Original Article

Relationship of Metalloproteinase-1 and its Tissue Inhibitor to Neuropathic Diabetic Foot Ulcerations.

Mamdouh R. El - Nahas[1], Amany A. Mousa[1], Ahmed M. Al - Borollosy[2], Ehsan M. Rizk[1]

Faculty of Medicine, Mansoura University [1]; Mansoura International Hospital [2].

Abstract:

Background: Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an essential role in the process of wound healing. However, their role in impaired healing of neuropathic diabetic foot ulcers is still unclear. Also peripheral nervous system dysfunction results in a well-documented up regulation of MMPs. **Objectives**: The aim of this work was to study serum levels of matrix metalloproteinase-1 (MMP-1) and its tissue inhibitor (TIMP-1) in diabetic patients with neuropathic foot ulcers in comparison to diabetic patients with and without peripheral nerve dysfunction. **Methods:** The study was conducted on eighty diabetic patients including; forty patients with neuropathic foot ulcers, twenty patients with diabetic peripheral neuropathy and twenty diabetic subjects without ulcer or neuropathy, recruited from diabetic foot clinic and diabetes outpatient clinics at Mansoura specialized medical hospital. All patients were consented and informed of the study purposes. Data were collected (including clinical, demographic and laboratory data) and serum samples were taken for measurement of serum MMP-1 and TIMP-1. All patients with ulcer were treated with debridement and appropriate offloading. Ulcer healing was assessed weekly and ulcer size reduction >40% after 4 weeks was considered good healing. Statistical analysis was carried out for all collected data using SPSS 18. Statistical significance was determined at a p-value < 0.05. **Results:** The results revealed that the MMP-1: TIMP-1 ratio is significantly higher in the neuropathy group than ulcer and diabetes groups **(**Median= 0.0284, 0.0245, 0.016; p= 0.041, 0.046 respectively). Ulcer duration was positively correlated with TIMP-1 (r = 0.594, p < 0.001) and negatively correlated with MMP-1: TIMP-1 ratio (r = -0.37, p= 0.019). MMP-1, MMP-1: TIMP-1, and MMP-9: TIMP-1 ratios were significantly increased in poor healers (Median = 160ng/ml, 0.0184, 0.0135 respectively) than in good healers (Median= 100ng/ml, 0.0112, 0.0097 respectively) (p= 0.009, 0.015 and 0.003 respectively). **Conclusion:** MMP-1 and TIMP-1 seem to have an important role in neuropathic diabetic foot ulcerations. Serum levels of MMP-1and MMP-1: TIMP-1 ratio could be used as predictors of wound healing in diabetic patients with neuropathic foot ulcers.

Introduction:

Diabetic foot ulcers represent a major public health problem, being the leading cause of non-traumatic amputation in developed countries. Their medical treatment remains a challenge. However, better understanding of their pathophysiology would improve their management. [(1)] For wounds to heal, the extracellular matrix (ECM) needs to be laid down and then progressively remodeled to reach maturity. The enzymes primarily involved in the degenerative arm of this turnover process are the matrix metalloproteinases MMPs. They comprise a family of some distinct but structurally related enzymes that, when acting together, can degrade almost all ECM components.[(2,3)] They help in elimination of damaged protein, destruction of the provisional extracellular matrix, remodeling of granulation tissue, angiogenesis control and also regulation of some growth factors activity.[(1)] Their activities are also tightly regulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[(4,5)] Abnormal MMPs activity has been implicated in many diseases characterized by disorganization of the extracellular matrix, such as rheumatoid arthritis and tumor development, and their importance as a therapeutic approach will be dependent upon careful analysis of their role in different disease stages. Overall, an imbalance between the MMPs and TIMPs is a consistent finding in non-healing wounds.[(6, 7)]

The aim of this work was to study serum levels of MMP-1 and its TIMP-1 in diabetic patients with neuropathic foot ulcers in comparison to diabetic patients with and

without peripheral nerve dysfunction and study their role in ulcer healing.

Subjects and Methods:

The study included eighty diabetic patients selected from Diabetic Foot Clinic and diabetes outpatient clinics in Specialized Medical Hospital, Mansoura University, from April 2013 to November 2013. The study was ethically approved and all patients were given written consent to participate in the study.

Patients were classified into 3 groups:

Group I (Ulcer group): Forty patients with neuropathic diabetic foot ulcer (DFU) (24 males and 16 females), with mean age (43.6 ± 9.3) years.

Group II (Neuropathy group): Twenty patients with peripheral nerve dysfunction without foot ulceration (9 males and 11 females), with mean age (42.1 ± 9.65) years.

Group III (Diabetic group): Twenty diabetic patients without peripheral nerve dysfunction and without ulcers (11 males and 9 females), with mean age (40.6 ± 10.65) years.

Exclusion criteria:

Smokers; patients with anemia (Hemoglobin <10mg/dl); Patients with, liver, renal and ischemic heart diseases; diabetic patients with foot ulcerations of different etiology rather than neuropathy including ischemic ulcers and patients with infected diabetic foot ulcers.

All patients were subjected to thorough history taking and clinical examination.

Diagnosis of PN was based on loss of pressure perception (at two or more sites) using a monofilament test and/or loss of vibration perception (>50 Volts) using the neurothesiometer. Doppler ultrasound was used to measure the ankle/brachial pressure index (ABPI) and toe/brachial index (TBI) to exclude patients with PAD. Patients with ABPI < 0.9 and TBI < 0.7 were excluded.

Ulcers were graded on a 1 to 3 scale according to the Texas Grading System. We used gridded paper to measure the size of the ulcers and ulcer area reduction on follow up. Patients with ulcers are followed up for 4 weeks and according to ulcer size reduction they were subdivided into two subgroups (Good healers and Poor healers; Patients with ulcer size reduction >40% were considered good healers) (8,9).

Laboratory analysis:

Six ml of venous blood was obtained in the fasting state from every patient and distributed as follow: one ml into EDTA tube for determination of CBC (using automated counter, Sysmex KX-21, USA) and HbA1c (Using Dimension Xpand plus chemistry autoanalyzer, Siemens); Five ml into plain tube and left to clot then serum was separated into two aliquots, one used for routine analysis (glucose, liver profile, lipid profile and creatinine) using commercially available kits and the other was stored at -20°C for MMP-1 and TIMP-1 analysis. Estimation of MMP-1and TIMP-1 were done using Raybio^R Human MMP-1and TIMP-1ELISA Kits, Ray-Biotech, Inc. USA for quantitative detection of MMP-1 and TIMP-1 respectively by enzyme linked immunosorbent assay.

Statistical Analysis:

The collected data were organized, tabulated and statistically analyzed using software statistical computer package (SPSS) version 16. For quantitative data, the range, mean and standard deviation were calculated. For qualitative data, comparison between two groups and more was done using Chi-square test ($\chi 2$). For comparison between means of two groups of parametric data, student t-test was used. For comparison between more than two means, the F value of analysis of variance (ANOVA) was calculated, where Scheffe test was performed to compare between each two means if F value was significant. Correlation between variables was evaluated using Pearson's correlation coefficient (r). Sensitivity, Specificity, positive predictive and negative predictive values and accuracy of measuring serum level of MMP-1 and TIMP-1 as prognostic markers were calculated. Significance was adopted at < 0.05 for interpretation of results of tests of significance.

Results:

Demographic and clinical data in the study groups (table I) showed that the duration of diabetes was significantly higher ($p< 0.001$) in the ulcer and neuropathy groups than in diabetes group (Median 15, 12.5 versus 2.5 years respectively). Body mass index (BMI) in the ulcer group (33.93 ±7.3 kg/m^2) was statistically different ($p= 0.03$) when compared with neuropathy and diabetes groups (31.76 ± 3, 30.33 + 4.37 kg/m^2 respectively). VPT

was significantly higher (P < 0.001) in the ulcer and neuropathy groups in comparison to the diabetes group (43.55 ± 4.47, 40.9 ± 7.91 vs. 1 5.4± 3.19 volts, respectively).

Table (II) showed the laboratory data in the study groups: There were insignificant differences in MMP-1 or TIMP-1 among the three different groups. However, there was a significant difference in MMP-1: TIMP-1 ratio in neuropathy group (Median= 0.0284) and diabetes group (Median= 0.0245) when compared to ulcer group.

Correlation between MMP-1, TIMP-1, MMP-1: TIMP-1 ratio and clinical variables in Ulcer group (table III) showed that Age and TBI was significantly correlated with MMP-1 levels (r = -0.327, p = 0.039; r = -0.366, p= 0.02 respectively). Also their VPT was significantly correlated with TIMP-1 levels (r = -0.457, p= 0.003). Ulcer duration was significantly correlated to MMP-1: TIMP-1 ratio (r = - 0.37, p = 0.019), and also showed a highly positive correlation with TIMP-1 (r = 0.594, p < 0.0001). By dividing patients with foot ulcer into poor and good healers (table IV): Poor healing was associated with significantly higher MMP-1 and MMP-1: TIMP-1 ratio (p= 0.009, 0.015, respectively). Table (V) showed analysis of ROC curve results denoting sensitivity, specificity, positive predictive value, negative predictive value and accuracy of MMP-1, MMP-1: TIMP-1 ratio in predicting healing. MMP-1 more than 130 ng/ml was 80% sensitive and 80% specific and MMP-1: TIMP-1 ratio more than 0.0116 was 100% sensitive and 60% specific for predicting poor healing among patients with diabetic foot ulcers.

Table (I): Demographic and clinical data in the study groups

		Group I (Ulcer group) 40 patients	Group II (Neuropathy group) 20 patients	Group III (Diabetes group) 20 patients	P	P1	P2	P3
Age (Years; Mean ± SD)		43.6 ± 9.3	42.1 ±9.65	40.6 ±10.65	0.3	NS		
Sex	**Male (%)**	24(60%)	9 (45%)	11 (55%)	0.5	NS		
	Female (%)	16 (40%)	11 (55%)	9 (45%)				
DM duration (Years; Median, IQR)		15 (10-18.5)	12.5 (9-19.5)	2.5 (2-3)	≤0.001	0.98	≤0.001	≤0.001
DM Therapy	**Insulin (%)**	30 (75%)	17 (85%)	8 (40%)	≤0.001	NS		
	OHG (%)	2 (5%)	3 (15%)	12 (60%)				
	Insulin+ OHG (%)	8 (20%)	Nil	Nil				
BMI (Mean ± SD)		33.93 ± 7.3	31.76 ± 3	30.33 ±4.37	0.03	0.55	0.03	0.4
Waist circumference (Centimeters; Mean ± SD)		112.1 ±8.48	108 ± 8.5	108.6±9.79	0.3	NS		
VPT (Volts; Mean ± SD)		43.55 ± 4.47	40.9 ± 7.91	15.4 ± 3.19	≤0.001	0.16	≤0.001	≤0.001
ABPI (Mean ± SD)		1.14 ± 0.1	1.13 ± 0.11	1.1 ± 0.1	0.3	NS		
TBI (Mean ± SD)		1.23 ± 0.13	1.2 ± 0.13	1.29 ± 0.12	0.06	NS		
HTN	**Yes (%)**	16 (40%)	8 (40%)	8 (40%)	1	NS		
	No (%)	24 (60%)	12 (60%)	12 (60%)				

Where, SD = Standard deviation; IQR = Interquartile range; P = Significance; NS = Non-Significant; P1 = Significance between groups I, II ; P2 = Significance between groups I,III ; P3 = Significance between groups II,III

Table (II): Laboratory data in the study groups

	Group I (Ulcer group)	Group II (Neuropathy group)	Group III (Diabetes group)	P	P1	P2	P3
HbA1c (%; Mean±SD)	8.42 ± 1.47	8.89 ± 1.40	8.35 ± 1.52	0.4	NS		
Serum creatinine (mg/dl ; Mean ± SD)	0.97 ± 0.19	0.97 ± 0.24	0.91 ± 0.17	0.5	NS		
Serum cholesterol (mg/dl ; Mean ± SD)	198.9 ± 36.28	197.7 ± 31.9	199.05 ± 24.52	0.9	NS		
Serum triglycerides (mg/dl ; Mean ± SD)	163.95 ± 39.86	160.85 ± 47.3	169.4 ± 40.21	0.8	NS		
Serum MMP-1 (ng/ml; Median, IQR)	150 (79-185)	140 (110-270)	180 (140-210)	0.15	NS		
Serum TIMP-1 (ng/ml; Median, IQR)	10000 (4050-12250)	9750 (4750-11000)	7000 (1900-10000)	0.08	NS		
MMP-1: TIMP-1 (Median, IQR)	0.016 (0.0094-0.0348)	0.0284 (0.0113-0.0414)	0.0245 (0.016-0.07)	0.049	0.041	0.046	0.7

Table (III): Correlation between MMP-1, TIMP-1, MMP-1: TIMP-1 ratio and clinical variables in Ulcer group

	MMP-1		TIMP-1		MMP-1:TIMP-1	
	r	P	r	P	r	P
Age	-0.327	0.039*	0.07	0.666	-0.202	0.212
DM Duration	0.271	0.091	-0.132	0.417	0.192	0.236
HbA1c	0.15	0.356	-0.274	0.087	0.25	0.12
BMI	-0.078	0.630	-0.117	0.473	-0.015	0.927
VPT	0.011	0.944	-0.457	0.003*	0.303	0.058
ABPI	-0.230	0.154	0.143	0.378	-0.166	0.305
TBI	-0.366	0.02*	-0.219	0.174	-0.116	0.477
Ulcer size	0.192	0.234	-0.062	0.704	0.193	0.232
Ulcer duration	0.002	0.993	0.594	0.000*	-0.370	0.019*
Creatinine	0.265	0.098	-0.004	0.979	0.131	0.420
S.Cholesterol	0.199	0.219	-0.003	0.985	0.074	0.651
S.Triglycerides	0.284	0.075	-0.142	0.382	0.281	0.08

*** =** Significant (<0.05)

Table (IV): Relationship of MMP-1, TIMP-1and MMP1:TIMP1 ratio to healing in patients with ulcers

	Healing				P
	Poor		Good		
	Median	Range	Median	Range	
MMP_1	160	70-190	100	40-140	0.009
TIMP1	5000	3800-13000	12500	3800-14000	0.2
MMP1:TIMP1	0.0184	0.012-0.0428	0.0112	0.0028-0.0315	0.015

Test used: Mann-Whitney
P significance when <0.05

Table (V): Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of MMP-1and MMP-1: TIMP-1 ratio as predictors of poor healing

	Area under curve (CI 95%)	Cut-off value (ng/ml)	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value	Accuracy
MMP-1	84.0(63.3-100)	>130	80	80	80	80	80
MMP1:TIMP1	82.0(63.3-100)	> 0.0116	100	60	71.4	100	80

Discussion:

Matrix metalloproteinases and their tissue inhibitors have been previously studied in diabetic foot ulcers, both in wound fluid (Muller et al., 2008; Liu et al., 2009) and in serum.[12]

In our study, there were no significant differences in MMP-1or TIMP-1levels between the studied groups. Howev er, MMP-1: TIMP-1 ratio was significantly different (p=0.041) in both group 2 and 3 in comparison to ulcer group (0.284, 0.0245 versus 0.016). Age of the patients of the ulcer group was negatively correlated with MMP-1 (r=-0.327, p=0.039). Similar correlation was obtained by others[13] and this was explained by the superimposition of a disease processes in the older subject may result in different ECM remodeling than that is seen in a younger subject. Also, Bonnema et al., (2007) studied the effect of age on MMP-9 and TIMP-1. They found that, as subject age increased, MMP-9 decreased and TIMP-1 increased.[14]

All participating subjects were overweight or obese evidenced by the abnormally increased BMI in all the study groups. BMI of patients within the ulcer group (33.93 + 7.3) was significantly higher than that in diabetes group (30.33 +4.37) (p= 0.03). Sohn et al., (2011) studied the association between BMI and foot ulceration risk. They found that higher BMI was significantly related to DFU occurrence.[15] BMI was not significantly correlated with MMP-1 or TIMP-1 in our DFU group. In another study enrolled diabetic subjects without foot ulcers, and followed them for the occurrence of foot ulcer. They found that increased body weight, along with other risk factors such as insensitivity to 5.07 monofilament, were significantly related to foot ulcer risk. [16]

Incorporating vibration perception threshold (VPT) testing into clinical practice has the potential to significantly improve the outcomes in patients with DPN, thereby reducing the

socio-economic burden of this common and challenging disease.[17] In our study, VPT was significantly higher in group 1 and 2 than in diabetic patients without DPN and without ulcers (43.55 ± 4.47, 40.9 ± 7.91, and 15.4 ± 3.19 volts, respectively) (P<0.001). Jayaprakash et al., (2011) carried out a study to evaluate the usefulness of VPT measurement in patients with DPN. They found that VPT was positively correlated with the patients' symptoms and signs.[18] In another study that aimed to assess the ability of VPT to predict the development of DFUs, VPT < 15 volts had a cumulative incidence of foot ulceration of 2.9% compared with 19.8% in patients with a VPT > 25 v. They concluded that VPT is an effective predictor of the risk of foot ulceration in diabetes.[19] Studies correlating MMP-1 or TIMP-1 to VPT is lacking in the literature. However, TIMP-1 in our patients with foot ulcers had a significant negative correlation with their VPT measurement (r= -0.457, p= 0.003).

In healing of wounds, ECM needs to be laid down then must be able to undergo degradation and remodeling to form a mature tissue with appropriate strength. Proteases, namely matrix metalloproteinases are known to degrade almost all the extracellular matrix components. They are known to be involved in fibroblast and keratinocyte migration, tissue re-organization, inflammation and remodeling of the wound tissue.[20] MMP-1 is the major collagenase implicated in wound healing: it has been shown that its specific proteolysis of type I collagen (an essential component of the dermis) is essential for keratinocyte migration. [21]

In our study, ulcer duration was positively correlated with TIMP-1 (r= 0.594, p<0.001) and negatively correlated with MMP-1: TIMP-1 ratio (r= -0.37, p= 0.019). Moreover, the process of ulcer healing was assessed by measurement of the ulcer size after 4 weeks and according to ulcer size reduction, patients were subdivided into good healers (Ulcer size reduction > 40%) and poor healers (Ulcer size reduction < 40%). We correlated both sub-groups to MMP-1and MMP-1: TIMP-1 ratio. We found that, poor healing was significantly related to higher levels of MMP-1and MMP-1: TIMP-1 ratio (p= 0.009 and 0.015 respectively). Moreover, MMP-1 level > 130ng/ml had 80% sensitivity and 80% specificity and MMP-1: TIMP-1 ratio > 0.0116 showed sensitivity 100%, specificity 60% in the prediction of poor ulcer healing

We hypothesize that higher level of MMP-1 is harmful to the healing process. It has been suggested that the contribution of increased MMPs and MMPs to TIMPs ratio to poor wound healing can be explained by the deleterious effects exerted by excess MMPs on the healing process, which is believed to be related to exaggeration of ECM degradation[22] and/or prolongation of the inflammatory phase of healing.[23] According to the available literature, studies investigating the relationship of MMPs or TIMPs to Diabetic foot ulcers (DFUs) have always focused on the gelatinases (MMP-2 and MMP-9) rather than any other MMPs isoform[6,11, 12, 24] and used the wound fluid, rather than serum, as sampling methods.[11,24] Our study is considered a unique one that explored the relationship of serum MMP-1 and TIMP-1concentrations to neuropathic DFUs and correlated these parameters to some clinical variables. Ladwig et al. (2002) described a higher level of activated MMP-9 in a group of poorly healing pressure ulcers compared to a group of good healers. In their 56 patients, the MMP-9/ TIMP-1 ratio was positively correlated with poor healing, which underlies the deleterious effect of an MMP-9 excess in chronic wounds.[25] Also, Li et al., (2013) studied the serum levels of MMP-9 in patients with DFUs and they found that the serum MMP-9 level decreases as the ulcer duration increases, and in agreement with us, they found that the MMP-9: TIMP-1 ratio is significantly related to poor healing.[12] Only

Muller et al., (2008) studied the relationship of MMP-1: TIMP-1 ratio to neuropathic DFUs but they found no significant differences in MMP-1: TIMP-1 ratio nor MMP-9: TIMP-1 ratio between good and poor healers.[10] Their study differs from ours in that they only enrolled 16 subjects and used wound fluid as a sampling method.

In conclusion: MMP-1and MMP-1: TIMP-1 ratio seem to have an important role in neuropathic diabetic foot ulcerations. They might be used as predictors of wound healing in diabetic patients with neuropathic foot ulcers and better understanding of this area will help in the development of possible therapeutic strategies.

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