Original article

DOI: https://doi.org/10.18320/JIMD/201805.0130

Journal of International Medicine and Dentistry 2018; 5(1): 30-38

JOURNAL OF INTERNATIONAL MEDICINE AND DENTISTRY

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JIMD

p-ISSN: 2454-8847
e-ISSN: 2350-045X

The influence of salivary pH and calcium/phosphate ions concentration on salivary gland stones' formation

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Abstract:
Sialolithiasis is a multi-factorial pathology that accounts for more than 50% of salivary gland disease. However, the exact impact of salivary pH and calcium (Ca^{2+}) and phosphate (PO_4^{3-}) on the sialoliths' formation still remains unknown. **Materials and Methods:** Seven removed sialoliths were put into flasks with artificial saliva solution that differed in concentrations of Ca^{2+} and PO_4^{3-}. The changes in weights of sialoliths and alterations in pH had been measured for 13 weeks. Concentrations of Ca^{2+} and PO_4^{3-} in the artificial saliva solution were estimated at the beginning and at the end of the experiment. **Results:** Sialoliths' weight decrease was observed from the 2nd to 7th week. It occurred parallel with natural pH decrease (from 7 to 6.5 pH). Sialoliths' weights started increasing after pH was adjusted with NaOH. Analyzing the concentration changes of Ca^{2+} in every flask, it was determined that from natural or double Ca^{2+} concentration, it decreased till similar amount (7–14 times). Analyzing the concentration changes of PO_4^{3-}, it was determined that from natural or double PO_4^{3-} concentration it decreased 24 times. **Conclusions:** Sialoliths' weights decreased parallel with natural pH decrease and sialoliths grew when pH was adjusted. PO_4^{3-} ions concentration decreased relatively more than Ca^{2+} ions concentration.

.**Key words:** Sialolithiasis, salivary calculi, salivary stones, sialoliths

Introduction:

Sialolithiasis is a multi-factorial pathology that accounts for more than 50 percent of salivary gland disease[1]. It is diagnosed when calcified mass, called sialolith or salivary gland stone, forms in the body of salivary gland or its duct, causing postprandial recurrent swelling, tenderness or pain and saliva blockage[2]. Sialoliths are found in submandibular salivary gland or its duct (80 - 95%), but can also appear in parotid, sublingual and very rarely in minor salivary glands[3]. More commonly, sialoliths are diagnosed in the duct of salivary gland (72%) than in the gland itself[4]. There are a lot of etiological factors that have been associated with a higher risk of having sialoliths. These include the changes in salivary pH and in Ca^{2+} and PO_4^{3-} ions concentrations, which were proved to be significantly higher in patients having sialolithiasis than in healthy individuals[5, 6]. Physiologic salivary Ca^{2+}ions concentration varies from 1.03 to 3.6 mmol/L, while physiologic salivary PO_4^{3-} ions concentration is 4.5-6 mmol/l[7, 8]. Although the range of physiologic values is wide and ions concentrations rarely exceed these norms, it is known that elevation in salivary Ca^{2+} and PO_4^{3-} ions concentrations may occur in case of primary hyperparathyroidism[9], followed by hyperphosphaturia and hypercalciuria. Agha-Hosseini et al[10] showed that increased concentration of salivary Ca^{2+} was associated with oral dryness and menopause, excreting the excess of calcium in serum, caused by elevated concentration of parathyroid hormone, through saliva and urine. Sewóna et al[11] also presented a positive correlation between smoking and salivary calcium and phosphate ions concentrations, which increase in conjunction with the amount of cigarettes per day. All these factors, increasing Ca^{2+} and PO_4^{3-}concentration in saliva are very important, because for crystallization to appear, a higher calcium phosphate supersaturation is essential as a thermodynamic factor [5]. Other etiologic factors include bacterial infection[12], concomitant systemic diseases such as diabetes mellitus, arterial hypertension[4], Sjögren syndrome[13,14], hyperparathyroidism[15], as well as anatomy of major salivary glands[16,17,18,19], medication [20, 21, 22] and gender.[17]

However, it was shown that local factors, such as bacterial infection, alterations in salivary pH, anatomy of duct are more influential for developing salivary gland stones[1, 3, 23]. Recently, Shih-Han Hung et al[23] showed a positive correlation between chronic periodontitis and sialolithiasis, which reinforce the idea of retrograde theory and inflammation factors[9]. The most commonly found hypothesis is related to stagnation of calcium and phosphate rich saliva, followed by changes in salivary pH, which are considered to be the initial factors for the calcification process to begin[1, 5]. Naturally, pH of saliva varies from 6.2 to 7.6 [24], but increased pH value is associated with salivary stone formation as the solubility of $Ca_3(PO4)_2$ decreases in more alkaline environment. Nonetheless, this theory encounters with controversial opinions and that awakes researchers' curiosity for further investigations. In order to find the exact role of calcium and phosphate ions in forming sialoliths, this in vitro research was performed using removed salivary gland stones and observing how they react to different concentrations of calcium and phosphate ions and changes in salivary pH. In this research, we present the results, which give a chance for new possible theories to come into view.

Materials and Methods:

1. Test groups

All salivary stones were received from the Hospital of Lithuanian University of Health Sciences, Department of Maxillofacial Surgery. Salivary glands stones were placed into artificial saliva substitute: 0.31 mmol/L $MgCl_2$, 14.819 mmol/L NaCl, 4.610 mmol/L K_2HPO_4, 2.396 mmol/L KH_2PO_4, 2.244mmol/L $CaCl_2$, pH 7 at 37°C. Before the in vitro experiment, sialoliths were autoclaved for 30 minutes at the temperature of 121°C in the artificial saliva substitute. Test tubes with salivary glands stones were randomly divided into 4 groups:

I) Control, artificial saliva substitute with physiological $Ca^{2+}$and PO_4^{3-} ions concentrations (accordingly: 2.244 and 7.006 mmol/L)

II) Artificial saliva substitute with physiological PO_4^{3-} ions concentration (7.006 mmol/L) and double Ca^{2+} ions concentration (4.488 mmol/L)

III) Artificial saliva substitute with physiological Ca^{2+} ions concentration (2.244 mmol/L) and double PO_4^{3-} ions concentration (14.012 mmol/L)

IV) Artificial saliva substitute with double Ca^{2+} and PO_4^{3-} ions concentrations (4.488 and 14.012 mmol/L).

2. Determination of Calcium and Phosphate ions concentrations

At the beginning of the experiment pH in all test tubes was 7.0 (physiological pH in human saliva). All test tubes were held in a thermostat with the temperature of 37°C for 13 weeks. The weight alteration was analyzed after 2, 3, 4, 6, 7, 9, 13 weeks. Every sialolith was held on a sterile wipe for 5 seconds and after that weighed three times. The pH change was calculated after 4, 6, 8, 9, 13 weeks. Ca^{2+} and PO_4^{3-} ions concentrations were calculated at the beginning of the study and measured at the end of the study in the Department of Physical and Inorganic Chemistry, Kaunas University of Technology. The concentrations were measured using T70+ UV/VIS spectrophotometer and PFP7 Flame Photometer JANWAY. The pH was re-established to the primary physiological pH (7.0) with the solution of NaOH in all test tubes in the 8th week.

3. Statistical analysis

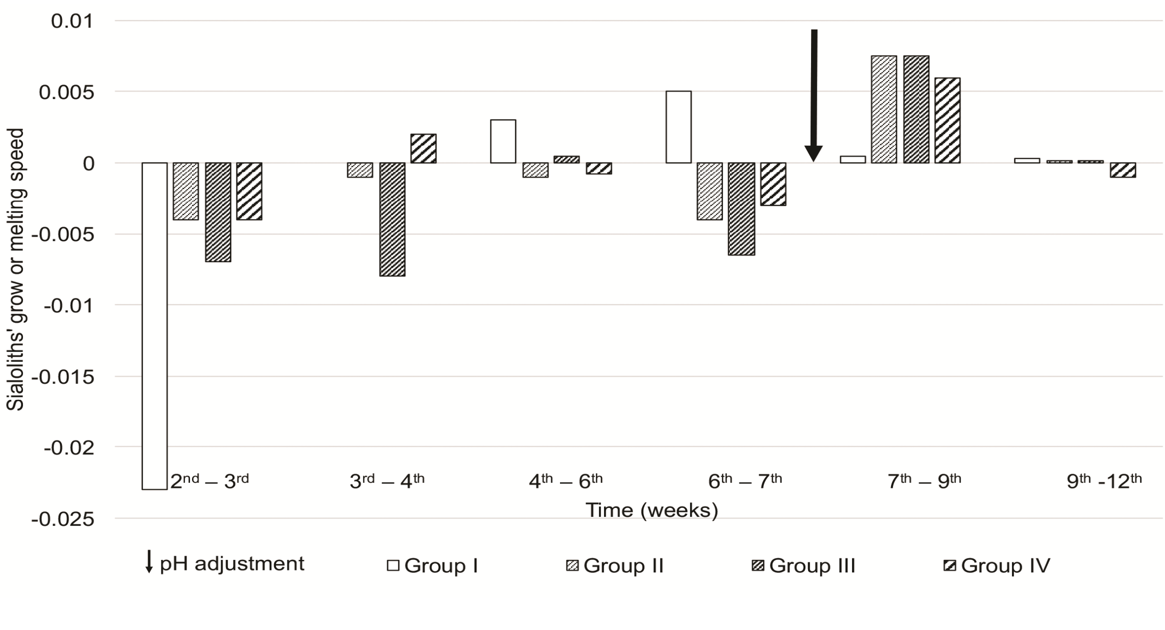
The results were assessed after estimating relative alteration in weight of salivary glands stones (the difference of weight in grams divided by the primary weight). All statistical analyses were performed using the IBM Statistical Package of Social Sciences 21.0. The statistical method used was Friedman test for testing the difference between related samples. The data is significant when $p < 0.05$.

Results:

Effects of the pH and Ca^{2+}/PO_4^{3-} ions concentrations on salivary glands stones' weights were analyzed during each week for 13 weeks. The tendency of decreasing weights was observed from the 2nd to the 7th week (a 1.24 % decrease) (**Figure I**). This tendency was manifested together with natural decrease of average pH in all test groups from 6.94 ± 0.01 (at the beginning of the experiment) to 6.69 ± 0.02 (the 7th week, when the smallest average pH was fixed). Solutions pH was adjusted in an artificial way in the 8th week. Since then, sialoliths weight started to increase. At the end of the experiment, average pH in all test groups was 6.83±0.02. Statistically significant difference was determined between all measurements of solutions pH (Friedman Exact test: $p < 0.001$)(**Figure II**).The smallest average weight of sialoliths was set in the 7th week (0.954 ± 0.425 g) and the biggest- in the 13th week (1.095 ± 0.425 g). Statistically significant difference was identified between the weight of sialoliths during the 2nd-13th week (Friedman Exact test: $p < 0.001$).

Statistically significant decrease of Ca^{2+} and PO_4^{3+} ions concentrations was determined comparing all test tubes solutions at the beginning and at the end of the experiment (accordingly: $p = 0.016$ and $p = 0.016$). Analyzing changes of Ca^{2+} ions concentrations in test tubes, it was identified that Ca^{2+} ions concentrations decreased everywhere to the same value (accordingly: from normal- 2.244 mmol/L or double- 4.488 mmol/L to 0.31-0.389 mmol/L), this is 7-12 times (**Figure III**).

Figure I: Sialolith's grow or melting speed



A statistically significant connection between primary sialolith weights and their grow or melting speed was not determined after the correlation analysis (Spearman test: $p>0.05$)

Figure II: Statistically significant difference was determined between all measurements of solutions pH without dividing into groups

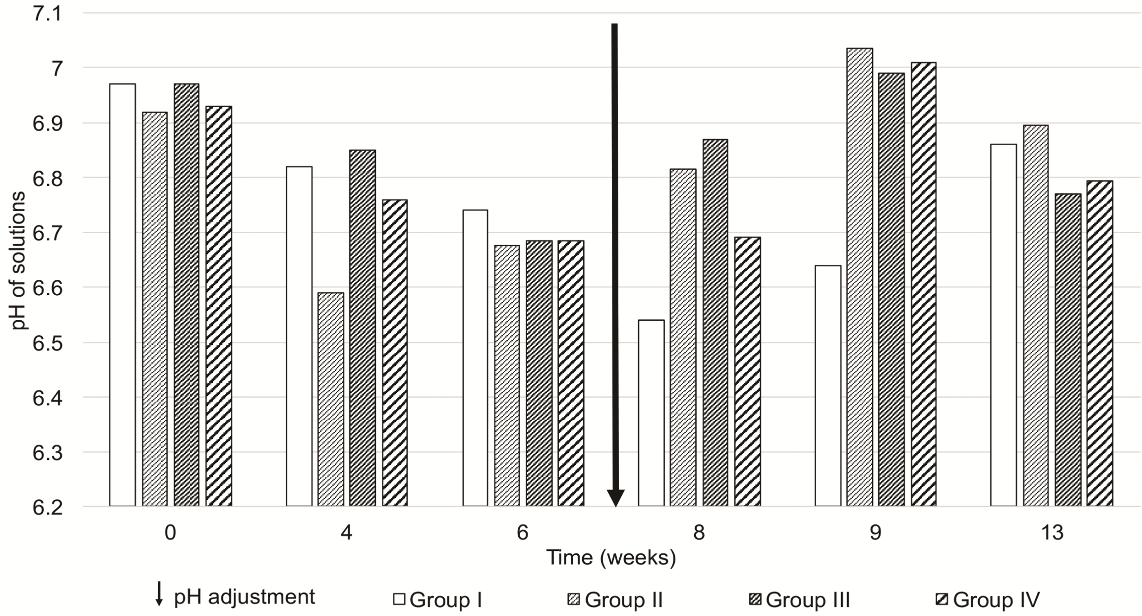


Figure III: Calcium ions (mmol/L) concentration in the artificial saliva substitute

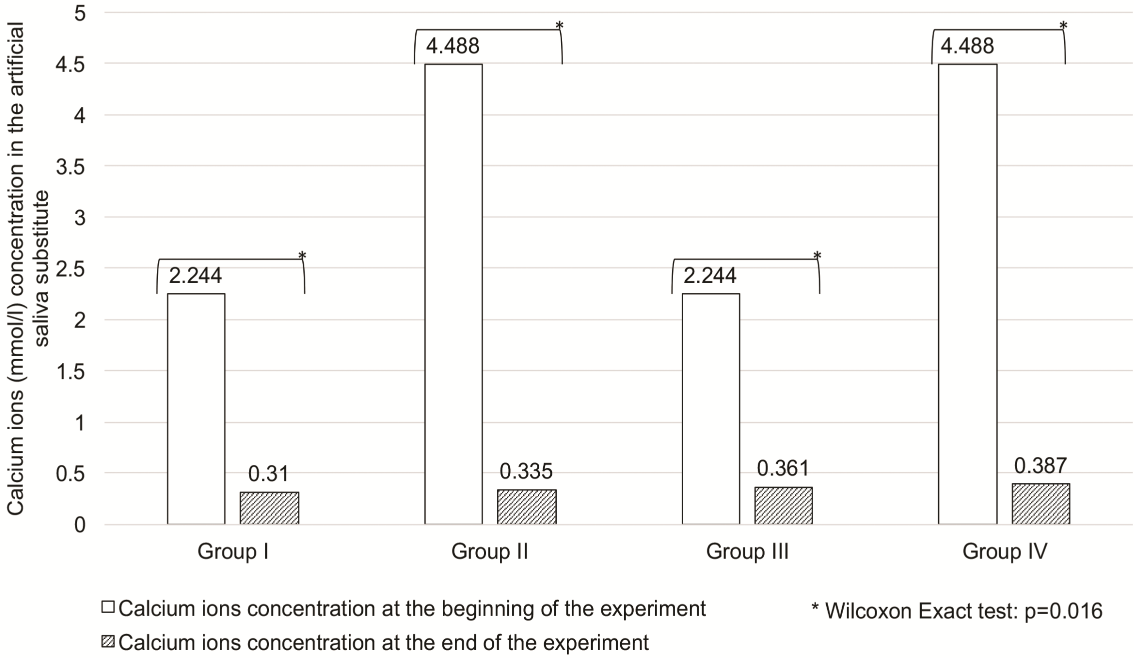
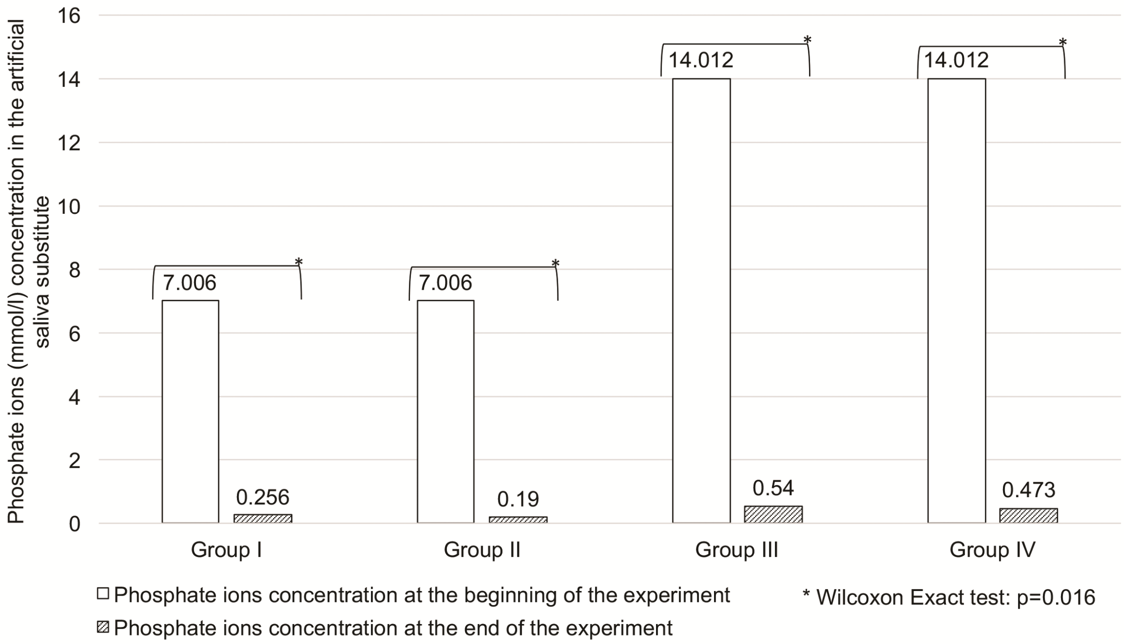


Figure IV: Phosphate ions (mmol/L) concentration in the artificial saliva substitute



p = 0.016

Analyzing the changes of PO_4^{3+} ions concentrations in all test tubes, it was determined that ions concentrations decreased everywhere 26-27 times (accordingly from 7.006 mmol/L to 0.256 mmol/L and from 14.012 mmol/L to 0.54 mmol/L) (**Figure IV**).

Analyzing the addiction between sialoliths weight changes and pH in different test tubes groups, it was ascertained that sialoliths weights were decreasing while solutions were acidifying till the 7th week in all test tubes with double Ca^{2+}/PO_4^{3+}ions concentrations and increased Ca^{2+} and PO_4^{3+} ions concentrations. Control (group No. I) sialoliths weight was decreasing from the 2nd to the 3rd week and increasing from the 4th to the 13th week despite changes of solution pH. Neutral artificial saliva pH was restored in the 8th week. From then ,average sialoliths weights were increasing in all test tubes (**Figure I**). At the end of the experiment significant weights accession (average sialoliths weight plus weight of formed participates) was determined in all samples when compared to their primary weight.

A statistically significant connection between primary sialolith weights and their grow or melting speed was not determined after the correlation analysis (Spearman test: $p>0.05$) (**Figure I**).

Discussion:

Our study indicated that calcium and phosphate ions have a significant role in preconditioning the formation of salivary gland stones. It supports previous investigations of Grases et al[5], which shows the increased salivary concentrations of these ions in patients with hydroxyapatite calculus compared to control patients. However, there are just few investigations previously done that define the association between diary calcium and its concentration in blood and salivary calcium. Schrøder et al[25] has recently found a positive correlation between calcium and magnesium ions levels in drinking water and their concentration in saliva. If differences in drinking water calcium and magnesium play a role in the incidence of sialolithiasis, presumably other nutrition sources also have an effect on salivary calcium and other ions concentrations. Our study showed that phosphate ions are used in the process of calcification relatively more than calcium ions. Decrease in Ca^{2+} and PO_4^{3-} concentrations in our experiment is explained as monocalcium, dicalcium and tricalcium phosphate precipitates formation and sialoliths weight grow. However, without knowing how these processes exactly interact, it is difficult to determine the etiology of increased calcium and phosphate ions in saliva. In addition to that, the relation between the calcium level changes in saliva and systemic diseases such as hyperparathyroidism also remains unclear. All that leads to the idea that local etiology factors are more critical for initiating sialolithiasis than systemic ones. A small amount of clinical cases when salivary gland stones are bilateral- 0.5-2.2% of all salivary stones[1]- also supports that idea.

Contrary to Shih-Han Hung et al [23] our investigations showed that salivary pH has a statistically significant impact on crystallization of sialoliths. According to our study, sialoliths are more prone to increase in weight when pH is 7.0 or higher. pH changes in vivo could be associated with retrograde theory[1,9] that bacteria from oral cavity can migrate into the ducts of salivary glands and cause inflammatory processes there leading to increased salivary pH which decreases the solubility of calcium salts, as well as performing as organic nidus that works as a frame for inorganic salts to precipitate, because of the affinity to calcium and phosphate ions.

The artificial saliva solution used in this study neither contained any organic substances, usually found in natural saliva, nor bacteria or desquamated epithelial cells that could possibly migrate from the oral cavity into salivary gland duct. That might be the reason why new sialoliths did not form during this experiment and only unstructured precipitates were found at the bottom of each flask. These results prove that organic factors are needed for sialoliths' formation.

Microscopic studies show that salivary gland stones grow in layers, which differ in composition. However, a lot of paradox can be found in the literature. Afshin Teymoortash et al [26] state that the nucleus of sialolith has inorganic composition and that outer layers are more organic, consisting mainly of glycoproteins, mucopolysaccharides, lipids and cell detritus[26]. Contrarily, Pollack et al [27] reported that the core of every sialolith is formed from organic substances, most commonly inflammatory cells, foreign bodies, mucous, which attract and bind inorganic salts. Our study proves that sialoliths grow even without organic factors, even though new sialoliths did not form.

Our findings showed that smaller sialoliths grew more in percentage (from 15.3% to 85.6%) than bigger ones (from 3.2% to 13.2%). That strengthens the previous investigations which showed that sialoliths grow in layers, usually by rotation of organic layer and inorganic layer[28, 29]. It gives an idea that smaller salivary gland stones are possibly more organic in composition, which gives a better affinity for inorganic salts to attach and the whole calcification process is more active.

Conclusions:

The results of the research have brought a clear view of a positive impact of calcium ions on sialoliths' formation encouraging more attention be paid to the role of phosphate ions, which our experiment shows to have even a bigger significance to the process of calcification. However, there is a need for more investigations to find the reasons of salivary pH changes in major salivary glands and role of bacteria, which could lead to more efficient prevention of sialolithiasis and earlier diagnosis of this disease.

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Acknowledgment: The authors would like to thank Dr. Rasa Šlinkšienė, Department of Inorganic and Physical Chemistry, Faculty of Chemical Technology, Kaunas Technology University, for her help and opportunity to perform spectrophotometric and photometric analysis during experiments.

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Conflict of interests: Nil Source of funding: Nil

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