Determination of Matrix Metalloproteinases-9 in Egyptian Patients with Pulmonary *Mycobacterium Tuberculosis*

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ABSTRACT

Matrix metalloproteinases (MMPs) constitute a large family of enzymes that degrade extracellular matrix proteins (ECM). MMPs are implicated in tissue remodeling processes such as wound healing, and pregnancies. MMPs also participate in some pathological conditions such as cancer. Recent studies have shown that MMPs are induced by Mycobacterium tuberculosis (MTB) during pulmonary infection. The aim of the present study was to determine the Matrix Metalloproteinases-9 (MMP-9) levels in Egyptian patients with MTB compared to their levels in healthy control individuals. Forty six patients with MTB (group I) and forty three healthy volunteers (Group II) were included in the study. The concentrations of MMP-9 in the serum samples of the two groups were determined quantitatively by human MMP-9 enzyme–linked immunoassay (ELISA) kit. The result showed that MMP-9 levels were significantly higher in MTB patients ($p<0.0001$), compared to their levels in healthy control group. MMP-9 levels were increased with increasing the severity of the disease, since, their concentrations were significantly increased in complicated cases compared with uncomplicated cases ($p<0.0001$). In conclusion, our study suggests that, the higher levels of MMP-9 in patients with tuberculosis may be due to overexpression by a variety of cells including mononuclear phagocytes and stimulated neutrophils. Also, MMP-9 levels were directly proportional with the severity of the disease. Their rising levels may be used as indicator of MTB activity.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is a facultative intracellular, aerobic, acid-fast bacillus naturally pathogenic for human. Its virulence is related to its ability to survive and proliferate in mononuclear phagocytes. MTB reduces the bactericidal activity of macrophages by preventing the fusion of enzyme containing lysosomes with phagosomes containing the bacilli [(1)].

The immune response is initiated when MTB arrives in the alveolar space where it encounters resident alveolar macrophages, after passing the protective mechanical barriers in the upper respiratory tract [(1,2)]. This initial interaction between the alveolar macrophages and mycobacteria can result in destruction of the organism

or persistence and replication of organism within the macrophage [3].

In pulmonary tuberculosis, macrophages play a key role by releasing many kinds of proteases and cytokines, and by introducing a protective cellular immune response[4]. Among the proteases secreted by macrophages, matrix metalloproteinases (MMPs) are particularly interesting, as these enzymes are able to degrade all extracellular matrices[5]. The proteolytic balance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) is important not only in normal tissue remodeling, but also in various pathological conditions[6-9]. Proteolytic processes may play a role in the formation of pleural effusions by increasing vascular permeability, and therefore by facilitating fluid influx into the pleural space [10].

The presence and enzymatic activities of MMPs and TIMPs have been identified in pleural effusions [11, 12]. Tissue damage is a characteristic manifestation of infection by MTB. Proteolysis by macrophage-secreted proteases has been implicated in such destructive processes[13,14]. In this regard, the proteolytic action of MMPs may be involved in the pathogenesis of tuberculosis, like many other diseases associated with tissue destruction. Several studies showed that macrophages release MMP-9 in response to MTB or its cellular components [15, 16]. An in vivo study demonstrated the activation of MMP-2 and MMP-9 in the lungs of mice infected with MTB [17]. **Chang et al.** [18] reported that the levels of MMP-9 were significantly higher in the broncho-alveolar fluid of patients with active cavitary tuberculosis. MMP-9 in patients with tuberculosis is not restricted to the site of infection, but it is also visible in circulation, as a result of stimulation of circulating leukocytes via cytokines released by activated macrophages.

The aim of the work, was to determine MMP-9 levels in Egyptian patients with MTB, using ELISA Kit, compared to their levels in healthy control individuals.

SUBJECTS & METHODS

All samples were collected after obtaining informed consent from the patients.

a) Patients (group I). Forty six patients (41 males and 5 females) with a confirmed diagnosis of pulmonary TB from Mansoura, and El-mahalla El-kobra Chest Hospitals were selected for the study. The median age of the patients was 31 years (range: 20 to 62 years). TB patients group was classified into 33 complicated cases (15 with pleural effusion, 12 with empyema, and 6 with apical cavitation) and 13 uncomplicated cases.

b) Healthy volunteers (group II). Forty three subjects (31 males and 12 females) of non tuberculosis patients with no previous symptoms or disease were selected for the study. The median age of the volunteers was 32 years (range: 20 to 53 years). The healthy volunteers were documented by a general clinical

examination and no respiratory symptoms were indicated.

Bacteriological culture:

Equal volumes of sputum samples were inoculated into tubes of Lowenstein-Jensen (L-J) medium with glycerol. The L-J tubes were then incubated for 6 - 8 weeks at 35-37^{o}C, and inspected for growth at weekly intervals. Suspicious growth was subjected to Ziehl-Neelsen staining to confirm diagnosis. Negative culture was discarded after 8 weeks.

Ziehl-Neelsen staining: This technique was used for identification of *Mycobacterium tuberculosis* in sputum samples and bacteriological cultures according to the method described by Jenkins [19].

Determination of serum MMP-9: MMP-9 levels were determined in serum samples of pulmonary tuberculosis patients and healthy subjects using a commercial specific Enzyme-Linked Immunosorbent Assay (ELISA) kit provided by RayBiotech, Inc., Parkway Lane, Norcross Georgia**,** USA [20-22]. MMP-9 kit is used for the quantitative measurement of human MMP-9 pro and active forms in serum. This assay employs a mono clonal antibody specific for human MMP-9 coated on a 96-well plate. Assay was processed according to the manufacture's specifications.

Statistical analysis:

Results were expressed as mean ± standard deviation (SD). Unpaired student "t" test and one-way ANOVA were performed using graph pad Instant version 3.00 for Windows 95, Graph pad software, San Diego California USA, to determine the significance. P value < 0.05 is considered to be significant. Interactive dot diagram was performed using MedCalc version 11.1 for Windows XP, MedCalc Software, Broekstraat 52 B-9030 Mariakerke Belgium, with a horizontal line indicates the cut-off point with the best separation between the two groups.

RESULTS

The characteristics of the studied groups were summarized in table 1. A total of 46 pulmonary TB patients and 43 healthy control individuals were included in the present study. The mean age of TB patients was 35.5 ± 12.5 years, with a range between 20 – 62 years. The mean age of healthy control individuals was 32.4 ± 8.9 years, with a range between 20 - 53 years. There was male predominance among the patients with TB, 41 males (89.1 %) versus 5 females (10.9 %), with a male-to-female ratio 8.2: 1. In the healthy control subjects there were 31 (72.1%) males versus 12 (27.9 %) females with a male-to-female ratio 2.6:1. there was a significance difference in the sex ratios of TB patients and healthy control group (p = 0.05). Regarding to cigarette smoking, 82.6 % of TB patients were cigarette smokers in comparison with non smoker healthy group which was statistically highly significant (p=0.0003). All TB patients were sputum smear and culture positive for acid fast Bacilli. All patients presented with clinical and radiological pictures suspicious of pulmonary TB as unilateral and bilateral pulmonary infiltrates with lung cavities (table 1).

Table 1: Characteristics of the studied groups:

	Healthy volunteers	**Tuberculosis patients**
Number	43	46
Sex: female/ male	12/ 31	5/ 41
Age (years)	32.4 ± 8.9	35.5 ± 12.5
Smokers	---	38
Chest-x rays	---	Cavities & opacities are observed
Bacteriological culture	43 Negative	46 Positive
Z.N. staining for AFB	43 Negative	46 Positive
MMP-9 levels (pg / ml)		
Range	270 - 3450	3455-8500
Mean ± SD	2180.9 ± 691.1	6575.2 ± 13 23.4

Determination of MMP-9:

MMP-9 levels were measured in the serum samples of 46 tuberculous patients and 43 healthy volunteers, using MMP-9 ELISA Kit. The results showed that TB patients had higher MMP-9 levels (ranged from 3455 to 8500 pg/ml), with the mean value of 6575.2 ± 1323.4 pg /ml, compared with its levels in healthy control group (ranged from 270 to 3450 pg/ml) with the mean value of 2180.9 ± 691.1 pg/ ml (table 1 & figure 1). Statistically, the levels of MMP-9 in TB patients were significantly higher than in healthy control group (P<0.0001) (table 1 and figure 1 and 2).

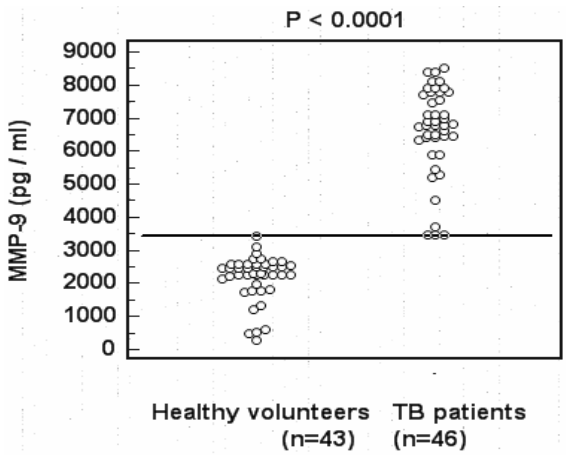

Fig. 1: distribution of the serum MMP-9 in 43 healthy volunteers and 46 TB patients. The horizontal line indicates the cut off point (>3450).

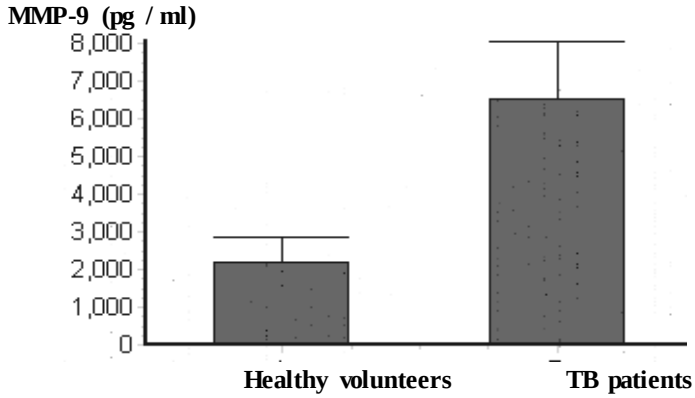

Fig. 2: MMP-9 levels in healthy volunteers and TB patients

Also, we investigated whether MMP-9 levels in serum samples of tuberculous patients is of clinical significance or not. For this purpose the patients were classified according to the severity of the diseases into complicated and uncomplicated subgroups. Complicated patients subgroup was divided into 15 patients with pleural effusion with mean value of MMP-9 (6625.5 ± 199.17 pg/ml), ranged from 6350 – 6900 pg / ml, 12 patients with empyema with mean value of MMP-9 (7819.2 ± 424.1 pg/ ml), ranged from 7100 – 8400 pg / ml, and 6 patients with apical cavitation with mean value of MMP-9 (7625 ± 560.1 pg / ml), ranged from 7000 – 8500 pg / ml. The complicated cases subdivisions had the highest mean value of MMP-9 compared to the mean value of MMP-9 (4884.5 ± 1013.5 pg / ml) in uncomplicated TB patients subgroup and ranged from 3455 – 5900 pg / ml. Statistically, the levels of MMP-9 in complicated TB patients were significantly higher than in uncomplicated TB patients ($P<0.0001$) (table 2, Figure 3).

Table 2: **MMP-9 levels in serum samples of uncomplicated TB patients and subdivisions of complicated TB patients.**

	TB patients (n=46)			
	Complicated cases (n= 33)			**Uncomplicated cases (n=13)**
	Pleural effusion (n=15)	**Empyema (n=12)**	**Apical cavitation (n=6)**	
MMP-9 range (pg/ml)	**6350 - 6900**	**7100 - 8400**	**7000 - 8500**	**3455 - 5900**
Mean ± SD (pg / ml)	**6625.5 ± 199.17**	**7819.2 ± 424.1**	**7625 ± 560.1**	**4884.5 ± 1013.5**
F value	**53.162**			

P value	< 0.0001

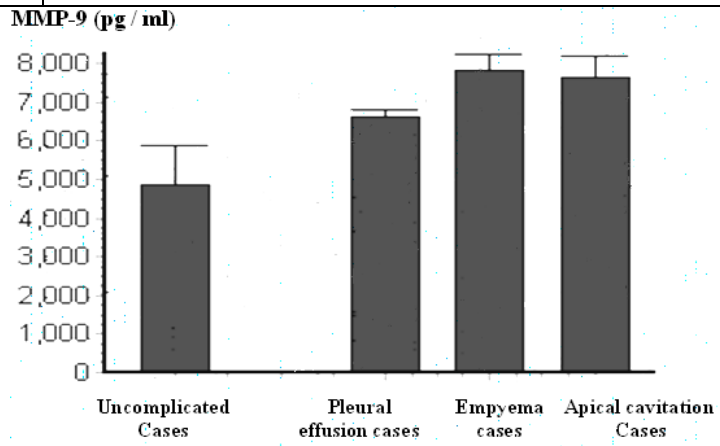

Fig. 3: MMP-9 levels in serum samples of uncomplicated TB patients and subdivisions of complicated TB patients.

DISCUSSION

The mechanism of MTB penetration into tissues is poorly understood but it is reasonable to assume that there is a contribution from proteases capable of disrupting the extracellular matrix of the pulmonary epithelium and the blood vessels[23]. It appears that the collagen degrading activity of *M. tuberculosis*, which is also important for bacterial penetration in tissues, contributes to a disruption of ECM of the pulmonary epithelium and blood vessels[23]. MTB infection induces the expression of host MMPs which are capable of tissue degradation [17]. So, the suggestion presented by Dannenberg[24] That MTB itself is non-toxic and only the host reactions and release of MMPs destroy the lung tissues, seems rather doubtful. MMP-9 induction is elicited by the direct interaction between cell wall components of MTB bacilli and human monocytes and macrophages. MMP-9 could play important roles in the pathogenesis of tuberculosis [17]. MMP-9 (gelatinase B) has been implicated in the pathogenesis of asthma[25], idiopathic pulmonary fibrosis [26], chronic obstructive pulmonary disease (COPD)[27], and acute lung injury [28].

In the present study, the results showed that, MMP-9 levels were significantly higher in tuberculosis patients than in healthy volunteers ($P < 0.0001$); this may be due to overexpression as a result of infection by a variety of cells including mononuclear phagocytes and stimulated neutrophils. This result is in agreement with a previous finding [15] which stated that MMP-9 levels are high in TB patients and the large number of infiltrating neutrophils may contribute to high levels of MMP-9, since neutrophils harbor preformed

MMP-9 in their granules that can be readily released, also there are many lung cells can synthesize and release MMP-9. The elevation of MMP-9 levels was also observed by Rivera-Marrero et al. after infection of cultured U937 human monocytes cells with an attenuated as well as a virulent strain of *M. tuberculosis* [17].

In this study we observed also that MMP-9 levels are directly proportional with the severity of tuberculosis cases from uncomplicated cases to complicated cases; this may be due to existence of large number of acid fast bacilli. This result is in agreement with a previous finding [15] which stated that the influence of large bacterial loads in the tissues may also be one of the reasons for the high MMP-9 activity.

In conclusion, MMP-9 was presented in a high concentration in serum samples of patients with MTB comparing with its levels in serum samples of healthy volunteers due to overexpression by a variety of cells including mononuclear phagocytes and stimulated neutrophils. MMP-9 concentrations were increased with increasing the severity of the disease as measured in complicated and uncomplicated cases so; the evaluation of the serum levels of this enzyme may be helpful for estimating the activity of pulmonary tuberculosis.

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تعيين الأنزيم ألبروتيني الفلزي القالبي رقم 9 في المرضى المصريين المصابين بعصويات الدرن الرئوي

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*** الهدف من الدراسة :-**

يهدف هذا البحث إلى دراسة مستوى الأنزيمات البروتينية الفلزية القالبية و خاصة الأنزيم الفلزي القالبي رقم 9 في المرضى المصابين بعصويات الدرن الرئوي و كذلك في الأصحاء.

:- بروتوكول العمل *

تم سحب عينات دم و فصل المصل من 46مريض بالدرن الرئوي و قد تأكد إصابتهم من خلال الفحص الميكروسكوبي وعمل مزارع لهم علماً بأنه لم يتم علاجهم بأية مضادات حيوية للدرن وكذلك تم سحب عينات دم و فصل المصل من 43شخص سليم، ثم تم تعيين مستويات الأنزيم الفلزي القالبي رقم 9 في المصل لكل من المرضى المصابين بالدرن والأشخاص الأصحاء باستخدام طريقة الإليزا.

*** نتائج الدراسة :-**

أظهرت نتائج مرضى الدرن زيادة ذات دلالة إحصائية في مستوي الأنزيم الفلزي القالبي رقم 9 مقارنة بالأشخاص الأصحاء، وعند تقسيم المرضى إلى ذوي حالات غير مصحوبة بمضاعفات و مرضى ذوي مضاعفات متقدمة استناداً على درجة خطورة المرض، ظهرت نتائج أيضاً ذات دلالة إحصائية في مستوي الأنزيم الفلزي القالبي رقم 9.

*** خلاصة الدراسة :-**

إصابة الأشخاص بمرض الدرن الرئوي يؤدي الى زيادة إفراز الأنزيمات البروتينية الفلزية القالبية و خاصة الأنزيم الفلزي القالبي رقم 9و ربما يكون ذلك من خلال خلايا النيتروفيل و خلايا المكروفاج مقارنة بالأشخاص الأصحاء كما أثبتت الدراسة أن مستوي الأنزيم يزيد بشكل ملحوظ في المرضى ذوي الحالات المصحوبة بمضاعفات عنه في الحالات الغير مصحوبة بمضاعفات.

*** توصيات الدراسة :-**

قد يفيد استخدام قياس مستوي الأنزيم الفلزي القالبي رقم 9في تقدير نشاط عصويات الدرن الرئوي في المرضى المصابين بها