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Original Article

Salivary enzymes in peptic ulcer disease

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ABSTRACT

Aim: Peptic ulcer, the common disease of the upper gastro-intestinal tract, occurs in about 5–10% of the world's population. Therefore, diagnosis of trace disease progression with a noninvasive method is of prime importance in the field of healthcare research. The aim of this study was to evaluate the validity of salivary enzymes as noninvasive biomarkers for peptic ulcer.

Materials and methods: In practice, 34 peptic ulcer patients and 30 healthy subjects donated their un-stimulated saliva samples after 8 h of fasting. The activity of some selected enzymes was measured using appropriate enzymatic assay methods.

Results: The results indicated an overall alternation in enzymatic activity of saliva in patients suffering from peptic ulcer. Biological activity of α-amylase, peroxidase and lactate dehydrogenase, showed significantly higher values in almost all patients as compared to control subjects.

Conclusions: Based on the results of salivary enzyme activity, it was concluded that besides the influence of their peptic ulcer on enzyme activity of saliva, the considerably higher activity of α-amylase could also be related to the major role of the enzyme on physiological oxidative stress.

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1. Introduction

Peptic ulcer disease is a common benign ulceration of the epithelial lining of the stomach (gastric ulcer) or duodenum (duodenal ulcer). Under normal conditions, a physiologic balance exists between peptic acid secretion and gastro duodenal mucosal defense. Mucosal injury and, thus, peptic ulcer occurs when the balance between the aggressive factors and the defensive mechanisms is disrupted. Peptic ulcer disease has been a major threat to the world's population over the past two centuries, with a high morbidity and

substantial mortality.¹ It has been found that *Helicobacter pylori* is the main cause of peptic ulcer.² However, understanding the rise and fall of the disease is still the subject of many research works in this area. Successful monitoring, especially in its early stages of the disease, may reduce any severe impacts on a patient's health and prevent complications. The ability to evaluate trace disease progression with a noninvasive method is one of the primary objectives in the field of healthcare research.

Saliva is the first biological medium confronted by external materials that are taken into the body as part of food, drink, or

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inhaled volatile ingredients. Human saliva is a complex liquid mixture of about 99% water, the remainder being mainly enzymes, glycoproteins, electrolytes and small organic molecules.³ It has multiple roles in gastrointestinal tract including bolus formation, enzymatic digestion, antioxidant action and buffering. Its ability to reflect both oral and systemic health conditions has made saliva an attractive noninvasive clinical tool during the last decade. However, in order to use saliva as a useful diagnostic body fluid, a sensitive and specific biomarker among the complicated composition of saliva must be specified for each case.⁴

Secretion of saliva, which is stimulated as food is chewed, is the primary step in the digestive process. Food particles are reduced in size by chewing and saliva then moistens and lubricates them into a bolus to be easily swallowed.⁵ In healthy individuals, the rate of saliva secretion is about 0.3–0.4 ml min⁻¹ in resting conditions, but during a meal the salivary glands are stimulated and saliva secretion increases to 1–2 ml min⁻¹.^{6,7} Thus, considerable quantities of saliva are mixed with the food that reaches the stomach.

Among its various physiological functions, human saliva acts as an antioxidant system due to the presence of its antioxidants including uric acid, peroxidase and superoxide dismutase.⁸ There are various classes of antioxidants in saliva including non-enzymatic antioxidants, and enzymes such as peroxidase (POD), catalase and superoxide dismutase (SOD).

Release of reactive oxygen species (ROS) is the natural defense mechanisms of neutrophils against bacteria. These are free radical species including mainly superoxide (O₂), hydroxyl (OH), hydrogen peroxide (H₂O₂), nitric oxide (NO), hypochlorous acid (HOCl), and singlet oxygen.⁹ Despite their vital role in continuation of the normal cellular metabolism, ROS can also initiate series of free radical chain reactions leading to significant tissue damage. In normal physiological conditions, an antioxidant defense system reacts against the harmful effects of ROS.

Peroxidase in saliva is secreted by the salivary glands and myeloperoxidases by polymorphonuclear neutrophils.¹⁰ Salivary SOD is a key antioxidant enzyme that efficiently and specifically scavenges O₂ by catalyzing its dismutation to H₂O₂ and O₂. Salivary antioxidant system is not only of high importance locally in the oral cavity, it has a critical role in the acidic stomach, where oxidative and nitrosative stress is considerable.

Lactate dehydrogenase (LDH) is an enzyme detectable in cytoplasm of almost every cell and becomes extracellular upon cell death. In an anaerobic condition, pyruvate, the normal product of glycolysis, is reduced to lactate by catalytic action of LDH in the presence of nicotinamide adenine dinucleotide (NAD) as a coenzyme. Therefore, its extracellular presence is always related to cell necrosis and tissue breakdown. It has been reported that parotid and submaxillary–sublingual glands contributed very little to LDH activity in whole saliva.¹¹ As it is a cellular necrosis marker, any increase of its activity in saliva, could be a specific indicator of oral or gastrointestinal mucosa breakdown.

This research investigated the enzymatic activity, pH and flow rate in saliva of patients suffered from peptic ulcers and compared their alternations with healthy controls. It was

expected that the results could explain the 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

The chemical reagents and solvents were of analytical grade and used as supplied by manufacturers without further purification. A commercially available direct α -amylase kit based on the hydrolysis of a substrate by α -amylase in the presence of chromogen was used (Chem Enzyme). 4-Amino antipyrine, phenol, hydrogen peroxide, horseradish peroxidase were purchased from Merck chemical company. Superoxide dismutase activity assessment kit (Cayman chemical, Cat No.706002, USA) was purchased from local importer representatives in Iran. All buffers were prepared freshly in Biochemistry laboratory, University of Guilan and their pH was double checked using pH meter. All general chemicals and reagents were of the highest purity available.

2.2. Subjects

The subjects were 52 patients (24 male and 28 female, aged 15–70 years) undergoing endoscopy in Gastrointestinal and Liver Disease Research Center, Guilan University of Medical Sciences and diagnosed with peptic ulcer (either chronic gastritis or duodenal ulcer in various degree of severity). The control group composed of 55 volunteers from staff or students in Razi Medical center, Rasht (26 male and 29 female, aged 18–62 years). By explaining the aim of the research project, both groups agreed to enter the study and donate their saliva samples. Informed consent was obtained from each volunteer and the study was approved by the Board of Ethics of the Guilan University of Medical Sciences.

2.3. Saliva collection and measurement of flow rate and pH value

An advantage of saliva compared to blood is that it is easily accessible and can be sampled noninvasively and relatively stress-free. Therefore, collection of saliva by patients and control volunteers needed only modest training and, in some cases, the repeated collection of samples took only a few minutes short-time. Firstly, the aim of research was explained the forms filled in with the aid of a technician. The subjects were then instructed to rinse their mouth with distilled water and try to store their un-stimulated saliva for exactly 3 min and pour the samples in clean, dry, and sterile tubes by spitting method.¹² The flow rate was then calculated in ml/min. Saliva was centrifuged for 10 min at 3000 rpm in an Eppendorf centrifuge at 4 °C to remove squamous cells and cell debris. The value of pH in resulting supernatant fluid was measured using a digital pH meter instrument (Model 3510, Jenway, UK). The clear samples were then stored at –80 °C for later assays.

2.4. Peroxidase assay

The biological activity of peroxidase on 4-amino antipyrine was measured by a spectrophotometric method using UV–visible spectrophotometer (Ultrospec 3000 UV/Vis, Pharmacia Biotech, Sweden). The oxidation of 4-amino antipyrine was measured at 25 °C in 3 ml of 0.3 M phosphate buffer, pH 7.4, containing 0.0010 M hydrogen peroxide, 0.002 M 4-amino antipyrine and 0.15 M phenol according to the procedure described in.¹³

2.5. Lactate dehydrogenase assay

The activity of LDH was assayed using a kit obtained from Pars Azmoon®, base on a photometric method proposed by the International Federation of Clinical Chemistry (IFCC), standardized to 30 °C, as recommended by the German Society of Clinical Chemistry.¹⁴ The principle of kit was based on measuring the oxidation rate of NADH, which is directly proportional to LDH activity. This was achieved by following the decrease in absorbance at 340 nm, the wavelength of NADH absorption.

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

2.6. Assay of α-amylase

Salivary α-amylase was measured using 2-chloro-4-nitrophenyl-α-D-maltotrioxide (CNPG3) as substrate, to which a chromogen 2-chloro-4-nitrophenyl was attached to a molecule of maltotrioxide. This is a direct amylase assay without using enzymes such as α-glucosidase/glucoamylase. CNPG3 is hydrolyzed by α-amylase producing 2-chloro-4-nitropheny (CNP) directly and the concentration of CNP is measured at 405 nm.¹⁵

The statistical differences are given in the result section. As no significant difference was observed between thawed and fresh samples, therefore, only the frozen samples were used for continuing studies.

2.7. Statistical analysis

Results (means ± SD) are expressed as percentages or weights, or on the basis of biological activity. Statistical significance was assayed by application of one-way ANOVA, followed by a ranking procedure based on the Student–Newman–Keuls test and calculated with SAS software (SAS Institute Inc., Cary, NC). The presented results are the means of triplicates, and in the figures, each error bar denotes the standard deviation.

3. Results

The characteristics of patients extracted from the filled informed consent are presented in Table 1. The control group

Table 1 – Main demographic characteristics of the peptic ulcer patients.

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

consisted of 25 male and 30 female and they were selected so that their age and general characteristics be similar to the patient group. Smokers, those taking medications or having systemic diseases such as diabetes were not included in this group. In Table 2 the results are summarized for patients and control group.

4. Discussions

According to the results obtained from this study, the flow rate of saliva is decreased significantly ($p < 0.05$) in patients suffering from peptic ulcers compared to healthy individuals (Table 2). It is known that the average daily flow of whole saliva varies in health between 1.0 and 1.5 L.¹⁶ A considerable volume of this salivary fluid is swallowed with food or on its own. The salivary mucus swallowed with food is protective because it decreases the flow rate of bile. It has been suggested that bile, and not hydrochloric acid, plays the causative role in pathogenesis of peptic ulceration.¹⁷ If bile is held in the gall bladder longer, it loses its alkalinity and will not be able to damage the mucous cells. Therefore, saliva can play an important role in the prevention of peptic ulcer and reduction in its flow rate may worsen the conditions of peptic ulcer. It has been reported that hormonal fluctuations could affect the flow rate of saliva, during events like puberty, menstruation, pregnancy and menopause.¹⁸ Although our study group was smaller than making a certain conclusion, however, a slight decrease in flow rate was observed in older volunteers that may be due to hormonal changes.

According to the results, a significant shift ($p < 0.05$) to lower pH was observed in saliva of peptic ulcer patients compared to the control group (Table 2). It can be suggested that when the flow rate is decreased less bicarbonate is released, leading to a decrease in value of pH. Variation in salivary pH observed in this study is supported by other research works which showed salivary flow rates vary widely between subjects.¹⁹ The decrease of salivary pH could be due to a decrease in the salivary flow rate. The reduction of pH could also be the result of decrease in buffering capacity of

Table 2 – Salivary parameters in patients and controls.

Salivary parameter	Patients (mean ± SD)	Controls (mean ± SD)	p-Value
Flow rate (ml/min)	0.62 ± 0.24	0.85 ± 0.25	0.006
pH value	6.25 ± 0.3	7.25 ± 0.3	0.008
Peroxidase (U/L)	611.1 ± 25	351.9 ± 44	0.009
LDH (U/L)	1662 ± 46	1635 ± 31	0.042
α-Amylase (U/ml)	3524 ± 1120	3119 ± 1010	0.014

saliva in patients with peptic ulcer. On the other hand, as one of the major causes of peptic ulcer disease is the presence of *H. pylori*, the increase in acidity may be the result of bacterial growth. It has been suggested that alterations in saliva composition or flow rate may reflect secondary systemic changes related to diseases, medications or treatments. For example, it has been found that diabetes is associated with both salivary flow rate and pH.²⁰

It was also noted that all three tested enzymes were significantly more active ($p < 0.05$) in patients when compared to controls (Table 2). Production of various antioxidants and increase in activity of antioxidant enzymes is the nature's response to the attack of free radicals. The mean activity of peroxidase (U/L) obtained in this research was 611.1 ± 25 (Table 2). Each test was repeated three times and the results were taken as the mean value of the tests. However, triplicate tests indicated that there was no significant ($p < 0.05$) differences between the three repeated samples. It can be seen that peroxidase activity is considerably higher in the peptic ulcer patients compared to healthy controls ($p < 0.05$). One possible explanation is that an increase in activity of peroxidase is due to a rise in oxidative status of oral cavity in ulcerative cases.²¹ Fluctuations in salivary peroxidase have been drawing increased attention during the last two decades^{22,23} as it plays important role in the oral defense mechanism and attack of free radicals. It has been reported that peroxidase in saliva can significantly inhibit the initiation and progression of oral cancer.²⁴ It has also been found that patients with oral lichen planus (a premalignant lesion) have a lower salivary antioxidant capability.²⁵ In addition, peroxidase in saliva can be activated in response to various internal and external factors. In a previous research we found that high intensity exercise can activate major salivary glands, leading to higher activity of peroxidase.¹³ The partial antioxidant effect of saliva can block oxidative damage of biological molecules at normal salivary pH (6.8–7.5). However, in stomach medium (pH 2–3) this type of antioxidant effect is not expected due to structural changes of effective molecules mostly antioxidant enzymes.²⁶ Therefore, although a number of salivary components have partially antioxidant effects, saliva cannot provide adequate protection against the deleterious processes in the stomach.

The activity of LDH obtained in this study was considerably higher ($p < 0.05$) in patients suffering from peptic ulcers (Table 2). The centrifuged saliva samples were stored at 4 °C until use since a gradual degradation of LDH may occur when stored at –20 °C.²⁷ It has been shown that LDH activity in saliva of adults is almost similar to that of young volunteers.^{28,29} We also observed that there was no change in LDH activity when various ages were compared, but due to small size of subjects it cannot be conclusive. In a study on aiming to find relationship between oral health and LDH activity in salivary fluid, it was found that the enzyme could be used as a possible biochemical marker of periodontal status.³⁰

Activity of salivary amylase was considerably higher ($p < 0.05$) in patients as compared to similar healthy cases (Table 2). Amylase activity in biological fluids is sometimes used as a marker of oxidative stress due to external factors.¹³ It has been reported that salivary α -amylase could be an indicator of alternations in body status due to any type of

oxidative stress.^{31,32} According to the results of present research, variation in the activity of amylase is much higher compared to other salivary factors studied here. This explains that the increase in amylase activity is the result of oxidative stress caused by the disease. In support of this reasoning, it has been reported that salivary amylase activity could be used as a powerful marker of catecholamines during the evaluation of patients in different stressful situations.³³ The researchers have even gone further purposing the possibility of using saliva to evaluate the general health of an individual.³⁴

5. Conclusions

Based on the results obtained it could be suggested that saliva could be thought of as a potential specimen for diagnosis and detection of peptic ulcer in early stages. 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. However, as our subjects were limited to about 50 in each patient and control groups, more studies are recommended with more patients and normal subjects in order to make more conclusive remarks.

Conflicts of interest

All authors have none to declare.

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