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Original Article

Salivary enzymes and flow rate: Markers of peptic ulcer

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ABSTRACT

Aim: Peptic ulcers are among the most common and most costly diseases known to human. Its diagnosis in the early stages would have great benefit in treatment of the disease. The aim of this study was to compare alternations of enzymes activity in salivary fluid between patients with peptic ulcer disease and healthy controls.

Materials and methods: Two groups each composed of 26 subjects (13 male and 13 female) patients with peptic and healthy controls entered. Activities of selective salivary enzymes were assayed by a spectrophotometric method.

Results: The results showed significant increased activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as well as total salivary protein (TSP) concentration in peptic ulcer patients as compared to control group. However, the enzymatic activity of alkaline phosphatase (ALP) also increased in peptic ulcer patients as compared to healthy control, but not statistically significant.

Conclusions: Based on the obtained results it was concluded that peptic ulcer may be early diagnosed by detecting activity of salivary enzymes. Therefore the saliva can be suggested as a diagnostic fluid.

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1. Introduction

Peptic ulcer disease (PUD) is a common medical problem in emergency medicine,¹ which seriously affects the quality of life for patients as well as the national healthcare systems.^{2,3} Peptic ulcers are open craters or sores that develop in the inner lining of the stomach (gastric ulcer) or the duodenum (duodenal ulcer). A coating of mucus and other chemicals normally shields the stomach and duodenum from digesting themselves. If the protective mechanisms are disrupted, strong digestive acids

can erode into the lining of these organs and cause peptic ulcers.⁴ Therefore, peptic ulcer could be the result of an imbalance between offensive and defensive factors in the gastric and duodenal mucosa. This may be caused by a number of factors; the principal causes by *Helicobacter pylori* infection, and the use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin.⁵ Smoking and age are among other risk factors which may alter mucosal resistance.⁶ Its morbidity and mortality is estimated around 5%–10% over the past decade.^{7,8}

Saliva, the aqueous fluid found in the oral cavity, is composed of a complex mixture of secretory products from

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the salivary glands and other substances originating from the oropharynx, upper airway, gastrointestinal reflux, gingival sulcus fluid, food deposits, and blood-derived compounds.⁹ It is composed of water (99.5%), proteins (0.3%) and inorganic and trace substances (0.2%).^{10–12} The proteins in saliva (1–2 mg/ml) are mainly constituted by glycoproteins,¹⁰ enzymes, immunoglobulins, and a wide range of peptides having antimicrobial activities.^{10,12–14} The inorganic fraction of saliva contains the usual electrolytes of the body fluids, but at different concentrations making saliva a hypotonic fluid. Human saliva plays an important role in esophageal physiology, the digestive process, and gastric cell protection. In the oral cavity, saliva takes part in mastication, speech, deglutition, gustatory sensitivity, tissue lubrication, mucosal protection against invasion, anti-bacterial, anti-fungal, and antiviral activity, and acid diffusion limitation.^{15–17} The amount and composition of secreted human saliva depends on many factors, i.e. the flow rate, circadian rhythm,^{18–21} type and size of the salivary gland,^{22,23} duration and type of the stimulus, diet, drugs, age, gender and blood type^{24,25} and physiological status.²⁶ Stimuli for increased salivation include the presence of food or irritating substances in the mouth, and the sight or the smell of food.²⁷ The rate of saliva secretion ranges from 0.3 to 7 ml saliva per minute²⁸ with about 0.5–1.5 L of saliva secreted per day.¹² Salivary pH can range from 6.2 to 7.4, with the higher pH exhibited upon increased secretion.

Saliva is a clinically informative, biological fluid that is useful for novel approaches to prognosis, laboratory or clinical diagnosis, and monitoring and management of patients with both oral and systemic diseases. It is easily collected and stored and ideal for early detection of disease as it contains specific soluble biological markers.²⁹

Intracellular enzymes are among the most important salivary biochemicals which are released in response to a variety of diseases. Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), tyrosinase, amylase and many other enzymes are found and reported in salivary fluid.

ALP (EC 3.1.3.1) is present in almost all body tissues, particularly in intestinal epithelium, kidney tubules, bone, liver, and placental. Alkaline phosphatase catalyzes removal of phosphate from a number of different molecules. The enzyme works best in an alkaline environment/

AST, also called serum glutamate-oxaloacetate transaminase (SGOT) is a pyridoxal phosphate (PLP)-dependent transaminase (EC 2.6.1.1). It catalyzes the reversible transfer of an α -amino group between aspartate and glutamate. Under normal conditions, AST is found in most tissues and fluids of the body except urine.

ALT (EC 2.6.1.2), also known serum glutamate-pyruvate transaminase (SGPT) is a pyridoxal enzyme which belongs to pyridoxal-phosphate-dependent aminotransferase family. It catalyzes the reversible interconversion of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. ALT is found in a variety of tissues, mainly in the liver.

In this study the enzymatic activity of ALP, ALT, AST as well as total protein concentration, flow rate and pH were compared in saliva of peptic ulcer patients with healthy controls. It was expected that the results could explain the possible role of saliva in prevalence of peptic ulcers. The other

goal was to establish the validity of salivary fluid as a noninvasive easily accessed body fluid in diagnosis of the disease.

2. Material and methods

2.1. Materials

All the chemicals and solvents were of reagent grade used without further purification unless otherwise stated. Assay kits for alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase were obtained from local importer representatives in Iran. Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Pharmacia Biotech (Ultrospec 3000) spectrophotometer was used throughout the experiments. All buffers were freshly prepared in our biochemistry laboratory.

2.2. Participants

Two groups each composed of 26 (13 male, 13 female, aged 19–62 years) were selected to enter the research. The first group was patients diagnosed (by endoscopy) as suffering from peptic ulcers, either chronic gastritis or duodenal ulcer in various degree of severity, Table 1 in Gastrointestinal and Liver Disease Research Center, Guilan University of Medical Sciences. The second group composed of healthy volunteers from staff or students in Razi Medical Center, Rasht used as controls. Before collecting their unstimulated saliva samples, the aim of research was explained to them informed consent obtained. The moral of the project was approved by of the Guilan University of Medical Sciences.

2.3. Saliva collection

As a diagnostic fluid, saliva offers superiority over serum due to both a noninvasive collection method, short sampling time and the cost-effective approach for screening of large populations. Collection of saliva offers a reduced risk of infection compared to serum. The subjects were asked to remain fasted for 8 h prior to sampling. They were instructed to rinse their mouth with distilled water and their oral cavity was examined for the lack of foreign material. They were then asked to store their unstimulated saliva for exactly 3 min and pour the samples in clean, dry, and sterile tubes by spitting method.²³ The volume of collected saliva was measured for determining the salivary flow rate (ml/min). The samples of saliva were centrifuged at 3000 rpm at 4 °C for 10 min for to remove insoluble material and cell debris. The value of pH in resulting supernatant fluid was measured using a digital pH meter

Table 1 – Demographic characteristics of patients and their peptic ulcers.

	Age (years)	Number of ulcers	Age of ulcer (months)
Mean	44.2	2.8	35.6
SD	24.4	0.6	8.5
Range	15–70	1–4	12–60

instrument (Model 3510, Jenway, UK). Aliquot quantities were stored at -80°C until biochemical analysis.

2.4. Alkaline phosphatase assay

The activity of ALP was assayed using a kit based on a photometric test recommended by the German Society of Clinical Chemistry (DGKC). The method, used *p*-nitrophenylphosphate (*p*-NPP) as substrate. In principle, *p*-NPP is hydrolyzed specifically by the ALP in pH 9.8 producing *p*-nitrophenol, which is measured in spectrophotometer at 405 nm. The rate of *p*-nitrophenol release is proportional to the enzyme activity.³⁰

The unit of enzyme activity was defined as the quantity of enzyme that catalyzes the reaction of 1 mmol of substrate per minute. The catalytic concentration was then expressed as U/L.

2.5. Aspartate aminotransferase assay

The activity of AST was measured according to the recommendations of the Expert Panel of the IFCC (International Federation of Clinical Chemistry) without pyridoxal phosphate activation. AST catalyzes the transfer of amino group between α -aspartate and α -ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH in the presence of malate dehydrogenase to form NAD^+ . The rate of oxidation of NADH to NAD^+ is then measured as a decrease in absorbance (340 nm) which is proportional to the SGOT activity in the sample.³¹

2.6. Assay of alanine aminotransferase

The activity of ALT was also assayed according to an optimized UV-test recommended by.³¹

The increase in pyruvate concentration is measured in a subsequent indicator reaction which is catalyzed by lactate dehydrogenase.

The rate of decrease in NADH (Measured photometrically in 340 nm) is directly proportional to the rate of formation of pyruvate, and thus the ALT activity.

2.7. Salivary total protein concentration

The total protein content of the centrifuged saliva samples was determined by Bradford using bovine serum albumin as standard.³²

2.8. Statistical analyses

The results were expressed as mean $\pm$ SD. Data were tested for normal distribution using Kolmogorov–Smirnov before statistical procedures were applied. Statistical differences between the two groups were determined according to student's *t*-test. A *p*-value < 0.05 was considered to be significant. Statistical analysis was performed using the SPSS statistical package for Windows version 21 (Statistical Package for Social Sciences (SPSS), Inc, Chicago, IL, USA).

3. Results

Table 2 has summarized the salivary flow rate, pH value, enzymatic activities and concentration of total salivary protein in patients and controls. Statistical analysis using the student *t*-test revealed the presence of a highly significant difference in many parameters between the study and the control groups.

3.1. Flow rate

A summary of all biochemical and physical parameters examined in this study is presented in Table 2. Salivary flow was significantly lower ($p < 0.05$) in peptic ulcer patients as compared to healthy controls (Table 2). In a normal condition the average rate of salivary flow has shown to alternate between 1.0 and 1.5 L.³³ However, a considerable percent of this volume is swallowed. Considering that bile secretion plays the causative role in pathogenesis of peptic ulceration, the salivary mucus swallowed with food could protect the gastrointestinal tract against peptic ulcer as it decreases the flow rate of bile.³⁴ According to the results (Table 2) the flow rate in both patients and control group decreased gradually with the age of volunteers (Fig. 1). Reduction in salivary flow rate with age has also been reported in response to hormonal changes during puberty, menstruation, pregnancy and menopause.³⁵

3.2. Salivary pH

Table 2 also presents the results of pH measurement for both groups. It was noted that salivary pH was significantly lower compared to the control group. The reduction in pH could be explained in terms of flow rate. When the rate of salivary flow is decreased less bicarbonate could be released, leading to a decrease in value of pH. It has also been shown that pH of saliva could be varied widely between subjects possibly due to

Table 2 – Differences between salivary parameters examined in patients and controls.

Salivary parameter	Patients (mean $\pm$ SD)	Controls (mean $\pm$ SD)	<i>p</i> -Value
Flow rate (ml/min)	0.62 $\pm$ 0.24	0.85 $\pm$ 0.25	0.022
pH	6.25 $\pm$ 0.3	7.25 $\pm$ 0.3	0.028
AST (U/L)	23.79 $\pm$ 4.7	21.03 $\pm$ 3.2	0.018
ALT (U/L)	19.11 $\pm$ 3.44	16.95 $\pm$ 3.17	0.023
ALP (U/L)	176.32 $\pm$ 14.32	168.4 $\pm$ 13.32	0.044
TSP (mg/ml)	0.41 $\pm$ 0.06	0.36 $\pm$ 0.05	0.003

Fig. 1 – Dependence of salivary flow rate with age of both groups of subjects.

a decrease in the salivary flow rate.¹² A decrease in buffering capacity of saliva in patients with peptic ulcer may lead to their lower pH values compared to healthy subjects. However, lower pH may be the result of *H. pylori* growth, one of the major causes of peptic ulcer disease. It is worth indicating that pH value of saliva showed no relationship with age or sex of subjects and only slightly different within subjects (Fig. 2). Supportive to this result, it has been found that pH of saliva remains constant during the different stages of life for regardless of age.³⁶ The pH value of saliva is reported to be among a series of salivary markers associated with end stage renal disease.^{37,38}

However, any alternation of salivary pH among different people could also be the result of food intake, diseases such as peptic ulcers and presence of infections in the oral cavity.³⁶

3.3. Salivary enzymes

It was found that the three tested enzymes had higher values in saliva of patients compared to the control group.

In the present research, all salivary parameters were tested triplicate for each case and the value of all patients were summarized as presented in Table 2. It was found that the mean activity of alkaline phosphatase, 176.32 U/L in patients

had significantly higher ($p < 0.05$) value as compared to control group (168.4 U/L). It was noted that almost no relationship existed between age of subjects and activity of their salivary ALP. The range ALP activity was from 168 to 182 U/L regardless of age and sex of patients. On the other hand, this range was 160–176 U/L in the case of control group and once again no relationship was observed which could relate the values to age or sex of volunteers. A few other research works were found to have obtained similar results, i.e. increase of salivary ALP in disease or abnormal conditions.^{39,40} Most of studies on salivary ALP activity are related to the disorders in the oral cavity itself including periodontitis.⁴¹ It has been shown that the activity of ALP in the acute phase of periodontal disease, and after the periodontal therapy, the activity restored to the value as found with the healthy persons. In the case of gastrointestinal disorders, ALP has not been studied and this is the first report of its kind.

On the other hand, the mean activity of AST was 23.79 U/L in patients which is significantly higher ($p < 0.05$) as compared to control group (21.03 U/L). One of the host responses to any cell destructive disease is production, increase of activity or release of different enzymes by the inflammatory cells. It is, therefore, expected that the activity of these types of enzymes are increased followed by their decrease after remediation. We suggest that increase observed in AST in peptic ulcer patients may be declined if the ulcers are healed. Supportive to this study, positive correlations have been reported for the activity of AST in saliva of patients suffering from periodontitis.⁴² However, it has been found that after conventional periodontal therapy the activity of all salivary enzymes was significantly decreased.

According to the results of the present study, the mean activity of ALT was 19.11 U/L in patients which is significantly higher ($p < 0.05$) as compared to control group (16.95 U/L). The important point to be noted was that alternation observed in ALT activity was a function of health status i.e. peptic ulcer or healthy subject, but it showed no relation to the age and sex of all subjects. In the case of this enzyme, too, the studies so far have concentrated on the cell destructions in the oral cavity mainly due to periodontal problems.⁴² The activity of alanine aminotransferase showed a higher value in peptic ulcer patients. It can be suggested that cell destructions occurred due to ulcers have caused the release of the intracellular enzymes into the saliva and blood. A few rare studies have reported the activity of ALT in salivary fluid and they are commonly related to problems in the oral cavity.⁴² Our investigations of these types of enzymes which are released into the biological fluids in response to a cell destruction is continuing with the aim of opening a simpler way to investigate internal diseases at their early stages.

3.4. Total protein

Measurement of total protein is a non-specific method, suitable to indicate the amount of all proteins present in a solution. It is used to examine changes in overall protein secretion in blood or saliva that are associated with a certain disease. According to the results of this study, the mean concentration total protein in saliva of patients significantly higher ($p = 0.003$) than its mean value in control group (0.41 compared

Fig. 2 – pH value of saliva for the two groups showing the absence of its relationship with age or sex.

to 0.36). The increase in concentration of certain substance in saliva is expected when one considers the lower volume of saliva in patients due to slower flow rate. Difference in salivary protein concentrations has been investigated in HIV-infected patients with respect to their disease phase with a follow up for 2 years.⁴³ It has been found that total salivary protein levels were significantly higher in all phases of HIV infection. The follow up research showed that after 2 years, salivary total proteins were still higher in all HIV phases when compared with the control group. The amount of total protein has also been measured in salivary fluid of patients with oral squamous cell carcinoma.⁴⁴ It has been found that salivary level of total proteins was significantly higher in oral squamous cell carcinoma cases than controls.

4. Discussions

The physicochemical and biochemical properties of saliva along with its complex composition endowed this fluid with multiple functions, including: anti-bacterial, anti-viral and anti-fungal properties; buffering capacity for plaque acids; digestive activity (amylase, protease, nuclease enzymes) needed for food mastication; mineralizing agents for protection and repair of hard tissues; lubricant and viscoelastic properties essential for the maintenance of oral health; and protective and repairing fluid for mucosal surfaces. The thin layer of epithelial cells separating the salivary ducts from the systemic circulation enables the transfer of substances to the saliva by active transport, or passive diffusion via a concentration gradient. One of the special advantages of using saliva as a diagnostic media is that its sampling is easy and noninvasive, thus eliminating any discomfort and pain associated with blood collection while also avoiding privacy issues associated with urine collection. The biochemical composition of saliva is not as complex or varying as serum, and should more accurately reflect the current condition of the body. It is worth emphasizing that a noninvasive collection method not only simplifies a patient's ability to take repeated samples for long-term disease monitoring, but significantly reduces the pain and anxiety that is typically associated with blood tests. Various salivary chemical constituents including enzymes, uric acid vitamins and hormones have been analyzed in autoimmune diseases, systemic diseases, Sjögren's syndrome,⁴⁵ oral squamous cell carcinoma^{44,46} and hormonal disorders.⁴⁷ However, to best of our knowledge, this is the first study to evaluate the salivary transaminases in peptic ulcer patients in order to show their relation with relation to the disease. Considering that intracellular enzymes including AST, ALP and ALT released from any damaged cell into the blood could be transported to saliva, the use of saliva as an alternative to blood sampling could not only reflect the periodontal alternation inside the oral cavity, it also could guide a physician for diagnosis of cell damages in other parts of body caused by various diseases.

5. Conclusions

Based on the findings from the present study, it may be concluded that saliva can be used as a possible noninvasive

body fluid for diagnosis and detection of peptic ulcer in early stages. It can be assumed that activity of these enzymes in saliva could be useful as biochemical markers for damage caused by peptic ulcers and may be help early diagnosis, prognosis and evaluation of therapy effectiveness in peptic ulcer disease.

Conflicts of interest

All authors have none to declare.

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REFERENCES

1. Yavorski RT, Wong RK, Maydonovitch C, Battin LS, Furnia A, Amundson DE. Analysis of 3,294 cases of upper gastrointestinal bleeding in military medical facilities. *Am J Gastroenterol.* 1995;90:568–573.
2. Barkun A, Leontiadis G. Systematic review of the symptom burden, quality of life impairment and costs associated with peptic ulcer disease. *Am J Med.* 2010;123(358–366):e2.
3. Viviane A, Alan BN. Estimates of costs of hospital stay for variceal and nonvariceal upper gastrointestinal bleeding in the United States. *Value Health.* 2008;11:1–3.
4. Feldman M, et al. *Sleisenger & Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management.* 9th ed. Philadelphia, PA: Saunders Elsevier; 2010.
5. Yuan Y, Padol IT, Hunt RH. Peptic ulcer disease today. *Nat Clin Pract Gastroenterol Hepatol.* 2006;3:80–89.
6. Lockrey G, Lim L. Peptic ulcer disease in older people. *J Pharm Pract Res.* 2011;41:58–61.
7. Lim CH, Vani D, Shah SG, Everett SM, Rembacken BJ. The outcome of suspected upper gastrointestinal bleeding with 24-hour access to upper gastrointestinal endoscopy: a prospective cohort study. *Endoscopy.* 2006;38:581–585.
8. Barkun A, Sabbah S, Enns R, et al. The Canadian Registry on Nonvariceal Upper Gastrointestinal Bleeding and Endoscopy (RUGBE): endoscopic hemostasis and proton pump inhibition are associated with improved outcomes in a real-life setting. *Am J Gastroenterol.* 2004;99:1238–1246.
9. Dodds MW, Jonson DA, Yeh CK. Health benefits of saliva: a review. *J Dent.* 2005;33:223–233.
10. Van Nieuw Amerongen A, Bolscher JGM, Veerman ECI. Salivary proteins: protective and diagnostic value in cardiology? *Caries Res.* 2004;38:247–253.
11. Zalewska A, Zwierz K, Zólkowski K, Gindziński A. Structure and biosynthesis of human salivary mucins. *Acta Biochim Pol.* 2000;47:1067–1079.
12. Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. *J Prosthet Dent.* 2001;85:162–169.
13. Troxler RF, Offner GD, Xu T, Vanderspek JC, Oppenheim FG. Structural relationship between human salivary histatins. *J Dent Res.* 1990;69:2–6.
14. De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. *Biotechnol Lett.* 2005;27:1337–1347.

15. Mandel ID. The role of saliva in maintaining oral homeostasis. *J Am Dent Assoc.* 1989;119:298–304.
16. Sreebny L. Saliva: its role in health and disease. *Int Dent J.* 1992;42:291–304.
17. Denny PC, Denny PA, Klauser DK, Hong SH, Navazesh M, Tabak LA. Age-related changes in mucins from human whole saliva. *J Dent Res.* 1991;70:1320–1327.
18. Dawes C. Circadian rhythms in human salivary flow rate and composition. *J Physiol.* 1972;220:529–545.
19. Ferguson DB, Fort A, Elliott AL, Potts AJ. Circadian rhythms in human parotid saliva flow rate and composition. *Arch Oral Biol.* 1973;18:1155–1173.
20. Ferguson DB, Botchway CA. A comparison of circadian variation in the flow rate and composition of stimulated human parotid, submandibular and whole salivas from the same individuals. *Arch Oral Biol.* 1980;25:559–568.
21. Castagnola M, Cabras T, Denotti G, et al. Circadian rhythms of histatin 1, histatin 3, histatin 5, statherin and uric acid in whole human saliva secretion. *Biol Rhythm Res.* 2002;33:213–222.
22. Gusman H, Leone C, Helmerhorst EJ, et al. Human salivary gland-specific daily variations in histatin concentrations determined by a novel quantitation technique. *Arch Oral Biol.* 2004;49:11–22.
23. Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV. Human glandular salivas: their separate collection and analysis. *Eur J Oral Sci.* 1996;104:346–352.
24. Mandel ID. Relation of saliva and plaque to caries. *J Dent Res.* 1974;53:246–266.
25. Shannon IL, Suddick RP, Dowd Jr FJ. Saliva: composition and secretion. *Monogr Oral Sci.* 1974;2:1.
26. Battino M, Ferreiro MS, Gallardo I, Newman HN, Bullon P. The antioxidant capacity of saliva. *J Clin Periodontol.* 2002;29:189–194.
27. Christensen CM, Navazesh M. Anticipatory salivary flow to the sight of different foods. *Appetite.* 1984;5:307–315.
28. Edgar WM. Saliva and dental health. *Br Dent J.* 1990;169:96–98.
29. Malamud D, Rodriguez-Chavez IR. Saliva as a diagnostic fluid. *Dent Clin North Am.* 2011;55:159–178.
30. German Society for Clinical Chemistry. Standard method for determination for alkaline phosphatase (ALP) activity. *J Clin Chem Clin Biochem.* 1972;10:290.
31. Thomas L. Alanine aminotransferase (ALT), aspartate aminotransferase (AST). In: Thomas L, ed. *Clinical Laboratory Diagnostics*. 1st ed. Frankfurt: TH-Books Verlagsgesellschaft; 1998:55–65.
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248–254.
33. Ginsburg I, Koren E, Shalish M, Kanner J, Kohen R. Saliva increases the availability of lipophilic polyphenols as antioxidants and enhances their retention in the oral cavity. *Arch Oral Biol.* 2012;57(10):1327–1334.
34. Malhotra SL. New approaches to the pathogenesis of peptic ulcer based on the protective action of saliva with special reference to roughage, vegetable fiber and fermented milk products. *Med Hypotheses.* 1978;4(1):1–14.
35. Lukacs JR, Largaespada LL. Explaining sex differences in dental caries prevalence: saliva, hormones, and “life-history” etiologies. *Am J Hum Biol.* 2006;18(4):540–555.
36. Sreebny LM. Saliva in health and disease: an appraisal and update. *Int Dent J.* 2000;50(3):140–161.
37. Walt DR, Blicharz TM, Hayman RB, et al. Microsensor arrays for saliva diagnostics. *Ann N Y Acad Sci.* 2007;1098:389–400.
38. Arregger AL, Cardoso EM, Tumilasci O, et al. Diagnostic value of salivary cortisol in end stage renal disease. *Steroids.* 2008;73(1):77–82.
39. Chapple ILC, Socransky SS, Dibart S, Glenwright IL, Mathews JB. Chemiluminescent assay to alkaline phosphatases in human gingival cervical fluid: investigations with on experimental gingivitis model and studies on the source of the enzyme within cervical fluid. *J Clin Periodontol.* 1996;23:587–594.
40. Nakashima K, Roechrich N, Cimasoni G. Osteocalcin, prostaglandin E2 and alkaline phosphatases in gingival cervical fluid: their relation to periodontal status. *J Clin Periodontol.* 1994;21:327–333.
41. Yan F. Alkaline phosphatase level in gingival cervical fluid of periodontitis before and after periodontal treatment. *Chung Hua Kou Chiang Hseueh Tsa Chin.* 1995;30:255–266.
42. Todorovic T, Dozic I, Barrero MV, et al. Salivary enzymes and periodontal disease. *Med Oral Patol Oral Cir Bucal.* 2006;11:E115–E119.
43. Mellanen L, Sorsa T, Lähdevirta J, Helenius M, Kari K, Meurman JH. Salivary albumin, total protein, IgA, IgG and IgM concentrations and occurrence of some periodontopathogens in HIV-infected patients: a 2-year follow-up study. *J Oral Pathol Med.* 2001 Oct;30(9):553–559.
44. Shivashankara AR, Kavya Prabhu M. Salivary total protein, sialic acid, lipid peroxidation and glutathione in oral squamous cell carcinoma. *Biomed Res.* 2011;22(3):355–359.
45. Streckfus CF, Bigler R, Navazesh M, Al-Hashimi I. Cytokine concentrations in stimulated whole saliva among patients with primary Sjögren's syndrome, and primary Sjögren's with secondary Sjögren's syndrome, and primary Sjögren's syndrome receiving varying doses of interferon for symptomatic treatment of the condition: a preliminary study. *J Clin Oral Invest.* 2001;5:133–135.
46. SahebJamee M, Eslami M, AtarbashmiMoghadam F, Sarafnejad A. Salivary concentration of TNF alpha, IL 1 alpha, IL 6, and IL 8 in oral squamous cell carcinoma. *Med Oral Patol Oral Cir Bucal.* 2008;13:E292–E295.
47. Groschl M. Current status of salivary hormone analysis. *Clin Chem.* 2008;54:1759–1769.