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TOTAL CONTENT OF CALCIUM AND PHOSPHORUS IN SERUM OF WISTAR RATS BY AN OPTIMIZED METHOD OF ICP-MS

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ABSTRACT

In the Odontology field, the modeling process of alveolar bone has been a topic of investigation for the last years since the lost bone after tooth extraction can preclude aesthetic and function. In this practice, the volume of the tissue to be modeled depends on, among other factors, the serum levels of calcium and phosphorus, and it is of interest to monitor these elements to better understand the mechanism of tissue regeneration. These elements are routinely analyzed for clinical purposes, as serum constituents, by UV-VIS spectrophotometry after chromogenic reactions for each metal with different reagents. Depending on the analytical demands and work objectives, it is necessary to have methods based on technologies that allow multi-element quantification in a single experiment. In this context, ICP-MS has been considered a powerful tool for rapid multi-element analysis due to its high selectivity and sensitivity. However, it has been reported that samples with high content of salts and proteins, such as serum samples, cause matrix-induced interferences due to weak sample preparation and the presence of polyatomic ions. The present study aimed to identify and mitigate matrix effects in rat serum samples during the simultaneous quantification of calcium and phosphate using ICP-MS and evaluate its capability of differentiating element levels from Wistar rats subjected to tooth extraction with and without alveolar bone grafting. In addition, the optimized method can be used for more comprehensive mineralomics studies related to the bone modeling process in future studies.

Keywords: *Bone mineralization, rat serum, sample preparation, ICP-MS*

1. INTRODUCTION

The modeling and remodeling process of alveolar bone after tooth extraction has been a topic of investigation for the last years to maintain the volume of the tissue since tooth extraction results in bone loss, which can affect aesthetic and function (Stavropoulos *et al.,* 2015; Lopes *et al.,* 2018). The use of bone grafting during the surgical procedure favors this process (Stavropoulos *et al.,* 2015). Three main types of cells are involved in bone remodeling: osteoclasts, which resorb bone; osteoblasts, which deposit new bone, and osteocytes, responsible for mechanical senses perception and stimulus of bone remodeling (Alford *et al.,* 2015). These cells are regulated by local (mechanical or cytokines) and systemic (hormones) signals (Lopes *et al.,* 2018). The bone tissue is composed of organic and inorganic matrices. Collagen fibers are the main constituents of the organic part. At the same time, hydroxyapatite - a molecule structured by calcium phosphate - is the key element of the inorganic matrix (Tobeiha *et al.,* 2020). Bone neoformation starts with the deposition of collagen fibrils by osteoblasts, forming a scaffold, which is then mineralized by the precipitation of ions of calcium and phosphate, promoting the hydroxyapatite crystals' formation. The process requires normalized levels of calcium and phosphate in the blood. Otherwise, there can be inadequate bone mineralization with an excess of non-calcified

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bone matrix (Alford *et al.,* 2015; Murshed *et al.,* 2018). Indeed, the knowledge of seric calcium and phosphorus levels is important to better understand the process of alveolar bone modeling. Therefore, using a preclinical *in vivo* model analog to the human. To study the alveolar healing after tooth extraction, rodents are the most used *in vivo* model, since they are cheaper and faster than larger animals and allow harvest of specimens to analysis, which is not possible in human studies, added to issues related to practical and safety, ethics and regulatory concerns (Stavropoulos *et al.,* 2015).

Serum levels of calcium and phosphorus are routinely quantified, for clinical purposes, by UV-VIS spectrophotometry after chromogenic reactions for each metal with different reagents (Bueno & Czepielewski, 2010). However, depending on the analytical demands and work objectives, such as the determination of free or total elements, sample type, and the number of samples, it is necessary to have methods based on technologies that allow multi-element quantification in a single experiment with good selectivity. In this context, ICP-MS has been considered a powerful tool for rapid multi-element analysis due to its high selectivity and sensitivity (Laur et al., 2020). However, it has been reported that samples with high salts and proteins, such as serum samples, struggle with suppression and enhancement of analytic signal on ICP-MS methodologies, especially due to weak sample preparation and the presence of polyatomic ions (Lu *et al.,* 2015). Some works have been published to overcome these problems presenting different sample treatments, such as digestion conditions (Lu *et al.,* 2015) and types of equipment with different configurations (Konz *et al.,* 2017). Although good results were demonstrated, some adaptations are occasionally required to reach specific analysis needs.

The present study aimed to identify and mitigate matrix effects caused by rat serum samples during the simultaneous quantification of calcium and phosphate by ICP-MS and evaluate its capability of differentiating element levels from Wistar rats submitted to tooth extraction with or without alveolar bone grafting.

2. MATERIALS AND METHODS

2.1. Chemicals

Sub-boiling distilled Nitric acid 65% EMSURE® Reagent Ph Eur, ISO (Merck, Germany); Type 1 water obtained by Milli-Q UV system (Millipore, USA), were used. In addition, multi-element standard solution XVI (Merck Darmstadt, Germany), Calcium and phosphorus TraceCert standard at 1000 mg/L in 2% nitric acid and water, respectively; ketamine and xylazine were purchased from Syntec (Syntec do Brasil LTDA, Brazil).

2.2. Animals

This study was approved by the Ethics Committee on Animals Use of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS, protocol #9108), and performed following the guidelines for the National Council for Animal Experimentation Control (CONCEA). Ten adult female Wistar rats (±220 g) were kept in cages with temperature-controlled rooms and with food and water offered *ad libitum*. After an acclimatization period, the animals were subjected to tooth extraction of molars of the right side of the jaw under general anesthesia with a mixture of ketamine and xylazine. Five rats had the socket filled with bone grafting (group 1), and five animals did not receive grafting (group 2). The animals were euthanized after 35 days by blood exsanguination and anesthetic overdose. The collected blood was centrifuged at 3000 rpm for 10 min after the formation of the clot. The serum was stored at -20 ºC until analysis. In addition, the maxilla was collected for histological examination of the alveolar socket.

2.3. Sample Preparation

Serum samples were prepared according to described elsewhere (Lu *et al.,* 2015), with modifications. Briefly, thawed samples were transferred to 15 mL polymeric vials with a screw cap and added by sub-boiling nitric acid. The vials were heated to the water boiling point in a thermostated water bath. After digestion, all samples were added by type 1 water at a final volume of 10 mL and were injected into the ICP-MS system. To evaluate the digestion efficiency, a group of five distinct serum samples was added by standards at a final concentration of 50 mg/L and nitric acid at the sample: acid ratio of 100:308; 50:308; 33:308; 25:308; and 20:308 µL (v/v), representing the dilution factors of 1, 2, 3, 4, and 5 respectively. These factors were used to normalize the analyte signal and evaluate matrix effects. The digestion time was evaluated using another group of five serum samples added by standards and nitric acid at a ratio of 20:308 µL (v/v), which were digested at the times of 0, 15, 30, 60, 120, and 180 min. Calibration curves were constructed at concentrations of 50, 100, 250, 500,

and 750 µg/L, prepared in 2% nitric acid. All test samples were analyzed in triplicate.

The the collected jaws were fixed, decalcified, and embedded in paraffin. One section of 4 µm was cut in the middle of the alveolar socket and stained with hematoxylin and eosin (HE) for histological evaluation.

2.4. ICP-MS analysis

It was used an ICP-MS 7700 (Agilent Technologies, Tokyo, Japan) system equipped with an autosampler, 2.5mm quartz torch, Ni sampler, and skimmer cones, i.d. 1.0 and 0.4 mm. The operating conditions were 1550W plasma RF power, 1.3 L/min argon carrier gas, and 4 L/min helium collision gas flow. The isotopes monitored for calcium were ^{42}Ca and ^{44}Ca, and phosphorus was ^{31}P, with a dwell time of 0,3 s. The oxide species (CeO/Ce) level was below 1.5%, and doubly charged (Ce^{2+}/Ce) was below 3%. The quantification was performed with external calibration curves in gas (He) and no gas mode.

3. RESULTS AND DISCUSSION:

The parameters optimized for sample digestion were the digestion time and the sample: acid ratio. After optimization, the proposed method was applied to determine the total content of phosphorus and calcium in Wistar rat serum by ICP-MS. As presented in Figure 1, effective sample digestion was obtained after 60 min. Considering the amount of analyte released from the matrix. The optimal sample:acid ratio was obtained from the dilution factor of 3 (33:308 µL, v/v). However, we fixed the factor of 5 to avoid matrix effects. Matrix effects were evaluated by examining the signal suppression or enhancement after reducing the amount of sample concerning the amount of nitric acid in the digestion step and after normalizing analytes signals by the dilution factor.

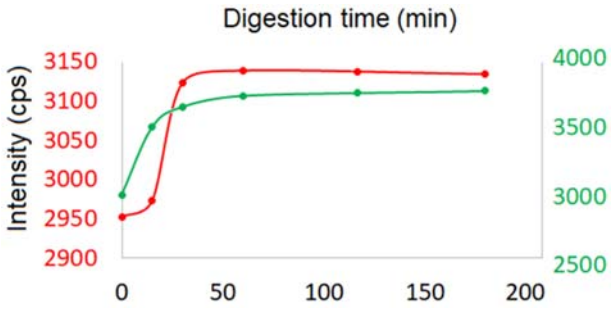

Figure 1. *Digestion efficiency due to contact time between the sample and nitric acid. Total serum calcium (average) was presented in red, and total serum phosphorus (average) was presented in red. Y-axis as signal intensity (cps).*

As presented in Figure 2, this effect was observed for sample:acid ratios of 100:308 and 50:308 µL (v/v), in which analyte signals were higher than the other ratios. We also added multi-element standards into the samples to evaluate the effects caused by polyatomic interferences over phosphorus and calcium signals.

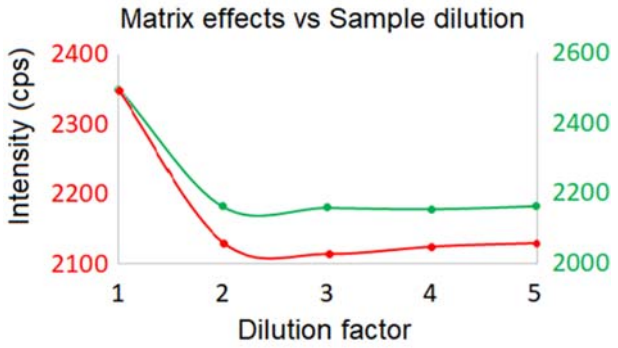

Figure 2. *Matrix effects correction with sample dilution. Total serum calcium (average) was presented in red, and total serum phosphorus (average) was presented in red. Y-axis as signal intensity (cps).*

Indeed, we observed an enhancement of calcium signal due to the presence of magnesium in the standard, which interferes with ($^{26}Mg^{16}O^{+}$ and $^{26}Mg^{18}O^{+}$) in both calcium isotopes used in the present work (^{42}Ca and ^{44}Ca). For this purpose, calibration curves were constructed with single-element standards.

We tested the capability of the optimized method to differentiate element levels from Wistar rats submitted to tooth extraction with (group 1) or without (group 2) bone grafting inside the socket. As presented in Figure 3, the group that received grafting treatment presented a lower concentration of total calcium and a higher amount of phosphorus. The calcium analysis showed a significant difference between groups ($p<0.05$, Mann Whitney test), indicating a higher mobilization of calcium to the alveolar bone. However, it is important to emphasize that the levels of this mineral remained inside normal ranges in serum of rats (Hernández-Becerra *et al.,* 2020) and, therefore, bone remodeling could happen in a standard way in both groups, which was observed in histological evaluation. Calcium is an essential mineral, and serum levels between 92 – 140 mg/L are considered normal (Mulyaningsih *et al.,* 2019). Figure 4 shows the alveolar bone of group 2.

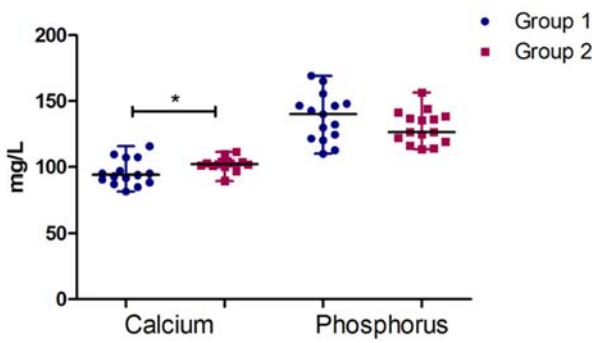

Figure 3. *Concentration of calcium and phosphorus in serum according to the groups. Data is shown as median and range*.

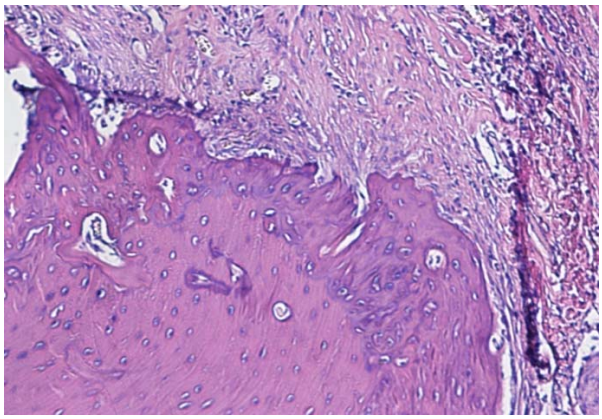

Figure 4. *Alveolar bone formed after tooth extraction in group 2*. HE stains, 100x

Considering that total calcium and phosphorus content in human and rat blood is similar (Zaksas *et al.,* 2010), the results obtained in the present work were relatively similar to the findings reported by other authors that also used ICP-MS (Konz *et al.,* 2017; Tao *et al.,* 2019, Xia *et al.,* 2021). However, total calcium levels were slightly higher than reported in the literature, probably due to magnesium in the serum, which ranged from 0.5 to 21.3 mg/L (Laur *et al*., 2020).

4. CONCLUSIONS:

A simple and effective acid digestion method was proposed here to determine the total content of calcium and phosphorus in rat serum samples with reduced matrix effects. The methodology pointed a small difference between the groups that stayed inside normal ranges of the minerals to provide normal bone remodeling. The optimized method was considered useful for monitoring calcium and phosphorus in rat serum samples and propitious for more comprehensive mineralogical studies related to the bone modeling process in future studies.

5. ACKNOWLEDGMENTS:

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