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Chronic Aspiration of Gastric and Duodenal Contents and Their Effects on Inflammatory Cytokine Production in Respiratory System of Rats

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ABSTRACT

Gastroesophageal reflux disease (GERD) is defined with clinical symptoms of heart burning and regurgitation. It may be associated with external esophageal symptoms such as chronic cough, asthma, laryngitis, chronic lung disease, sinusitis and pulmonary fibrosis. In the present study, rats with chronic aspiration of gastroduodenal contentswere studied for cellular phenotypes and cytokine concentrations in bronchoalveolar lavage and lung tissue.

Thirty-six male Albino N-MRI rats were randomly divided into six groups. After anesthesia and tracheal intubation, the animals received either 0.5ml/kg of normal saline (control), gastric juice, pepsin, hydrochloric acid or bile salts by injection into their lungs twice a week for 8 weeks. In sham group nothing was injected. Thereafter, cellular phenotypes and cytokine concentrations of Interleukine (IL)-1α, IL-1β, Transforming Growth Factor (TGF)-β, Tumor Necrosis Factor (TNF)-α, and IL-6 were assessed in bronchoalveolar lavage and lung tissue homogenates.

The numbers of epithelial cells, macrophages, neutrophils and lymphocytes in BAL and levels of cytokines IL-1α, IL-6, TNF-α and TGF-β in BAL and lung tissue of test groups were significantly higher than the control group. Aspiration of bile salts caused more cytokine levels and inflammatory cells compared to other reflux components.

It can be concluded that GERD with increased cytokines and inflammatory cells in lung could cause or exacerbate asthma and pulmonary fibrosis.

Keywords: Bronchoalveolar lavage; Cells; Cytokines; Gastroesophageal reflux; Inflammatory; Lung

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INTRODUCTION

Gastroesophageal reflux disease (GERD) manifests with clinical symptoms of heart burning and

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regurgitation. This disease can be associated with external esophageal symptoms including upper and lower pulmonary complications such as chronic cough, asthma, laryngitis, chronic obstructive pulmonary disease (COPD), voice harshening, sinusitis, otitis media, recurrent pneumonia and larynx cancer. External esophageal symptoms may be due to aspiration of gastric contents and vagus nerve stimulation.[1] In Western countries the prevalence of GERD is about 45% in adults.[2] Idiopathic pulmonary fibrosis is a chronic progressive pulmonary disease with mean survival rate of 2-3 years from diagnosis. It is believed that chronic microaspiration of small amounts of gastric juice following gastroesophagial reflux may cause repetitive subclinical injuries and finally pulmonary fibrosis.[3] A systematic review has shown that prevalence of gastroesophagial reflux or esophagus dismotility is higher in patients with different interstitial and lung diseases compared to control group patients. In idiopathic pulmonary fibrosis, the abnormal contact of acid with esophagus is 67-76 percent.[4] Another study in asthma patients has proven a considerable reduction in respiratory parameters of FEV1, FVC, PEF and DLCO. [5] The signs of gastroesophagial reflux has been reported to be 30% in mild, 76% in moderate and 70% in severe asthma patients.[6]

A systematic review of 28 epidemiologic studies on adults has clarified that 59.2% of asthma patients have GERD symptoms in comparison to 38.1% of control group.[7] Trajkov et al proved association between cytokine gene polymorphisms and bronchial asthma.[8] In our previous study in rat, intra-tracheal administration of gastric juice and its components caused histopathological signs of inflammation and fibrosis in all groups compared to saline administration.[9] Due to significant role of cytokines in inflammatory responses, we decided to investigate the effects of aspiration of gastric juice, pepsin, hydrochloridric acid and bile salts individually on cellular phenotypes and inflammatory cytokines in BAL fluid and lung tissue of rats in order to find the effect of gastroesophageal reflux components on inflammatory cells and cytokine production following aspiration to the lung.

MATERIALS AND METHODS

The study was performed on 36 male Albino rats weighed 250-300g. Animals were kept in the animal house of Afzalipour School of Medicine, Kerman/Iran At 20-22° C and 12h light-dark cycle. They had free access to water and food. All procedures and animal care were approved by ethics committee (Ethic committee permission no 90/390 KA) of Kerman University of Medical Sciences.

Animals were randomly divided into six groups (n=6 in each group):

Group 1: Sham: Animals underwent tracheal cannulation, but no substance was injected into the trachea.

Group 2: Control: After tracheal cannulation, 0.5ml/kg normal saline was injected into the trachea.

Group 3: Gastric juice: After tracheal cannulation, 0.5ml/kg gastric juice was injected into the trachea.

Group 4: Hydrochloric acid: After tracheal cannulation, 0.5ml/kg HCl (pH 1.5-2) was injected into the trachea.

Group 5: Pepsin: After tracheal cannulation, 0.5ml/kg pepsin (concentration 2.5µg/ml) was injected into the trachea.

Group 6: Bile salts: After tracheal cannulation, 0.5ml/kg bile salts (concentration 2.5µg/ml) was injected into the trachea.

The applied concentrations were based on previous studies in which the concentration of these substances in gastric juice had been determined.[10,11] Since in normal conditions gastric juice has no bile salts, the amount of bile salts were chosen based on the concentration of pepsin.

Collection of Gastric Juice

This part was done on six extrarats out of the above mentioned groups. Animals were kept fasted for 12 hours and were anesthetized by intraperitoneal injection of 50mg/kg sodium thiopental in the early morning. Then abdomen was opened and distal part of esophagus was closed tightly by a thread. The intestine (duodenum) was cut few centimeters after pyloric sphincter and then 4ml distilled water was injected into the stomach through duodenum via a tube connected to a syringe. After 20 minutes, gastric content was collected by the syringe and transferred into the Eppendorf tubes to be stored at -80°C until the time of experiments.[10,11]

Chronic Aspiration

Following anesthesia with ether, the animal was placed on its back on a 45-degree inclined plane with its head bent from the top of the plane. The throat was lighted by using a probe connected to the cold light

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source (15W, 6V lamp). Then the animal's mouth was opened and by observing the opening of air passage at inhalation and exhalation, intubation was performed through mouth. Then, based on the animals' grouping, 0.5ml/kg of the related substance was slowly injected into the trachea with syringe connected to a PE10 cannula. Then the animal was laid on right lateral position with its head upper than the body to allow the injected substance enter the right lung and to prevent entering into the left lung causing unwanted death of the animal. During this time, the animal was kept anesthetized by using an ether mask. Aspirations were performed under short-term anesthesia with ether twice a week between 8 to 10 AM for 8 weeks. The animals were under care for one hour after recovery from anesthesia and then were transformed into the cage.[10-11]

Collection of BAL and Lung Tissue Specimens

One week after the last injection (the 9th week), animals were anesthetized by intraperitoneal injection of 50 mg/kg Sodium thiopental and 1ml normal saline was injected into the trachea through tracheal cannulation, and bronchoalveolar (BAL) fluid was collected by syringe and transferred into Eppendorf tubes to be kept at –80°C until being used for determining cellular phenotypes and inflammatory cytokines. In the day of experiment, BAL specimens were thawed and centrifuged. The precipitate was sent to the pathology laboratory for identification of inflammatory cells and the supernatant was used for cytokine analysis (see below). [10-11] After staining, a pathologist who was blind to animals' grouping, evaluated the slides for the number of macrophages, neutrophils, lymphocytes and epithelial cells. Cell counts wereperformed in at least 10 microscopic fields at a magnification of ×400 and the results were presented as mean cell numbers.

After BAL collection, each animal was sacrificed, the chest was opened and the right lung was removed. A part of the lung was transferred into the Eppendorf tube and kept at -80°C for determining inflammatory cytokines in lung tissue.

Cytokine Analysis

Lung samples were homogenized using Hielscher homogenizer (UP200H, Germany). For this purpose, 100mg of the right lung was added to 1ml buffer solution containing protease inhibitor (10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L Ethylene Diamine Tetraacetic Acid (EDTA), 0.5 mmol/L Ethylene Glycol Tetraacetic Acid (EGTA), 1 mmol/L Dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 20 mmol/L glycerophosphate, and 1 mmol/L sodium vanadate in phosphate buffered saline solution (pH 7.2) containing 0.5% Triton X-100) and was homogenized on ice (4°C). The homogenized sample was incubated at 4°C for one hour and then was centrifuged for one minute at 4°C and 12000rpm (S430R, Eppendorf Co., Germany). TNF-α, IL-6, IL-1β, IL-1α and TGF-β were determined by Enzyme Linked Immunosorbent Assay (ELISA) (MAT2000, ELM2000, DRG USA) in the supernatants of BAL and homogenized lung by relevant kits for rat (eBioscience-Germany).

Data Analysis

Quantitative data were presented as mean±SD. Data were investigated by Shapiro Wilks test in regard to their normal distribution. Since none of the inflammatory cell indices, tissue cytokines and BAL cytokines had normal distribution, non-parametric test of Kruskal Wallis was used for comparison of groups. The homogeneity of variance was determined by Leven test and in the case of being present, sheffe as a post hoc test was used. In the case of non-homogeneity, Tamhane's T2 was used for comparison of paired groups. Data analysis was performed by SPSS18 software package and *p*-values <0.05 were considered as statistically significant.

RESULTS

Inflammatory Cells in BAL

As it is presented in Figure 1, in comparison to sham and control (normal saline) groups, all experimental groups showed significant increase in the number of epithelial cells, macrophages, neutrophils and lymphocytes in BAL fluid ($p<0.001$). Among these the number of cells in bile salt group was significantly higher compared to other three groups ($p<0.05$).

Inflammatory Cytokines in Lung Tissue

The results of measurement of cytokines in lung tissue are shown in Table 1. TNF-α, IL-1α, IL-6 and IL-1β in lung tissue showed significant increase in all experimental groups in comparison to the sham or control group ($p<0.001$). TNF-α was significantly

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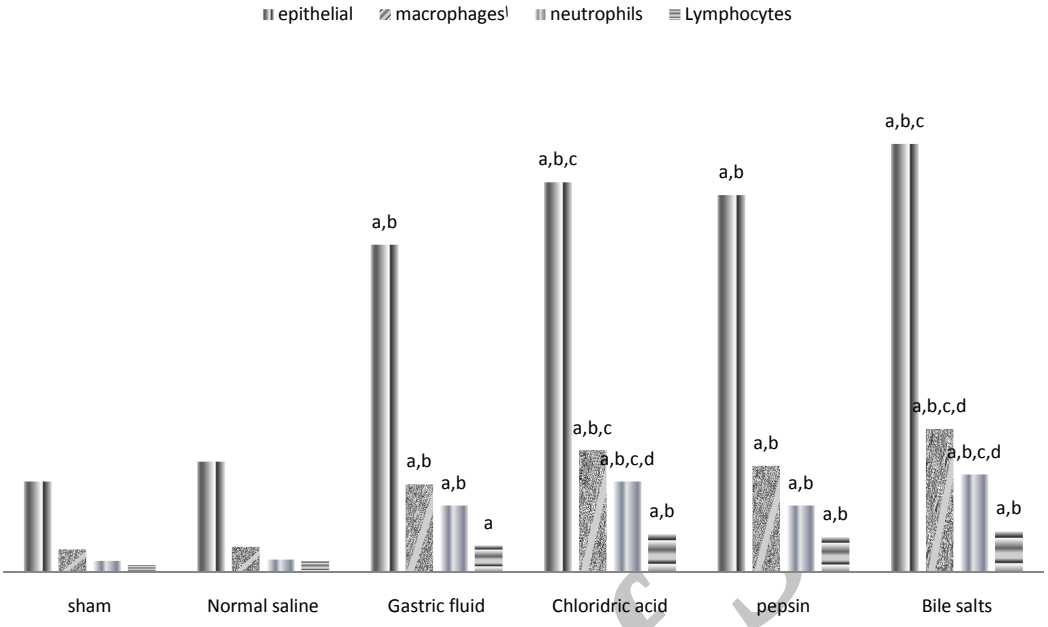

Figure 1. Comparison of inflammatory cells in BAL fluid in the studied groups. Cell counts in microscopic fields at a magnification of ×400 for each slide. a: $p<0.05$ compared to the sham group, b: $p<0.05$ compared to the normal saline group, c: $p<0.05$ compared to the gastric fluid group, d: $p<0.05$ compared the pepsin group.

higher in bile salt group compared to the other three groups ($p<0.05$).

TGF-β in lung tissue was significantly higher in hydrochloridric acid, pepsin and bile salt groups compared to sham and normal saline groups ($p<0.001$) (Table 1).

Inflammatory Cytokines in BAL

Table 2 shows the result of measurement of cytokines in BAL. The amount of IL-1β and IL-6 in BALwere significantly higher in hydrochloridric acid, pepsin and bile salt groups in comparison to sham and contrrol groups ($p<0.05$). Also, IL-1α and TNF-α in

Table 1. The amount of cytokines (mean ±SD) in lung tissue of the studied groups.

Cytokines / group	IL-6 (pg/mL)	TNF-α (pg/mL)	TGF-β (ng/mL)	IL-1α (pg/mL)	IL-1β (pg/mL)
Sham	4384.4±869.0	4267.3±893.8	1722.9±422.1	10887.9±4413.3	940.7±228.9
Normal saline	5811.7±788.2	5379.5±641.9	1688.9±460.7	13511.3±5592.6	1037.7±115.9
Gastric juice	7711.8±836.8 [a,b,d]	6450.1±1331.1 [a]	2492.8±345.3	18058.8±3872.0 [a]	1724.5±314.4 [a,b]
Chloridric acid	6036.4±850.8 [a]	6203.7±1137.3 [a]	3764.9±1399.9 [a,b,c]	23257.9±4460.9 [a,b]	1887.8±432.3 [a,b]
Pepsin	6066.4±702.3 [a]	6602.8±818.1 [a]	2930.8±335.8 [a,b]	24628.8±2456.7 [a,b]	2160.9±463.1 [a,b]
Bile salt	6917.6±675.0 [a]	8682.7±1320.3 [a,b,c,d]	3105.8±613.4 [a,b]	22942.5±2670.0 [a,b]	1885.4±541.2 [a,b]
P value (among groups)	<0.001	<0.001	<0.001	<0.001	<0.001

a: $p<0.05$ compared to the sham group, b: $p<0.05$ compared to the normal saline group, c: $p<0.05$ compared to the gastric juice group, d: $p<0.05$ compared to gastric acid and pepsin groups

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Table 2. The amount of cytokines (mean ±SD) in BAL fluid of studied groups.

Cytokines / Groups	IL-6 (pg/mL)	TNF-α (pg/mL)	TGF-β (pg/mL)	IL-1α (pg/mL)	IL-1β (pg/mL)
Sham	370.0±75.8	379.0±145.7	66.4±16.3	738.4±665.3	63.9±52.0
Normal saline	412.5±190.6	379.0±144.7	77.9±7.3	785.6±778.8	30.2±31.1
Gastric juice	747.4±204.2	701.0±60.6	81.5±11.3	2155.6±1807.3	144.5±53.5
Chloridric acid	1286.6±756.3 [a,b]	557.0±165.6	89.8±37.3	1961.6±1233.7	250.3±90.3 [a,b]
Pepsin	1218.3±352.7 [a,b]	733.0±163.6	139.5±76.8[a]	2566.4±1332.3	247.7±76.0 [a,b]
Bile salt	1165.0±287.1 [a,b]	763.0±329.3 [a,b]	91.3±20.1	3180.4±889.1 [a,b]	312.6±181.0 [a,b]
P value (among groups)	<0.001	<0.001	<0.001	<0.001	<0.001

a: $p<0.05$ compared to the sham group, b: $p<0.05$ compared to the control (normal saline) group.

bile salt group were significantly higher compared to sham and control groups ($p<0.001$). Pepsin group showed significant increase in TGF-β ($p=0.045$) compared to the sham group.

DISCUSSION

In this experimental study, chronic aspiration of different gastroduodenal reflux components was investigated in rat lung. Among these components, bile salts caused the most significant increases inflammatory cytokines in BAL and lung tissue and in the number of inflammatory cells in BAL. This finding was in agreement with the results of our previous study in rats that inflammation and tissue fibrosis in bile salt group were significantly higher than the gastric juice, pepsin and hydrochloric acid groups.[9] This probably means that bile salts among reflux components are the main harmful component for the lungs. SinceTNF-α in lung tissue and BAL in bile salt group showed significant increase in comparison with other groups (see Tables 1 and 2), and this cytokine is cytotoxic, its presence in respiratory system causes a wide spectrum of pathologic, metabolic and hemodynamic complications.[12-13] Therefore, it can be asserted that one of the reasons of more lung injuries from the aspiration of bile salts compared to the other components[9] might be due to this cytokine.

In the present study, TGF-β in lung tissue of experimental groups showed significant increase in comparison to sham and normal saline groups. TGF-β potency in multiplication of fibroblasts and extracellular matrix production has been well known.[14] Perng et al have proved that aspiration of bile salts causes fibroblast multiplication and fibrosis through TGF-β synthesis by epithelial cells.[15] Increase in TGF-β in all test groups in the present study was also in agreement with the findings of Apple and Hartwig in which this cytokinewas increased along with other cytokines due to gastric juice reflux.[10,16] Although TGF-β is known as a regenerative (anti-inflammatory) factor produced by the damaged tissues to oppose with the effect of inflammatory ones, it causes chemotaxis of fibroblasts to the tissue and an increase in extracellular matrix leading to fibrosis.[17] Therefore, the high level of this cytokine in lung tissue homogenates (Table 1) and the large numbers of macrophages in BAL (Fig 1) may be the second reason for more intensive fibrosis found in bile salt group.[9] It may be postulated that fibrosis through destruction of lung tissue causes increase in tension on mediastinal structures and disturbance in lower esophageal sphincter function and facilitating reflux of gastrodeudenal contents.[18] This in turn intensifies fibrosis generation, creating a positive vicious cycle.

Chronic aspiration of gastric juice significantly increased macrophages, lymphocytes, neutrophils and epithelial cells in BAL fluid. In the Apple and Downing studies too, the numbers of macrophages and white blood cells in BAL fluid were significantly higher in gastric juice group.[10-11] Although in the present study, the number of inflammatory cells was significantly higher in gastric juice group compared to control and sham groups (Fig 1),among all groups, the highest cell count belonged to the bile salt group. This finding also parallels higher cytokine and fibrosis production in this group mentioned before.

The non significant difference between cytokines and inflammatory cells in the sham and normal saline groups showed that our interventions (intubation and

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fluid injection into the airways) were not the cause of increase in cytokines and inflammatory cells in lungs and the observed complications wereonly related to the gastro-duodenal reflux components.

In the present study, the concentration of the cytokines in lung tissue were higher compared to BAL fluid. This might be due to the dilution of cytokines following fluid injection into the lung and the fact that lung inflammatory cells are the source of these cytokines.

The findings that cytokines and inflammatory cells increased due to chronic aspiration of gastro-duodenal juice provide a better knowledge in understanding the pathogenesis of pulmonary inflammatory diseases such of asthma and pulmonary fibrosis complicated by GERD. We believe that frequent micro-aspirations of bile salts in conjunction with gastric juice components are the origin of above mentioned complications.

The small number of weekly aspirations in the present study was one of our study limitations that might not be representative of clinical GERD in which reflux happens more frequently. Although more severe complications are expected in the case of increasing the number of aspirations, it was not possible in the present study since based on our pilot study, increasing the number of aspirations might increase the mortality rate of animals due to the invasiveness of the procedure (anesthesia, intubation and substance injection).

Overall the findings of this study showed that asthma, fibrosis and other pulmonary complications of GERD may be due to the arousal of inflammatory responses by reflux components, and bile salts are the main harmful component. The finding that non-acid reflux components may have more important role implies that the current treatment strategy for GERD that is mostly based on anti-acid prescription should be revised and fundoplication surgery in addition to antiacid drugs should be considered. Also reflux control in early stages of the disease might improve or prevent the disease progress.

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