would be recommended, since the precision of the assays was highly improved. Overall, once the saliva sample treatment is chosen: (1) the same sample treatment should be performed during the whole study in order to permit comparisons of the results; and (2) reference intervals should be used according to the treatment performed.

In conclusion, the centrifugation of saliva samples highly improved the precision of the assays, but resulted in significantly decreased CK and AST, whereas the use of cotton produced a decrease in LDH. In all cases, the values of the three enzymes showed a significant correlation between differentially treated samples. All together, these data demonstrated the importance of standardization of the pre-analytical phase when CK, AST, and LDH in human saliva samples are measured.

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