Pepsin and Bile Acid Concentrations in Sputum of Mustard Gas Exposed Patients
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ABSTRACT

Background/Aim: Gastro-esophageal reflux has been suggested to be associated with several pulmonary complications such as asthma, and post-transplant bronchiolitis obliterans (BO). Pepsin or bile salts in the sputum is shown to be an optimal molecular marker of gastric contents macro/micro aspiration. In this study, we investigated sputum pepsin as a marker of micro-aspiration in sulfur mustard (SM) exposed cases compared to healthy controls. Materials and Methods: In a case controlled study, 26 cases with BO and 12 matched healthy controls were recruited and all cases were symptomatic and their exposure to SM was previously documented during Iran-Iraq conflict. Pepsin levels in sputum and total bile acids were measured using enzymatic assay. The severity of respiratory disorder was categorized based upon the spirometric values. Result: The average concentration of pepsin in sputum was higher in the case group (0.29 ± 0.23) compared with healthy subjects (0.13 ± 0.07; P = 0.003). Moreover, the average concentration of bile acids in the sputum cases was not significantly different in comparison to the controls (P = 0.5). Conclusion: Higher pepsin concentrations in sputum of SM exposed patients compared with healthy control subjects indicate the occurrence of significantly more gastric micro-aspiration in SM exposed patients.

Key Words: Bile acid, gastro esophageal reflux, pepsin, sulfur mustard

Gastro-esophageal reflux disease (GERD) is a common disorder caused by the reflux of gastric contents into the esophagus causing esophageal and extra-esophageal symptoms. Respiratory manifestations of GERD represent as one of the most considerable extra-esophageal symptoms.[1] GERD accompanied by regurgitation and aspiration has been suggested to contribute to several pulmonary complications including, asthma, chronic cough, recurrent pneumonitis and pathologic conditions of the pharynx and larynx such as laryngitis, chronic hoarseness, pharyngitis, and dental diseases.[2-4] It has also been associated with idiopathic pulmonary fibrosis, cystic fibrosis, connective tissue disease, and obstructive lung disease[5] and the development of post-transplant bronchiolitis obliterans (BO).[6-8]

Several mechanisms by which GERD causes pulmonary damage have been suggested. First, the refluxed content may stimulate chemoreceptors in the distal esophagus that can cause bronchial constriction and asthma-like symptoms via vasovagal activation.[9,11] Second pathway is the inflammatory reaction in the airways, mediated by release of interleukin (IL)-8 and recruitment of polymorpho
nuclear leukocytes, as a result of the direct contact of gastric content to the lower airways.\[^{12,13}\] However, more evidence is available in favor of the second mechanism.\[^{14,15}\] Certain lung disorders such as cystic fibrosis and lung transplantation may accompany prolonged exposure of the bronchial epithelium to gastric contents due to impaired clearance mechanisms and mucous bronchial secretion function\[^{16}\] that initiate an inflammatory reaction and further lung impairment through a defective cycle.

The major limitation of the study of reflux and aspiration in patients with pulmonary disease is the absence of a reliable diagnostic tool. Esophageal pH measurements are not sufficient for the evaluation of GERD.\[^{15,17}\] Impedance monitoring may be a superior measure of aspiration risk as it measures both acid and non-acid reflux episodes but it is hard to determine whether the gastric content have reached to the lungs to cause respiratory complications or not.

Molecular markers of aspiration, such as pepsin or bile salts in the saliva or sputum, are reported to be the optimal diagnostic tests. Pepsin measurement in the sputum/saliva collected at the time of symptoms provides a sensitive, non-invasive method for diagnosing GERD in patients with clinically suspected atypical GERD symptoms and its presence in sputum or saliva is considered to be pathologic.\[^{18}\] Bile acids are also believed to cause lung injury and cytotoxicity, but their value as an aspiration marker remains controversial.\[^{19,20}\]

Respiratory problems are the greatest cause of long-term disability among patients with exposure to sulfur mustard (SM) and are mostly presented by a triad of cough, expectoration, and dyspnea. Previous collaborative studies have revealed that BO and bronchiolitis obliterans organizing pneumonia is the main long-term respiratory pathology in SM exposed cases\[^{21,22}\] and it is also demonstrated that reflux esophagitis is more frequent in these patients.\[^{23}\] However, to date no study has been conducted to assess micro-aspiration in these patients. The aim of the present study was to investigate, the presence of pepsin as a marker of micro-aspiration in SM exposed patients and assessing their relationship with degree and severity of respiratory symptoms.

**MATERIALS AND METHODS**

**Study design and participants**

A total of 26 BO subjects with a history of SM exposure and 12 matched healthy cases were recruited between November 2008 and May 2009 at the Baqiyatallah Hospital, Tehran. Only SM exposed patients with atypical manifestations of GERD such as non-cardiac chest pain, hoarseness, chronic cough, and belching were considered for enrollment in this study. The diagnosis of BO was previously established with physical examination, spirometry and high resolute computed tomography (CT). A detailed questionnaire was completed by an investigator for each patient in order to obtain symptom information for typical and atypical symptoms.

All patients had been referred to the Departments of Cardiology, Otorhinolaryngology, or Pulmonology before enrollment in the present study to rule out other causes of respiratory symptoms. Exclusion criteria; any abnormality in chest radiograph, indicating the evidence for BO, a respiratory infection within 4 weeks of inclusion, gastric or esophageal surgery, evidence of upper gastrointestinal (GI) malignancy or other severe concomitant disease and heavy smoking. The control group consisted of 12 healthy non-smokers with negative allergic history, no clinical symptoms of upper or lower airway disease, and no typical GERD symptoms. Informed consent was obtained from all study subjects. Subjects were required to discontinue their anti-acid medications during a 2-week period. The study was approved by the Ethical Committee of Baqiyatallah University of Medical Sciences, Tehran, Iran.

**Laboratory and spirometric assessment**

Sputum samples were collected at the time of nocturnal cough, dyspnea attacks and after meal, from patients for pepsin measurement. Subjects were instructed to cough and clear the sputum, if there was lack of sputum, from the back of their throat and spit it into one of the tubes. Citric acid was used to maintain the sample at acidic pH after collection to stabilize the pepsin and to act as a simple antibacterial agent for the sample. Pepsin levels in sputum and total bile acids were measured by ELISA kit by adding the same concentration of dichlorodiphenyltrichloroethane to the standard curve samples as used in the sputum supernatants. The severity of respiratory disorder was categorized based upon forced expiratory volume in 1 second (FEV1) values as “mild” (FEV1 > 50%) “moderate” (50%>FEV1 > 30%) and “severe” (FEV1 < 35%). Para clinical examinations such as chest CT scan, pulmonary function tests and gastric endoscopy were conducted for all patients.

**Statistical analysis**

Data are presented as mean and standard deviation. Differences between the patients and controls were estimated using independent samples t-test, Mann-Whitney U test, Chi-square and or by the Kruskale Wallis test (for more than 2 groups), as appropriate. Bivariate correlations were assessed using Pearson’s and Spearman’s correlation coefficients for normally and abnormally distributed data, respectively. All data were analyzed using SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). A probability of $P < 0.05$ was considered as significant.
RESULTS

In total, 26 patients and 12 controls were evaluated in this study. The mean age of case group was 44.5 ± 6.2 years. All participants were male. 9 (36.6%) patients had mild, 14 (53.8%) moderate and 3 (11.5%) had severe respiratory impairment. High resolution computed tomography (HRCT) findings revealed air-trapping in 1-25% in 18 (72%) cases and in 25-50% in 4 (16%) patients. Esophageal wall thickening was the most prevalent finding in HRCT. Atypical reflux symptoms were observed in 84.6% of patients. Endoscopic findings included, erosive gastritis (60%), grade a reflux (42%), hiatal hernia (36%), grade b of reflux (16%) and grade c reflux (4%) in our patients [Table 1].

Pepsin and bile assay

The average concentration of pepsin in sputum was higher in the case group (0.29 ± 0.23) compared with healthy subjects (0/13 ± 0.07), (P = 0.003).

The average concentration of bile acids in the sputum of mustard gas (MG) exposed patients was not significantly different in comparison to the normal group (0.287 ± 0.271) compared with healthy people.[12] In the presence of acid, pepsinogen is transformed to pepsin and digests protein. Pepsin exhibits enzymatic activity at a pH level above 4 and is only irreversibly inactivated at a pH level greater than 6.5.

However, we couldn’t find a significant association between clinical severity and pepsin (P = 0.7) or bile acid (P = 0.4) concentration.

DISCUSSION

In this study, we concluded that SM exposed patients with chronic cough, suspected to have GERD appear to have more features of micro-aspiration than control subjects. Sputum bile acid concentrations were not significantly different between our patients and controls.

Gastric refluxed contents contain acid and pepsin both of which are deleterious to the laryngeal and pharyngeal mucosa. In previous studies, pepsin was found to reliably detect micro-aspiration in patients after lung transplantation in comparison to healthy people.[12] In the presence of acid, pepsinogen is transformed to pepsin and digests protein. Pepsin exhibits enzymatic activity at a pH level above 4 and is only irreversibly inactivated at a pH level greater than 6.5.

Pepsin has been reported to be present in the saliva and sputum of patients investigated for reflux.[26‑28] The micro-aspiration of gastro esophageal content and their pathogenic potential to induce severe lung damage was suggested in animal studies and some clinical studies of rare diseases.[26‑28]

Bronchial epithelium is a recognized source of cytokines, chemokines, and growth factors, which play an important role in normal lung function. However, several conditions are thought to impair its integrity. Blondeau et al., showed frequent presence of bile acids in saliva of cystic fibrosis (CF) patients and increased the presence of bile acids in bronchitis obliterans syndrome bronchoalveolar lavage (BAL) of lung transplantation patients with bronchitis obliterans syndrome (BOS) or chronic rejection. D’Ovidio et al., showed that the presence of bile acids in bronchoalveolar lavage fluid (BALF) is associated with increased levels of IL-8 and neutrophilia and risk of development of BOS. Other studies have shown an association between the presence of gastric contents in bronchoalveolar lavage fluid of these patients and development of BOS.[26,29‑31] A possible role of retrograde aspiration after gastroesophageal reflux (GER) in the early development of BOS has been implicitly documented in the recent work from Cantu et al., in which they show that, in lung transplant patients with known GER disease, early fundoplication led to significant improvements in freedom from the development of BOS. GER disease has also been associated with the development of BOS.

Table 1: Demographic and respiratory disorders data of volunteers

| Age, year  | Control (n=12) | Mild MG exposed (n=9) | Moderate MG exposed (n=14) | Severe MG exposed (n=3) |
|-----------|---------------|----------------------|--------------------------|------------------------|
| Sex, M:F  |               |                      |                          |                        |
| FEV1, % predicted | 44.5±6.2 | 49.8±3.2 | 43±4.9 | 49±3.2 |
| FEV1/FVC % | <0.72 | <0.98 | <0.63 | <0.52 |
| Dyspnea intensity on ATS scale (stage) | 0-I | I-II | II | III-IV |
| Air trapping intensity % | 0 | 14±4 | 31±5 | 68±7 |

Table 2: The mean concentration of pepsin and total bile acids in all groups in comparison to controls

| Factor analysis | Control (n=12) | Mild MG exposed (n=9) | Moderate MG exposed (n=14) | Severe MG exposed (n=3) |
|----------------|---------------|----------------------|--------------------------|------------------------|
| Pepsin in sputum | Mean | Mean | SD | Asymp. significant | Mean | SD | Asymp. significant | Mean | SD | Asymp. significant |
| TBA in sputum | 0.29±0.23 | 0.215 | 0.233 | 0.004 | 0.287 | 0.271 | 0.003 | 0.312 | 0.295 | 0.002 |

TBA: Total bile acids, MG: Mustard gas, SD: Standard deviation, pepsin and TBA unit measurements respectively are µg/protein and µg/mg
of BO–associated pneumonia or diffuse bronchiolitis in the non-lung transplant population.\cite{32,33}

In a previous study, we found that the frequency of both endoscopic and pathologic defined esophagitis was significantly higher in the patients with mustard-gas exposed patients than in controls. MG may directly damage the gastrointestinal via cholinergic effect, inflammatory reaction of mucosa, and delayed radiometric effects.\cite{34} In addition, pulmonary defense mechanisms, including the cough reflex and mucociliary clearance of foreign bodies, are markedly impaired in SM exposed patients. It is conceivable that a prolonged contact time of aspirated gastric contents may therefore lead to substantially greater lung parenchymal injury. Moreover, while GERD may cause direct lung injury, it is also possible that it may play a role in augmenting the alloimmune response by creating an unregulated inflammatory environment in these patients.

It should be noted that the finding of pepsin or other possible markers of micro-aspiration in BALF does not necessarily conclude a causal relationship between GERD and pulmonary symptoms. On the other hand, other findings suggest that GERD could be secondary to anatomic changes following respiratory problems.\cite{35,36} In fact, pulmonary diseases promote the occurrence of esophageal reflux due to abnormal breathing physiology, thus forming the pathologic circle. A limitation of our study is that, the pepsin concentration of sputum may be under the influence of the procedure itself, as reported by Ervine and coworkers in a recent study on healthy children.\cite{37,38}

**CONCLUSION**

Our findings of higher pepsin concentrations in induced sputum of MG exposed patients than healthy control subjects support the occurrence of significantly more gastric micro-aspiration in GER-related MG exposed patients. However, pepsin concentration in sputum does not appear to be correlated with severity of respiratory symptoms. Larger observational studies are needed to determine the clinical significance of aspiration in these patients and the diagnostic test that best predicts adverse outcomes.

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