Myeloperoxidase activity in infection complicated and uninfected diabetic patients

T V Suchithra and K F Zuhara*
Department of Life Sciences, University of Calicut, Calicut University (P.O), Malappuram (Dt), Kerala 673 635, India

Received 30 October 2007; revised 31 March 2008

The activity of myeloperoxidase (MPO) in the blood and tissue neutrophils of three groups viz., Group I: patients of diabetes mellitus (DM) without infection and related complications; Group II: patients of DM with infections and related complications, and Group III: non-diabetic normal persons as control subjects was estimated to find out the influence of the MPO status on the occurrence of diabetes and also on the infection and related complications found in some of these patients. Group III category showed the highest blood MPO compared to the two diabetic groups \( (P<0.001) \). Group II patients exhibited higher MPO activity than group I \( (P<0.001) \). The study revealed that increase in MPO in group II was achieved both by increased production of the enzyme at cellular level and also of leukocytes in the system. Estimation of MPO in infected tissue was performed in group II in relation to the histological features and compared with group III, as an index of neutrophil infiltration to the infected sites. Tissue MPO was found higher in group II than group III subjects. Histological analysis revealed that the elevated MPO in infected cases was due to the increased neutrophil infiltration to the infected site. Blood sugar status, diabetic management measures, wound healing ability etc. of diabetic patients was also studied in relation to MPO. MPO activity was higher in normal subjects having normal sugar. In group II, MPO was low in the uncontrolled sugar group compared to the controlled, and in group I vice versa. Insulin-treated diabetic patients showed higher MPO activity than drug-treated and combined therapy groups. Also, patients with healing impairment showed comparatively high MPO activity.

Keywords: Myeloperoxidase, Diabetes mellitus

Infection is one of the major challenges in diabetes mellitus (DM), in particular foot ulcer, which is associated with significant morbidity, disability and impairment in a diabetic patient's life quality. Patients with DM are more prone to infections and the increased susceptibility is multi-factorial. The associated metabolic disturbances impair all steps of neutrophils functioning in diabetic patients, which may increase the risk of vascular complications and infectious episodes. The cellular and humoral elements of immune system against germ invasion are disturbed in these patients.

Myeloperoxidase (MPO; EC 1.11.1.7), a mammalian heme enzyme, is the major component of microbicidal armamentarium of neutrophils and a key player in cellular innate host defense. It is found in azurophilic cytoplasmic granules of neutrophils and in lysosomes of the monocytes. It is abundant in neutrophils, while monocytes and macrophages contain only about 1/3rd of that present in the neutrophils. Commonly known as myeloperoxidase hydrogen peroxide chloride (MPO-H\(_2\)O\(_2\)-Cl) system, it is one of the key enzymes that has role in phagocytic oxygen-dependent antimicrobial system. The product of this system is hypochlorous acid. This most powerful neutrophil oxidant contributes to both bacterial killing and oxidative injury of host tissue. MPO is extremely cytotoxic and reacts readily with most biological molecules, thereby promoting inflammatory tissue damage caused by neutrophils in diabetic patients.

In this study, the MPO level was estimated in infected and non-infected diabetic categories, to find out the influence of the enzyme status on the occurrence of diabetes and on the infection and related complications found in some of these patients. This is significant since no similar studies, including the parameters studied here, were conducted so far in diabetic subjects, except a few related ones. In addition, the MPO level in relation to the histological features of the infected tissue of diabetic subjects was also studied in comparison with the normal, which is an index of neutrophil infiltration to the infected sites. Other relevant parameters like blood sugar status,
diabetic management measures, wound healing ability etc. in relation to the MPO level of the diabetic patients were also included in this study.

**Materials and Methods**

**Subjects**

The study subjects were drawn from Diabcare Clinic, Manjeri, which is a specialty clinic for diabetes and diabetic foot care. The group consisted of 3 categories: Group I: 50 patients of diabetes mellitus (DM), without infection and related complications; Group II: 50 patients of DM with infections and related complications; and Group III: 50 non-diabetic normal persons. Informed written consent was obtained from each patient and normal person, before including in the study. Patients were screened for the presence of diabetes as per the American Diabetes Association’s new criteria for the diagnosis of DM. The case history of each patient was recorded, which included sex, age, mode of diabetic management, infection status, presence/absence of infection and wound healing ability etc.

**Sample collection and preparation**

The venous blood (4 ml) was collected from each individual in collection tube containing acid-citrate-dextrose solution and each blood sample was processed separately for MPO extraction and blood sugar estimation. Tissue samples were collected for the analysis of tissue MPO, which is an index of neutrophil infiltration to tissues. For the purpose, tissues were collected by lavage (4-15 mg tissue) during the dressing of wound of diabetic foot patients. Normal tissue samples were collected from biopsy specimen of patients subjected for surgery for hernia, varicose vein etc. One part of tissue was fixed with 20% formaldehyde and then subjected for paraffin microtome sectioning for histological studies and rest was processed for MPO extraction.

**Estimation of cellular MPO in blood and tissues**

Leukocytes from the anticoagulated blood were separated and purified as described previously. MPO from the leukocytes and tissue was extracted. The activity of MPO was estimated using 4-aminoantipyrine as the hydrogen donor. The increase in absorbance at 510 nm was recorded at 30 s intervals for 5 min using kinetic mode of spectrophotometer (UVmini-1240, Shimadzu, Japan).

The MPO activity in each case was expressed both as unit MPO in total leukocytes of 1 ml of blood, and also as unit MPO in a single leukocyte. One unit of MPO was defined as the amount of enzyme that catalyzed the transformation of 1 µ mole of hydrogen peroxide per min at pH 6.1 and 25°C. Since the number of leukocytes varies in each individual, the total cellular MPO of 1 ml blood would depend on the leukocyte count. The enzymatic activity per single leukocyte could be calculated from the unit cellular MPO activity of 1 ml of blood of an individual and the total WBC count present in 1 ml of blood. The number of WBCs of each blood sample was counted by manual method and expressed as cells/mm³ of undiluted blood, for this purpose. The unit MPO activity in tissues was expressed in units per mg of weight.

**Estimation of blood sugar**

Random blood sugar status was determined by enzymatic glucose oxidase-peroxidase (GOD-POD), end-point colorimetric single reagent chemistry method using Preci Chem glucose GOD kit. Absorbance was read at 490-550 nm using Autospan Semi-autoanalyzer.

**MPO activity in relation to other parameters**

The unit MPO activity at single leukocyte level was analyzed in relation to the relevant parameters like blood sugar status, mode of disease management (using hypoglycemic drugs/insulin) and wound healing ability of diabetic patients.

**Statistical analysis**

The results were analyzed using a one-way ANOVA with SPSS (version 11) and data expressed as mean ± SE of the mean. \( P \leq 0.05 \) was considered significant.

**Results and Discussion**

**Estimation of cellular MPO**

MPO activity was higher in group III compared to the diabetic groups (group I and II). Since these normal people were healthy and free of infection at the time of study, the mean MPO activity observed in these subjects could be considered as a level effective to confer protection against infections. The standard or optimum level/range of MPO required for a host to be safely protected from infections, is still unknown. Previous study has shown that the MPO level in normal people boosts up further when infection occurs.
Of the three groups, MPO level was least in the group I. The reduction in the MPO activity, compared to group III was statistically highly significant ($P<0.001$) both at 1 ml blood and single leukocyte levels. Reduced MPO activity has been reported earlier in diabetic patients, hence as per this and previous studies, low level of MPO is a feature of diabetes.

In group II, ulcer foot infection was a common challenge. It is mainly caused by gram negative bacilli etc. The MPO level was distinct in group II, falling between that of group III and I. The leukocytic MPO activity/ml blood of group II was near to that of group III, but differed significantly ($P<0.001$) from that of group I. But, at single leukocytic level, MPO activity of the group II was near the average to that of group III and group I, indicating that at single cell level, MPO activity of group II was significantly lower to group III ($P<0.001$) and higher to group I ($P<0.001$). Earlier study also reported an increased MPO in diabetic patients with a high oral yeast count. The MPO status in leukocytes of 1 ml blood and in single leukocyte of the three groups is shown in Figs 1 and 2 and data are presented in Table 1.

As per this and previous studies, a reduction in MPO was observed as a feature of the diabetic groups (group I & II) compared to the normal. But, in group II, compared to group I, an increase in MPO was achieved both by an increased production of the enzyme at cellular level and also of leukocytes in the system. This was corroborated by increased production of leukocytes that was observed in the WBC count of group II patients, compared to the other groups. The group II showed a mean WBC count as high as 10229 cells/mm$^3$ ± SE 827, compared to 5652 cells/mm$^3$ ± SE 254 in group I and 6648 cells/mm$^3$ ± SE 271 in group III. In group I, a general lowering in the WBC count was observed to the extent that no case with count above the normal range was observed, while in group II 28% had WBC above the range.

As per the observation here, at an infection, diabetic system tries to tide over the inherent deficit of MPO through stringent steps. But, despite this, MPO level could not reach even that of uninfected normal in these patients. Infected normal was found to have increased MPO in earlier study. The low MPO observed here might be due to the effect of the underlying diabetic condition that predisposes impairment in the production of MPO.

Earlier study proposed the protection of an auxiliary mechanism that acts in normal individuals in the deficiency of MPO. But DM patients were exempted from this protection by the study, since they suffered from severe candidial disease. As per this observation, the group II cases of this study will not get the protection of either the MPO (since it is low in the group) or the auxiliary mechanism (due to the prevailing infection), hence subjected to the worst effect of the infection.

<table>
<thead>
<tr>
<th>Category</th>
<th>Leukocytic MPO per ml blood</th>
<th>MPO in single leukocyte</th>
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<tbody>
<tr>
<td>Group I</td>
<td>$1.636 \times 10^{-2}$ ± SE $1.589 \times 10^{-3}$</td>
<td>$2.887 \times 10^{-9}$ ± SE $2.601 \times 10^{-10}$</td>
</tr>
<tr>
<td>Group II</td>
<td>$4.309 \times 10^{-2}$ ± SE $3.773 \times 10^{-3}$</td>
<td>$4.633 \times 10^{-9}$ ± SE $3.441 \times 10^{-10}$</td>
</tr>
<tr>
<td>Group III</td>
<td>$4.376 \times 10^{-2}$ ± SE $4.415 \times 10^{-3}$</td>
<td>$6.484 \times 10^{-9}$ ± SE $4.907 \times 10^{-10}$</td>
</tr>
</tbody>
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Fig. 1—Leukocytic MPO per ml blood [Highly significant $P<0.001$]

Fig. 2—MPO in single leukocyte [Highly significant $P<0.001$]
Estimation of tissue MPO

Analysis of tissue MPO revealed that although the level of leukocyte MPO (units/cell) in blood of group II (4.629 × 10^-9 ± SE 3.23 × 10^-10) was lower as compared to group III (6.484 × 10^-9 ± SE 4.907 × 10^-10), the tissue MPO (units/mg) at the infected area (1.51 × 10^-2 ± SE 6.25 × 10^-3) was far above that of group III (6.92 × 10^-4 ± SE 1.310 × 10^-4). Data are presented in Fig. 3.

The elevated level of MPO in tissue might be due to the large-scale infiltration of neutrophils to the infected area in group II patients. The histopathological study of ulcer foot tissues showed regions containing dense neutrophil infiltration associated with edema. Apparent marked matrix dissociation with >95% of neutrophils and occasional large macrophages was also observed. In the deeper regions, there was an increased density of blood vessels, and many contained rounded endothelial cells, surrounded by migrating neutrophils. The high MPO activity in tissues would be correlated with the large neutrophil counts observed in the ulcer tissue sections (Fig. 4). Earlier 17, the correlation of MPO and infiltrated neutrophils was reported in chronic pressure ulcers. The neutrophil infiltration was not observed in group III tissue.

MPO activity in relation to blood sugar status

The individuals were grouped based on their random blood sugar into controlled (≤ 140 mg/dL) and uncontrolled (>140 mg/dL) sugar groups 18. Random blood sugar level of all (100%) in group III fell within the normal range (70-140 mg/dL). In group I, majority (82%) had uncontrolled sugar level, while only 18% had it controlled to the normal range, while in group II, it was 78% and 22% respectively.

When the unit MPO activity per cell of controlled and uncontrolled blood sugar groups of each diabetic category was analyzed in comparison with the normal highest MPO activity (6.484 × 10^-9 ± SE 4.908 × 10^-10) was observed in the group III subjects with normal sugar level. In group II cases, MPO activity was low (4.256 × 10^-9 ± SE 4.389 × 10^-10) in uncontrolled sugar group, compared to controlled group (5.329 × 10^-9 ± SE 5.488 × 10^-10). Group II, whose leukocytes were infection stimulated had an elevated level of MPO production compared to group I as per this study. As some microorganisms become more virulent in a high glucose environment 19, the uncontrolled diabetic cases with high glucose level are likely to have an elevated production of MPO to manage the situation; but on the contrary, an increased production of MPO was observed when the sugar level was normal. This indicated that the cell metabolism proceeded well, leading to a higher MPO production, when the cells had normal sugar status.

In contrast to the above observations, group I cases had a low level (1.788 × 10^-9 ± SE 3.333 × 10^-10) of MPO at controlled sugar level, compared to the uncontrolled (2.886 × 10^-9 ± SE 2.601 × 10^-10).

Effect of diabetic management on MPO activity

The common methods used for control of blood sugar in diabetic patients were considered here in relation to their MPO level. In group I cases, MPO was higher in 16 cases of insulin-treated (4.294 × 10^-9 ± SE 9.328 × 10^-10), compared to the 24 drug-treated (2.899 × 10^-9 ± SE 2.687 × 10^-10) and 10 cases of combined therapy group (1.171 × 10^-9 ± SE 2.226 × 10^-10). All the group II cases that had a mean MPO activity of 4.633 × 10^-9 were under insulin treatment and hence had no scope for comparison.

Here, despite having high MPO activity, infection prevailed in insulin treated diabetic patients. Earlier 20,
it was observed that although phagocytosis was normal in insulin-treated subjects, intracellular killing by granulocytes was significantly reduced in them compared to normal subjects. It was also observed in an in vitro study\textsuperscript{21} that insulin administered was interfered to some extent with the MPO activity.

**MPO activity and impaired wound healing**

Impaired wound healing is a well-documented phenomenon both in experimental and clinical diabetes. Although the group I cases had no infection, 12% of the patients were suffering from delayed healing of wound, which were not infected. Patients with healing impairment showed comparatively higher mean unit MPO activity ($3.015 \times 10^{-9} \pm SE 2.874 \times 10^{-10}$) than with proper wound healing ($1.941 \times 10^{-9} \pm SE 3.35 \times 10^{-10}$). As already observed, the group II cases which had infection and poor wound healing also had the MPO raised ($4.633 \times 10^{-9} \pm SE 3.441 \times 10^{-10}$). Earlier, delayed wound healing was reported together with low collagen content, breaking strength, and increased malondialdehyde levels (an end product of lipid peroxidation due to MPO activity) in diabetic mice, compared to healthy ones. The study suggested that an increased lipid peroxidation in diabetic mice might have a role in determining a defect of wound repair\textsuperscript{8}.

Apart from being a potent antimicrobial system, the oxidizing activity of the MPO-H\textsubscript{2}O\textsubscript{2}-halide system could elicit inflammatory reactions and tissue injury\textsuperscript{22}. Also, antioxidant status is impaired in diabetics compared to normals\textsuperscript{23,24}. Due to this oxidative stress and increased MPO activity, diabetic patients fail to kill the pathogens and heal the wounds in foot ulcer. This may further lead to amputation.

**Acknowledgement**

The authors thank Dr. P Sureshkumar, Diabcare Clinic, Manjeri for his help rendered in sample collection.

**References**