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Research Paper

The effects of storage time and temperature on the stability of salivary phosphatases, transaminases and dehydrogenase

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ABSTRACT

Objectives: To investigate the influence of temperature and storage time on salivary acid phosphatase (ACP), tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and lactate dehydrogenase (LDH).
Design: Unstimulated whole expectorated saliva was collected from healthy men and women subjects (n = 26) between 8 and 10 a.m. The saliva samples were centrifuged, and the supernatants were measured for ACP, TRAP, ALP, AST, ALT and LDH activities immediately (without freezing) [baseline values] and after time intervals of 3, 7, 14 and 28 days (d) of storage at −20 °C and −80 °C using spectrophotometric methods The influence of storage time was analyzed by one-way ANOVA followed by the Dunnett post-test, while the paired Student's-*t*-test was used to compare the differences between the temperature ($p < 0.05$).
Results: There was significant decline in the activities of all enzymes at −20 °C with increasing storage time. This decrease was relevant from day 14 onward for the majority of the enzymes, with the exception of AST. After day 28, the more sensitive enzymes were ALP and LDH, which showed residual activity of 39% and 16%, respectively, compared with baseline values. There were considerable, but insignificant changes, in the activities of all enzymes after storage at −80 °C for 28 days.
Conclusions: Frozen samples should be kept at −80 °C to preserve these activities, but there are restrictions for the enzymes ALP, ALT and LDH. Storage of samples at −20 °C could introduce high error variance in measured activities.

1. Introduction

Total saliva is composed of water, electrolytes, proteins, enzymes, volatile compounds, hormones of endogenous origin and cellular components such as desquamated epithelial mucosa and microorganisms (Al Kawas, Rahim, & Ferguson, 2012; Naumova et al., 2012). Saliva is involved in the processes of lubrication, digestion, neutralization of acids and bases, protection against demineralization, and has antimicrobial function (Pannunzio et al., 2010). Saliva is considered a diagnostic tool capable of providing molecular biomarkers for prevention, monitoring and diagnosis of various oral and systemic diseases and conditions, such as caries, periodontitis, oral cancer, diseases of the salivary glands and systemic disorders such as hepatitis and HIV. The advantages of using saliva as a diagnostic fluid are easy collection; noninvasive, low cost-effectiveness, and prompt availability (Nagler, Hershkovich, Lischinsky, Diamond, & Reznick, 2002). However, the success of analyte measurement requires optimal conditions and procedures for collection, processing and storage (Henson & Wong, 2010), because many factors may influence the stability of the analyte and accuracy of the test.

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Storage time and temperature, and oral health condition are some of the factors that may influence the stability of saliva components, thus being sources of errors in laboratory determinations. Storage and temperature are factors that influence steroid hormone concentrations because of their degradation by the action of salivary enzymes and the decomposing action of oral microorganisms (Kaufmann et al., 1999). A large range of errors in the measurement of testosterone and estradiol occur when saliva is stored at temperatures above −20 °C (Toone et al., 2013). In contrast, cortisol concentration is stable for up to 3 months when stored at 5 °C, −20 °C and −80 °C (Garde & Hansen, 2005). In turn, the concentration of Immunoglobulin A – Secretory (IgA-S) in saliva decreased as a function of storage time, while degradation was more accentuated at lower temperatures (Presser, Simuyandi, & Brown, 2014). On the other hand, lysozyme concentrations have been shown to be stable for up to 3 months when stored at −30 °C (Ng, Koh, Fu, & Chia, 2003). The patient's oral health condition also influences the stability of analytes; for example, storing saliva from patients with periodontal disease is not recommended when malonaldehyde and total protein levels are measured (Emekli-Alturfan et al., 2013).

The influence of storage time and temperature on most salivary components, even those with importance for diagnosis and monitoring of systemic and local changes, remain unknown. Example are Acid phosphatase (ACP, EC 3.1.3.2), tartrate-resistant acid phosphatase (TRAP; EC EC 3.1.3.2), alkaline phosphatase (ALP, EC 3.1.3.1), lactate dehydrogenase (LDH, EC 1.1.1.27), aspartate aminotransferase (AST, EC 2.6.1.1) and alanine aminotransferase (ALT, EC 2.6.1.2), enzymes found in saliva and that are outstanding for their importance in the diagnosis and monitoring of oral and systemic diseases. The activity of the phosphatases has been evaluated in the saliva of diabetic patients (López et al., 2003), smokers (Prakash et al., 2016), cystic fibrosis (Oglesbee et al., 1984), post-menopausal women (Sophia, Suresh, Sudhakar, Jayakumar, & Mathew, 2017) and healthy children (Chaves-Neto, Sassaki, & Nakmune, 2011). The increased activity of these enzymes in saliva has also been associated with the consequence of the destructive process of the alveolar bone and the degradation of tissues of in the advanced stage of periodontal disease (Dabra, China, & Kaushik, 2012; Dabra & Singh, 2012; Kaufman & Lamster, 2000; Yoshie et al., 2007).

ALT, AST and LDH are markers of cellular damage and inflammation. There is evidence of the association between the salivary levels of these enzymes and periodontitis (Alptekin, Kurtoglu, Serpek, Duran, & Gözlü, 2000; Cesco, Ito, & de Albuquerque, 2003; Dabra et al., 2012; Fiorellini, Nevins, Sekler, Chung, & Oringer, 2000; Kugahara, Shosenji, & Ohashi, 2008; Totan, Greabu, Totan, & Spinu, 2006), while the activity of ALT, AST and LDH tends to decrease in saliva after non-surgical treatment of periodontitis (Yoshie et al., 2007). Higher levels of salivary LDH, AST, and ALT were attributed to autoimmunological damage associated with the pathophysiology of diabetes mellitus (Malicka, Skoskiewicz-Malinowska, & Kaczmarek, 2016) and are therefore considered for monitoring the diabetic involvement of salivary glands (Musumeci et al., 1993; Verma et al., 2014). Furthermore, higher levels of salivary AST, and ALT may help with the early diagnosis of peptic ulcer (Boghori, Aghamaali, Sariri, Mohamadpour, & Ghafouri, 2014).

Considering the need to store saliva samples correctly, to avoid compromising the accuracy of the laboratory determinations, this study was proposed. We hypothesized that temperature and time of storage may affect the stability of ACP, TRAP, ALP, ALT, AST and LDH. To evaluate these factors, we investigated the activity of these salivary enzymes, after storage at −20 °C and up to −80 °C immediately after collection, and after time intervals of 3, 7, 14 and 28 days of storage.

2. Materials and methods

2.1. Participants

Saliva samples were obtained from healthy adult men (n = 14) and women (n = 12) volunteers aged 18–36 years. The research protocol was approved by the Human Ethics Committee on Research with Human Beings of the School of Dentistry, Araçatuba, São Paulo State University – UNESP (Permission Number CAAE 44627915.0.0000.5420 and 50712215.3.0000.5420). None of the volunteers had a history of chronic somatic illnesses such as autoimmune disease; diabetes; cancer; metabolic disturbances, or obesity; no history of neurological or psychiatric disorders; or alcohol abuse; and all of them were drug- free. In addition, participants had to be free of fever and/or cold; non-smokers; and have good oral hygiene, while participants with gingival and periodontal inflammation were excluded. To minimize any contamination of samples and to obtain a relatively constant baseline, participants were asked to refrain from brushing their teeth and eating or drinking in the 60 min prior to sample collection.

2.2. Collection, processing and storage of saliva samples

Unstimulated whole expectorated saliva (5 ml) was collected from each subject between 8 and 10 a.m., considering the circadian rhythm, according to a modification in the method described by Navazesh (1993). Subjects rinsed their mouth with water 10 min prior to sampling. The first expectoration was discarded to eliminate food debris and unwanted substances capable of contaminating the saliva, which might have caused analytical inaccuracy. The saliva samples were then expectorated into sterile tubes kept on ice, while the subject was seated in an upright position. The samples were kept on ice to minimize degradation of salivary proteins until further processing. Immediately after saliva collection, pH and buffer capacity were determined using a portable pH-meter. The buffer capacity was measured by titration using 1 ml saliva, adding 0.2 ml of 0.01 mol/L HCl. The process of adding 0.2 ml of 0.01 N HCl was repeated, and pH was recorded until a pH level of 4.0 or less was reached (Bassoukou, Nicolau, & dos Santos, 2009). To estimate the unstimulated whole saliva flow rate, saliva density was assumed to be 1 g/ml as suggested by Flink, Tegelberg, and Lagerlöf (2005). Salivary flow rate was calculated by dividing the sample volume (ml) by the time (min) taken to produce it. The unstimulated whole saliva samples were centrifuged (10,000 × *g* at 4 °C for 10 min) to remove cellular debris and to minimize the turbidity of saliva, which could negatively impact on the accuracy of analysis (Schipper, Silletti, & Vingerhoeds, 2007). The supernatant from each volunteer was divided into nine aliquots. The biochemical parameters of one aliquot were analyzed immediately after centrifugation (without freezing) [baseline values]. The remaining aliquots from each volunteer were stably stored at −20 °C (standard freezer) and −80 °C (ultra-low temperature freezer), n = 4 aliquots for each temperature, until their analyses after time intervals of 3, 7, 14 and 28 days of storage. Aliquots were thawed once when needed and then discarded; refreezing was not allowed.

2.3. Biochemical analysis of saliva

The salivary total protein concentrations were determined by using the modified Lowry method by Hartree (1972) and was expressed as mg/L. TAP activity was measured by the method based on hydrolyses of the substrate *p*-nitrophenyl-phosphate (*p*NPP) to *p*-nitrophenol (*p*NP) at pH 5.0, which has an intense yellow color at an alkaline pH (Granjeiro, Taga, & Aoyama, 1997). TRAP activity was measured by hydrolyses of *p*NPP to *p*NP at pH 5.8, in the presence of sodium tartarate and *p*-hydroxy mercury benzoate (Granjeiro et al., 1997; Janckila et al., 2005), the latter acts by inhibiting the low molecular weight acid phosphatases (Laidler, Taga, & Van Etten, 1982). The TALP activity was measured