ORIGINAL ARTICLE

Study of the level of sputum matrix metalloproteinase-9 (MMP-9) and tissue inhibitor metalloproteinase-1 (TIMP-1) in COPD patients

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KEYWORDS
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Abstract  Background: Chronic obstructive pulmonary disease (COPD) is a major cause of respiratory morbidity and mortality worldwide. One of the main hypotheses concerning the pathogenesis of emphysema, a key cause of morbidity and mortality in COPD, is the protease antiprotease imbalance. Irreversible airflow obstruction in Chronic Obstructive Pulmonary Disease (COPD) is thought to result from airway remodeling associated with aberrant inflammation.

This study examined changes in sputum as regards MMP-9, TIMP-1 and levels of inflammatory cells in COPD patients compared with sputum of healthy smokers and non-smokers.

Methods: Forty patients were included in this study. FEV1 before and after salbutamol inhalation, MMP-9, TIMP-1 and inflammatory cell count in the sputum of COPD patients, healthy smokers and non-smokers were investigated.

Results: MMP-9 was significantly increased in both COPD patients (194.4 ± 100.6), and healthy smokers (104.5 ± 42.1) compared with healthy non-smokers (34.5 ± 36.1). TIMP-1 was increased more in healthy non-smokers (192.7 ± 37.7) than COPD patients (115 ± 55.5) and healthy smokers (145.3 ± 35.1). MMP-9/TIMP-1 was high in COPD patients (1.7 ± 0.9) and healthy smokers (0.7 ± 0.3) compared with healthy non-smokers (0.2 ± 0.2). Mean sputum total leucocytic count (TLC) was highly statistically significantly different between the three groups. COPD group showed the highest means value while non-smokers group showed the lowest one.

Conclusions: COPD is characterized by an imbalance between MMP-9 and TIMP-1 which may play an important role in the pathogenesis of tissue remodeling and airway obstruction. © 2014 The Egyptian Society of Chest Diseases and Tuberculosis. Production and hosting by Elsevier B.V. All rights reserved.

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Background

Chronic Obstructive Pulmonary Disease (COPD) is a common preventable and treatable disease characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases [1]. COPD is more common in smokers and ex-smokers than in nonsmokers. Cigarette smokers have more respiratory symptoms, lung function abnormalities, decline in FEV1, and COPD mortality than non-smokers. The starting age, total pack-years smoked and current smoking status are predictive of COPD morbidity and mortality [2]. COPD is a classic disease of airway damage and remodeling. Extracellular matrix (ECM) deposition is one pathological form of the tissue remodeling detected in the airways [3–5]. Matrix metalloproteinases (MMPs) have an important role in the breakdown of ECM and they are considered as biomarkers of tissue damage in several smoking related lung diseases [4–6].

MMPs are a family of zinc containing endopeptidases, which is a subset of the metzincin superfamily of metalloproteinases. These regulatory proteases are the extracellular matrix (ECM) remodelers characterized by their substrate specificity to degrade ECM proteins [7]. Approximately 20 different types of MMPs have been discovered and classified based on their pre-synthetic region on chromosomes and their various substrate specificities. Number designations MMP-1 to MMP-28 are used for classification [8]. Many of MMPs are activated by smoking and/or oxidative stress [9,10]. They are produced by a range of stromal cells and by two of the major inflammatory cells implicated in COPD – neutrophils and alveolar macrophages [11,12]. MMPs can be separated into 6 main classes according to their substrate specificity, cellular location and primary structure: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and others [13,14]. Recent studies have shown that levels of MMPs, especially MMP-9, are elevated in the bronchoalveolar lavage (BAL) fluid from patients with COPD, compared to normal controls [11,12], and high levels of both MMP-9 and its cognate inhibitor TIMP-1 have been found in sputum from chronic bronchitis [15] and correlated with decrease in lung function [16,17]. MMP-9 may play important physiologic roles in lung extracellular matrix remodeling and repair, and in regulating the lung inflammatory response to injury [18]. However, MMP-9 has also been implicated in the pathogenesis of various lung diseases including chronic obstructive pulmonary diseases [11,19,20].

Tissue inhibitors of metalloproteinases are a family of secretory proteins that are able to inhibit matrix metalloproteinase activities through non-covalent binding of pre- or active forms of MMPs at molar equivalence. By inhibiting MMPs, TIMPs may influence also MMP-mediated processes such as processing of cytokines, degradation of growth factor binding proteins, and the release of ECM-bound growth factors [21–24]. Tissue inhibitors of matrix metalloproteinases (TIMP) make up a family of four inhibitors (TIMP-1, -2, -3 and -4) [25]. All members of the TIMP-family inhibit MMPs by forming covalent 1:1 complexes [13,26], and selective inhibition of some of the MMPs exists [27]. The actions of TIMPs are not restricted to MMP-inhibition, and their effect depends on the cell context and model studied. TIMPs can modulate cellular processes such as cell growth, apoptosis and migration, and can be both anti- and protumourigenic [13,26,27]. TIMP-1 works as a natural inhibitor of MMP-9 and is found in most tissues and body fluids. By inhibiting MMPs activities, TIMPs are involved in tissue remodeling and regulation of ECM metabolism. The TIMP family consists of four members sharing important structural features as well as the ability of MMP inhibition [13].

Induced sputum (IS) collection is a non-invasive method for the assessment of inflammation in the airways [28,29]. Healthy smokers had a higher concentration of total MMP-9 and that concentration was correlated with their exposure to tobacco smoke. Maintenance of the active MMP-9/TIMP-1 ratio in healthy smokers may explain the absence of progressive airway obstruction [30].

Subjects methods

Subjects

Forty subjects were included in this study and were classified into the following three groups: Group I (COPD group): included 20 patients with smoking related clinically stable COPD [using (GOLD 2013) criteria]. These patients had a stable airflow limitation with FEV1/FVC < 70% predicted with a reversibility of <12% predicted followed by inhaled β2-agonist administration. COPD group was classified as mild, moderate, severe and very severe according to GOLD, 2013 classification as follows: mild COPD (FEV1 ≥ 80% predicted) with FEV1/FVC < 0.70, moderate COPD (50% ≤ FEV1 < 80%) with FEV1/FVC < 0.70, severe COPD (30% ≤ FEV1 < 50%) with FEV1/FVC < 0.70, very severe COPD (FEV1 < 30% predicted) with FEV1/FVC < 0.70. Patients were excluded if they had a history of respiratory disease other than COPD and respiratory tract infection during the last 4 weeks before the study. Group II (Healthy smokers group): included 10 subjects with smoking history and normal FEV1 > 70% of predicted and no medical history of lung disease. Group III (Healthy non smokers group): included 10 subjects who never smoked before. The patients included in this study were inpatients in the Benha university hospital.

Methods

A written informed consent was obtained from all subjects. All the three groups underwent full history taking including history of smoking (current, ex, and non smoking), history of chest symptoms (cough, expectoration, wheezes and dyspnea), number of exacerbation in the last year and history of any other co morbidities. Also full clinical examination (general, full local respiratory system examination including inspection, palpation, percussion and auscultation, with special regards to manifestations of right sided heart failure as lower limbs edema, congested neck veins, tenderness over right hypochondrium, and dullness on right parasternal area) and chest X ray. Physiological assessment was also done using spirometry. Flow/volume loop was performed to all cases. The test was done before and 20 min after 2 puffs of Salbutamol (200 µg) via a metered dose inhaler for the COPD group. All data were expressed in percent of predicted value (FEV1%, FVC%, FEV1/FVC%). Sputum induction was done and was processed
to assess matrix metalloproteinase-9 (MMP-9) by ELISA (MMP-9 BIOTRAK ELISA), tissue inhibitor of metalloproteinase-1 (TIMP-1) by ELISA (TIMP-1 BIOTRAK ELISA), cytological examination and cellular count. The total and the differential cell counts were expressed as corrected percentage. Routine laboratory investigations (CBC, Liver enzymes, serum creatinine) were also done.

Sputum induction and processing

Sputum was collected either spontaneously or induced with hypertonic saline nebulization from all subjects. Prior to sputum induction, patients inhaled 200 μg of salbutamol to minimize broncho-constriction during the induction procedure. Sputum was induced by inhalation of 3% hypertonic saline solution for 5 min (DeVilbiss 65 ultrasonic nebulizer; DeVilbiss, Somerset, PA, USA), and the subjects were encouraged to cough and expectorate sputum into sterile containers between each dose of nebulized saline. This procedure continued until an adequate sample containing >0.5 ml visible mucocellular material was obtained. If a satisfactory sputum sample was not obtained at the time the FEV1 had fallen >20% compared to baseline values occurred or if troublesome symptoms appeared the procedure should be stopped [31]. The sputum was processed with Dithiothreitol (putolysin) at a final concentration of 0.1% and then sputum diluted and centrifuged (1500 rpm) [32] and the supernatants was collected. The levels of MMP-9 and TIMP-1 of the sputum were measured with enzyme-linked immunosorbent assay (ELISA). Cell viability was assessed in unfixed cells by Trypan blue staining and Hemocytometry. A differential cell count of Neutrophils, Macrophages, Eosinophils and Lymphocytes cells was then performed using the May–Grünwald–Giemsa stain.

The assay of matrix metalloproteinases-9 in sputum is done by ELISA

Principles of assay

The RayBio® human MMP-9 ELISA (enzyme-linked immunosorbent assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human MMP-9. This assay employs an antibody specific for human MMP-9 coated on a 96-well plate. Standards and samples are pipetted into the wells and MMP-9 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human MMP-9 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted into the wells. The wells are washed again, and a TMB substrate solution is added to the wells and color develops in proportion to the amount of MMP-9 bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm [33].

Assay procedure

All reagents and samples were brought to room temperature (18–25 °C) before use. 100 μl of each standard (see Reagent Preparation step 2) and sample were added into appropriate wells. Covered well and incubated for 2.5 h at room temperature. The wells were washed 4 times with 1x wash solution (200 μl each). 100 μl of 1x prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 μl each). 100 μl of prepared Streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded and washed 5 times with 1x wash solution (200 μl each). 100 μl of TMB One-Step Substrate Reagent was added to each well and incubated for 30 min at room temperature in the dark. 50 μl of Stop Solution was added to each well then the absorbance was read at 450 nm immediately.

Calculation of results

The concentration of MMP-9 was calculated from standard curve.

The assay of tissue inhibitor metalloproteinases-1 in sputum is done by ELISA

Principles of assay

The RayBio® human TIMP-1 ELISA (enzyme-linked immunosorbent assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human TIMP-1. This assay employs an antibody specific for human TIMP-1 coated on a 96-well plate. Standards and samples are pipetted into the wells and TIMP-1 present in the sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TIMP-1 antibody is added. After washing away the unbound biotinylated antibody, HRP-conjugated Streptavidin is pipetted into the wells. The wells are washed again, and a TMB substrate solution is added to the wells and color develops in proportion to the amount of TIMP-1 bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm [34].

Assay procedure

All reagents and samples were brought to room temperature (18–25 °C) before use. 100 μl of each standard (see Reagent Preparation step 2) and sample were added into appropriate wells. Covered well and incubated for 2.5 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 μl each). 100 μl of 1x prepared biotinylated antibody was added to each well and incubated for 1 h at room temperature. The solution was discarded and washed 4 times with 1x wash solution (200 μl each). 100 μl of prepared Streptavidin solution was added to each well and incubated for 45 min at room temperature. The solution was discarded and washed 5 times with 1x wash solution (200 μl each). 100 μl of TMB One-Step Substrate Reagent was added to each well and incubated for 30 min at room temperature in the dark. 50 μl of Stop Solution was added to each well then the absorbance was read at 450 nm immediately.

Calculation of results

The concentration of TIMP-1 was calculated from the standard curve.
### Statistical analysis

Quantitative data were presented as means and standard deviation (SD) values. Student’s *t*-test was used for comparisons between means of two groups. Mann–Whitney *U*-test was used to compare between smoking indices of symptomatic and asymptomatic smokers. Paired *t*-test was used to compare between pulmonary function tests before and after the use of bronchodilators in the smokers (with symptoms) group. One-way ANOVA (analysis of Variance) was used to compare between means of the three groups. Qualitative data were presented as frequencies and percentages. Chi-square ($x^2$) test was used for studying the comparisons between different qualitative variables. Pearson’s correlation coefficient was used to determine significant correlations between the different variables. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with SPSS version 19.0 (statistical package for scientific studies) for windows.

### Results

Forty subjects were included in this study and were classified into 3 groups; 20 patients known to have COPD who were subdivided into mild, moderate, severe and very severe COPD cases according to (GOLD, 2013), 10 apparently healthy smokers and 10 healthy non smoker individuals (control). Patients demographic and laboratory data were seen in Table 1. There was no statistical significant difference between the three groups as regards age. All patients and healthy subjects included in this study were males. Mean number of cigarettes smoked in COPD patients and smoking indices were statistically significantly higher than the healthy smokers group (Table 1).

In the COPD group all spirometric parameters were decreased. But in the healthy smokers group the FEV$_1$%, FVC% and FEV1/FVC% were preserved in contrast to FEF$_{25-75}$%, FEF$_{50}$%, PEF% and MVV% were decreased (Table 2).

### Discussion

COPD is associated with a chronic inflammatory response, predominantly in small airways and lung parenchyma, which is characterized by increased number of macrophages, neutrophils, and T lymphocytes. Many proteases are also involved in the inflammatory process and are responsible for the destruction of elastin fibers in the lung parenchyma, which is the hallmark of emphysema. The identification of inflammatory mediators and understanding their interactions are important for the development of anti-inflammatory treatments for this important disease [6].

Healthy non smokers group and healthy smokers group had statistically significantly higher mean FEV$_1$%, FVC% & FEV1/FVC% ratio than the COPD group. It also showed that the non smokers group had statistically significantly higher mean FEF$_{25-75}$%, FEF$_{50}$%, PEF% and MVV% followed by the healthy smokers group and the COPD group which showed the lowest values which means that there is early affection of the small airways in the healthy smokers group which is in line with [35] (table 2).

As regards MMP-9, the COPD group showed highly statistically significant highest mean MMP9, and MMP9/TIMP-1 ratio compared to healthy smokers group and healthy non smokers group (table 3). These findings were in line with [36] who found that sputum MMP-9 concentrations were increased in COPD subjects compared to healthy never smoked but were similar to healthy smokers. In addition, [37] in his study about noninvasive biomarkers for early smoking related lung disease, concluded that long-term smoking increased the levels of MMP-9 and MMP-9/TIMP-1, suggesting that an imbalance...
of MMP-9 and TIMP-1 may play a key role not only in local, but also in the systemic inflammatory process.

As regards TIMP-1, it was found that healthy non smokers group showed statistically significant highest mean TIMP-1
compared to COPD group and healthy smokers group (table 3). This result was in agreement with many studies [30,38].

In the COPD group, mean sputum MMP9/TIMP-1 ratio was statistically significant different between COPD cases with the highest mean was found in cases with very severe COPD cases and gradually decrease in sever, moderate and mild cases (table 4).

Table 5 shows the correlation between sputum MMP9 and different variables in the three groups. There was statistically significant negative correlation between sputum MMP9 and (FEV\(_1\)%, FEF\(_{25-75}\)%). This result was in line with [36,38–41].

Table 6. shows the correlation between sputum TIMP-1 and different variables in the three groups. There was a statistically significant negative correlation between sputum TIMP-1 and smoking index in COPD group.

Table 7 shows the correlation between sputum MMP9/TIMP-1 ratio and different variables in the three groups. There was a statistically significant positive correlation with FEF\(_{25-75}\)% in healthy non smokers group. These results agreed with [30,41].

Conclusions

Sputum MMP-9 level in COPD patients showed statistically significant highest means followed by healthy smokers followed by healthy non smokers group while sputum TIMP-1 level showed statistically significant highest means in healthy non smokers group followed by healthy smokers group followed by COPD group. Sputum MMP-9/TIMP-1 ratio and sputum total leucocytic counts showed statistically significant highest means in smokers (COPD and healthy smoker groups) compared to non smokers group.

COPD is characterized by an imbalance between MMP-9 and TIMP-1 which may play an important role in the pathogenesis of tissue remodeling and airway obstruction.

Conflict of interest

We have no conflict of interest to declare.

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Levels of sputum MMP-9 and TIMP-1 in COPD patients


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