Salivary biomarkers of cellular damage and oxidative stress following of lower third molar surgical removal

Biomarcadores salivares de dano celular e estresse oxidativo após a remoção cirúrgica do terceiro molar inferior

Abstract

Background: The aims of this study were the temporal analysis of salivary biomarkers of cellular damage and oxidative stress following of lower third molar surgical removal from healthy patient and without postoperative complications. Material and Methods: Three whole unstimulated saliva samples were collected from each of 17 patients (8 men, 9 women) before surgery, 1 and 7 days after lower third molar surgical removal using the expectation (or ‘spit’) method. Salivary flow rate (SFR), pH, buffer capacity (BC) were measured, immediately after collection. The samples were centrifuged and the supernatants were stored in aliquots at -80°C until analysis. Salivary thiobarbituric reacting substances (TBARs), total antioxidant capacity (TAC), hemoglobin (Hb), total protein (TP), uric acid (UA), acid phosphatase (ACP), tartrate-resistant acid phosphatase (TRAP), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) were measured by spectrophotometric method. Results: There were no significant differences between pre- and post-surgical SFR, pH, BC or TP. One day after extraction were detected a significant increases in Hb, TBARs, ACP, TRAP, ALP, AST, ALT, and LDH activities, and decreases of UA and TAC levels were observed. Seven days after extraction, only AST (higher) remained increased compared to pre-surgical levels. Conclusions: The surgical removal of impacted lower third molars increases salivary biomarkers of cellular damage and oxidative stress, and decreases the TAC in the early postoperative. Considering these issues, our data open new perspectives of a possible use of these parameters as biomarkers for screening and monitoring of patients vulnerable to the development of postoperative complications.

Descriptors: Saliva; Biomarkers; Oral Surgical Procedures; Oxidative Stress; Enzymes; Thiobarbituric Acid Reactive Substances.

Resumo

Justificativa: O objetivo deste estudo foi a análise temporal de biomarcadores salivares de dano celular e estresse oxidativo após a remoção cirúrgica do terceiro molar inferior de paciente saudável e sem complicações pós-operatórias. Material e Método: Três amostras de saliva total não estimulada foram coletadas de cada um dos 17 pacientes (8 homens, 9 mulheres) antes da cirurgia, 1 e 7 dias após a remoção cirúrgica do terceiro molar inferior usando o método da expectoração. A taxa de fluxo salivar (TFS), pH, capacidade tampão (CT) foram medidos, imediatamente após a coleta. As amostras foram centrifugadas e os sobrenadantes foram armazenados em aliquotos a -80 °C até a análise. Substâncias reativas com o ácido tiobarbitúrico (SRAT), capacidade antioxidante total (CAT), hemoglobina (Hb), proteína total (PT), ácido úrico (AU), fosfatase ácida resistente ao tartarato (FART), fosfatase alcalina (FAI), aspartato aminotransferase (AST), alanina aminotransferase (ALT), e lactato desidrogenase (LDH) foram medidos por métodos espectrofotométricos nas amostras de saliva. Resultados: Não houve diferenças significativas entre TFS, pH, BC e TP comparando-se as amostras coletadas no pré e pós-cirúrgico. Um dia após a extração do terceiro molar inferior, foram observados aumentos significativos nos parâmetros Hb, SRAT, FART, FAI, AST, ALT e LDH, e reduções nos níveis de AU e CAT. Sete dias após a extração, apenas AST (maior) permaneceu aumentada em comparação aos pré-cirúrgicos. Conclusão: A remoção cirúrgica dos terceiros molares inferiores impactados aumenta os biomarcadores salivares de dano celular e estresse oxidativo e diminui a CAT no pós-operatório precoce. Considerando essas questões, nossos dados abrem novas perspectivas de um possível uso desses parâmetros como biomarcadores para triagem e monitoramento de pacientes vulneráveis ao desenvolvimento de complicações pós-operatórias.

Descritores: Saliva; Biomarcadores; Cirurgia Bucal; Estresse Oxidativo; Enzimas; Substâncias Reactivas com Ácido Tiobarbitúrico.

INTRODUCTION

The surgical removal of impacted lower third molars is one of the most common oral surgery. This surgical procedure can prevent clinical symptoms such as pericoronitis and oral malodor, however, it also may be affects patient's quality of life due various postoperative side effects and/or complication, for example, pain, bleeding, variable swelling, trismus, alveolitis, hygiene and food...
difficulties. Its postoperative recovery process generally takes approximately 7 days and concurrently initiates the socket healing, a complex that involves a coordinated cascade of events that include inflammatory, proliferative, and modellling/remodelling phases. In this context, the saliva has proven to be necessary in the early stages of post–tooth extraction wound healing for optimal healing, as it was found to modulate the expression and activity of inflammatory mediators in alveolar bone tissue repair at the studied time points.

Saliva is considered a diagnostic tool capable of providing molecular biomarkers of various oral and systemic diseases. During the inflammatory phase of alveolar wound healing, immunobiologically active substances regulate the cellular movement and infiltration necessary for tissue repair. Growth factors, enzymes, cytokines, and chemokines produced by the stromal, epithelial, and inflammatory cells that accumulate in the wound space during the inflammatory phase, can be released into the oral cavity and influence the composition and function of saliva. The characterization of these salivary biochemical changes occurring during the early phase of alveolar wound healing could provide biomarkers molecular for prevention, monitoring and diagnosis of postoperative complications following of third molar surgical removal.

Lactate dehydrogenase (LDH, EC 1.1.1.27), aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2), acid phosphatase (ACP, EC 3.1.3.2), tartrate-resistant acid phosphatase (TRAP; EC 3.1.3.2), and alkaline phosphatase (ALP, EC 3.1.3.1) are biomarkers of cellular damage and inflammation in saliva. The increased activity of these enzymes in saliva may be due to the destructive process of alveolar bone and the degradation of advanced periodontal disease. Despite these facts, there have been no investigations of the alterations of these enzymes during the early stages of post–tooth extraction wound healing.

Oxidative stress is a result of a physiological imbalance between the production of reactive oxygen species (ROS) and their deactivation by enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) and non-enzymatic (uric acid, vitamins E and C) antioxidant systems. Antioxidants protect against endogenously formed free radicals and are present in all tissues and body fluids, including saliva. Nutrition, trauma, stress, and immune disturbance are some factors that contribute to disturbances of the oxidant/antioxidant balance of organisms. An excess of production of ROS has been correlated with several oral diseases. Saliva constitutes a first-line defence against oxidative stress and has protective effects against microorganisms, toxins, and oxidants. Uric acid (UA) is one of the most important non-enzymatic antioxidant and contributes approximately 70% of the salivary total antioxidant capacity (TAC). Salivary UA and TAC can be used as a biomarker to evaluate the health of periodontium. In these situation, salivary thioarbituric reacting substances (TBARS) has been used as a biomarker for the measurement of oxidative damage. Although previous studies have reported that tooth extraction can trigger an increase of oxidative stress in the plasma, it is still unknown if similar changes occur in saliva.

Unlike other oral dysfunctions and pathologic condition, in which monitoring salivary biomarkers is central to analyse of symptoms, quality of life and the successful treatment of patients, the changes in this fluid after the surgical removal of impacted lower third molars are not yet investigated. The present study was proposed in this context. Our hypothesis was that biomarkers of cellular damage and oxidative stress parameters are increased in saliva following surgical removal of lower third molars at time dependent manner.

MATERIAL AND METHOD

 Patients

Patients requiring impacted third molar surgical removal were initially considered for inclusion. Each patient underwent clinical and radiographic studies to ensure that the surgery was as simple as possible. To standardize the duration and difficulty of each operation, the Winter’s Classification was used to classify the position of the impacted third molar relative to the long axis of the second molar. The final study included 8 men (18-26 years) and 9 women (20-26 years).

None of the volunteers had a history of chronic somatic illnesses such as autoimmune diseases, diabetes, cancer, metabolic disturbances, or obesity; none had a history of neurological or psychiatric disorders or alcohol abuse; and all were drug-free. In addition, participants had to be free of fever and/or cold, non-smokers, and have good oral hygiene; participants with gingival and periodontal inflammation were excluded. 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 no. CAAE 44625815.8.0000.5420) in accordance with ethical principles of the World Medical Association Declaration. All participants were requested to provide informed consent following a detailed explanation of the study.

Surgical procedure

The surgical removal took place at the Oral and Maxillofacial Clinic of the Department of Surgery and Integrated Clinic of the School of Dentistry, Araçatuba – UNESP. One postgraduate student in a Master’s degree programme in Oral and Maxillofacial Surgery, in the Department of Surgery, Araçatuba, São Paulo State University – UNESP, was responsible for the surgical removal of lower third molars. The surgical procedure was explained to the patient and the patient was informed about the possible complications following of this procedure. Preoperative and postoperative clinical assessments were performed by the same investigator (study period).

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Maxillofacial Surgery and Traumatology performed the procedures. One lower third molar was extracted per session. The first specimens of saliva were collected before the third molar was removed. In the preoperative period, the patient used 10 mL of mouthwash with 0.12% chlorhexidine digluconate for 30 s. Afterward, the patient received local anaesthesia (mepivacaine 2%, epinephrine 1:100,000). A relaxing incision was made in the vestibular region of the second lower molar with a no. 15 blade and the total flap was detached with a Molt no. 9. Due to the position and localization of the third molar, all cases required an osteotomy, which was performed using a high-speed drill (conical bit no. 702) under irrigation. Some cases required tooth sectioning. After molar surgical removal, the incision was closed with a simple suture with non-absorbable nylon 4-0 wire to facilitate soft tissue healing and avoid infection.

Nimesulide (100 mg every 12 h for 3 days) and amoxicillin (500 mg every 8 h for 5 days) were prescribed as anti-inflammatory agents during the postsurgical period. Dipyrrone (500 mg every 8 h for 2 days) was prescribed only for pain. Each patient received an explanation of hygiene techniques and recommendations for the postoperative period. All the participants were requested to rinse their mouth with 15 mL of 0.2% chlorhexidine twice a day for 1 week postoperatively. Patients with postoperative complications such as suture dehiscence, paraesthesia, alveolitis, or infection were excluded from the study.

**Collection, processing, and storage of saliva samples**

As mentioned above, saliva samples were collected at three different time-points: before surgery and 1 day and 1 week after. Participants were asked to refrain from eating, drinking and oral hygiene procedures for at least 1-hour prior to saliva collection. Unstimulated whole expectorated saliva (5 mL) was collected from each subject between 8 and 10 a.m., considering the circadian rhythm. Subjects rinsed their mouths with water 10 min prior to sampling. The first expectoration was discarded to eliminate food debris and unwanted substances capable of contaminating the sample, which might have caused analytical inaccuracy. The subsequent sample was 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 (BC) were determined using a portable pH meter. The buffer capacity was measured by titration using 1 mL saliva and adding 0.2 mL of 0.01 N HCl. The addition of HCl was repeated, and the pH was recorded until a pH of 4.0 or less was reached\(^1\). To estimate the salivary flow rate (SFR), the saliva density was assumed to be 1 g/mL. The SRF was calculated by dividing the sample volume (mL) by the time (min) taken to produce it. Immediately after saliva collection, the saliva samples were centrifuged (10,000 \( \times \) g at 4°C for 10 min) to remove cellular debris and minimize the turbidity of the saliva, which could negatively impact analytical accuracy. The supernatants were stored in aliquots at -80°C. Each aliquot was frozen and thawed only once.

**Biochemical analysis of saliva**

Salivary haemoglobin (Hb) levels were measured using hemoglobin cyanide method, according to the manufacturer’s instructions (Labtest Diagnostica S.A., MG, Brazil). Saliva samples with 0.1% (1000 µg/mL) blood contamination were excluded from analyses, since salivary biomarkers of oxidative stress can be altered by blood-contaminated saliva\(^15\).

The salivary TP concentrations were determined using the modified Lowry method according to Hartree\(^16\) and were expressed in g/L. ACP activity was measured based on hydrolysis of the substrate p-nitrophenyl-phosphate (pNPP) to p-nitrophenol (pNP) at pH 5.0, which has an intense yellow colour at an alkaline pH (17). TRAP activity was measured by hydrolysis of pNPP to pNP at pH 5.8 in the presence of sodium tartrate and p-hydroxy mercury benzoate\(^17\); the latter acts by inhibiting acid phosphatases of low molecular weight. ALP activity was measured based on the enzyme’s ability to catalyse the hydrolysis of pNPP to pNP at pH 9.4 in the presence of magnesium chloride. In both determinations, the formation of pNP was determined spectrophotometrically by reading the absorbance at 405 nm using a molar extinction coefficient of 18,000 M\(^-1\)cm\(^-1\). Controls without enzyme were included in each assay to adjust for non-enzymatic hydrolysis of pNPP. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse 1 µmol of pNPP per min at 37°C. ACP, TRAP, and ALP activity was expressed as specific activity (U/g of total protein).

Salivary LDH, AST, and ALT activities were assessed using Labtest kits (Labtest Diagnostica S.A., MG, Brazil). AST activity was measured based on the transfer of an amino group from L-aspartate to \( \alpha \)-ketoglutarate with formation of oxaloacetate and L-glutamate; afterwards, oxaloacetate was reduced to malate by malate dehydrogenase with simultaneous oxidation of NADH to NAD\(^+\). ALT activity, similar to AST, was analysed using the method based on transfer of an amino group from L-alanine to \( \alpha \)-ketoglutarate with formation of pyruvate, which was then reduced to L-lactate by LDH with simultaneous oxidation of NADH to NAD\(^+\). In both cases, the reduction of the absorbance at 340 nm in the sample was due to NADH oxidation, and was proportional to ALT and AST activity. LDH activity in the
supernatant was determined by the enzymatic catalysis of the interconversion of pyruvate and lactate in the presence of NADH. The reduction of the absorbance at 340 nm due to NADH oxidation was proportional to the LDH activity in the sample. The LDH, ALT, and AST activities were expressed as specific activity (U/g of total protein).

Salivary lipid peroxidation products were determined based on TBARS levels as previously described. Briefly, aliquots were mixed with 15% (w/v) trichloroacetic acid plus 0.375% (w/v) thiobarbituric acid and heated at 100 °C for 45 min. Samples were cooled to room temperature, centrifuged at 1,000 × g for 15 min, and their absorbance at 535 nm was measured. The molar absorption coefficient was 1.56 × 105 M−1 cm−1 and values were normalized to TP concentrations in each sample and expressed in μmol/g of protein.

Salivary TAC was determined using the ferric reducing antioxidant power FRAP assay as previously described by Benzie and Strain, based on the reduction of Fe3+ to Fe2+, which is chelated by 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) to form a Fe2+-TPTZ complex with a maximum absorbance at 593 nm. The results were calculated using a standard curve constructed using different concentrations of FeSO4 solutions and values were normalized to TP concentrations in each sample and expressed in mmol Fe2+/g of total protein.

Salivary UA was evaluated using a Labtest kit (Labtest Diagnostica S.A., MG, Brazil) based on the enzymatic method of Trinder, per the manufacturer’s instructions. Results were normalized to TP concentrations in each sample and expressed in mg/g of total protein.

Statistical methods

Data were expressed as mean ± standard deviation. Parametric data were analysed by one-way ANOVA followed by the Tukey post-hoc test, while non-parametric data were analysed by Kruskal-Wallis test followed by the Dunn post-hoc test.

RESULTS

Table 1 expresses the average values of SFR, pH, and volume of acid (0.01 N HCl) used to determine the pH range, TP, and Hb concentration of whole saliva samples. Data are provided as mean ± standard deviation. Parametric data were analysed by one-way ANOVA followed by the Tukey post-hoc test, while non-parametric data were analysed by Kruskal-Wallis test followed by the Dunn post-hoc test.

Table 1. SFR, pH, and volume of acid (0.01 N HCl) used to determine the pH range, TP, and Hb concentration of whole saliva samples

<table>
<thead>
<tr>
<th>Variables</th>
<th>Before surgery</th>
<th>After 1 day</th>
<th>After 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFR (mL/min)</td>
<td>0.66 ± 0.30</td>
<td>0.65 ± 0.31</td>
<td>0.73 ± 0.36</td>
</tr>
<tr>
<td>pH</td>
<td>6.85 ± 0.35</td>
<td>6.94 ± 0.39</td>
<td>7.01 ± 0.26</td>
</tr>
<tr>
<td>Buffer capacity (mL acid/mL saliva)</td>
<td>0.09 ± 0.15</td>
<td>0.12 ± 0.16</td>
<td>0.13 ± 0.16</td>
</tr>
<tr>
<td>pH 4.9-6.0</td>
<td>0.56 ± 0.20</td>
<td>0.43 ± 0.20</td>
<td>0.55 ± 0.21</td>
</tr>
<tr>
<td>pH 5.9-5.0</td>
<td>0.25 ± 0.11</td>
<td>0.29 ± 0.12</td>
<td>0.31 ± 0.15</td>
</tr>
<tr>
<td>pH 4.9-4.0</td>
<td>0.24 ± 0.16</td>
<td>0.22 ± 0.19</td>
<td>0.16 ± 0.15</td>
</tr>
<tr>
<td>TP concentration (µg/mL)</td>
<td>1.02 ± 0.23</td>
<td>0.94 ± 0.27</td>
<td>0.97 ± 0.19</td>
</tr>
<tr>
<td>Hb concentration (µg/mL)</td>
<td>53.94 ± 22.43</td>
<td>47.02 ± 63.67**</td>
<td>55.33 ± 22.50**</td>
</tr>
</tbody>
</table>

* p < 0.01 vs. before surgery; # p < 0.01 vs. 1 day after surgery

The data were compared using a one-way ANOVA with a post-hoc Tukey test.

Figure 1: Activities of salivary acid phosphatase (ACP), tartrate-resistant acid phosphatase (TRAP), and alkaline phosphatase (ALP) throughout the perioperative period (before to 7 days after surgery). The data are expressed as mean ± standard deviation. Data were analysed using a one-way ANOVA followed by a post-hoc Tukey test (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).
DISCUSSION

Considering that all patients received the same guidelines and postoperative medications, and none presented complications within the first week post-surgery, we consider that the salivary changes reflect normal events during early post-tooth extraction tissue repair. We did not detect significant variations between pre- and post-surgical values of SRF, pH, BC values, or TP concentration. In contrast, the salivary enzymes of tissue degradation were significantly higher 1 day after the procedure and had returned to baseline values after 7 days. In the same way, we observed a significant increase in lipid oxidative damage in the first postoperative day, but this parameter was similar to pre-surgical values after one week.

SRF, BC and pH can be important predictors in periodontium health status. Although our findings do not show significant changes in these parameters within the first postoperative week, salivary pH is assumed to have a reverse correlation with third molar post-surgical removal pain. The exclusion of volunteers had a history of chronic illnesses, poor oral hygiene and participants with gingival and periodontal inflammation can contribute to this results.

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Salivary proteins play an important role in acquired pellicle formation and subsequent bacterial accumulation within the oral cavity. In addition, salivary proteins are involved in the inflammatory response, antimicrobial and immune defence, lubrication, buffering, and remineralisation capacities. Although salivary assays show that TP levels are higher in gingivitis and chronic periodontitis patients, our data showed that injury from surgical removal of impacted lower third molars did not change this parameter, which corroborates a previous study of Gutiérrez-Corrales et al. that also evaluated the effect of the acute inflammatory process occurring in the salivary TP after surgery.

The ability to reflect both oral and systemic health conditions has made saliva an attractive, easily collected, low-cost, and noninvasive clinical tool. However, in order to use saliva as a diagnostic body fluid, a sensitive and specific biomarker among the complicated composition of saliva must be specified for each case. In this context, salivary enzymes, oral microbiorganisms, polymorphonuclear leukocytes, and oral epithelial cells derived from gingival crevicular fluid have been highlighted as biomarkers, especially LDH, AST, ALT, ACP, TRAP, and ALP, which may be a consequence of the destructive process of the alveolar bone and the degradation of advanced stage tissues of periodontal disease. Our findings demonstrate for the first time that these enzymes also are involved in the early stages of post-tooth surgical removal wound healing.
ALT, AST, LDH, ACP, and TRAP localized in the cytosol and/or cytoplasmic organelles, while ALP is associated with the plasma cell membrane. These enzymes are involved in various cell metabolic processes, and they are mostly present in the cells of hard and soft tissues. Increased activities of these enzymes in saliva are considered to be biomarkers of cellular damage and inflammation. After the surgical removal of impacted lower third molars, several potential sources of these enzymes emerge in the oral cavity, such as stromal, epithelial and inflammatory cells involved in the gingival flap and socket healing; blood clots within the alveolar socket; desquamated epithelial mucosa; and biofilms. The latter are accentuated due to the difficulty of oral hygiene after surgery. In addition, AST, ALT, and phosphatases may contribute to the intrinsic maturation of acquired enamel pellicle proteins\(^\text{28,29}\), the first step of oral biofilm formation and subsequent adhesion of opportunistic periodontal pathogens, which leads us to emphasize oral hygiene in the postoperative period to prevent infectious complications.

In normally healing wounds, ROS are required for defense against pathogens and act as messengers to stimulate processes associated with wound healing, including cell motility, cytokine action, and angiogenesis. However, an increased level of ROS may overlap the positive effects and cause additional tissue damage\(^\text{30}\). Previous studies have also reported that tooth extraction can trigger an increase of oxidative stress in the plasma\(^\text{31}\); however, there are no published reports related to salivary oxidative stress interactions after tooth extraction. To our knowledge, this is the first demonstration that the peak salivary oxidative stress response occurs between the immediate postoperative period and 24 hours after tooth surgical removal, as evidenced by the increased salivary TBARS and reduction of TAC levels. Seven days after surgery, TBARS levels had returned to near pre-surgical levels. This could be explained by the neutralizing action and subsequent restoration of antioxidant defense non-enzymatic, especially UA concentration, and/or reduction of free radical generation during wound healing and regeneration of gingival tissue surrounding the alveolar bone. This finding suggests a perspective for assessing whether complementary antioxidant therapies can benefit the repair process after tooth surgical removal.

The present data must be interpreted considering the following methodological limitations. First, small sample size was the main limitation of this study. Secondly, the results were not correlated with the parameters of the acute inflammatory process that occurred after surgery. Future researchers could consider whether the results obtained in the present study remain when using antiseptic mouth rinse after surgery in patients with pre-existing conditions to oral surgery, such as poor oral hygiene, gingivitis, or periodontitis, smokers, diabetes and hypertension and/or if the results are associated with inflammatory responses to surgery such as swelling and pain.

Our results confirm this hypothesis with significant increase of the salivary enzymes of the tissue degradation and lipid damage oxidation, and decreased the TAC in the early postoperative period after surgical removal of lower third molar from healthy patient and without postoperative complications. Considering these issues, our data open new perspectives of a possible use of these parameters as biomarkers for screening and monitoring of patients vulnerable to the development of postoperative complications.

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CONFLICTS OF INTERESTS
The authors declare no conflicts of interests.

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