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Research Article

EVALUATION THE VALUE OF SALIVARY PEPSIN IN DIAGNOSIS OF GASTROESOPHAGEAL REFLUX DISEASE

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ABSTRACT

Background: Gastroesophageal reflux disease is among the common healthy problem in developed and developing countries, we evaluated the value of salivary pepsin in the diagnosis of gastroesophageal reflux disease

Material and Methods: In this study a total of 463 individuals were selected of which 128 are healthy controls and 335 are GERD patients. All participants were selected in accordance with the set criterions. Salivary pepsin concentration was analyzed and determined by ELISA in line with manufactures manual

Results: In this study, high pepsin concentration was detected in GERD group compared to healthy control group with significant statistical difference ($P < 0.05$). Basing on different timing points a statistical significance ($P < 0.001$) was observed between gastroesophageal reflux disease and health controls. On stratification of GERD group into reflux esophagitis (RE) and non-erosive reflux disease (NERD), the timing point at waking and symptom onset were not significant ($p > 0.05$)

Conclusion: Salivary pepsin has an important significance for the diagnosis of gastroesophageal reflux disease

Key words; Humans; Pepsin; Control group; Gastroesophageal Reflux

INTRODUCTION

Gastroesophageal reflux disease is common health problem associated with involuntary back flow of gastric content containing acid, enzymes and bile from duodenum and stomach into the esophagus. The reflux contents can be in acidic or alkaline form, liquid, or gaseous in nature [1]. Mainly distal part of about 8-10cm esophagus involved. Correspondingly, the frequency and duration of episodes of reflux as well as the destination of the gastroesophageal refluxate influence the severity of GERD [2].

It is a common disease with increasing prevalence on a global scale and estimated prevalence of 22.95% in North America, 17.35% in Europe and 5.15% in East Asia with a significant morbidity though its mortality is sporadic[3]. Typically, GERD is manifested with heartburn and acid regurgitation. Nevertheless, atypical symptoms such as Hoarseness or laryngitis, Persistent cough, asthma-like symptoms, frequent throat clearing, sore throat, burning in the mouth or throat have been described [4, 5].Although physical examination and history taking have critically been used in the diagnosis of gastroesophageal reflux disease, sensitive and specific diagnostic tools have surfaced. Presently many publications have suggested endoscopy, **proton-pump inhibitor (PPI)**, PH-metry as important ways for evaluating pepsin concentrations in various samples [6, 7].The limited sensitivity and specificity of endoscopy and PH testing have posed a challenge in diagnosis of GERD, however, the introduction of cost effective noninvasive saliva pepsin testing is promising step in the diagnosis and management of GERD[8].A part from saliva, also secretions from lungs, middle ear, sinus and trachea can be used for pepsin detection. In the pathological point of view, various studies have revealed the presence of space ultra structural lesions in patients with GERD associated with dilated intercellular spaces as one of the common changes, these spaces usually seen in the basal layer of GERD patients.

Pepsin is an aspartic acid protease enzyme that produced by chief cell in the stomach. It's an active form of pepsinogen responsible for the hydrolysis of polypeptides or proteins. Its presence in the saliva confirms the backflow of gastric contents into the esophagus [2, 9].The existence of pepsin in saliva has been suggested as a marker in the diagnosis of gastroesophageal reflux disease, and both immunological and enzymatic techniques have been employed in detection of pepsin in saliva or sputum. From studies, high levels of pepsin have been detected in the bronchoalveolar lavage and tracheal aspirates [10], evidence from reports suggests that pepsin detection in saliva is vital in the diagnosis of GERD. In addition, during evaluation of proximal esophageal reflux, salivary pepsin had sensitivity and specificity of 57% and 91% respectively[11].Testing for saliva pepsin in patients with suggestive symptoms for GERD may assist in confirmation of the diseases reducing unnecessary use of drugs and the use of discomfort invasive approaches. Although salivary pepsin appears to be an attractive option because of ease of sampling, no such study has ever been done in a northern Chinese population. Therefore, in this study we evaluated the values of saliva pepsin in diagnosis of gastroesophageal reflux disease in the sample of the Chinese population.

METHODS AND MATERIALS

Study subjects and Design:

The study included a total of 463 individuals, of which 335 were patients presented with acid reflux heartburn, non-cardiogenic chest pain, long-term score throat, chronic cough, belching, unexplained caries, seeking medical attention from the Department of Gastroenterology, Shengjing Hospital Affiliated to China Medical University from November 2016 to November 2017. One hundred twenty-eight healthy volunteers without any GI symptoms during the last 3 months were also recruited for comparison. Signed informed consent was obtained from each individual prior to study inclusion. Other considered Inclusion criteria: Age group of population: 18-80; GerdQ grade: ≥8; finish gastro endoscopy examination; Taking saliva sample regularly.

Exclusion criteria:

Patients were excluded if any one of the following conditions was present: peptic ulcer, erosive gastritis, or GI tumors detected by endoscopy; taking proton pump inhibitors; history of gastroesophageal operation; Zolinger Ellison syndrome; Achalasia of cardia; women in pregnancy or lactation; and severe systemic diseases such as severe heart, liver, kidney, brain, and other organ damage.

Sample collection:

The patients were instructed to have an overnight fasting, and followed by gastroscopy in the next morning. The gastroscopy equipment was Fujineng electronic gastroscope, and the examiners were all experienced gastroenterologists.

Saliva collection and pepsin detection: Each patient was given 5EP tubes with PH of 2.5 and concentration of 0.1 mmol/l citric acid of 0.5ml. The tube capacity was 2ml. It was used to collect saliva samples from patients when they woke up, 1 hour after breakfast, 1hour after lunch, 1hour after dinner and at the time of symptom onset

Pepsin analysis:

The pepsin concentration was determined by **enzyme-linked immunosorbent assay** (ELISA)

Standard sample adding: Set standard hole and sample hole, and added 50μl of different concentration to each standard hole. Sample adding: Set blank hole and sample hole to be tested. Added 40μl of sample diluents and 10μl of sample to be tested in the sample hole on the enzyme label coated plate. Added the sample to the bottom of the enzyme plate hole, shake gently and mixed well. Warm incubation: Sealed the plate with sealing film and left it at 37°C for 30 minutes. Solution preparation: Diluted 30 times of concentrated washing solution with 30 of distilled water for standby. Washing: Carefully removed the sealing film, discarded the liquid and dried it. Filled each hole with washing liquid and left it for 30 seconds. Repeated for 5 times and dried the plate.

Enzyme addition: added 50μl of enzyme standard reagent into each pore, except for the blank pore, left to warm up at Incubation and Washed. Color development: First added color reagent A50μl to each hole,

then added color reagent B50μl, shaken gently and mixed well, and developed color at 37°C in dark for 15minutes. Termination: Added 50μl of the termination solution to each hole to terminate the reaction (at this time, the blue color turns to yellow vertically). Determination: Measured the absorbance (0ptical Density value) of each hole in the sequence with the blank air conditioning zero and 450 nm wavelength. The determination was carried out within 15minutes after the termination solution added.

Statistical analysis:

In this study, we used Statistical Package for Social Scientists (SPSS) version 21.0 to analyze the data. Counting data were analyzed X^2. Otherwise, the mean (Q1-Q3, percentile) is used to represent the obtained data. The single-sample Kolmogorov-Smirnov test was used for normality test. Levene test was used for homogeneity of variance test. One-way ANOVA T test and LSD T test were used for normal distribution. Mann-whitneyU test Kruskal-Wallis H test were adopted for the non-normally distributed independent samples, and Spearman test and Friedman test were adopted for the relevant sample data. The differences of pepsin concentration distribution at different sampling time points between different groups were analyzed, and the corresponding ROC curve was drawn to evaluate the diagnostic value of pepsin concentration at different time points for GERD. P<0.05 was statistically significant.

RESULTS

Sixty-seven symptomatic patients (Gerd Q scores≥8, GERD group) and thirty-two healthy controls (HC; 12 male and 20 female; median age, 50.00 (37.00-62.25) years) were recruited. In the GERD group, there are thirty-five in RE sub-group (18 male and 17 female; median age, 55.00 (48.00-59.00) years) and thirty-two in NERD sub-group (18 male and 14 female; median age, 54.50 (44.75-63.75) years). Differences in the sex distribution and ages of subjects among the three groups were not significant ($P>0.05$ for both). There was significant difference in the body mass index (BMI) among the three groups ($P=0.038$), however, there was no difference between the RE sub-group and NERD sub-group ($P>0.05$). Besides, there were also no significant differences in course of disease and Gerd Q scores ($P>0.05$ for both).

Salivary pepsin results between HC and GERD groups

Pepsin was measured in a total of 463 saliva samples, including 128 (27.65%) in the healthy control group and 335 (72.35%) in the GERD group. Pepsin concentrations in all samples ranged from 0 to 171.72 ng/mL. The GERD group had significantly higher pepsin concentrations than the HC group (88.22 vs. 10.81 ng/mL, $P< 0.001$) (Table 1). In the HC group, there was no difference among the four timing points on pepsin concentration ($P=0.076$). And in the GERD group, there were significant differences among the five timing points ($P<0.001$), in which the concentration of 1 h after meals had no obvious difference between each other ($P>0.05$), and just waking in the morning had the highest concentration of pepsin, with significant differences to other timing points ($P<0.001$) (Table 2).

Group	N	median (IQR)	minimum	maximum	*P*
HC	128	10.81（5.96-18.80）	0.00	68.28	＜0.001
GERD	335	88.22（69.94-100.34）	0.26	171.72	

Table 1: Characteristics of whole pepsin concentration between two groups (ng/mL)

HC : healthy control;*GERD* : gastro-esophageal reflux disease

timing point	HC	GERD	*P*
waking	14.63（6.15-22.75）	96.64（85.37-108.15）	＜0.001
1 h after breakfast	9.33（5.63-17.59）	87.47（60.83-97.66）	＜0.001
1 h after lunch	14.63（6.15-22.75）	82.39（60.88-98.66）	＜0.001
1 h after dinner	11.27（6.40-17.87）	77.67（54.63-96.92）	＜0.001
symptom onset	-	88.67（74.98-103.43）	-
P	0.076	＜0.001	

Table 2: Characteristics of pepsin concentration at each timing point of two groups (ng/mL)

HC : healthy control;*GERD* : gastro-esophageal reflux disease

Salivary pepsin results between RE and NERD subgroups:

Of the335saliva samples from GERD patients, 175 (52.24%) were from RE subgroup and 253 (44.86%) were from NERD subgroup. The overall pepsin concentration in RE group was higher than that in NRED group (95.09 vs. 78.40 ng/mL, *P*< 0.001) (Table 3). Among them, there were no significant differences in pepsin concentrations between the two groups at the time of early morning awakening, 1 h after breakfast, 1 h after dinner and symptoms (*P*> 0.05), while at the 1 h after lunch, pepsin concentrations in RE group were significantly higher than those in NERD group (92.44 vs. 76.91 ng/mL, P < 0.001) (Table 4). Comparing the concentration at different five timing in the same subgroup, it was similar to that at the time of symptom onset and 1 h after three meals (breakfast, lunch and dinner) and at the time of waking in the morning was significantly higher than others' time (P=0.001, 0.007, < 0.001 and 0.015) in RE subgroup (Sig. 1). In NERD subgroup, there was no significant difference among the 1 h after three meals (P > 0.05), while the concentration of salivary pepsin at the waking was higher than that at 1 h after three meals (P=0.012, < 0.001 and < 0.001), and similar to that at the onset of symptoms (P=0.398).

subgroup	N	median (IQR)	minimum	maximum	*P*
RE	175	95.09（78.35-106.27）	0.26	171.72	＜0.001
NERD	160	78.40（60.51-91.47）	10.76	122.35	

Table 3: Characteristics of whole pepsin concentration between two subgroups (ng/mL)

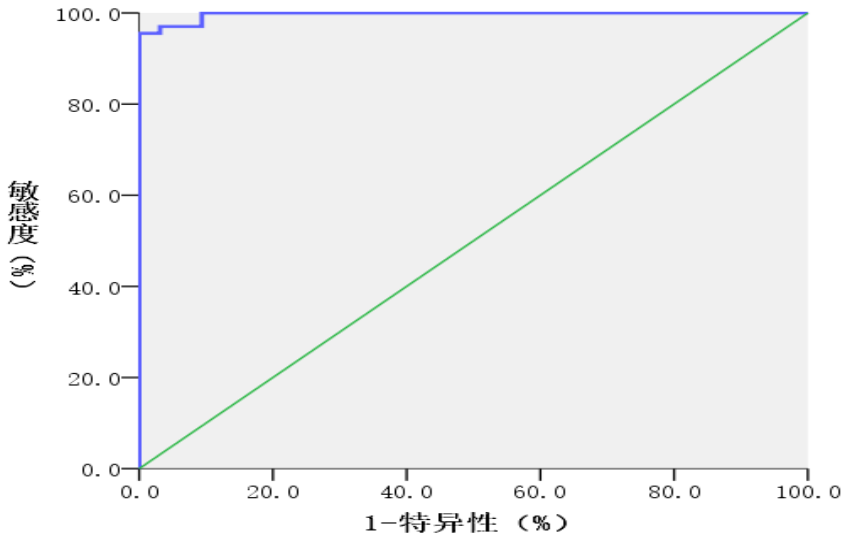
RE : reflux esophagitis;*NERD* : non-erosive reflux disease

timing point	RE	NERD	*P*
waking	101.85（85.63-117.27）	91.11（77.87-100.34）	0.060
1 h after breakfast	94.76（80.65-106.27）	76.56（58.78-90.86）	0.197
1 h after lunch	92.44（76.65-109.83）	74.49（54.41-86.12）	＜0.001
1 h after dinner	94.88（55.84-100.26）	76.91（46.36-86.70）	0.060
symptom onset	92.32（77.87-103.43）	80.02（70.61-103.99）	0.173
P	＜0.001	＜0.001	

Table 4: Characteristics of pepsin concentration at each timing point of two groups (ng/mL)

RE : reflux œsophagites; NERD : non-erosive reflux disease

Value of pepsin concentration in saliva:



Using Gerd Q score as the standard, ROC curve of pepsin concentration in saliva on the wakening in the morning was obtained (Sig. 1). It is not difficult to find the concentration in saliva on the waking obtain a good value. Therefore, the threshold concentration of pepsin in saliva was 53.76 ng/mL, the sensitivity was 95.52%, the specificity was 100%, PPV was 100%, NPV was 91.43%, Youden's index was 0.96, and Kappa value was 0.93.Sig. 1The ROC curve of saliva pepsin concentration when waking up in the morning

DISCUSSION

Gastroesophageal disease has steadily increased by prevalence both in developed and developing countries. It is commonly associated with increased significant esophageal acid exposure with reduced mucosal integrity[12, 13]particularly manifested with heartburn and regurgitation.Consequently**,** several risk factors for gastrooesophageal reflex diseases have been postulated and identified, to mention; age, gender, smoking,

depression, race, eating habits, lack of exercise and body mass index[14]. In our study, the latter was significantly different in healthy control group and gastroesophageal reflux disease group suggesting to be a predisposing factor for the development of GERD**.** Although various diagnostic techniques for GERD have been established, there is still a need for simpler and cost effective method that can be used in the clinical settings. Several publications have suggested saliva pepsin testing as an approach for GERD diagnosis[15] however, greater variation in pepsin concentration still observed and serious question remains unanswered whether salivary pepsin estimates mucosal damage.

Pepsin is considered as a specific biomarker for GERD produced chiefly by the stomach cells [5, 16]. Apart from its detection in saliva, it can also be found in sputum and tears specimens [2]. Saliva is considered as diagnostic fluid for a number of biological molecules that enter saliva from blood through active transport and passive transport. In contrast to other bimolecular found in saliva, pepsin is present in saliva as admixture with gastric juice from the stomach through upper esophageal sphincter into the gullet [17, 18]. Moreover, pepsin detection has surfaced as a noninvasive technique in the diagnosis of gastroesophageal disease as compared to other invasive methods, meaning it's easy to perform with less inconvenience to patients. From published literatures, pepsin detection using enzymatic techniques, western blotting and enzyme linked immunosorbent assay has been considered vital in the diagnosis of gastroesophageal reflux disease[19, 20].As a matter of fact, rapid lateral flow kits are commercially available currently for detection of pepsin in saliva samples

In this study, we evaluated the value of salivary pepsin in diagnosis of gastreoesophageal reflux disease where enzyme linked immunosorbent assay was used to determine the pepsin concentration in the saliva samples.

From our study results, it was found that the pepsin detection had a sensitivity of 95.52%, specificity of 100%, PPV was 100%, NPV was 91.43%, highly differing results from other salivary pepsin studies[5].This result heterogeneity could be due to difference in study design and enrollment criteria for patients.

In addition, the area under the receiver operator curve analysis (ROC) was 0.997 for pepsin concentration in saliva on the wakening in the morning, this confirms that pepsin detection in saliva can be used as a tool to aid in the diagnosis and management of gastrooesophageal reflux disease, however this in contrary to another study where pepsin was found not play utility role in the diagnosis of GERD[2], the observed discrepancy could be due lack of protocol standardization and differences in the study samples.

In the current study, we found that GERD group had high pepsin concentration as compared to healthy controls meaning that the pathophysiology of GERD is responsible for reflux of quantitative detectable amount of pepsin from the stomach into the oral route, In the similar agreement a publications also found high pepsin concentration in GERD patients[21]. Therefore, pepsin availability in saliva at any concentration is absolute signal that reflux took place [17]

Further, timing points in healthy control group demonstrated no statistical difference for pepsin concentration nevertheless the difference was observed with GERD group, however on further analysis of

GERD group, no significance detected at waking up, 1 hour after breakfast, 1hour after dinner and on symptom onset signifying a similar gastric pepsin secretion in reflux esophagitis and non-erosive reflux disease at all the time intervals. The present results show that the salivary pepsin concentration was high in the morning waking sample for both healthy control group and GERD group in contrary to other publications were high pepsin concentration was significantly detected in the postprandial samples compared to morning waking samples[14]indicating stomach pepsin is pooled into the oral cavity during long resting hours and only morning specimen could be used for clinical diagnosis.

Moreover, from our results, the high sensitivity and predictive values suggest that pepsin detection in saliva may be at least sensitive method for diagnosis of GERD; also, on the other hand the high predictive value may indicate the high prevalence of the disease. Considering the studied patients, GERD group had high pepsin concentration than the healthy group. This outcome could be attributed to the high incidence of transit lower esophageal sphincter relaxation rate in patient group. Markedly, there are number of factors that contribute to the viability of pepsin concentration in saliva. Among them, the volume of gastric content in the reflux that reaches the mouth, the speed at which salivation occurs, and rate of swallowing decreases the concentration of pepsin.

CONCLUSION

In summary, pepsin detection with immunosorbent method is seen as a rapid, convenient, noninvasive, and easily-interpretable means of diagnosing GERD. From this study, saliva pepsin revealed to play a fundamental role in diagnosis of gastrooesophageal reflux disease. However, further studies are still needed to evaluate saliva pepsin sensitivity and specificity under controlled protocols and with large sample size.

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