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Salivary Enzymes in Peptic Ulcer Disease

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ABSTRACT

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 non invasive biomarkers for peptic ulcer. 34 peptic ulcer patients and 30 healthy subjects donated their un-stimulated saliva samples after 8 hours of fasting. The activity of some selected enzymes was measured using appropriate enzymatic assay methods. The results indicated an overall alternation in enzymatic activity of saliva in patients suffering from peptic ulcer. Biological activity of a-amylase, peroxidase and lactate dehydrogenase, showed significantly higher values in almost all patients as compared to the control subjects. 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 a-amylase could also be related to the major role of the enzyme on physiological oxidative stress.

Key words: Peptic ulcer, saliva, lactate dehydrogenase, peroxidase, amylase.

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 *H. 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 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 non-invasive 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 (O_2), hydroxyl (OH), hydrogen peroxide (H_2O_2), 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 O_2 by catalyzing its dismutation to H_2O_2 and O_2. 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 non-invasive easily accessed body fluid in diagnosis of the disease.

MATERIAL AND METHODS

Materials

The chemical reagents and solvents were of analytical grade and used as supplied by manufacturers without further purification. A commercially available direct ±-amylase kit based on the hydrolysis of a substrate by ±-amylase in the presence of a 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.

Subjects

The subjects were 34 patients (19 male and 15 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 30

volunteers from staff or students in Razi Medical center, Rasht (15 male and 15 female, aged 18-55 years). By explaining the aim of the research project, both groups agreed to enter the study by donating 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.

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 non-invasively 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 minutes 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 subjected to centrifugation 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.

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 0.3M phosphate buffer, pH 7.4, containing 0.0010 M hydrogen peroxide, 0.002 M 4-amino antipyrine and 0.15 M phenol. 40ml of enzyme solution (6 $\times 10^{-4}$ mg/ml in 0.3 M phosphate buffer pH 7.4) was then added and the change in absorption at 510 nm (DA/min) was recorded. The change in absorbance at 510 nm is due to the formation of a chromogen product with a λ_{max} at 510 nm. One unit of activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions[13].

Lactate dehydrogenase Assay

The activity of LDH was assayed using a kit obtained from Pars Azmon®, base on a photometric method proposed by the International Federation of Clinical Chemistry (IFCC), standardised 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.

LDH

$$\text{Pyruvate} + \text{NADH} + H^+ \rightarrow \text{Lactate} + NAD^+$$

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

Assay of α-amylase

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

$$10\ \text{CNPG3} \rightarrow 9\ \text{CNP} + \text{CNPG2} + 9\ \text{G3} + \text{G}$$

In a typical reaction mixture at 37°C, 25 µl of the saliva sample was added to 1 ml of the substrate reagent and mixed rapidly. Absorption was measured at 405 nm after exactly one minute followed by a second measurement after 5 minutes. The increase in absorption was related to the activity of α-amylase.

$$\alpha - \text{amylase activity (U/ml)} = \frac{\text{Increase in absorption} \times 1025}{12.9 \times 25 \times 5}$$

The value of 12.9 is absorption coefficient for 1 mM of CNP at 405 nm. The terms 1025 and 25 are the total and sample volumes respectively. The

statistical differences are given in the result section. One unit of activity was defined as the amount of enzyme that caused an absorbance change of 0.001 per min under standard conditions. To test the effect of freezing on the biological activity of salivary amylase, the enzyme activity was also measured on some fresh supernatant of saliva samples. No significant difference was observed between thawed and fresh samples. Therefore, only the frozen samples were used for continuing studies.

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.

RESULTS

The characteristics of patients extracted from the filled informed consent are presented in Table 1. Age, duration of disease and type of ulcer were the variable considered in peptic ulcer patients.

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

Table 2: Salivary parameters in patients and controls

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

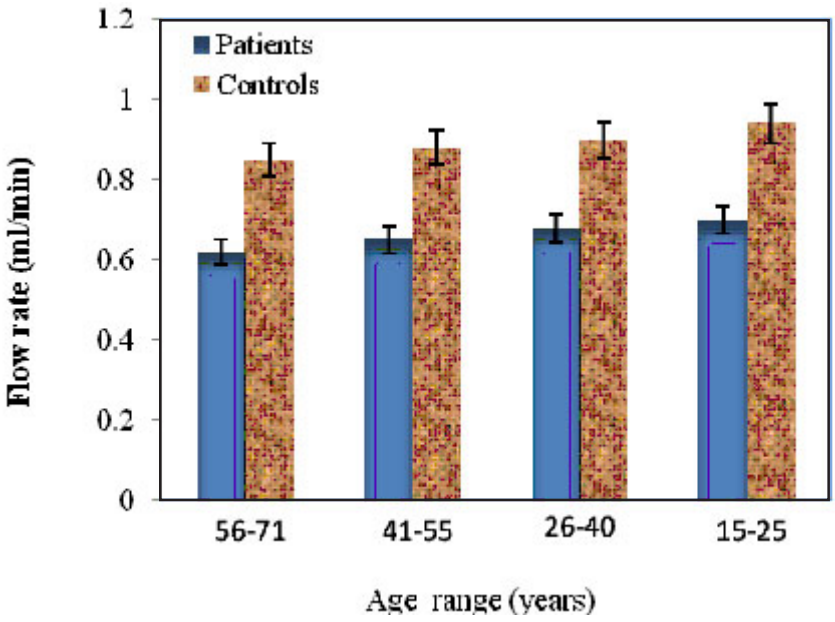

Fig. 1: Flow rate in patients compared to control group

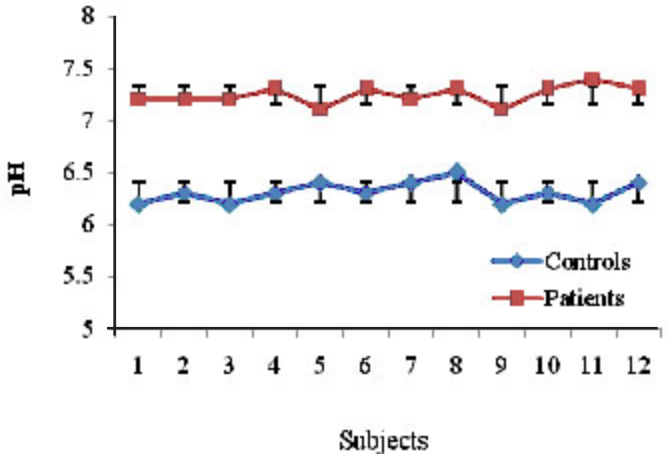

Fig. 2: pH value for a range of selected subjects

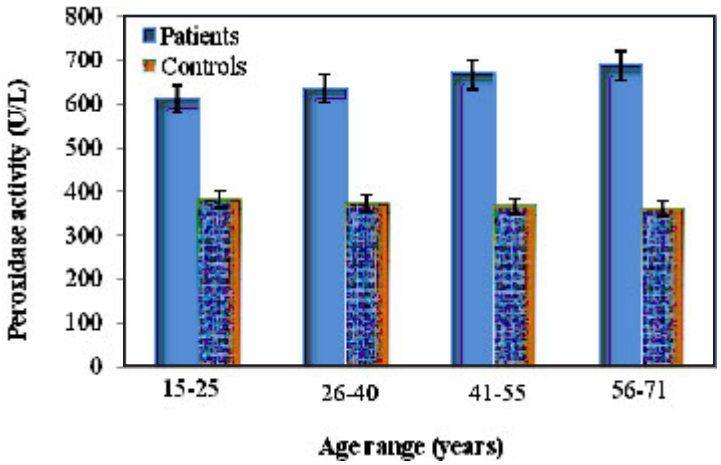

Fig. 3: The activity of peroxidase in peptic ulcer patients and control subjects

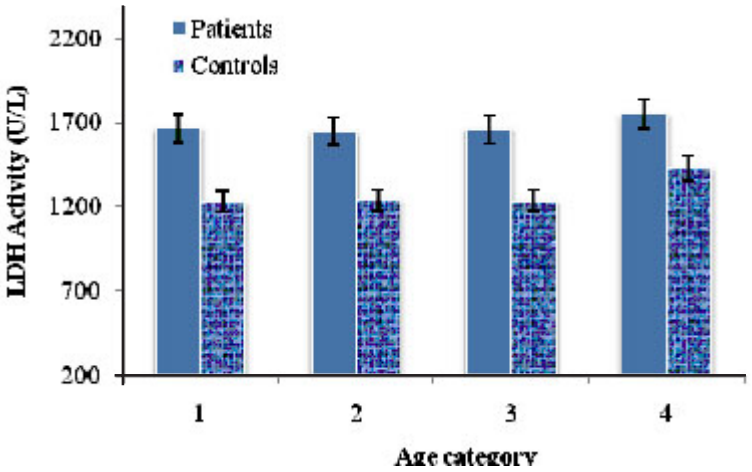

Fig. 4: Biological activity of LDH in saliva from peptic ulcer patients and control subjects aged 1) 15-25, 2) 26-40, 3) 41-55 and 4) 56-71 years

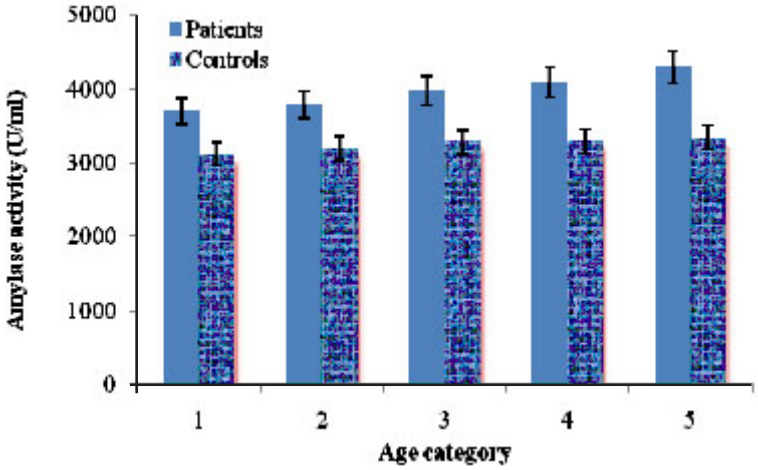

Fig. 5: Comparison between α-amylase activity in salivary fluid in peptic ulcer patients and control subjects aged 1) 15-25, 2) 26-40, 3) 41-55 and 4) 56-71 years

The control group consisted of 15 male and 15 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. Figures 3-5 show alternation in activity of salivary peroxidase, lactate dehydrogenase and a-amylase respectively. In Table II the results are summarized for patients and control group.

DISCUSSIONS

According to the results obtained from this study, the flow rate of saliva is decreased in patients suffering from peptic ulcers compared to healthy individuals (Table II). 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, salvia can play an important role in the prevention of peptic ulcer and reduction in its flow rate may worsen the conditions of peptic ulcer. According to the results (Fig. 1) the flow rate in both patients and control group decreases slightly with the age of volunteers. Supportive to our results, a similar alternation has been observed in response of hormonal fluctuations during events like puberty, menstruation, pregnancy and menopause[18].

The results obtained from this study showed a shift in salivary pH in peptic ulcer sufferers towards lower values compared to the control group (Table II). 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 is also the result of decrease in buffering capacity of 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. However, no correlation was observed in pH value of saliva and age or sex of subjects (Fig. 2). This result is similar to what has been reported by Sreebny [6] who found that pH of saliva remains constant during the different stages of life for any individual. In any case, an important consequence of pH fall is increasing the risk of teeth erosion and periodontal disorders. The normal, resting, pH of the mouth does not usually fall much below pH 6.3 and the reason for

this is the buffering effect of bicarbonate in saliva. 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].

In terms of enzymatic activity, it was noted that all three tested enzymes were significantly more active in patients when compared to controls (Figures 3-5 and 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 is presented in 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. However, the severity of the disease may affect its remaining at high level of activity. The fact that an increase in activity of peroxidase is the response of rising oxidative status of oral cavity in ulcerative cases[21] may be a reasonable explanation for this result. Fluctuations in salivary peroxidase have been drawing increased attention during the last two decades[22, 23]. The enzyme plays important role in the oral defense mechanism, especially against the attack of free radicals and reactive oxygen species (ROS). It has been reported that peroxidase in saliva can significantly inhibit the initiation and progression of oral cancer[24]. Moreover, it was recently reported 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 amount of salivary peroxidase[13]. Therefore, physical activity in any age could be beneficial in preventing gastrointestinal disorders including peptic ulcers by capturing free radicals through the action of salivary peroxidase. To develop a profile of age contribution in the salivary factors, the saliva samples were grouped in terms of age range. It was found that oral peroxidase activity gradually increased by aging in peptic ulcer patients, while normal subjects showed less enzyme activity when aged. This can be explained by the considering that more free radical damage caused by the ulcer in older patients. 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. However, increasing the polyphenol concentration in salivary fluid and stomach medium by consumption of fresh vegetable and fruit can largely inhibit lipid peroxidation and possibly protect against peptic ulcer.

We also found that the activity of LDH is considerably higher in patients suffering from peptic ulcers (Table 2 and Figure 4). 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]. When the activity of the enzymes was compared within each group, no differences were found due to age. This finding is supported by a research work indicating that LDH activity in saliva of adults was almost similar to that of young volunteers [28]. We also observed that a few individuals in older age range did not follow this pattern which is probably due to higher percent of cellular necrosis. This finding is similar to the result obtained for selected serum enzymes in a population more than 60 years of age[29]. It is known that LDH activity in serum increases as a marker of cellular necrosis. It was expected that the same pattern must also be observed for the activity of this enzyme salivary fluid. Therefore, LDH activity in saliva could constitute a specific indicator of oral mucosal and possibly gastrointestinal lesions problems including periodontitis and peptic ulcers. 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 in patients as compared to similar healthy cases (Table 2 and Fig. 5). However, no considerable correlation was found due to age ranging. Amylase is an extracellular enzyme produced in the salivary glands and is responsible for early digestion of carbohydrates. Its activity in biological fluids is sometimes used as a marker of oxidative stress due to external factors[13]. The results obtained from the present study showed inter-personal range of amylase activity levels in salivary fluid which is comparable with results reported by others[31].

Mental stress could cause many problems including neurodegenerative diseases as well as gastrointestinal disorders. It has been found that salivary a-amylase could be an indicator of alternations in body status due to any type of oxidative stress[32]. Fig. 5 indicates higher amylase activity in saliva of peptic ulcer patients as compared to healthy controls. Variations in the activity of enzyme are 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 nature of 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].

CONCLUSIONS

In conclusion, saliva can be used as a potential specimen for diagnosis and detection of peptic ulcer in early stages. 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.

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