Phosphate Concentration in Unstimulated Saliva of Patients with Type 2 Diabetes Mellitus

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Abstract

Patients with type 2 diabetes mellitus (T2DM) are at a high risk of dental caries. Phosphate plays a primary role in the buffer capacity of unstimulated saliva; thus, the concentration of phosphate is associated with individual risk factors for caries. Ketosis and hyperparathyroidism, conditions that frequently develop with T2DM, can reduce the phosphate buffer levels in the body, consequently reducing the concentration of phosphate in unstimulated saliva.

The aim of this study is to determine the concentration of unstimulated saliva from patients with type 2 diabetes mellitus (T2DM).

Unstimulated saliva samples from fifteen individuals with T2DM and 15 nondiabetic subjects were collected, and the concentration of phosphate was measured using the phosphomolybdate method with an ultraviolet–visible spectrophotometer.

There were significant differences in the phosphate concentrations ($p < 0.05$) in the saliva of the test and control subjects. The phosphate concentration in the unstimulated saliva of patients with type 2 diabetes mellitus (T2DM) ($0.27 \pm 0.05$ mmol/L) is significantly lower than that of individuals without diabetes mellitus ($2.16 \pm 0.22$ mmol/L).


Keywords: Buffer capacity, Phosphate concentration, Type 2 Diabetes mellitus, Unstimulated saliva, Patients, Phosphate.

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Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by high blood glucose levels (hyperglycemia) due to abnormal insulin secretion, insulin function, or both.¹ According to the International Diabetes Federation, more than 240 million people worldwide had DM in 2010. Type 2 diabetes mellitus (T2DM) is the most common form of DM, comprising 90–95% of the total patient population. The incidence of T2DM increases with age, but it is generally rare in children; therefore, T2DM is often referred to as a metabolic abnormality that occurs in adults. Research has shown that T2DM can cause an increase in the incidence and severity of caries (Ministry of Health, 2013).

Caries refers to an imbalance in the demineralization and remineralization of enamel and tooth dentine in the oral cavity. Cavities eventually develop owing to the occurrence of more demineralization than remineralization. Caries is a multifactorial disease with primary risk factors and modifying. Bacteria inside the plaque can ferment carbohydrate substrates (sucrose and glucose) to produce acids and cause the plaque pH to drop below 5. This decrease in plaque pH continuously causes tooth surface demineralization.³

Saliva secreted into the oral cavity is one of the main factors that protect teeth against caries, because the saliva has a buffer capacity to neutralize acids in the oral cavity. Saliva is produced from blood plasma filtration in the salivary gland acinar cells and possesses a buffer capacity because it contains phosphate ($\text{HPO}_4^{2-}$) and bicarbonate ($\text{HCO}_3^-$).² Individuals with a low salivary buffer capacity are more susceptible to caries, whereas those with a higher buffer capacity are more resistant.⁴ Saliva can be divided into two types: unstimulated and stimulated saliva. Phosphate plays a major role.
in the salivary buffer capacity in unstimulated saliva, whereas in stimulated saliva bicarbonate plays the primary role of providing a buffer effect. In resting conditions, the saliva secreted into the oral cavity is unstimulated saliva, and this is the case for most of the day.\(^5\)

Type 2 diabetes mellitus is characterized by hyperglycemia and can cause various systemic complications, such as ketosis and hyperparathyroidism, which reduce the level of the phosphate buffer in the body. The body’s compensatory mechanism responds to increased acidity in the body (i.e., ketosis) by neutralizing the acid. This process requires a phosphate buffer system and bicarbonate, and thus the body’s phosphate buffer is necessary to compensate for ketosis. Hyperparathyroidism, which is characterized by an increase in parathyroid hormones (PTHs), increases the rate of phosphate excretion into the urine by reducing phosphate reabsorption in the tubules of the kidney. With the decrease of the phosphate buffer level in the body, the phosphate levels in the saliva acting in the capacity of a buffer also decrease. As a result, patients with T2DM are more likely to experience dental caries.\(^1\)

Jawed et al. conducted a study using the colorimetric method to measure the phosphate levels in 398 unstimulated saliva samples from patients with T2DM and 395 unstimulated salivary control samples (nondiabetic). They reported that there was a significant decrease in the phosphate levels in the unstimulated saliva from patients with T2DM.\(^6\) Additionally, Pacheco et al. (in Aravindha) evaluated 30 subjects with type 1 DM, 30 subjects with T2DM, and 30 nondiabetic subjects. They found differences in the unstimulated saliva compositions of the three groups, reporting significantly higher phosphate levels for the patients with T2DM compared with the nondiabetic subjects.\(^7\) Owing of the results of this study, researchers have attempted to determine the effect of T2DM on the phosphate levels in unstimulated saliva.

Type 2 diabetes mellitus occurs as a result of insulin resistance and is often referred to as relative insulin deficiency. Individuals with this condition do not require extra insulin intake (non-insulin-dependent DM) because insulin is available but does not function properly.\(^8\) Type 2 is the most common category of DM, comprising 90–95% of the total diabetic population. The pathogenesis is influenced by hereditary, environmental, and age-related factors. Individuals who have only one parent with T2DM are at a 38% risk of developing the disease, whereas those who have both parents with T2DM are at a 60% risk. For identical twins, the risk is close to 100% if one twin develops the disease. Obesity and lack of physical activity are environmental factors that increase the likelihood of T2DM. Additionally, the condition is more common in individuals above 40 years of age (Little, 2013). Continuous hyperglycemia can cause neural complications in sympathetic and parasympathetic nerves, both of which play a role in controlling salivary secretion and can lead to decreased salivary secretion. Hyperglycemia in patients with T2DM can manifest in various ways in the oral cavity, namely, periodontal problems in the form of gingivitis and periodontitis and dental problems in the form of caries. Hyperglycemia also causes various physiological disorders, such as microangiopathy, atherosclerosis, nephropathy, cardiovascular disorders, ketosis, and hyperparathyroidism. Ketosis and hyperparathyroidism can cause the body’s phosphate buffer level to decrease.

The primary mineral component of hard tooth tissue is hydroxyapatite (HA), whose chemical structure is \(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\). Under neutral conditions, HA exists in a balanced state with \(\text{Ca}^{2+}\) and \(\text{PO}_4^{3-}\) ions dissolved in saliva.\(^2\) When intraoral pH drops to 5.5, HA reacts with hydrogen ions (\(\text{H}^+\)), causing the HA crystals to dissolve. This event is called demineralization. Apatite can re-form HA through remineralization by releasing \(\text{H}^+\) ions. When the demineralization process is greater than remineralization, dental caries occurs.\(^3\)

Salivary phosphate buffers can neutralize acids in the oral cavity by binding to \(\text{H}^+\) ions. Under the conditions of adequate phosphate levels in the saliva, the acid formed by the fermentation of carbohydrates by bacteria in the plaque can be neutralized, minimizing the demineralization process. However, under the conditions of low phosphate levels in the saliva, the acid in the oral cavity is \(\text{H}^+\) ion cannot be neutralized. As a result, the \(\text{H}^+\) ions will continue to cause the demineralization of the enamel and dental dentine.\(^3\)

Goldenberg and Fernandez (1966) measured the phosphate levels in the saliva via molybdenum reactions using spectrophotometric methods. This process was modified by Bardow...
et al. (2000) using an ultraviolet–visible (UV–Vis) spectrophotometer. Briefly, a reaction mixture (5 N sulfuric acid, 40 g/L ammonium paramolybdate, and 2.8 g/L K-Sb-tartrate) was added to the saliva sample. After 10 min of centrifugation, the supernatant was added to a 50 g/L ascorbic acid solution in deionized water. The phosphate molecules in the saliva can bind to ammonium paramolybdate to form bluish compounds that increase the salivary turbidity. The level of turbidity can be tested and converted to determine the phosphate level in the saliva. In order to perform this test, a 10 mL saliva sample and a standard solution are needed. The test is carried out at a wavelength of 680 nm, and the results of the test indicate the phosphate levels in the samples.  

Materials and methods

Fifteen people with T2DM (test group) and 15 nondiabetic subjects (control group) who met the inclusion criteria were provided with an explanation of the research to be conducted and asked to sign an informed consent form if they understood and consented to be research subjects. The test and control subjects were asked to refrain from eating and drinking for 2 h before the collection of unstimulated saliva samples and were asked to come to the Yudia Diabetes Clinic in Bekasi, Indonesia, on the agreed upon date between 9:00 and 11:00 a.m.

On the day of the test, the subjects were asked to sit back and allow unstimulated saliva to accumulate at the base of their oral cavities. They were then asked to spit into a collection tube every 5 min until the volume in the tube reached 10 mL over a 30 min period. The collected saliva was stored in a cooler box until testing. The control subjects were all allowed to rest as needed.

In order to test the phosphate levels in the unstimulated saliva samples, the samples were homogenized with a vortexer and diluted with aquadest. Afterwards, 20 mL of the diluted sample was added to 1 mL of an ascorbic acid solution and homogenized with a vortexer. The sample was then added to 4 mL of combination reagents and left for 30 min until the mixture turned blue. The sample was tested using a UV–Vis spectrophotometer at a wavelength of 780 nm to determine the phosphate level in the sample by comparing it to a standard phosphate solution with a known phosphate concentration.

The data obtained was analyzed statistically using SPSS version 21 software (IBM Corp., Armonk, NY, USA), and a data normality test was performed using the Shapiro–Wilk test. If the data distribution was normal, an independent t-test was performed with a significance value of 5%. However, if the data distribution was not normal, the Mann–Whitney U test was performed with a significance value of 5%.

Results

Data on the phosphate levels in unstimulated saliva from patients with T2DM and individuals without DM were tested for normality using the Shapiro–Wilk test because the number of samples was less than 50. The distribution was normal for both groups (diabetic group, \( p = 0.302 \); nondiabetic group, \( p = 0.231 \)). The phosphate content was subsequently evaluated with the independent t-test, which yielded a p-value of 0.00. Table 1 shows the significance of the phosphate levels in the unstimulated saliva from the study subjects. There was a significant difference in the levels of phosphate in the unstimulated saliva from the two groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>( N )</th>
<th>Mean ± SD</th>
<th>Average differences (IK95%)</th>
<th>Sig. (( \rho ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2DM</td>
<td>15</td>
<td>0.27 ± 0.05</td>
<td>1.89 (1.77–2.01)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td>15</td>
<td>0.16 ± 0.22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD: standard deviation; IK95%: ---.

Table 1. Significance of the Phosphate Levels in Unstimulated Saliva from Individuals with and without T2DM Evaluated Using the Independent t-Test.

Discussion

This study was conducted with the aim of analyzing the phosphate levels in unstimulated saliva from patients with T2DM to support already existing results regarding the increased risk of dental caries in this population. The results of this study are expected to contribute to the development of dentistry, especially regarding the phosphate level in unstimulated saliva and its relationship with the salivary buffer capacity in patients with T2DM. The study will also
contribute to clinical dentistry by providing insights into the prevention of caries in patients with T2DM as well as providing information to the public regarding the risk factors of caries related to the saliva.

The minimum sample size was determined from manual calculations and was strengthened by calculations in the GPower 3.1 application. Both produced the same results, namely, nine samples for each group. Therefore, the data obtained can represent the actual clinical situation and covers samples that cannot be read in laboratory tests. The research subjects were selected from patients who visited the Yudia Diabetes Clinic in Bekasi, Indonesia, and met the predetermined inclusion and exclusion criteria.

Researchers used unstimulated saliva because, in the resting condition without any stimulus, only unstimulated saliva is secreted into the oral cavity, making it the dominant type of saliva secreted throughout most of the day. Phosphate levels were evaluated because phosphate molecules play a role in the salivary buffer capacity with bicarbonate molecules and proteins, particularly in unstimulated saliva. The buffer capacity neutralizes the acid produced by acidogenic bacteria; thus, it can prevent and minimize the process of demineralization in the tooth structure, which leads to the occurrence of dental caries.

Individuals who had T2DM for at least 10 years were chosen for this study because Kihm (2016) reported that ketosis and hyperparathyroidism are found after 10 years of the disease. In addition, T2DM generally occurs in individuals above 35 years of age. Therefore, the test subjects in this study were above 45 years of age. Smoking and drinking alcoholic beverages are associated with disruptions in the human physiological system. The subjects in this study had no habits of smoking or drinking alcoholic beverages. In order to minimize the occurrence of discomfort, subjects were excluded if the saliva collected within 30 min was less than 10 mL. The control group comprised individuals of the same age and gender as the test group subjects who did not suffer from T2DM or systemic diseases so that the data could be compared.

The research was cross-sectional. Sampling was carried out in the morning between 09:00 and 11:00 a.m., taking circadian rhythms into account, as the quantity of unstimulated saliva secreted reaches its peak during this period. Subjects were asked not to eat or drink for 2 h before sampling to prevent contamination of the saliva in the oral cavity, which can lead to bias in the data due to the presence of phosphate molecules derived from food or beverages consumed by the subject.

During sample collection, the subjects were asked to sit back passively and relax. They were asked to spit out the saliva that had accumulated at the bottom of their mouths into a glass funnel until the collected saliva reached 10 mL over a period of 30 min. This 30 min time window was chosen owing to the expectation that the salivary flow rate ranges from 0.3 to 0.5 mL/min in accordance with the article published by Edgar et al. (2012). According to their results, 10 mL of unstimulated saliva can be collected within 20–30 min.

The collected samples were stored in a cooler box containing ice cubes to minimize the chemical and biological processes and sent to the Analytical Chemistry Laboratory at the Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia. At the laboratory, the samples were tested with a UV–Vis spectrophotometer using the phosphomolybdic acid method to quantify the phosphate levels in the sample in units of millimoles per liter.

The independent t-test showed that there was a significant difference in the level of phosphate in the unstimulated saliva for the two groups evaluated. The average phosphate level in the unstimulated saliva from patients with T2DM was significantly lower than that for the control group. These results are in accordance with the hypothesis of this study that the phosphate levels in unstimulated saliva are lower in patients with T2DM.

In this study, the average phosphate level in the unstimulated saliva from patients with DM was 0.27 ± 0.05 mmol/L, whereas the level from nondiabetic individuals was 2.16 ± 0.22 mmol/L (Table 1). These values were slightly higher than those obtained in the study conducted by Lorenzo et al. (2014) for both the test and the control groups (0.21 ± 0.03 mmol/L and 1.04 ± 0.01 mmol/L, respectively). In other words, the research subjects in this study, namely, Indonesian people suffering from T2DM, experienced a more significant decrease in
unstimulated phosphate levels compared to Lorenzo’s research subjects, who were Americans. This is likely due to the differences in the food and lifestyle of Indonesians and Americans. Thus, the nutrient intake levels of phosphate are also different. Surdilovic et al. (2009) reported that the phosphate level in individuals aged 20–35 who did not suffer from any systemic disease was 7–10 mmol/L. Dodds et al. (2012) stated that the composition of phosphate in the human saliva decreases along with advancing age. The results of this study are in line with these two studies. The results also suggested that individuals without DM, aged above 45, experience a decrease in the phosphate levels in unstimulated saliva, although less than the decrease experienced by patients with T2DM. Therefore, the restoration of the phosphate buffer function in unstimulated saliva is also important for individuals above 45 years of age.

The results of the present study can be attributed to the fact that there is a disruption in the fat metabolism in patients who have had T2DM for more than 10 years. During this process, the oxaloacetate needed to bind to the acetyl-CoA in the Krebs cycle is hydrogenated to malate in the liver. As a result, the acetyl-CoA is converted into β-hydroxybutyric acid, acetoacetic acid, and other ketogenic acids (ketones). These acidic ketones are released into the bloodstream, a condition called ketonemia, which is followed by urinary ketone excretion, also known as ketonuria. When ketonemia and ketonuria occur simultaneously, the condition is called ketosis. Physiologically, the body activates a compensatory mechanism using its own phosphate and bicarbonate buffer system to neutralize the acid sourced from the ketone. As a result, the level of phosphate in the blood decreases, followed by a decrease in the phosphate levels in the saliva as blood plasma filtrate.

A decrease in the phosphate levels in unstimulated saliva can also occur because of osmotic diuresis, which causes hyperparathyroidism or an increase in the PTH. Under osmotic conditions, the body experiences a lack of calcium in the blood due to the large amount of calcium wasted through the urine (Sherwood, 2013). Physiologically, the parathyroid gland secretes the PTH, which can increase the calcium levels in the blood through increased absorption and calcium reabsorption. However, in addition to increasing calcium absorption, the PTH also increases phosphate excretion in the kidneys to prevent calcium from binding to phosphate and depositing in the bone. As a result, the phosphate levels in the blood decrease, followed by a decrease in the phosphate levels in the saliva as blood plasma filtrate.

The weakness of this study is that it took the subjects a long time to collect 10 mL of unstimulated saliva and that many subjects gave up because they could not reach 10 mL within 30 min. These individuals were replaced by others to maintain the expected number of subjects, namely, 30 individuals. A sample volume of 10 mL was collected in anticipation of repetitions or unread tests. However, the laboratory reported that all samples collected could be read with good phosphate levels in one test.

Another limitation is that, in this study, we only investigated the buffer capacity of unstimulated saliva and not the overall salivary buffer capacity. In order to address this limitation, additional evaluation with saliva buffer kit 2 is needed.

A decrease in the phosphate levels in unstimulated saliva can reduce the salivary buffer capacity, making the acids produced by acidogenic bacteria on the tooth surface difficult to neutralize. This prevents acidic H+ ions from binding to phosphate molecules, which causes the oral cavity to become acidic and leads to tooth structure demineralization. In other words, there is an increased risk of caries in the affected individuals. The results of this study demonstrate that the average phosphate level in unstimulated saliva from patients with T2DM is significantly lower than that from normal individuals. This is in agreement with various studies showing that people with T2DM are more susceptible to dental caries and also exhibit a high incidence of dental caries.

Minimal intervention dentistry (MID) is a philosophy used in the practice of modern dentistry. This philosophy is aimed at maintaining the original tooth structure as much as possible by evaluating the risk factors for caries, prioritizing early prevention, and inhibiting the development of caries. MID consists of four main principles: recognition, reduction, regeneration, and repair. The principle of
recognition emphasizes the identification of the risk factors of caries early through lifestyle analyses, saliva diagnostic tests, and plaque. The reduction principle focuses on the elimination or minimization of the risk factors of caries through diet and lifestyle changes and increasing the pH of the oral cavity to near-neutral levels. The principle of regeneration is to limit the development of caries and restore the tooth structure if it is still in the reversible stage. Finally, the principle of repair is applied when a cavity has already formed, that is, by conservatively removing the noninvasive caries tissue to maximize restoration and maintain tooth structure.\(^\text{20}\)

In accordance with the MID, patients with T2DM need to be educated on their high risk of caries. Dental caries is a preventable disease, and the best strategy for treating this condition is to prevent it before clinical signs and symptoms appear. Therefore, individuals above 45 years of age with or without T2DM should be advised to change their lifestyle, such as improving their oral hygiene and starting a low-sugar diet. Studies conducted by Mickenaustch et al. demonstrated that chewing xylitol gum after meals can reduce the progression of carious lesions.\(^\text{21}\) Clinicians can also provide topical fluoride to reduce the risk of caries.

In order to help remineralize and restore the phosphate buffer function, teeth can be treated with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), which has been shown to have anticariogenic effects in various studies in laboratories (\textit{in vitro}) as well as in animals and humans (\textit{in vivo}). CPP-ACP is useful for phosphate replacement because it can create an environment in the oral cavity that is rich in calcium and phosphate, increasing the phosphate levels in the saliva and the saliva buffer capacity of patients with T2DM.\(^\text{22}\) The appropriate CPP-ACP dosage for diabetic patients varies.\(^\text{2}\)

CPP-ACP reacts with acids, forming dissolved calcium and phosphate stabilized by casein phosphopeptide, to provide phosphate and calcium molecules in the saliva, especially in the form of CaHPO\(_4\), which can improve tooth remineralization. CPP-ACP buffers plaque pH and produces calcium and phosphate, particularly CaHPO\(_4\). The increase in CaHPO\(_4\) in the plaque offsets the decrease in pH, preventing the demineralization of teeth. Acids are also produced in the plaque as phosphoric acid (H\(_3\)PO\(_4\)) along with the formation of HA crystals during remineralization. However, CPP-ACP uses the resulting phosphoric acid to produce more CaHPO\(_4\), ensuring that the reaction continues toward the remineralization of the teeth.

Patients with T2DM who have ketosis and hyperparathyroidism must be well controlled. They can also be given drugs to reduce their PTH secretion. In addition, specialists should evaluate these individuals for disorders of the parathyroid gland. Patients with T2DM who experience hyposalivation can receive long-term care with pilocarpine, a drug that can increase the flow rate of the saliva to improve the self-cleansing mechanism. These patients can be treated with artificial saliva containing various kinds of electrolytes that are normally present in the saliva, including phosphate. Artificial saliva has the same viscosity as the actual saliva and is comfortable to use. In addition, moisturizing gels containing phosphate can be used to replace the saliva\(^\text{2}\) phosphate buffer.

\section*{Conclusions}

Phosphate levels in unstimulated saliva from patients with T2DM are significantly lower compared to individuals without DM.

\section*{Suggestions}

(1) For further research on salivary buffer capacity in patients with T2DM, a test kit buffer can be used to test the buffer capacity of stimulated saliva.

(2) For studies in which the phosphate levels in the saliva are tested, the amount of saliva needed for only one laboratory test should be collected to minimize the sampling time and discomfort for the research subjects.

\section*{List of abbreviations}

\begin{itemize}
  \item DM Diabetes mellitus
  \item MID Minimal intervention dentistry
\end{itemize}

\section*{Declaration of Interest}

The authors report no conflict of interest.
References