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

Tomás BARRANCO,*1 Jose J. CERÓN,*1 Pía LÓPEZ-JORNET,*1 Josep PASTOR,*2 Jose M. CARRILLO,*3 Mónica RUBIO,*3 Pedro L. TORNEL,*4 Ramón CUGAT,*5 Fernando TECLES,*1*† and Asta TVARJONAVICIUTE*1

*1 Interdisciplinary Laboratory, Interlab-UMU, Campus of Excellence Mare Nostrum, University of Murcia, Spain
*2 Departament de Medicina i Cirugia Animals, Universitat Autònoma de Barcelona, Barcelona, Spain
*3 Department of Animal Medicine and Surgery, Universidad CEU Cardenal Herrera, Edificio Seminario s/n, 46113, Moncada, Valencia, Spain
*4 Clinical Analysis, University Hospital “Virgen de la Arrixaca”, Murcia, Spain
*5 Department of Orthopaedic Surgery and Traumatology, Hospital Quirón, Plaça d’Alfonso Comín, 5-7, 08023, Barcelona, Spain

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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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, 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 (first aliquot, C1), the second (C2) was centrifuged 10000 × g for 5 min at 4°C, the third 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 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 and mixed in a vortex for 30 s; thus, the enzymatic activities in 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 11%, 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 | 256.7 | 313.5 |
| Centrifuged | 14.3 | 27.7 | 378.9 |
| Cotton | 13.6 | 18.6 | 168.2 |
| 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 (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)  |
| CK IU/L  | 63.0 (20.4 – 106.5) | 41.8 (16.6 – 43.5) | 105.9 (25.2 – 145.8)  |
| CK IU/mg | 95.7 (41.4 – 424.4) | 22.7 (6.8 – 76.9) | 25.1 (3.3 – 48.8)  |
| LDH IU/L | 125.6 (111.7 – 751.3) | 47.8 (24.5 – 131.5) | 173.1 (21.2 – 854.9)  |
| LDH IU/mg | 487.4 (105.1 – 660.6) | 437.6 (84.8 – 585.5) | 50.5 (22.0 – 67.1)  |
| LDH 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. 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 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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