INFECTION SUSCEPTIBILITY OF DIABETIC PATIENTS BASED ON MYELOPEROXIDASE ACTIVITY

Thesis submitted to the University of Calicut in partial fulfillment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

UNDER THE FACULTY OF SCIENCE

By

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May 2007

CERTIFICATE

This is to certify that the thesis entitled **"Infection susceptibility of diabetic patients based on myeloperoxidase activity"** is an authentic record of research work carried out by Miss. Suchithra T.V. under my supervision and guidance in the Department of Life Sciences, University of Calicut in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and that no part of this work has been presented before for the award of any degree, diploma, associateship in any university or institution.

18/5/07

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DECLARATION

I hereby declare that the thesis entitled **"Infection susceptibility of diabetic patients based on myeloperoxidase activity"** is a genuine record of the research work done by me under the supervision of Dr. Fathimathu Zuhara K, Reader in Microbiology, Department of Life Sciences, University of Calicut and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title of any university or institution.

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Suchithra T.V.

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ABBREVIATIONS

ACS	:	Acute coronary syndrome
Br^-	:	Bromide ion
CAD	:	Coronary artery disease
CGD	:	Chronic granulomatous disease
Cl^-	:	Chloride ion
dH_2O	:	Distilled water
DM	:	Diabetes mellitus
ETOH	:	Ethyl alcohol
FPG	:	Fasting plasma glucose
gm/dL	:	Gram/ deciliter
H_2O_2	:	Hydrogen peroxide
HbA1C	:	Glycosylated haemoglobin
HOCl	:	Hypochlorous acid
HOX	:	Hypohalous acid
HTAB	:	Hexadecyl trimethyl ammonium bromide
I^-	:	Iodide ion
IDDM	:	Insulin-dependent diabetes mellitus (Type 1)
IGT	:	Impaired glucose tolerant
kDa	:	Kilo dalton
LDL	:	Low density lipoprotein
MDA	:	Malondialdehyde
mg	:	Milligram
ml	:	Milliliter
mm^3	:	Cubic millimeter
mmol/L	:	Millimole per liter
MPO	:	Myeloperoxidase
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NIDDM	:	Non insulin dependent diabetes mellitus (Type 2)
NO	:	Nitrogen monoxide
NO_2	:	Nitrogen dioxide
NO_2Cl	:	Nitryl chloride
O_2^-	:	Superoxide radicle

OGTT	:	Oral glucose tolerance test
$OH^{\cdot}$	:	Hydroxyl radical
PMN	:	Polymorphonuclear leucocytes or neutrophils
RBS	:	Random blood sugar
ROS	:	Reactive oxygen species
SCN	:	Thiocyanate
SE	:	Standard error
SOD	:	Superoxide dismutase
TBA	:	Tertiary butyl alcohol
TNF	:	Tumor necrosis factor
WBC	:	White blood cells
X^{-}	:	Halide
x g	:	Gravitational force
µl	:	Micromole

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Introduction

When pathogens enter a host body, white blood cells, otherwise called leukocytes, confront these harmful invaders on the front line to do sentry duty to prevent diseases from occurring. These cells patrol through body's blood stream, to gobble up invading germs by mounting search-and-destroy missions, with powerful enzymatic and non-enzymatic weapons, going into all the nooks and crannies of the body. They are born in the bone marrow, about a million in every second, and emerge to mature and form distinct divisions: phagocytes and lymphocytes. As part of defense mechanism, the inflammatory response is immediately brought into play and leads to the recruitment of phagocytes to the site of inflammation.

Phagocytes are capable of engulfment, and destruction of microorganisms that are responsible for inciting the inflammatory response. First to accumulate around the invaders and initiate the phagocytic processes are the neutrophils. Later, macrophages also migrate to the tissue site and initiate phagocytosis. Once recruited to an inflammatory site, neutrophils migrate rapidly from blood to tissue, which is otherwise devoid of neutrophils.[1] Neutrophils (also known as polymorphonuclear leucocytes or PMNs) and macrophages are sometimes referred to as professional phagocytes for their roles in this process.

The abundance of neutrophils in blood and the efficiency of their antimicrobial action make these cells an essential first line of defense of the innate immune system. PMNs are highly specialized white blood cells characterized by a multilobed nucleus and a granular cytoplasm. They

constitute 30 -70 % of the circulating white blood cells (leukocytes). They live only for a few days, but during an infection, their numbers increase five fold. Each neutrophil may engulf and destroy up to 25 bacteria and then die, but replacements come in a steady stream. They are especially important when fighting microorganisms (e.g., bacteria and certain fungi) have outstripped local mucosal-epithelial defenses and can rapidly multiply and disseminate out of host cells.

PMNs are equipped with four types of granules containing different proteins and enzymes. Peroxidase positive: (i) azurophilic or primary granules are characterized by the content of myeloperoxidase and may be divided into larger defensin-rich and smaller defensin-poor granules. The peroxidase negative may be divided into (ii) specific or secondary granules and (iii) gelatinase or tertiary granules on the basis of their relative content of lactoferrin and gelatinase. All the granules contain lysozyme. The secretory vesicles (iv) share some of their membrane protein with peroxidase negative granules, where as others are unique to secretory vesicles. This classification of granules is arbitrary, because granules form a continuum from azurophil granule to gelatinase granule, sharing some proteins, whereas other proteins can be chosen as specific markers of one particular subset e.g., myeloperoxidase of azurophilic granules.[2]

The recruitment of neutrophils is by chemotactic gradients, to inflammatory loci, where they recognize and phagocytize bacteria and other extrinsic microorganisms. The granule content is gradually released either into the formed phagosomes or into the extracellular space upon activation of cells. These proteins and enzymes are helpful to smooth the way for PMNs through the closely packed tissue material to the inflammatory loci. The coordination of granule/vesicle compositional diversity with selective exocytosis permits the timely delivery of many pre-formed as well as newly generated neutrophil constituents as and when needed.[1]

Macrophages (also called mononuclear phagocytes) also come up from bone marrow stem cells that give rise to promonocytes, which develop into monocytes that are released into the blood stream. Monocytes make up 3 -7% of the circulating white blood cells. The monocytes are actively phagocytic and bactericidal. Within 2 days or so, the blood stream monocytes (sometimes called wandering macrophages) emigrate into the tissues where they settle down, enlarge and become fixed macrophages (tissue histiocytes), which also have phagocytic potential. Macrophages are more active in phagocytosis than monocytes and develop many more granules containing hydrolytic enzymes. New macrophages can develop by cell division under inflammatory stimuli, but most macrophages are matured blood monocytes. Monocytes from the blood migrate into virtually every organ in the body where they mature into fixed macrophages. The total pool of macrophages is referred to as the system of mononuclear phagocytes. The system is scattered throughout connective tissue, basement membranes of small blood vessels, liver sinusoids, the spleen, lung, bone marrow and lymph nodes.

Compared to neutrophils, macrophages are long-lived cells. As phagocytes, neutrophils play a more important role in the acute stages of an infection. Neutrophils circulate in the blood stream, and during an acute inflammatory response they migrate through the endothelial cell junctions as part of the inflammatory exudate. They migrate to the focus of the infection and ingest or phagocytose the foreign agents. Neutrophils which have become engorged with bacteria usually die and largely make up the material of pus.

The microbicidal activities of phagocytes have historically been divided into two broad categories: oxygen-independent (non-oxidative) and oxygen-dependent (oxidative). The terms "oxygen-dependent" and "oxygen-independent" killing have often been mistaken to constitute cytotoxicity that is either oxidative or nonoxidative. However, cytotoxicity which is oxygen dependent, for maximum efficacy may also include actions of nonoxidative

neutrophil weapons acting either in parallel or somehow enhanced in their actions by oxidative activity. Conversely, killing that seems fully in the nonoxidative activity may still require oxidative activity and reactive oxygen species (ROS) for more complete digestion and elimination of microbial remnants.[1]

The oxygen-independent (non-oxidative) mechanism includes both catalytic (e.g., lysozyme, proteases, phospholipase A2) and non-catalytic (e.g., defensins, cathelicidins, bactericidal/permeability-increasing protein) polypeptides ranging in size from <2 to >50 kDa.[1] Virtually all non-oxidative granule-associated antimicrobial polypeptides are highly cationic and may be tethered, during storage, to the polyanionic glycosaminoglycan matrix of the granules.[3]

As a part of the oxygen-dependent (oxidative) mechanism the phagocyte begins to increase oxygen consumption during phagocytosis, termed, the "respiratory burst." In the neutrophil, very little of this consumed oxygen is used for energy needs. Instead, oxygen is consumed to form reduced oxygen metabolites, which will be used to kill the target microbe. This oxidative response is known to be mediated by a leukocyte oxidase (NADPH oxidase) that transfers electrons to molecular oxygen, that reduces O_2 to O_2^- (superoxide). Superoxide can be reduced to $OH^\cdot$ (hydroxyl radical) or dismutated to H_2O_2 (hydrogen peroxide) by superoxide dismutase. These oxygen radicals are converted to hydrogen peroxide and, by the action of a unique protein of azurophilic granules called **myeloperoxidase (MPO)**, to hypochlorous acid—microbicidal agents that have long been used as commercial and household antimicrobials.[4]

1.1 Myeloperoxidase

Myeloperoxidase is the major component of oxidative microbicidal armamentarium of neutrophils and a major player in host defense. This is an abundantly expressed mammalian heme enzyme, found in the azurophilic

cytoplasmic granules of polymorphonuclear leucocytes (PMN) or neutrophils and in the lysosomes of the monocytes [5]. It is abundant in neutrophils while in monocytes and macrophages,[6, 7] there is only about 1/3 of it present in the neutrophils.[8] It constitutes 2 % to 5 % of neutrophil protein by weight.[9]

1.1.1. Enzyme structure

Myeloperoxidase (MPO; donor, hydrogen peroxide oxidoreductase, EC 1.11.1.7)[10] is an~150 kDa symmetric, glycosylated homodimer ($\alpha\beta_2$). Each identical half composed of heavy (α) and light (β) subunits of 59 kDa and 13.5 kDa respectively. Each two identical dimers covalently is linked by a disulfide bond [9, 11, 12] where the latter contains a protoporphyrin IX group with a carbohydrate and a central iron ion. Both heme groups of MPO are functionally identical[13] (Fig.1). The hemes are covalently attached to the apoprotein by two ester linkages and one sulfonium ion linkage.[12]This three fold linkage of heme is unique compared to other heme proteins. It makes the porphyrin ring slightly curved and is discussed as the reason for the red shift of the Soret band to 428 nm in MPO. Different binding sites for halides have been identified.

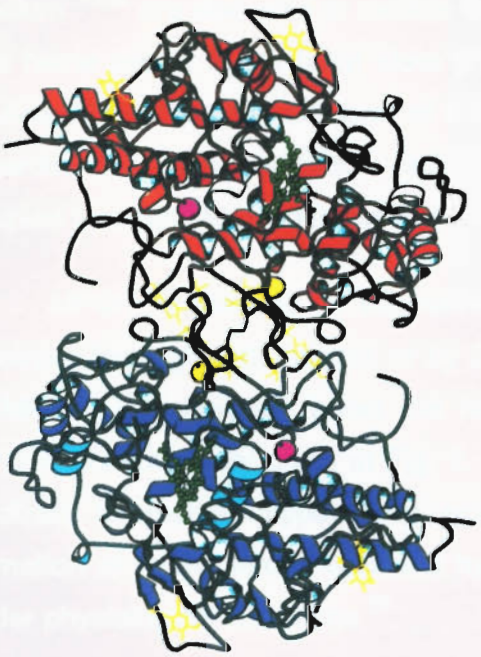


Fig. 1. Entire MPO dimer, viewed along the molecular dyad axis. The large polypeptides of the two halves are colored *red* and *blue*, whereas the small polypeptides are in *lighter shades* of the same colors. Other color coded features include: hemes (*green*), carbohydrate (*orange*), calcium (*purple*), and chloride (*yellow*). At the center of the molecule the disulfide linking the two halves is shown in *black*.[12]

1.1.2. Properties of Myeloperoxidase

1.1.2.1. Formation of strong oxidants by MPO

Myeloperoxidase is able to form a wide variety of oxidants. Furthermore, activated states of the enzyme [most of all compound I(MPO-I), but also compound II (MPO-II)] are also able to oxidize different substrates. In the native enzyme, the heme part of MPO is in the ferric state.

$MPO + H2O_2 \leftrightarrow MPO\text{-}I + H_2O$ (1)

$MPO\text{-}I + H_2O_2 \rightarrow MPO\text{-}II + HO_2^{\cdot}$ (2)

$MPO\text{-}I + X^- + H^+ \rightarrow MPO + HOX + H_2O$ (X^- Halide, HOX – Hypohalous acid) (3)

$MPO + HOCl \rightarrow MPO\text{-}I + H^+$ (4)

$MPO + O_2^{\cdot} \rightarrow MPO\text{-}III$ (Oxyperoxidase) (5)

Hydrogen peroxide is reduced to water by native MPO upon the formation of MPO-I (Reaction 1). MPO-I can also reacts with hydrogen peroxide, to form MPO-II (Reaction 2). Alternatively, different organic hydroperoxides [14] as well as hypochlorous acid (Reaction 3) in chloride-free medium [15-18] are also able to oxidize the native MPO to MPO-I (Reaction 4). The heme iron is in the ferryl state (Fe^{4+}) in MPO I and a further oxidizing equivalent is present in the form of a porphyryl radical. Thus, MPO I can be regarded as a ferryl *pi*-cation radical species, where an oxygen atom is coupled by a double bond to the iron [19].

MPO-I is reduced to the native enzyme either by abstracting two electrons from (pseudo)halides or by 2 one-electron steps via the formation of MPO-II. An overview about the halogenation and peroxidase cycle of MPO is schematically given in Fig. 2. In the first case, (pseudo)halides are oxidized to (pseudo)hypohalous acids. The myeloperoxidase-catalyzed formation of hypochlorous acid and hypothiocyanate is especially important under physiological conditions [20].

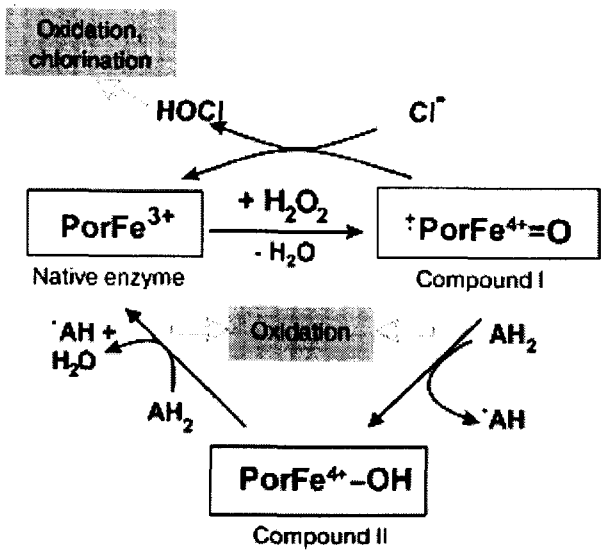


Fig.2 Halogenation and peroxidase cycles of myeloperoxidase. Both cycles utilize hydrogen peroxide to oxidize the native enzyme to compound I. Por denotes porphyrin. AH_2 and ·AH represent substrates being oxidized and the formed radical product, respectively. Instead of Cl^- other (pseudo)halides such as Br^-, I^-, and SCN^- will also be oxidized by compound I. Other pathways of myeloperoxidase are only denoted[19].

Many different substrates are known to be oxidized by compound I (and also at lower rates by MPO II) by abstracting only one electron on the formation of radical products. These substrates include tyrosine, tryptophan, sulfhydryls, phenol and indole derivatives, nitrite, hydrogen peroxide, xenobiotics, and others[21-24]. Numerous radical products, (pseudo) hypohalous acids and further products are involved in the damage of biological macromolecules and tissue degradation.

Other reactions of ferric myeloperoxidase are the reduction to the ferrous state and the reaction with nitric monoxide or superoxide anion radicals. Native MPO binds superoxide anion radicals and forms compound III (MPO-III) of the enzyme (Reaction 5), which is apparently involved in the hydroxylation of aromatic substrates[25].These pathways are denoted in Fig.2.

1.1.2.2. Formation of further reactive oxygen species by MPO

Some reactions of hypochlorous acid, formed by MPO, are lead to further reactive oxygen species with high potential for tissue damage.[26] Hypochlorous acid reacts with hydrogen peroxide on the formation of singlet oxygen (Reaction 6).[19] Hydroxyl radicals are not only formed as the result of the Fenton reaction (Reaction 7) but also derived from hypochlorous acid, either by their reaction with superoxide anion radicals (Reaction 8)[27, 28] or Fe^{2+} [29]. Hypochlorous acid also reacts with nitrite to yield the powerful chlorinating and nitrating compound NO_2Cl (Reaction 9).[30]

	+ H_2O_2	→	$^1O_2 + H_2O + H^+ + Cl^-$	.....	(6)
	+ Fe^{2+}	→	$^{\cdot}OH + Fe^{2+} + Cl^-$	.....	(7)
HOCl	+ O_2^-	→	$^{\cdot}OH + O2 + Cl^-$	.....	(8)
	+ NO^{2-}	→	$NO_2Cl + {}^-OH$	.....	(9)

1.1.2.3 Redox properties

Many reactions catalyzed by myeloperoxidase are redox reactions. The standard reduction potential of the redox couple MPO I /native MPO has recently been determined.[31] The couple MPO II/native MPO has a lower value for the standard reduction potential of 0.97 V at pH 7.0.[32] Consequently, the standard reduction potential of the couple MPO I/MPO II of myeloperoxidase was calculated to be 1.35 V. [32] This value is one of the highest reduction potentials found in cellular systems. These data reflect also big differences in thermodynamic properties between MPO I and II. Thus, myeloperoxidase is able to oxidize various biological molecules with significant rates.

1.1.2.4 Reactions of hypochlorous acid

Oxidative equivalents formed by MPO are involved in numerous processes of tissue damage. Hypochlorous acid is known to oxidize the

sulfhydryl and thioether groups of proteins at a significant rate.[33] [34] It chlorinates amino groups to chloramines.[35, 36] Because of its strong cationic properties, MPO is known to be easily attached to negatively charged biological membranes.[37] Thus, reactions with unsaturated bonds of different phospholipids are also quite possible. The formation of chlorohydrins as well as lysophospholipids in unsaturated phosphatidylcholines by hypochlorous acid and by the MPO-H_2O_2-Cl^- system has been recently described.[38-40] MPO products are also involved in the initiation of lipid peroxidation. Peroxidation by hypochlorous acid is favored by the presence of hydroperoxides previously accumulated in lipid material.[41, 42] The one-electron oxidation of different substrates by complex I of MPO causes radical products such as tyrosyl radical, which is also known to initiate lipid peroxidation processes.[43, 44]

1.1.2.5. Attachment of myeloperoxidase to membranes

Plasma levels of free MPO are often elevated in patients during inflammatory conditions.[45] This strong cationic protein binds to the negatively charged endothelial plasma membrane, whereby this binding depends on the presence of heparin/heparin-containing glycosamino- glycans.[46, 47] Moreover, cell-bound myeloperoxidase rapidly transcytoses the intact endothelium and localizes at the basolateral site of the endothelium closely associated with interstitial matrix proteins such as fibronectin. [47]

1.1.2.6. Protein nitration by myeloperoxidase

Nitration of free and protein-bound tyrosine correlates well with myeloperoxidase activity under inflammatory conditions.[48] MPO-I is able to oxidize nitrite to the nitrating species of nitrogen dioxide (NO_2) at a significant rate [22]. In inflammatory models, the immunoreactivity of MPO strongly co-localizes with the formation of nitrotyrosine in subendothelial and epithelial tissue regions.[49]

1.1.2.7. Modulation of the vessel tonus

Myeloperoxidase impairs NO-dependent blood vessel relaxation and guanylate cyclase activation in an inflammatory model by regulating the availability of nitric oxide [50]. This effect is favored by endothelial localization of secreted MPO [49] and the high rate of NO oxidation by radical products of MPO catalysis [51, 50, 52].

1.1.2.8. Termination of PMN responses

Myeloperoxidase also modulates a variety of aspects of the inflammatory response. It is assumed that the MPO-H_2O_2-halide system inactivates some of the secreted granule contents, decreases the binding of formylated peptides to chemotactic receptors, and influences other functions in stimulated PMNs [53] and auto-oxidize and inactivate products of PMNs, such as α 1-proteinase inhibitor and chemotaxins. Thus, myeloperoxidase contributes to physiological feedback of recruitment of PMNs. It contributes to the termination of the influx of PMNs in inflammatory loci. The MPO–hydrogen peroxide – Cl system is also believed to be involved in terminating the respiratory burst, because MPO-deficient PMNs usually exhibit a stronger and more prolonged respiratory burst.[54, 55]

1.1.2.9. Myeloperoxidase and pathologies

Myeloperoxidase is assumed to be involved in the pathology of different diseases such as atherosclerosis, cancer, multiple sclerosis, and degenerative neurological diseases like Alzheimer's disease.[56-59] MPO has been found in arteriosclerotic plaques.[60, 61] The -463 Guanine/Adenine polymorphism of the MPO gene influences the risk of coronary artery disease (CAD). Allele A of the MPO gene was less frequent in cases with CAD. Recessive model patients with the AA genotype had a decreased risk of CAD. This effect may be mediated by the effect of this polymorphism on the transcription level of the MPO gene.[62] In patients with acute coronary syndromes, MPO serum levels powerfully predict an increased risk for

subsequent cardiovascular events and extend the prognostic information gained from traditional biochemical markers. Given its proinflammatory properties, MPO may serve as both a marker and mediator of vascular inflammation, which further points toward the significance of PMN activation in the pathophysiology of acute coronary syndrome.

1.2. MPO–hydrogen peroxide – Cl microbicidal system.

When the neutrophils become activated, which can happen in conjunction with phagocytosis, they undergo a process referred to as respiratory burst where there is increased oxygen consumption leading to the production of superoxide anion ($O2^{-}$) by NADPH oxidase (Fig.3). MPO (released from cytoplasmic granules of neutrophils and monocytes by a degranulation process [63]), reacts with the hydrogen peroxide, (formed by dismutation of $O2^{-}$), and halides (Cl^{-}, I^{-}, Br^{-}, as well as pseudo halide, thiocyanate [SCN^{-}]) to their corresponding hypohalous acid (HOX).[64-67] Because the plasma concentration of Cl^{-} is more than 1000 times than that of other halides, hydrogen peroxide –MPO system probably uses Cl^{-} in most sites and produce hypochlorous acid (HOCl), with the subsequent formation of chlorine and chloramines.

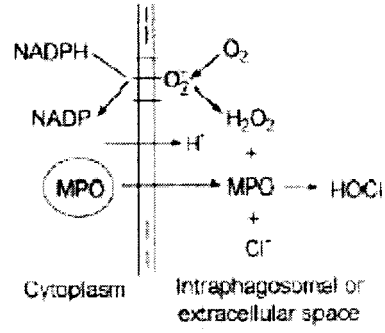


Figure 3: MPO-mediated antimicrobial system

These products of the MPO- H_2O_2 -chloride system are powerful oxidants that rapidly attacks a wide range of biologically relevant molecules. Potential targets include amines, amino acids, thiols, thioethers, nucleotides, heme proteins and polyenoic acids.[68] Hypochlorous acid is 50 times more

potent in microbial killing than hydrogen peroxide.[69-71] This antimicrobial system is effective against bacteria,[72] viruses,[73] fungi,[74] tumor cells,[75] natural killer cells and platelets.[71]

The release of MPO has also been shown to occur during periods of inflammation. During inflammation, plasma levels of MPO are usually increased and it binds to the endothelial plasma membrane. The neutrophilic granulocytes are deficient during early bacterial infection, possibly because of deficient synthesis of antibacterial proteins in the bone marrow, and that neutrophil toxic granulation is the visual counterpart of this defect. The plasma concentrations of myeloperoxidase was high at the start of infection and quickly decreased towards normal values.[76] MPO and H_2O_2 also can be released to the outside of the cell where a reaction with chloride can induce damage to adjacent tissue and, thus, contribute to the pathogenesis of disease. It has been suggested that pulmonary injury, renal glomerular damage, and the initiation of atherosclerotic lesions may be caused by the MPO system.

MPO deficiency has been reported worldwide. It may be partial or complete. Partial deficiency is characterized by the presence of MPO in half the normal amount. This condition can be hereditary or acquired. MPO deficiency in acquired cases is usually transient and generally resolved once the inciting condition improves. In addition, it is usually partial and involves only a fraction of PMNs.

Recent studies have identified several mutations in the myeloperoxidase gene.[5] Complete deficiency is characterized by lack of mature MPO protein.[69, 77] Several mutations have been described in MPO deficiency, making it genetically heterogeneous. Cytosine to thymine substitution at nucleotide 8089 of exon 10 leads to prevention of apoproMPO from becoming mature and active. A missence mutation in the gene leads to a cysteine in place of a tyrosine at position 173 of the amino acid sequence and allows new disulfide bonds to form in the light subunit. This prevents the

protein from undergoing proteolytic processing. In addition, a thymine to cytosine substitution was found to replace a methionine with threonine at amino acid position 251 in Italian patients with complete MPO deficiency.[78]

Neutrophils from patients with chronic granulomatous disease (CGD) have a microbicidal defect that is associated with the absence of a respiratory burst and, thus, H_2O_2 production. Neutrophils from patients with a hereditary MPO deficiency, who lack MPO, also have a microbicidal defect, although it is not as severe as that seen in CGD.

1.3. Diabetes mellitus and infection susceptibility

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia (high blood sugar) and other signs, as distinct from a single disease or condition. The World Health Organization recognizes two main forms of diabetes: type 1 and type 2, which have similar signs, symptoms, and consequences, but different causes and population distributions. Type 1 is usually due to autoimmune destruction of the pancreatic beta cells, which produce insulin. Type 2 is characterized by tissue-wide insulin resistance and varies widely; it sometimes progresses to loss of beta cell function.

Diabetes can cause many complications. Serious long-term complications include cardiovascular disease (doubled risk), chronic renal failure (the main cause of dialysis in developed world adults), retinal damage (which can lead to blindness and is the most significant cause of adult blindness in the non-elderly in the developed world), nerve damage (of several kinds), and microvascular damage, which may cause erectile dysfunction (impotence) and poor healing.

Patients with diabetes mellitus are more prone to infections than those without DM. The course of the infections is also more complicated in this patient group. A few infections, such as malignant otitis externa, rhinocerebral mucormycosis, emphysematous cystitis, emphysematous cholecystitis, emphysematous pyelonephritis, perinephric abscess, papillary

necrosis, necrotizing cellulites and osteomyelitis occur almost exclusively in patients with diabetes. Infections, such as staphylococcal sepsis, occur more frequently and result in greater mortality in patients with diabetes than in others. Details of some selected infections in diabetic patients such as clinical features and causative organisms is given in page 15.

Diabetic foot infections are the most common soft tissue infection associated with DM, with disease-related peripheral neuropathy and peripheral vascular disease playing major roles in this complication of diabetes. The ability of the skin to act as a barrier to infection may be compromised when the diminished sensation of diabetic neuropathy results in unnoticed injury. More serious complications include osteomyelitis, amputation, and death. Infection begins after minor trauma and may progress to cellulitis, soft tissue necrosis, and extension into bone. These infections range from local fungal infections of the nails to necrotizing limb or life-threatening infections. Cellulitis and minor web-space infections may progress more rapidly in diabetics. Foot ulceration is the most common precursor of amputation. The presence or absence of infection and/or ischemia, footwear and pressure relief, and overall glycaemic control influence the healing of ulcers. The depth of an ulcer is another important factor that affects the outcome of diabetic foot ulcers.

This increased susceptibility to infection is multifactorial in diabetic condition. Infections may precipitate metabolic derangements and, conversely, the metabolic derangements of diabetes may facilitate infection. One of the possible causes of this increased prevalence of infections is a defect in immunity. Besides some decreased cellular responses *in vitro*, no disturbances in adaptive immunity in diabetic patients have been described. Different disturbances (low complement factor 4, decreased cytokine response after stimulation) in humoral innate immunity have been described in diabetic patients. Concerning cellular innate immunity, most studies show decreased functions (chemotaxis, phagocytosis, killing) of diabetic

Clinical features and causative organisms of selected infections in patients with Diabetes mellitus			
Infection	**Clinical features**	**Organisms**	**Comments**
Respiratory tract			
Community acquired pneumonia	Cough, fever	*Streptococcus pneumoniae, Staphylococcus aureus, Hemophilus influenzae*, other gram negative bacilli, atypical pathogens	Pneumococcal infection carries a higher risk of death in diabetics than in non-diabetics
Urinary tract			
Acute bacterial cystitis	Increased urinary frequency, dysuria, supra pubic pain	*E.coli*, Proteus Species	Bacteriuria more common in diabetic than in non-diabetic women
Acute polynephritis	Fever, flank pain	*E.coli*, Proteus Species	Emphysematous infection
Emphysematous polynephritis	Fever, flank pain, poor response to antibiotics	*E.coli* and other negative bacilli	Emergency nephrectomy often required
Perinephric abscess	Fever, flank pain, poor response to antibiotics	*E.coli* and other negative bacilli	Surgical drainage usually required
Fungal cystitis	Same as acute bacterial cystitis	Candida species	Difficult to distinguish colonization from infection
Soft tissue			
Mild, non –limb threatening	Shallow ulcer; less than 2 cm cellulites; no evidence of fascitis & ischemia, abscess, or osteomyelitis; good metabolic control	Primarily aerobic gram positive cocci (e.g., *S.aureus*, streptococci)	Initial management- oral antibiotics and wound care
Limb threatening	Deep ulcer; more than 2 cm cellulites; suspected deep infection ; ischemia; poor metabolic control	Polymicrobial, aerobic gram positive cocci (e.g., *Bacteroides fragilis*) and gram negative bacteria (e.g., *E.coli*)	Initial management- broad-spectrum intravenous antibiotics, immediate hospitalization and surgical consultation.
Necrotizing fascitis	Local pain, redness, crepitus, bullous skin lesions	Gram negative bacilli, anaerobes (type I) or group A streptococci (type 2)	High mortality; emergency surgery required
Other			
Invasive otitis externa	Ear pain otorrhea, hearing loss, cellulites	*Pseudomonas aeuruginosa*	Prompt otolaryngologic consultation recommended.
Rhinocerebral mucormycosis	Facial or ocular pain, fever, lethargy, black nasal eschar	Mucor and Rhizopus species	Strong association with ketoacidosis; emergency surgery required.
Abdomen			
Emphysematous Cholecystitis	Fever, right-upper quadrant abdominal pain , systemic toxicity	Gram negative bacilli anaerobes	High mortality; gall stones in 50 %; emergency cystectomy required

polymorphonuclear cells and diabetic monocytes/macrophages compared to cells of controls.[79]

Although diabetes can compromise all aspects of the host's defenses against infection, diabetic patients are not equally susceptible to infection. Hyperglycaemia and acidemia exacerbate impairments in humoral immunity, polymorphonuclear leukocyte and lymphocyte functions but are substantially, if not entirely, reversed when pH and blood glucose levels return to normal. Furthermore, some microorganisms become more virulent in a high glucose environment. Another mechanism which can lead to the increased prevalence of infections in diabetic patients is an increased adherence of microorganisms to diabetic compared to nondiabetic cells.

Hyperglycaemia reduces oxidative killing capacity because of increased glucose metabolism through polyol pathway depleting NADPH which is necessary for the generation of superoxide free radicals.[80, 81] The associated metabolic disturbances impair the phagocytic function – mobilization and chemo taxis, adherence, phagocytosis and intracellular killing with bactericidal activities. All steps of PMN functioning are altered in diabetic patients, which may increase the risk of vascular complications and infectious episodes.[82] Even though there is no significant difference in the phagocytic activity of granulocytes than normals, decreased activity of intracellular killing of bacteria in granulocytes is one of the mechanisms of increased susceptibility to infection in patients with advanced stages of diabetes mellitus.[83] Patients with long-standing diabetes tend to have microvascular and macrovascular disease with resulting poor diapedesis and increased risk of infection.

In addition, the partial or complete MPO deficiency or ineffective MPO activity worsens the condition because of the inability to utilize/ generate potent oxidants for microbial killing. Previous investigators like Sato *et al.*,[84], Uchimura, K. *et al.*,[85] also had reported the reduced MPO activity in diabetic patients. But when infection rate is increased, neutrophils, recruited to tissue

sites of inflammation, release elevated levels of a variety of oxidants by the MPO system. The most powerful neutrophil oxidant, hypochlorous acid contributes to both bacterial killing and oxidative injury of host tissue. HOCl is extremely cytotoxic and reacts readily with most biological molecules thereby also promoting inflammatory tissue damage caused by neutrophils in diabetic patients. Fibronectin, an insoluble glycoprotein dimer that serves as a linker in the extracellular matrix, is extensively modified by the myeloperoxidase system. It has implications for the mechanism of tissue injury by neutrophils in inflammation, since a loss of functional fibronectin would result in cell detachment and a distortion of normal tissue organization. This oxidative stress causes the worsening of the infection complications in diabetic patients.

2

Relevance of the study

As the pathogens have many portals of entry to the body, host body has its specific and non-specific defense mechanisms against the invading organisms. Generally our immune system is very alert to the infection and capable to throw it out at any stage of its progress to a great extent. But in certain conditions like diabetes mellitus, leukemia etc., there is impairment in the normal functioning of the immune system. Diabetic patients suffer from recurrent episodes of infections in their debilitated condition. This increased susceptibility in diabetic condition is multifactorial. Neuropathy and vascular damage promote the development of wounds and inhibit their healing. Neuropathy, angiopathy, retinopathy, nephropathy and other diabetic complications can be triggered and aggravated during the course of an infection. Altered motility of the gastrointestinal and the urinary tract lead to increased penetration of bacteria in these systems. Electrolyte disturbances and reduced general conditions also increase susceptibility to infection. On the other hand, an infection deteriorates the metabolic situation in diabetes, resulting in the need for higher insulin doses, or insulin injections in patients who are normally on oral medication.

One of the major challenges in diabetic patients is infection, the important being foot ulcer problem, which is associated with significant morbidity, disability and impairment in a diabetic patients' life quality. In recent years, with global increase in prevalence of type 2 diabetes, there has been a concomitant increase in diabetic foot ulcers. According to estimates, at least 15 % of patients with diabetes develop ulcers of the lower extremity during their lifetimes. If improperly or inadequately treated, these ulcers become infected or gangrenous and may ultimately lead to amputation of the affected limb. The annual rate of amputation varies from 41 % to 77 % per

10,000 patients with diabetes and accounts for nearly half of all the lower-limb amputations in hospitalized patients. In recent years, there has been a significant increase in the prevalence of diabetes in India, particularly in the urban population. In 2000, there were an estimated 31.7 million cases of diabetes reported in India, making it the country with the largest diabetic population. This figure is expected to increase to 79.4 million cases by the year 2030. A parallel increase in the incidence of infection and diabetic foot ulcers is expected[86].

The cellular and the humoral elements of the defense system against germ invasion are disturbed by the diabetic metabolism. Myeloperoxidase is one of the principal enzymes that has a role in phagocytic oxygen dependent antimicrobial system. Commonly it is called as myeloperoxidase- hydrogen peroxide–chloride (MPO-H_2O_2-Cl) system. It is found in neutrophils and monocyte/ macrophages, which have great role in the innate immune system. In the light of the immunity impairment that has long been noticed in diabetic conditions and the fact that MPO has a significant role in the phagocytic antimicrobial system, this study has specifically been designed to estimate the MPO levels in diabetics without infection and also in infection - complicated diabetes to study it in relation to the immunity impairment associated with the disease. The data has been compared with non-diabetic, normal individuals. A knowledge of significant factors underlying the disease may help tackle this debilitated condition more effectively and successfully.

3

Review of Literature

Myeloperoxidase was originally called verdoperoxidase by Agner (1941)[10] who first purified this from purulent fluids of tubercular emphema, and was to reflect both its green colour and ability to catalyze peroxidative reactions. In 1967, Klebanoff [66] has demonstrated MPO system to be strongly bactericidal, and the enzyme an important component of the neutrophil antimicrobial armory.

In the next year, Klebanoff [67] described the antibacterial effect of MPO, a halide (such as iodide, bromide or chloride ion) and H_2O_2 on *Escherichia coli* and *Lactobacillus acidophilus* and reported that activity decreased by catalase, cyanide, azide, tapazole and thiosulphate. Rosen and Klebanoff [54] in 1976 investigated the role of superoxide anion- and myeloperoxidase-dependent reactions in the light emission (chemiluminescence) in phagocytosing polymorphonuclear leukocytes, using leukocytes that lack myeloperoxidase, inhibitors (azide, superoxide dismutase), and model systems. They suggested that light emission by phagocytosing polymorphonuclear leukocytes is dependent on both myeloperoxidase-catalyzed reactions and the superoxide anion, and involves in part the excitation of the ingested particle.

In 1976 Hansen *et al.*,[76] conducted sequential studies on changes in intraneutrophilic and plasma concentrations of the three antibacterial proteins- lysozyme, lactoferrin, and myeloperoxidase during acute bacterial infection in nine patients. Intraneutrophilic concentrations of the three proteins were decreased by more than 50 % during the 1st week of infection,

followed by a slow increase over the following 2 weeks. The data suggests that neutrophilic granulocytes are deficient of these proteins during early bacterial infection, possibly because of deficient synthesis of antibacterial proteins in the bone marrow, and that neutrophil toxic granulation is the visual counterpart of this defect. While the plasma lysozyme did not show any sequential change, plasma myeloperoxidase was high at the start of infection and quickly decreased towards normal values, and plasma lactoferrin, high in the first samples, showed a secondary peak 1 week after the onset of the disease, before normalization was seen. These differences in enzymes may result from differences in the signals, which are specific for the individual antibacterial protein and not for the different types of neutrophil granules.

Christensen and Rothstein [87] (1985) reported two situations in which the concentration of MPO/neutrophil was found to change; during the growth and development of animals and during bacterial infection. During fatal bacterial infection, MPO/neutrophil fell rapidly, often to undetectable levels, but during sublethal infections, following a 24-h lag period in adults and a 48-h lag in neonates, the concentration increased to twice the normal. For that they determined the quantity of myeloperoxidase in a volume of whole blood, and the neutrophil concentration in that same volume in experimental animals and the results expressed as units of MPO (10^{-7}/neutrophil).

Hofstra and Uetrecht [24] (1993) studied the myeloperoxidase-mediated activation of xenobiotics by human leukocytes and reported that MPO- or MPO-generated oxidants are capable of oxidizing a wide variety of compounds and a broad range of functional groups, especially those that contain nitrogen and sulfur. The powerful oxidant is hypochlorous acid. Leukocytes have a role in immune response; therefore, reactive intermediates generated by leukocyte metabolism of xenobiotics may have a role in idiosyncratic drug reactions, particularly those that are immune-mediated such as drug-induced lupus or agranulocytosis [24].

In 1995 Lincoln [88] demonstrated that the exogenously added MPO at physiological levels, enhance both phagocytosis and killing of *Escherichia coli* by macrophages. Both superoxide dismutase and catalase ablated MPO-induced bactericidal activity. They suggested that soluble MPO, released from neutrophils at a site of infection or inflammation, could enhance both phagocytosis and killing of microorganisms.

Marquez and Dunford [21] (1995) found that myeloperoxidase is the most efficient catalyst of tyrosine oxidation at physiological pH, when compared with horseradish peroxidase, thyroid peroxidase, and lactoperoxidase. Although chloride is considered as the major myeloperoxidase substrate, tyrosine is able to compete effectively for compound I (one of activated state of MPO). Steady state inhibition studies demonstrate that chloride binds very weakly to the tyrosine-binding site of the enzyme. Both Heinecke *et al.*, (1993) [43] and Marquez and Dunford (1995)[21] reported the dityrosine, the highly fluorescent stable compound, as a possible marker for proteins oxidatively damaged by myeloperoxidase in phagocyte-rich inflammatory lesions.

The neutrophil myeloperoxidase uses H_2O_2 to oxidize chloride, bromide, iodide and thiocyanate to their respective hypohalous acids. In 1997 Van Dalen *et al.*,[20] analyzed whether myeloperoxidase oxidizes thiocyanate in the presence of chloride at physiological concentrations of these substrates and determined the relative specificity constants for chloride, bromide and thiocyanate as 1:60:730 respectively, indicating that thiocyanate is by far the most favoured substrate for myeloperoxidase. Regardless of where the enzyme acts, thiocyanate is a major physiological substrate of myeloperoxidase.

Kabutomori *et al.*,[89] (1999) measured the mean myeloperoxidase index (MPXI) of neutrophils in normal subjects, with an automated hematology system, which differentiates white blood cells. MPO activity was represented by the MPO staining intensity. They found that the MPXI in

normal women was significantly higher than that in normal men and the menstrual cycle affects the MPXI. This sex difference suggested that some microbicidal activity may be stronger in women than in men. The MPXI may be useful as a partial index for microbicidal activity of neutrophils.

MPO has several roles in biological functions. Since it is an inevitable factor, its deficiency causes many problems like increased infection susceptibility, decreased tumor surveillance etc. The MPO deficiency can occur in two forms, hereditary and acquired. It may be partial or complete. In 1977 Cech *et al.,*[90] reported of hereditary MPO deficiency in the granulocytes of patients with diabetes mellitus suffering from *Candida albicans* hepatic abscess. Their functional granulocyte studies had revealed normal chemotactic and phagocytic activity although the bactericidal activity is partially diminished with regard to *Staphylococcus aureus* and almost nil with regard to *Candida albicans*. After two years (1979), they studied a case of hereditary myeloperoxidase deficiency in a diabetic patient suffering from *Candida albicans* liver abscess and found that peroxidase activity is completely absent in the neutrophils and monocytes although it is present in the eosinophils. [91]

In the study on 'MPO deficiency and its prevalence and clinical significance', by Parry *et al.,*[92] (1981) noted only minor defects in killing of *Staphylococcus aureus* by MPO deficient leukocytes, where as candidicidal activity was much more impaired. In the same year (1981), the study on hereditary MPO deficiency was conducted by Kitahara *et al.,*[93] and revealed that partial deficiency have impaired bactericidal activity against *S.aureus.* Their findings have supported the concept of multiple leukocyte bacterial killing systems. Another work supporting the multiple leukocytic bactericidal systems, was done by Lanza [94] (1998) on the clinical manifestations of MPO deficiency and reported that the total or partial deficient individuals does not have an increased frequency of infections.

Stendahl, *et al.*,[55] (1984) opined in support of their finding in a patient with complete MPO deficiency suffering from generalized pustular psoriasis, do not usually show any increased susceptibility to infection or altered inflammatory response, in contrast to several other biochemical defects in polymorphonuclear neutrophils. They found that the MPO-deficient neutrophils showed enhanced phagocytosis than in normal persons and prolonged stimulation of superoxide production. When MPO was added to the hyperactive MPO-deficient cells, phagocytosis was reduced more rapidly. Catalase, azide, and methionine eliminated the inhibitory effect, and catalase and methionine, in fact, enhanced the phagocytic activity of adherent neutrophils. Apart from being a potent antimicrobial system, the oxidizing activity of the MPO- H_2O_2-halide system may modulate the inflammatory response by impairing certain receptor-mediated recognition mechanisms of phagocytic cells, which otherwise could elicit inflammatory reactions and tissue injury.

3.1 Myeloperoxidase and diabetes mellitus

In 1975 Niethammer *et al.*,[95] had reported the impairment of granulocyte function in juvenile diabetes. They did not find a phagocytic defect in the ingestion of particles, but the capacity of intracellular killing of *Staphylococcus aureus* was impaired. Chemotaxis was also reduced whereas intracellular killing of *Candida albicans* were normal. Better control of diabetes led to an improvement of bactericidal killing capacity.

Kaneshige *et al.*,[83] (1982) also examined the phagocytosis and intracellular killing of *Staphylococcus aureus* by granulocytes in diabetic patients. There was no significant difference in the phagocytic activity of granulocytes between control and diabetic-subjects. However, intracellular killing by granulocytes was significantly reduced in insulin-treated diabetic patients compared with control subjects. No significant difference observed between controls and diet-treated diabetic patients. It is suggested that decreased activity of intracellular killing of bacteria in granulocytes is one of

the mechanisms of increased susceptibility to infection in patients with advanced stages of diabetes mellitus.

In 1993, Wykretowicz *et al.*,[96] noticed normal phagocytosis to staphylococci, decreased intracellular killing, impaired stimulated superoxide production and H_2O_2 production and low intracellular MPO activity. Their data indicated that the decreased bacterial killing by PMNs isolated from diabetics is partly at least related to an impairment of oxygen dependent bactericidal mechanism. They opined that impairment of the oxygen-dependent microbicidal mechanisms of polymorphonuclear neutrophils in patients with type 2 diabetes is not associated with increased susceptibility to infection. A previous observation of Nauseef [53] (1988) also revealed that though MPO activity is critical for optimal microbicidal activity of normal PMNs, in the absence of MPO, auxiliary mechanisms protect most MPO deficient hosts from clinically significant sequence except for some persons with diabetes mellitus who suffered from severe candidial disease.

In 1996 Zozulinska, *et al.*,[97] reported that toxic oxygen species production might be influenced by disease duration in patients with insulin-dependent diabetes mellitus (IDDM). The production of H_2O_2 by unstimulated PMNs is increased in diabetic patients while generation of $O2^-$ superoxide anions by stimulated neutrophils is markedly impaired.

In 1977, De Toni *et al.*,[81] observed that PMN function in diabetic patients is multifactorial in origin and is probably correlated to the glucose level and to glycation of PMN protein such as NADPH oxidase or MPO. In diabetic condition, the competition for NADPH, the coenzyme for the respiratory burst, reduces the superoxide production.

Kemona *et al.*,[98] (1985) determined the cytochemical indices of leukocytes in patients with diabetes mellitus in the period of uncontrolled and controlled. In unbalanced diabetics an evident decrease in the activity of acid phosphatase and MPO could be noted as well as a decrease of

glycogen content and an increase of lipid content. Balancing this disease induced the increase of all parameters in granulocytes except MPO activity. Their findings clearly indicate the role of metabolic disorders in diabetes mellitus on the activity of some neutrophilic enzymes and the glycogen and the content of lipids in neutrophils.

Sato *et al.*,[84] (1992) have reported the decreased MPO activity in poorly controlled diabetes mellitus patients and there is a significant correlation between glycosylated hemoglobin (HbA1c) levels. They demonstrated that every step in leukocytic generation of reactive oxygen intermediates should be reduced in the leukocytes from poorly controlled diabetes patients.

In 1997, Delamaire *et al.*,[82] evaluated polymorphonuclear neutrophil (PMN) cell performance in type 1 and type 2 diabetic patients free of infection, using tests that explore all the functional steps of PMN: adherence, chemotaxis, phagocytosis, and bactericidal activity. PMN chemotaxis was significantly lower in patients than in the healthy control group ($p < 0.001$) and associated with spontaneous adherence and increased expression of adhesion molecules. There is spontaneous activation of PMN cells and increased free radical production; after stimulation, response was lower than in the control group. The type of diabetes, the age of patients, HbA1C level and disease duration did not affect the responses. Chemotaxis was further reduced in patients with vascular complications and hyperglycaemia. They concluded that all steps of PMN functioning are altered in diabetic patients, which may increase the risk of vascular complications and infectious episodes.

In the same year Sato *et al.*,[99] opined that in poorly controlled non insulin dependent diabetes mellitus(NIDDM ,Type 2) patients, granulocyte-colony stimulating factor (G-CSF, a growth factor that stimulate the bone marrow for production and function of granulocytes) improves the impaired production of oxygen derived free radicals by neutrophils. They also found a

positive correlation between HbA1 and improving the effect of G-CSF on MPO activity. They suggested that G-CSF may be useful as a drug to prevent the aggravation of bacterial infections by improving neutrophil function, especially through H_2O_2 – MPO- OCl^- mechanism in poorly controlled diabetic patients.

Uchimura *et al.*,[85] (1999) evaluated the changes in superoxide dismutase activities and concentrations and myeloperoxidase activities in leukocytes from patients with diabetes mellitus than the control group. Myeloperoxidase activity in leukocytes was significantly reduced in NIDDM patients whereas Cu-SOD and Zn-SOD (types of superoxide dismutase) showed no change than those in control group. These findings suggested that changes in these enzymes may affect the susceptibility to infection and immunocompetence of patients with diabetes.

In 2000, Dodds *et al.*,[100] (2000) observed a similar pattern of decreased saliva flow rates and increased concentrations of saliva proteins (stimulated parotid lactoferrin, myeloperoxidase, and salivary peroxidase, as well as submandibular/sublingual total protein, albumin, lactoferrin and secretory IgA) in three groups of diabetics, healthy control and hypertensives. But this decreased flow rates and increased protein concentrations consistently greater in diabetics than hypertensives, which suggested that disease-specific mechanisms may be responsible. They also found diabetics are more prone to oral dryness and infections than non-diabetics.

The prevalence of pathogens in diabetic foot infections was determined by Viswanathan[101] (2002), in relation to parameters like Wagner's grading, duration of diabetes and healing time. Diabetic foot infection is polymicrobial in nature. The healing time of wound infected with anaerobic pathogens was higher than those infected with aerobic pathogens. Neuropathy was common in diabetic patients infected with both

aerobic and anaerobic pathogens. Presence of neuropathy increased the risk of foot infection.

The inhibition of MPO activity may happen in many ways. In 1998 Saeed *et al.*,[102] reported the inhibition of neutrophil chemiluminescence during phagocytosis by abnormally elevated acetoacetate. The inhibition of MPO activity and chemiluminescence implicate metabolic ketosis in the inhibition of neutrophil microbicidal activity and thus increased susceptibility to infections. According to Mario Allegra *et al.*,[103] (2001) melatonin also can inhibit the chlorination activity of MPO in the reaction between human MPO and melatonin at both pH 7 and pH 5.

The study of Accardo-Palumbo *et al.*,[104] (1996) revealed the occurrence of anti-myeloperoxidase (anti-MPO) antibodies in 34 out of 88 (38 %) patients with type 1 diabetes mellitus but in only 3 of 55 (5.7 %) healthy subjects and in 4 of 20 patients with autoimmune disease. Specificity of anti-MPO antibodies was assessed by MPO inhibition studies. A state of chronic neutrophil activation has been described in diabetes mellitus. As anti-MPO antibodies can stimulate neutrophils to damage endothelial cells in systemic vasculitis, this suggests that a similar mechanism may be operative in the development of diabetic angiopathy.

Oldenborg[105] (1999) suggested that elevated levels of insulin do not affect the NADPH-oxidase activity but, together with superoxide anions, interfere with myeloperoxidase availability and a subsequent myeloperoxidase-dependent generation of reactive oxygen metabolites in fMet-Leu-Phe-stimulated normal human neutrophils. The insulin-induced reduction of the chemiluminescence response was reversed by the addition of exogenous peroxidase and was also paralleled by a reduced myeloperoxidase activity, with no effect on the elastase activity, in cell-free supernatants from fMet-Leu-Phe-stimulated neutrophils.

An increased incidence of heart problems was found in diabetic patients. The role of myeloperoxidase in heart diseases has been reported in many works. In 2000 Anitra *et al.*,[106] reported the property of MPO to bind with low density lipoprotein (LDL) and its potential implication in atherosclerosis. MPO catalyzes the formation of a number of reactive oxygen species that modify LDL to a form that converts macrophages in 'lipid laiden' or 'foam' cells, the hallmark of atherosclerosis.

In 2003, Baldus *et al.*,[107] observed that patients with elevated MPO levels experienced a markedly increased cardiac risk. They found that in patients with acute coronary syndrome (ACS), MPO serum levels powerfully predict an increased risk for subsequent cardiovascular events and extend the prognostic information gained from traditional biochemical markers. Given its proinflammatory properties, MPO may serve as both a marker and a mediator of vascular inflammation and further points towards the significance of PMN activation in the pathophysiology of ACS.

According to Zhang, *et al.*,[108] (2004), vascular non-leukocyte-derived reactive oxygen species (ROS), such as superoxide and H_2O_2, have emerged as important molecules in diabetic endothelial dysfunction. In addition, leukocyte-derived myeloperoxidase has been implicated in vascular injury, and its injury response is H_2O_2 dependent. It is well known that MPO can use leukocyte-derived H_2O_2; however, it is unknown whether the vascular-bound MPO can use high-glucose-stimulated, vascular non-leukocyte-derived H_2O_2 to induce diabetic endothelial dysfunction. They demonstrated that MPO activity is increased in vessels from diabetic rats and suggest that vascular-bound MPO could use high-glucose-stimulated H_2O_2 to amplify high-glucose-induced injury in the vascular wall. MPO/ H_2O_2/HOCl/chlorinating species may represent an important pathway in diabetes complications and a new mechanism in phagocyte- and systemic infection-induced exacerbation of diabetic vascular diseases.

In 2004, when Buraczynska, *et al.*, [109]determined the tumor necrosis factor (TNF) and MPO levels in plasma, found that, in diabetic nephropathy patients molecular variants of TNF are more frequent than in nondiabetic patients with chronic renal failure and this change might be associated with altered ability to TNF synthesis. Analysis of the myeloperoxidase genotypes showed significant difference in genotype distribution in dialyzed patients with diabetic nephropathy. Chronic renal failure patients show a significant reduction in the intracellular myeloperoxidase level.

According to Moutschen[110] (2005), polymorphonuclear neutrophils are clearly influenced by the diabetic state. On one hand, their antimicrobial function is inhibited by hyperglycaemia, due to inhibition of glucose-6-phosphate dehydrogenase or diversion of NADPH in the polyol pathway; on the other hand, the pathogenesis of chronic complications of diabetes amplify inflammatory systemic manifestations associated with infections and play a role in the higher mortality rate observed in diabetic subjects with severe infections. These observations argue for the systematic vaccination of all diabetic patients against influenza and Streptococcus pneumoniae, for the reappraisal of diabetes as a significant pejorative risk factor in community acquired pneumonia and for intensive insulin therapy in all diabetic patients with severe infection.

In addition to antimicrobial activity of myeloperoxidase system, or its main product HOCl by itself, it readily abrogate the ability of α 1-proteinase inhibitor to inhibit elastase. In 1979, Matheson *et al.*,[111] reported this inactivation property of MPO in presence of H_2O_2 and Cl^-. In 1981 they demonstrated that there is a direct dependence on the concentration of α-1-PI and H_2O_2 and Cl^- ion.[112]

In 1987, Borregaard [113] described that this inactivation of alpha 1-proteinase inhibitor was effectively prevented by N-acetyl cysteine, methionine and reduced glutathione. These results indicated that the sulphydryl compounds work as scavengers of the products of the

myeloperoxidase system, and might be useful in inflammatory disorders, to prevent tissue damage inflicted by this system.

Vissers and Winterbourn [114] (1991) demonstrated that the primary and tertiary structures of purified human plasma fibronectin extensively modified on exposure to the myeloperoxidase- H_2O_2-Cl- system of neutrophils or to reagent HOCl. It has implications on the mechanism of tissue injury by neutrophils in inflammation, since a loss of functional fibronectin would result in cell detachment and a distortion of normal tissue organization.

Ottonello *et al.*,[115] (1993) took a rational approach to the pharmacological control of neutrophil-mediated tissue injury and focused on the histoprotective potential of an anti-inflammatory drug, Nimesulide as a down regulator of the activity of the neutrophil myeloperoxidase pathway during inflammatory processes. They investigated that this agent reduced the function by MPO pathway, responsible for HOCl production, by exerting a cell-directed inhibitory activity, as shown by measurement of superoxide anion and hydrogen peroxide production. Nimesulide also inactivated hypochlorous acid directly and protected alpha 1-antitrypsin from the neutrophil-mediated oxidation. These data suggested that nimesulide may prevent tissue injury at sites of inflammation by maintaining natural host protective systems.

According to Vijayalingam [116] (1996) antioxidant status is poor in both impaired glucose tolerant (IGT) and NIDDM. Antioxidant enzymes - superoxide dismutase and catalase were significantly lower in red blood cells obtained from IGT and early hyperglycaemic groups. They were closer to the levels showed in NIDDM confirming that antioxidant deficiency is already present in IGT subjects. Lipid peroxidation product in plasma, erythrocyte, and erythrocyte cell membrane was found to be significantly elevated ($p<0.001$) in IGT, early hyperglycaemia and diabetes mellitus while glycosylated haemoglobin was also higher. Among the antioxidant

scavengers, reduced glutathione and ascorbic acid are reduced by 15% and 20 % in IGT and NIDDM, respectively.

Muchova *et al.*,[117] (1999) examined the effect of type 2 diabetes mellitus on enzymes of importance for oxygen-dependent killing of microorganisms by leucocyte. The superoxide dismutase activity was lower by 41% in polymorphonuclear leucocytes from patients with Type 2 DM than in control group. Glutathione peroxidase and glutathione reductase activities of type 2 DM patients were 73.04 % and 81.12 % respectively of control values. The catalase activity showed no significant difference. A significant increase in the concentration of thiobarbituric acid reactive products was observed. A positive correlation between thiobarbituric acid reactive products and glucose, glycosylated haemoglobin and fructosamine was observed in the serum of diabetic patients. These findings may explain some of the mechanisms underlying the increased susceptibility to certain infections in patients with type 2 diabetes mellitus.

In 2001 Majchrzak *et al.*,[118] found reduced glutathione level and markedly lower dismutase activity in well metabolically controlled diabetic patients, compared to the control group. The levels of glutathione peroxidase did not differ significantly from values obtained in healthy subjects. They did not observe any correlation between the analysed parameters and duration of diabetes, HbA1c or presence of chronic complications of the disease. Their results indicated that antioxidative systems in the state of good metabolic control of diabetes have adaptive properties.

Galeano *et al.*,[119] in 2001 observed a delayed wound healing together with low collagen content, breaking strength, and increased malondialdehyde (MDA) levels and MPO activity in diabetic mice when compared with their healthy littermates. They found that the raxofelast, a protective membrane antioxidant, restores wound healing nearly to normal levels in experimental diabetes-impaired wounds in diabetic mice through the stimulation of angiogenesis, reepithelialization, synthesis, and maturation

of extracellular matrix. Furthermore, raxofelast treatment significantly reduced MDA levels, MPO activity, and increased the breaking strength and collagen content.

Diegelmann *et al,.*[120] (2003) has reported that chronic ulcers contain the persistence of neutrophils and their destructive enzymes, especially neutrophil-derived matrix metalloproteinase-8 and elastase which appears responsible for the extensive matrix dissociation and thus contributes to the chronicity of these ulcers. They also found that a significant correlation of myeloperoxidase activity with actual neutrophil counts in the ulcer biopsies further confirm the dense presence of neutrophils with occasional large macrophages actively phagocytosing depleted neutrophils. Their studies directly showed that there is extensive neutrophil infiltration in chronic pressure ulcer granulation tissue.

In 2005, Jantschko *et al.,*[26] designed reversible mechanism of HOCl production mediated by MPO, for the development of drugs against HOCl-dependent tissue damage, with respect to the enzymology of MPO. It was based on the extraordinary and MPO-specific redox properties of its intermediates compound I and compound II.

4

Materials and Methods

The study population consisted of 50 patients of diabetes mellitus without infection related complications, 50 patients of diabetes mellitus having complications of infection and 50 non-diabetic normal persons as control subjects.

Patients for this study were selected from Diabcare Clinic, Manjeri. Diabcare Clinic is a speciality clinic for diabetes and diabetic foot care. Informed written consent from the patients and controls was obtained before including them in this study. Patients were screened for the presence of diabetes according to the American Diabetes Association's new criteria for the diagnosis of diabetes mellitus[121]. Care was taken to exclude the cases suffering from eosinophilia and those receiving antioxidant therapy. Inclusion criteria for the diagnosis of diabetes is as shown below.

1. Symptoms of diabetes and a casual plasma glucose level of 200 mg/dL (11.1 mmol/L). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria (excessive passage of urine), polydipsia (excessive thirst all the time) and unexplained weight loss.

OR

2. Fasting Plasma Glucose (FPG) 126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 hour before blood collection.

OR

3. 2-hour plasma glucose 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT). The test was performed as described by the World Health Organization, using a glucose load containing the equivalent of 75gm anhydrous glucose dissolved in water.

The case history was recorded for each individual, which included sex, age, blood sugar level, method adopted for diabetic management, infection status and presence of other complications like coronary artery disease, nephropathy, delayed wound healing etc. Blood sugar estimation, total WBC count, MPO estimation in blood and tissues of each individual were also performed and results were duly recorded.

4.1. Sample collection

Four milliliter of venous blood was collected in a collection tube containing acid-citrate-dextrose solution (167µl / 1 ml of blood). The plasma was separated from 1 ml of each anticoagulated blood sample within 30 minutes of sample collection and this plasma was used for determination of blood sugar status. The remaining 3 ml was subjected to myeloperoxidase enzyme estimation.

Tissue samples were collected from the infected area for the estimation of myeloperoxidase, which is an index of neutrophil and macrophage infiltration. For this purpose, tissues (4–15 mg) were collected by lavage, during the dressing of wound of diabetic foot patients. Normal tissue samples were collected from biopsy specimen of patients subjected for surgery due of hernia, varicose vein, etc. One part of the tissue was fixed with 20 % formaldehyde for histological characterization and the rest washed with phosphate buffered saline (pH 7.0) to remove the contaminating blood to directly process it for MPO extraction.

4.2. Estimation of blood sugar

The procedure used was enzymatic GOD-POD (Glucose oxidase – Peroxidase), end point colorimetry single reagent chemistry method. PreciChem Glucose GOD kit was used for the estimation of sugar in blood.

Principle:

Glucose oxidase oxidizes the glucose into gluconic acid and hydrogen peroxide. In a subsequent peroxidase catalyzed reaction, the chromogen system (Phenol and 4-Aminoantipyrine) accepts the liberated oxygen and gives a red coloured quinoneimine compound. The red colour so developed is measured at 505 nm and this is directly proportional to glucose concentration.

Reagents:

The following reagents are provided in the kit:

1. **Glucose reagent** (stored at 2–8^{0}C)

   Components: Glucose oxidase, peroxidase, 4-aminoantipyrine, buffers and stabilizers.

2. **Glucose standard (100 mg/dL)** (stored at 2–8^{0}C)

   Components: Dextrose and benzoic acid.

Procedure:

Pipetted into tubes marked as:	**Blank**	**Standard**	**Test**
Plasma	-	-	10 µl
Glucose standard	-	10 µl	-
Glucose reagent	1000 µl	1000 µl	1000 µl
Mixed well and incubated at 37^{0}C for 10 min or at room temperature for 30 min. Mixed well and read absorbance at 490 – 550 nm against a reagent blank.			

Equipment:

Autospan semi-autoanalyzer was used for reading the measurements between 490 – 550 nm filters.

Calculation:

$$\text{Glucose (mg/dl)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

4.3. Extraction and estimation of MPO from leukocytes and tissues

4.3.1 Preparation of leukocytes from blood

During standardization, two methods were tried for the separation of leukocytes. (i) Histopaque density gradient method and (ii) modified method for the separation of total leukocytes by Percy and Brady[122] . In the previous method, a double gradient was made by layering Histopaque®-1077 (Polysucrose, 5.7 gm/dL, and sodium diatrizoate, 9.0 gm/dL) over Histopaque®-1119 (Polysucrose, 6.0 gm/dL and sodium diatrizoate, 16.7 gm/dL) taken in equal volumes. Whole blood is carefully layered onto the upper Hlistopaque®- 1077 medium. Centrifuged the tubes at 700 x g for 30 minutes. Cells of the granulocytic series were found at the 1077/1119 interphase whereas lymphocytes, other mononuclear cells and platelets at the plasma/1077 interphase. Aspirated the layers of granulocytes and monocytes separately. Washed the cells by addition of 10 ml isotonic phosphate buffered saline to the tubes. Centrifuged for 10 minutes at 200 x g. Aspirated the supernatant and discarded.

A better recovery of leukocytes was obtained in the second method. Hence the modified method of Percy and Brady[122] was adopted in this study for isolation of leukocytes from the anticoagulated blood. Ninety percent of the leukocytes thus isolated were neutrophils.

Principle

When anticoagulated whole blood is allowed to stand at room temperature for 30 to 60 minutes, erythrocytes settles first; leukocytes,

whose buoyant density is less than that of erythrocytes, sediment more slowly and forms a "buffy coat" between the erythrocytes and plasma. When the density of the medium through which sedimentation occurs is increased, for example, by the addition of dextran, leukocytes remain suspended. Recovery of the supernatant and subsequent centrifugation at low centrifugal force results in a pellet made up largely of leukocytes. Contaminating erythrocytes are eliminated by selective shock treatment, since they are less resistant than white blood cells to lysis in hypotonic solutions.

Reagents:

1. **Sodium chloride solution, 0.7 gm/dL:** Took 0.7 gm of sodium chloride in 100 ml volumetric flask and diluted to mark with distilled water. Stored stable for 2 months at 4 ^{0}C.
2. **Dextran solution:** 5 gm/dL in sodium chloride solution, 0.7gm/dL.
3. **Sodium chloride solution, 0.9 gm/dL:** Placed 0.9 gm of sodium chloride into 100ml volumetric flask and diluted to mark with distilled water. Stored stable for 2 months at 4 ^{0}C.
4. **Sodium chloride solution, 1.8 gm/dL:** Placed 1.8 gm of sodium chloride into 100 ml volumetric flask and dilute to mark with distilled water. Stored stable for 2 months at 4 ^{0}C.

Procedure:

1. Three ml of anticoagulated blood sample was taken in a centrifuge tube.
2. The freshly prepared dextran solution added to the anticoagulated blood taken (0.2 ml/ ml of blood) and mixed gently by inversion.
3. Allowed to stand for 45 minutes for sedimentation of cells.
4. Drew off supernatant with a pasture pipette and discharged into another centrifuge tube.
5. Centrifuged at 500 x g, at 4 to 10 ^{0}C for 10 minutes.

6. Drew off supernatant and discarded.
7. Resuspended the button of white blood cells in 1.0 ml of cold sodium chloride, 0.9 gm/dL.
8. Shock treated the cells as follows: Added 3.0 ml of ice cold distilled water and mixed gently for 45 seconds. Immediately added 3.0 ml of cold sodium chloride solution, 1.8 gm/dl and mixed.
9. Centrifuged at 500 x g for 10 minutes.
10. Drew off supernatant and discarded.
11. Followed the steps 7 through 10 for second shock treatment.

4.3.2. Extraction of myeloperoxidase from purified leukocytes

During standardization of the procedure, four methods were adopted for the preparation of cell contents i.e., (i) by using 0.5 % hexadecyl trimethyl ammonium bromide (HTAB) (ii) by freezing and thawing (iii) by sonication and (iv) sonication with HTAB. It was found that sonication with HTAB was more effective for cell lysis and so this method was adopted here. Sonication was done in Sonics Vibra-Cell ultrasonic liquid processor Model VC-750, USA.

Myeloperoxidase enzyme was extracted from leukocytes, that were separated from blood and purified, by modified method of Sharon *et al.,*[123]

Procedure

1. Purified leukocytes of each sample was suspended separately in 1 ml of 0.5 % hexadecyl trimethyl ammonium bromide (HTAB).
2. Sonicated at 228 μm amplitude for 1 minute in ice bath.
3. Centrifuged at 30,000 x g at 4 ^{0}C in cold centrifuge (Remi – C24).
4. Drew off supernatant of each sample and discharged into eppendorf microcentrifuge tubes. Stored at –20 ^{0}C until the reagents were ready to analyze enzyme activity.

The estimation of myeloperoxidase enzyme activity of cell lysates and electrophoretic analysis for isozymes of MPO were done as early as possible, after extraction of the enzyme.

4.3.3. Extraction of myeloperoxidase from tissue

Myeloperoxidase activity in tissue, an index of polymorphonuclear leukocyte infiltration and accumulation, was determined as previously described by Galeano *et al.*,[119] The samples were first homogenized in a solution containing 20 mmol/L potassium phosphate buffer (pH 7.4) and 0.01 mol/L EDTA and then centrifuged for 30 minutes at 20,000 x *g* at 4℃. The supernatant of each sample was then discarded, and the pellet was immediately frozen to -20^0 C. The samples were kept at a temperature of 0℃ for 14 hours before sonication. After thawing, the resulting pellet was added to 1ml of ice-cold buffer solution consisting of 0.5% hexa-deacyl-trimethyl-ammonium bromide dissolved in 50 mmol/L potassium phosphate buffer (pH 6). Each sample was then sonicated for 5 minutes at 228 amplitude and at a temperature of 4℃. After sonica tion, the samples were chilled on ice for approximately 30 minutes and centrifuged for 30 minutes at 40,000 x g at 4℃. An aliquot of the supernatant wa s used for MPO analysis.

4.3.4. Estimation of myeloperoxidase activity

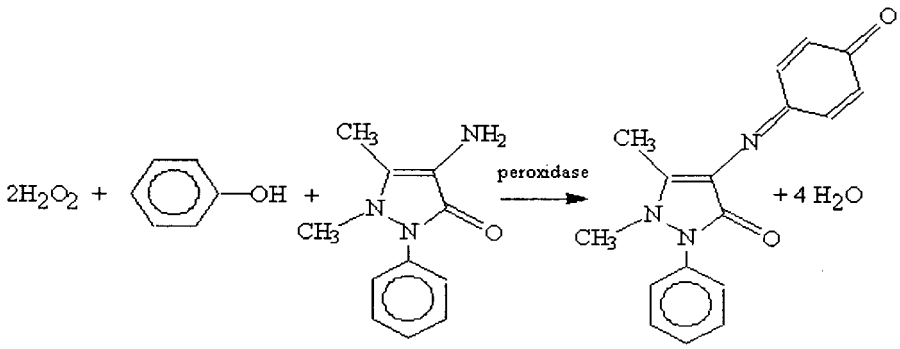
The activity of myeloperoxidase was estimated by the method of Matheson *et al.* [6] The measurement was done using 4-aminoantipyrine as the hydrogen donor.

Reagents:

1. **50 mmol/L Sodium Phosphate buffer (pH 6.1)**
2. **4-Aminoantipyrine:** Prepared the solution of 0.5mg 4-Amino antipyrine/ml in 0.17 M Phenol. It was stable for several weeks in dark at room temperature.

3. **Hydrogen peroxide:** Solution of 1.7 mmol/L was prepared fresh in sodium phosphate buffer.

Principle



Procedure:

1. Pipetted into each cuvette as follows:

4 Aminoantipyrine/ phenol solution	1500 µl
Sodium phosphate buffer	400 µl
1.7 mmol/L hydrogen peroxide	1000 µl

2. 100 µl of enzyme extract was added and the increase in absorbance at 510 nm at 30 seconds intervals for 5 minutes was recorded using kinetic mode of spectrophotometer (UVmini-1240, Shimadzu, Japan).
3. Increase in absorbance by each lysate sample was recorded.

For calculations the molar extinction coefficient (ε) of 6580 for quinoneimine at 510 nm was employed.

The unit MPO activity of blood was expressed in units per milliliter of blood and in units per cell. The unit MPO activity of tissue was expressed in units per milligram of weight.

4.4. Unit of enzyme activity

One unit of enzyme (U) is defined as the amount of the enzyme that catalyzes the conversion of 1 micromole of substrate per minute at specified conditions of temperature and pH. In the present study, one unit of peroxidase is defined as the amount of enzyme that catalyzes the transformation of 1 μ mole of hydrogen peroxide per minute at pH 6.1 and at 25 °C.

4.5. Calculation of unit MPO in the sample

4.5.1. Blood

Molar extinction coefficient of quinoneimine at 510 nm = 6580

For calculation, Δ OD_{obs}/ min is used.

That is, Δ OD of 6.58/ml reaction volume ≡ 1 μ mol quinoneimine

Volume of reaction mixture = 3 ml

∴ Δ OD of 6.58 in 3 ml = 3 μ mols quinoneimine

∴ quinoneimine formed for observed Δ OD at 510 in assay system

$$= \frac{\text{Observed } \Delta OD \times 3}{6.58} \mu\text{mols}$$

Volume of lysate used for assay system of 3 ml = 100 μl

∴ quinoneimine formed per ml lysate

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58} \mu\text{mols}$$

1 ml lysate ≡ 3 ml whole blood taken for the analysis

∴ quinoneimine formed per ml whole blood equivalent

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58 \times 3} \mu\text{mols}$$

$$= \frac{\Delta OD_{Obs} \times 10}{6.58} \mu\text{mols}$$

1 equivalent of quinoneimine formation requires 2 equivalence of H_2O_2 (see page no. 41)

$\therefore$ Unit MPO/ml equivalent of whole blood $= \frac{\Delta OD_{Obs} \times 10}{6.58 \times 2}$

Unit MPO / leukocyte is calculated from the above as follows:

No. of leukocytes / ml whole blood = N

$\therefore$ Unit MPO/ leukocyte $= \frac{\Delta OD_{Obs} \times 10}{6.58 \times 2 \times N}$

4.5.2. Tissue

Molar extinction coefficient of quinoneimine at 510 nm = 6580

That is, Δ OD of 6.58/ml reaction volume ≡ 1 μ mol quinoneimine

Volume of reaction mixture = 3 ml

$\therefore$ Δ OD of 6.58 in 3 ml = 3 μ mols quinoneimine

$\therefore$ quinoneimine formed for observed Δ OD at 510 in assay system

$$= \frac{\text{Observed } \Delta OD \times 3}{6.58} \mu\text{mols}$$

Weight of tissue sample taken for the analysis varied in each case (a range of 4–15 mg) and is denoted as **Y**. The final volume of tissue lysate in each case is 1.0 ml irrespective of the tissue weight.

Volume of lysate of tissue used for assay system of 3 ml = 100 μl

$\therefore$ quinoneimine formed per ml lysate

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58} \mu\text{mols}$$

1 ml lysate ≡ **Y** mg of tissue taken for the analysis

$\therefore$ Quinoneimine formed by total tissue equivalent

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58} \mu\text{mols}$$

$\therefore$ Quinoneimine formed per mg tissue equivalent

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58 \times Y} \mu\text{mols}$$

1 equivalent of quinoneimine formation requires 2 equivalence of H_2O_2 (see page no. 41)

∴ Unit MPO/mg tissue equivalent

$$= \frac{\Delta OD_{Obs} \times 3 \times 10}{6.58 \times Y \times 2}$$

4.6. Statistical Analysis

The experimental results were analyzed using one-way ANOVA, with SPSS (version 14). Data are expressed as mean ± standard error of the mean, and $p \leq 0.05$, which denotes significance.

4.7. Histological studies

Tissue processing

Requirements

1. **Fixative: 20% formaldehyde**
2. **Ethyl alcohol (ETOH)**
3. **Tertiary butyl alcohol (TBA)**

   TBA 1 (40% ETOH, 10% TBA, 50% dH_20)

   TBA 2 (50% ETOH, 20%TBA, 30% dH_20)

   TBA 3 (50% ETOH, 35% TBA, 15% dH_20)

   TBA 4 (40% ETOH, 55% TBA, 5% dH_20)

   TBA 5 (25% ETOH, 75% TBA)

   TBA 6 (100% TBA).
4. **Paraffin with ceresin**
5. **Mayer's egg albumin**

   Equal part of egg white and glycerin is mixed well and filtered through a filter paper. A pinch of thymol crystal was added as a preservative.

I. Fixation

The specimens were fixed for 24 hours in 20% formaldehyde. After 24 hours, they were kept in formaldehyde and a portion of each specimen was transferred to 70 % ETOH for storage.

II. Processing

A. Dehydration

The specimens were transferred from fixing solution to tertiary butyl alcohol (TBA). These were dehydrated through the graded TBA series, [TBA 1; TBA 2; TBA 3; TBA 4; TBA 5; TBA 6] with a minimum of 1 hour in each of the solutions. At all stages of the dehydration, the vials were tightly capped to prevent evaporation of the alcohol. Specimens were kept in TBA 6 overnight, on top of a slide-warming tray. Since absolute TBA solidifies at about 20°C, it was kept above this temperature, to remain in liquid form.

B. Clearing and impregnation

Added paraffin chips to fill the vial, capped it, and placed on the wax melter tray for a few hours. Put the tissue samples into the vials. When the paraffin began to dissolve into the TBA under these conditions, slowly infiltrated the tissues with a mixture of paraffin and alcohol.

1. The next day, uncapped the vial and placed it in a 56° C oven. The paraffin melted and the TBA slowly evaporated overnight.
2. The next day, made two paraffin exchanges, one early morning and the second about 3 hours later. To perform a paraffin exchange, removed the vial from the oven and carefully, but quickly, decanted the paraffin off into a disposal can next to the oven. Refilled the vial with fresh molten paraffin from the oven. At the end of this exchange, the specimens were ready to embed by late afternoon of the day.

3. To embed, decanted the molten fresh paraffin (with ceresin) to about half-of embedding boat. Flamed a spatula and moved it across the surface of the paraffin in the boat to keep the surface melted, while the paraffin begins to solidify at the bottom of the boat. Once the bottom fourth of the paraffin has solidified, quickly decanted the vial, with specimen, into the boat. Then oriented the specimen into the proper plane for sectioning, using a pair of flamed needles. Sections were prepared from the bottom of the boat upwards, so planed the orientation accordingly. After the paraffin has solidified around the specimen, floated the boat in a dish of ice water to complete the solidification process.
4. The paraffin embedded specimens were mounted onto sectioning stubs or wooden blocks, trimmed, and sectioned with a rotary microtome.
5. The trimmed blocks were fixed to the block holder and fitted to the microtome. Then moved the block holder until it just touched the blade. Ensured that the whole surface of the block moved parallel to the edge of the blade. Tightened all the adjusting screws of the microtome. Adjusted the gauge of the microtome about 7mm and trimmed the block.
6. Floated the sections on warm water to avoid the creasing of sections. Temperature maintained as equal to or 1-2^{0}C less than that off melting point of paraffin.
7. After the complete removal of creases, the sections were carried in clean albumin glue applied slides. Water was allowed to drain off and slides incubated at 60^{0}C for one hour. These slides were subjected to staining.

III. Staining and mounting

Reagents

1. **Xylene.**
2. **60% ethyl alcohol.**
3. **Absolute alcohol.**
4. **Haematoxylin**

Haematoxylin	:	2 gm
Ammonium or potassium alum	:	3 gm
Absolute alcohol	:	100 ml
Distilled water	:	100 ml
Glycerin	:	100 ml

Dissolved haematoxylin in 50 ml alcohol. Separately dissolved alum in distilled water. Mixed both and left exposed to light with a cotton plug for 3-5 days. Filtered and added 100 ml glycerin and 50 ml alcohol. Ripening period was 6-8 weeks or ripened for immediate use with 0.24 gm sodium iodate.

5. **Eosin Yellow stock solution**

Eosin yellow	:	1 gm
70 % Ethyl alcohol	:	1000 ml
Distilled water	:	100 ml
Glacial acetic acid	:	5 ml

6. **Eosin yellow working solution**

Diluted with equal volume of 70 % alcohol. Added 2-3 drops of acetic acid.

7. **DPX Mountant**

Procedure

1. Placed the section in xylene for 2 minutes to remove paraffin.
2. Slides were transferred to 100% ethyl alcohol and left it for one minute.
3. Placed the slide in haematoxylin for about 15-20 minutes. When the sections had absorbed excess stain, dipped those in acid alcohol solution.
4. Transferred the slides to 60 % alcohol and left for 2 minutes.
5. The slides were then transferred to Eosin, which is used as a counter stain, for 2 minutes.
6. The slides were left in 60 % ethyl alcohol for 2 minutes.
7. Then transferred the slides to absolute alcohol and left in it for one minute.
8. Placed the slides in xylene for one minute, dried and then subjected to mounting using DPX mountant.
9. Sections were observed under oil immersion objective and photographed using CXR III Research Microscope.

4.8. Determination of total white blood cell count

The number of white blood cells of each blood sample was counted by manual method and expressed as cells per cubic millimeter of undiluted blood.

Principle

Blood is diluted with acid solution, which removes the red blood cells by hemolysis and accentuates the nuclei of the white blood cells. The counting is done using a microscope under low power objective and with knowledge of the volume of fluid examined and the dilution of the blood obtained. The number of white cells per /mm^3 of undiluted whole blood is calculated.

Requirements

I. Apparatus

1. Microscope
2. Haemocytometer (WBC diluting pipette and counting chamber)
3. Lancet for sterile finger prick
4. Watch glass
5. Cover slip

II. WBC diluting fluid (Turk's fluid)

Composition

1. 1 % glacial acetic acid solution
2. 0.3 % w/v Gentian violet stain
3. Distilled water

Procedure

1. Kept the WBC pipette, watch glass, cover glass, cover slip and Neubauer's chamber as clean and dry.
2. Took enough WBC diluting fluid in a watch glass.
3. Pricked the finger under aseptic conditions and wiped off the first drop of blood. Allowed a good-sized blood drop to form on the finger tip spontaneously without squeezing.
4. Touched the blood drop with the tip of the pipette and sucked blood exactly up to the 0.5 mark.
5. Wiped the tip of pipette and maintained the blood level at the 0.5 mark by holding the pipette in a horizontal position.
6. Sucked WBC diluting fluid exactly up to the 11 mark.
7. Gently mixed the contents of the bulb and kept the pipette on table.
8. Placed the cover slip on platform of Neubauer's chamber.
9. Again mixed the contents of bulb of WBC pipette.

10. Discarded two drops of fluid from the pipette.
11. Touched the tip of pipette with the edge of cover slip.
12. Slowly released fluid from the pipette (fluid moves by capillary action) in such a way that fluid spread just beneath the cover slip and did not spill into the gutters and did not contain air bubbles.
13. Observed the charged Neubauer's chamber under low power objective, and checked for uniform distribution of the cells in the WBC squares.
14. Counted the total number of WBCs in the four corner squares under low power objective.
15. Calculated and recorded the final result.

Calculation

Area of 4 WBC squares = 4 x 1 =4 mm^2

Volume of 4 WBC squares = 4 x 1/10 = 4/10 /mm^3

Dilution factor = 1: 20

Cells in 4/10 /mm^3 volume of diluted blood = n

$\therefore$ Cells in 1 /mm^3 volume of diluted blood = n x 10/4

$\therefore$ Cells in 1 /mm^3 volume of undiluted blood = n x 10/4 x 20 = n x 50

$\therefore$ Cells in 1 ml volume of undiluted blood = n x 50 x 1,000

4.9. Electrophoretic analysis for MPO isozymes

The extracted enzyme was also used for the electrophoretic analysis of the isozymes. The principle behind the procedure is that different isoenzymic forms of myeloperoxidase get separated due to the difference in their charge density and size. For the purpose of separation of isozymes, native polyacrylamide gel electrophoresis was performed in slab gels. Activity of enzyme on the gel was detected by peroxidase staining using benzidine as a substrate. Guaiacol also was tried as a substrate. Native polyacrylamide gel electrophoresis for proteins in the extract was also performed. The procedure was repeated after lyophilizing the lysate sample.

5

Results

A total of 150 individuals were subjected for this study. The study population included patients having diabetes mellitus with complications of infections like ulcer foot syndrome, diabetes mellitus patients without any infection related complications and normal, healthy individuals as controls.

Out of 150 cases, 120 were males and 30 were females, ratio of male to female being 4:1. Based on the age, the study population was categorized as cases below 35 years, between 35 - 55 years and above 55 years. Their distribution under various age groups is presented in Graph:1. Mean age of the total 150 cases was 46.41 ± SE 1.27.

The Control or Normal Category (NC) included 50 normal persons. Number of normal individuals who had age below 35 years (< 35 years) was 38 (76 %), between 35 and 55 years – 9(18 %) and above 55 years (> 55 years) - 3(6 %). Mean age of the normal category was 32.36 ± SE 1.27 and ratio of male and female was 24:1. Their random blood sugar (RBS) level was within the normal range (70- 140 mg/dL). Details are shown in Table-1.

The uncomplicated diabetic cases (UD) were 50 in number. Diabetic patients, not complicated with infections, when categorized under the age groups under study, the distribution was as, < 35 years-3 (6 %), between 35 and 55 years –32(64 %) and > 55 years –15(30 %). Here the mean age of the category was 51.8 ± SE 1.704 and the male and female ratio, 1.7:1. The random sugar level in the blood when analyzed, majority (82 %) had it in

uncontrolled level. The remaining 18% cases had sugar level controlled. Details are presented in Table-2.

The complicated diabetic (CD) category also included 50 cases. Their distribution under the age groups was as < 35 years-3 (6%), between 35 and 55years – 28(56%) and > 55 years –19 (38%). Mean age of the category was 55.42 ± SE 1.649 and the male to female ratio as 9:1. The random sugar level when analyzed, majority (78%) had it above the normal limit while only 22% had sugar within the normal level. No hypoglycemic cases were observed in this group. Detailed data of each case is shown in Table-3.

Graph-1

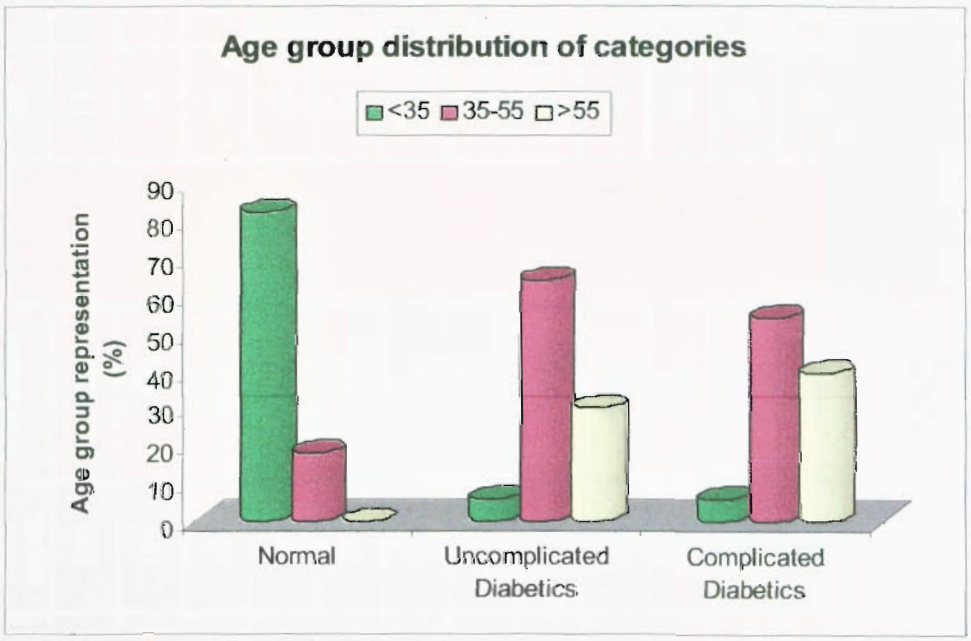


Table – 1. Details of Normal Category (NC)

No	ID	Age	Sex	RBS mg/dL	WBCs/mm^3
1	NC1	24	M	120	7800
2	NC2	32	M	135	8200
3	NC3	35	M	121	6600
4	NC4	78	M	130	7600
5	NC5	40	M	131	5800
6	NC6	33	M	129	4200
7	NC7	72	M	134	3800
8	NC8	35	M	125	11000
9	NC9	35	M	128	9800
10	NC10	50	M	136	9000
11	NC11	38	M	125	5800
12	NC12	23	M	138	6800

No	ID	Age	Sex	RBS mg/dL	WBCs/mm^3
13	NC13	26	M	126	6600
14	NC14	27	M	124	9600
15	NC15	35	M	140	8200
16	NC16	25	M	125	8200
17	NC17	22	M	129	5200
18	NC18	23	M	130	11000
19	NC19	39	M	131	8600
20	NC20	24	M	120	6400
21	NC21	23	M	121	8200
22	NC22	24	M	120	3400
23	NC23	22	M	122	4800
24	NC24	39	M	137	7600

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

No	ID	Age	Sex	RBS mg/dL	WBCs/mm^3
25	NC25	38	M	129	5400
26	NC26	24	M	136	8000
27	NC27	56	M	122	5600
28	NC28	28	M	126	8600
29	NC29	26	M	125	6800
30	NC30	30	M	132	7600
31	NC31	35	M	135	4000
32	NC32	25	M	121	5600
33	NC33	43	M	124	5200
34	NC34	27	M	125	6000
35	NC35	30	M	132	6800
36	NC36	30	M	131	7000
37	NC37	24	M	132	5800
38	NC38	22	M	133	4800

No	ID	Age	Sex	RBS mg/dL	WBCs/mm^3
39	NC39	23	M	125	8200
40	NC40	29	F	129	7400
41	NC41	38	F	137	5000
42	NC42	24	M	123	7400
43	NC43	24	M	140	4000
44	NC44	27	M	138	6400
45	NC45	30	M	134	3400
46	NC46	35	M	132	4000
47	NC47	26	M	139	7200
48	NC48	45	M	140	5200
49	NC49	22	M	129	5000
50	NC50	33	M	133	6800

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

Table – 2. Details of Uncomplicated Diabetic (UD) Category

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dL	WBCs/mm^3
1	UD1	85	F	2	57	5350
2	UD2	49	M	2	180	5150
3	UD3	66	M	2	180	4850
4	UD4	54	M	2	141	5000
5	UD5	65	F	2	127	7400
6	UD6	84	M	2	420	3000
7	UD7	50	F	2	193	4800
8	UD8	48	F	2	150	5400
9	UD9	46	F	2	127	5000
10	UD10	47	M	2	158	8800
11	UD11	42	M	2	142	4800
12	UD12	60	F	2	140	3200
13	UD13	50	F	2	298	6200
14	UD14	76	M	2	139	5000
15	UD15	68	M	2	230	5000
16	UD16	47	M	2	226	2600
17	UD17	42	M	2	120	5600
18	UD18	29	M	1	308	4800
19	UD19	63	F	2	149	10800
20	UD20	52	M	2	308	6600
21	UD21	50	F	2	116	4600
22	UD22	40	M	2	254	6200
23	UD23	48	F	2	152	7000
24	UD24	39	F	2	189	9800

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dL	WBCs/mm^3
25	UD25	55	F	2	180	4000
26	UD26	54	F	2	170	6400
27	UD27	65	F	2	282	5800
28	UD28	34	M	2	142	3800
29	UD29	56	M	2	250	4200
30	UD30	67	F	2	138	6600
31	UD31	60	M	2	242	7600
32	UD32	43	M	2	265	4800
33	UD33	55	M	2	300	2400
34	UD34	39	F	2	302	4600
35	UD35	50	F	1	229	4800
36	UD36	57	F	2	204	6400
37	UD37	56	F	2	260	5800

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dL	WBCs/mm^3
38	UD38	51	M	2	153	7000
39	UD39	51	F	2	296	6400
40	UD40	47	M	2	223	2800
41	UD41	43	M	2	128	5400
42	UD42	30	M	1	308	5000
43	UD43	40	M	2	254	6400
44	UD44	46	F	2	154	7000
45	UD45	38	F	2	189	9600
46	UD46	50	M	2	182	5000
47	UD47	66	M	2	180	4850
48	UD48	49	M	2	156	9000
49	UD49	41	M	2	143	5000
50	UD50	47	F	2	170	5000

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

Table – 3. Details of Complicated Diabetic (CD) Category

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dL	WBCs/mm^3
1	CD1	49	M	2	117	20800
2	CD2	48	M	2	204	21800
3	CD3	46	M	2	130	10600
4	CD4	52	M	2	130	6200
5	CD5	53	M	2	193	6600
6	CD6	27	M	1	399	12200
7	CD7	65	M	2	310	5000
8	CD8	55	F	2	204	8000
9	CD9	71	F	2	200	6400
10	CD10	50	F	2	103	5600
11	CD11	50	M	2	164	5200
12	CD12	60	M	2	490	11600

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dL	WBCs/mm^3
13	CD13	68	M	2	216	20200
14	CD14	57	M	2	389	9400
15	CD15	55	M	2	339	5600
16	CD16	58	M	2	180	7600
17	CD17	74	M	2	204	9400
18	CD18	50	M	2	174	14600
19	CD19	49	M	2	190	10600
20	CD20	50	M	2	244	10000
21	CD21	50	M	2	130	5600
22	CD22	62	M	2	202	10000
23	CD23	50	M	2	137	6000
24	CD24	53	M	2	210	6200

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dl	WBCs/mm^3
25	CD25	75	M	2	114	6000
26	CD26	34	M	2	317	7400
27	CD27	48	M	2	211	9400
28	CD28	54	M	2	221	10000
29	CD29	52	M	2	168	5600
30	CD30	48	M	2	140	5400
31	CD31	53	M	2	172	4000
32	CD32	55	M	2	141	10400
33	CD33	60	M	2	239	10000
34	CD34	30	F	2	147	10000
35	CD35	38	F	2	126	11400
36	CD36	50	M	2	480	7600
37	CD37	55	M	2	140	9400

No	ID	Age	Sex	Diabetes Type 1/2	RBS mg/dl	WBCs/mm^3
38	CD38	78	M	2	212	16000
39	CD39	48	M	2	146	21400
40	CD40	63	M	2	103	26400
41	CD41	45	M	2	212	14600
42	CD42	74	M	2	202	27400
43	CD43	76	M	2	127	5000
44	CD44	65	M	2	451	12200
45	CD45	64	M	2	287	6800
46	CD46	60	M	2	205	4000
47	CD47	73	M	2	395	10000
48	CD48	60	M	2	200	6400
49	CD49	48	M	2	214	14600
50	CD50	55	M	2	142	9400

ID- Case No., M- Male, F-female, RBS- Random blood sugar, WBC-White blood cells

Table – 4. Unit MPO in Leukocytes/ml blood of Normal Category (NC)

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
1	NC1	7800	4.1032×10^{-2}
2	NC2	8200	2.3241×10^{-2}
3	NC3	6600	3.9592×10^{-2}
4	NC4	7600	4.2472×10^{-2}
5	NC5	5800	3.2188×10^{-2}
6	NC6	4200	1.5837×10^{-2}
7	NC7	3800	2.0670×10^{-2}
8	NC8	11000	7.0032×10^{-2}
9	NC9	9800	6.5610×10^{-2}
10	NC10	9000	8.3709×10^{-2}
11	NC11	5800	2.3138×10^{-2}
12	NC12	6800	6.2730×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
13	NC13	6600	5.0390×10^{-2}
14	NC14	9600	4.9567×10^{-2}
15	NC15	8200	3.1777×10^{-2}
16	NC16	8200	4.3294×10^{-2}
17	NC17	5200	4.8539×10^{-2}
18	NC18	11000	2.0547E-01
19	NC19	8600	5.0184×10^{-2}
20	NC20	6400	4.2472×10^{-2}
21	NC21	8200	4.1443×10^{-2}
22	NC22	3400	1.9950×10^{-2}
23	NC23	4800	3.8770×10^{-2}
24	NC24	7600	4.3191×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
25	NC25	5400	2.9926×10^{-2}
26	NC26	8000	4.3706×10^{-2}
27	NC27	5600	3.3936×10^{-2}
28	NC28	8600	2.1102×10^{-2}
29	NC29	6800	3.4142×10^{-2}
30	NC30	7600	1.0551×10^{-1}
31	NC31	4000	2.5606×10^{-2}
32	NC32	5600	2.7149×10^{-2}
33	NC33	5200	2.6121×10^{-2}
34	NC34	6000	2.7869×10^{-2}
35	NC35	6800	5.8514×10^{-2}
36	NC36	7000	7.2294×10^{-2}
37	NC37	5800	3.7947×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
38	NC38	4800	1.0329×10^{-1}
39	NC39	8200	6.4067×10^{-2}
40	NC40	7400	2.8897×10^{-2}
41	NC41	5000	3.1365×10^{-2}
42	NC42	7400	1.5055×10^{-2}
43	NC43	4000	1.1004×10^{-2}
44	NC44	6400	2.5812×10^{-2}
45	NC45	3400	2.5092×10^{-2}
46	NC46	4000	1.5837×10^{-2}
47	NC47	7200	3.6610×10^{-2}
48	NC48	5200	4.1649×10^{-2}
49	NC49	5000	4.0929×10^{-2}
50	NC50	6800	1.9333×10^{-2}

Table – 5. Unit MPO in Leukocytes/ml blood of Uncomplicated Diabetic (UD) Category

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
1	UD1	5350	1.5426×10^{-4}
2	UD2	5150	9.3787×10^{-3}
3	UD3	4850	1.5220×10^{-2}
4	UD4	5000	1.7482×10^{-2}
5	UD5	7400	1.0078×10^{-2}
6	UD6	3000	9.0496×10^{-3}
7	UD7	4800	9.8723×10^{-3}
8	UD8	5400	1.7996×10^{-2}
9	UD9	5000	1.2855×10^{-2}
10	UD10	8800	9.1319×10^{-3}
11	UD11	4800	1.0489×10^{-2}
12	UD12	3200	7.2500×10^{-3}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
13	UD13	6200	9.8723×10^{-3}
14	UD14	5000	5.3475×10^{-3}
15	UD15	5000	4.0106×10^{-3}
16	UD16	2600	1.2340×10^{-3}
17	UD17	5600	9.1525×10^{-3}
18	UD18	4800	6.5816×10^{-3}
19	UD19	10800	3.2805×10^{-2}
20	UD20	6600	1.2546×10^{-2}
21	UD21	4600	8.4326×10^{-3}
22	UD22	6200	4.7099×10^{-2}
23	UD23	7000	2.8280×10^{-2}
24	UD24	9800	3.0851×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
25	UD25	4000	1.5323×10^{-2}
26	UD26	6400	8.5355×10^{-3}
27	UD27	5800	3.0028×10^{-2}
28	UD28	3800	8.2270×10^{-3}
29	UD29	4200	3.3422×10^{-2}
30	UD30	6600	2.3858×10^{-2}
31	UD31	7600	3.0234×10^{-2}
32	UD32	4800	3.1160×10^{-2}
33	UD33	2400	1.5220×10^{-2}
34	UD34	4600	1.0181×10^{-2}
35	UD35	4800	1.6505×10^{-2}
36	UD36	6400	2.7046×10^{-2}
37	UD37	5800	1.8202×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes /ml blood
38	UD38	7000	2.9926×10^{-2}
39	UD39	6400	9.7695×10^{-3}
40	UD40	2800	1.4397×10^{-3}
41	UD41	5400	9.2553×10^{-3}
42	UD42	5000	6.5816×10^{-3}
43	UD43	6400	4.6894×10^{-2}
44	UD44	7000	2.7252×10^{-2}
45	UD45	9600	3.0851×10^{-2}
46	UD46	5000	9.5021×10^{-3}
47	UD47	4850	1.5323×10^{-2}
48	UD48	9000	9.2553×10^{-3}
49	UD49	5000	1.0695×10^{-2}
50	UD50	5000	1.7996×10^{-2}

Table - 6. Unit MPO in Leukocytes /ml Blood of Complicated Diabetic (CD) Category

No	ID	WBCs/mm^3	Unit MPO in leukocytes/ml blood
1	CD1	20800	1.015×10^{-1}
2	CD2	21800	7.898×10^{-2}
3	CD3	10600	6.942×10^{-2}
4	CD4	6200	2.520×10^{-2}
5	CD5	6600	3.733×10^{-2}
6	CD6	12200	5.450×10^{-2}
7	CD7	5000	4.329×10^{-2}
8	CD8	8000	2.879×10^{-2}
9	CD9	6400	6.067×10^{-2}
10	CD10	5600	2.869×10^{-2}
11	CD11	5200	1.944×10^{-2}
12	CD12	11600	7.199×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes/ml blood
13	CD13	20200	8.947×10^{-2}
14	CD14	9400	2.458×10^{-2}
15	CD15	5600	3.620×10^{-2}
16	CD16	7600	2.540×10^{-2}
17	CD17	9400	4.216×10^{-2}
18	CD18	14600	3.178×10^{-2}
19	CD19	10600	9.050×10^{-3}
20	CD20	10000	6.129×10^{-2}
21	CD21	5600	2.509×10^{-2}
22	CD22	10000	2.005×10^{-2}
23	CD23	6000	2.458×10^{-2}
24	CD24	6200	5.903×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes/ml blood
25	CD25	6000	3.167×10^{-2}
26	CD26	7400	6.674×10^{-2}
27	CD27	9400	3.743×10^{-2}
28	CD28	10000	3.085×10^{-2}
29	CD29	5600	2.838×10^{-2}
30	CD30	5400	4.473×10^{-2}
31	CD31	4000	4.072×10^{-2}
32	CD32	10400	8.741×10^{-3}
33	CD33	10000	6.263×10^{-2}
34	CD34	10000	3.065×10^{-2}
35	CD35	11400	1.584×10^{-2}
36	CD36	7600	4.648×10^{-2}
37	CD37	9400	5.471×10^{-2}

No	ID	WBCs/mm^3	Unit MPO in leukocytes/ml blood
38	CD38	16000	5.296×10^{-2}
39	CD39	21400	3.517×10^{-2}
40	CD40	26400	1.381×10^{-1}
41	CD41	14600	5.193×10^{-2}
42	CD42	27400	8.062×10^{-2}
43	CD43	5000	3.743×10^{-2}
44	CD44	12200	2.160×10^{-2}
45	CD45	6800	5.039×10^{-3}
46	CD46	4000	8.021×10^{-3}
47	CD47	10000	5.070×10^{-2}
48	CD48	6400	1.861×10^{-2}
49	CD49	14600	5.209×10^{-2}
50	CD50	9400	5.468×10^{-2}

5.1. Estimation of cellular myeloperoxidase enzyme per ml of blood

The level of myeloperoxidase enzyme in the polymorphoneutrophils was estimated by extracting the enzyme from isolated cells. After separation, the WBC fraction was found to be containing 90 % of neutrophils. The unit MPO in leukocytes of one milliliter of blood was taken as an index of the overall MPO level in the body of an individual.

The estimation was done in 3 ml of blood collected from each individual. The MPO estimated was calculated to leukocytes of one ml blood in all the samples and the result is given in Tables - 4,5 and 6.

The unit myeloperoxidase in leukocytes of 1 ml blood was estimated in 50 controls (Table-4). The level was varying slightly in each case and ranged from 1.93×10^{-2} to 2.055×10^{-1},excepting very few extreme values that fell below this range. The mean MPO activity of the normal cases was $4.376 \times 10^{-2} \pm$ SE 4.415×10^{-3} in this study.

Table-5 shows the unit MPO in leukocytes of 1 ml blood of 50 uncomplicated diabetes cases in detail. The unit MPO/ml blood in uncomplicated cases ranged from 1.543×10^{-4} to 4.71×10^{-2}. The mean unit leukocytic MPO/ml blood of this category was $1.636 \times 10^{-2} \pm$ SE 1.589×10^{-3}.

The unit MPO in leukocytes of 1 ml blood of 50 complicated diabetes cases is shown in the Table-6. In complicated diabetic cases also, the unit activity differed from individual to individual and ranged from 5.039×10^{-3} to 1.381×10^{-1}. The mean activity of this category was $4.309 \times 10^{-2} \pm$ SE 3.773×10^{-3}

Table 7 shows the mean unit MPO in the leukocytes of one ml blood of all the 3 categories. Graph –2 is a representation of the comparative MPO in leukocytes per ml of blood level of the 3 categories. (Error bars show 95 % confidence interval of mean) The mean difference between categories in relation to each other and their p-values are shown in the Table-8.

Table-7 Mean MPO in leukocytes/ml blood

Category	Mean	Std. Error
Normal	4.376×10^{-2}	4.415×10^{-3}
Uncomplicated diabetics	1.636×10^{-2}	1.589×10^{-3}
Complicated diabetics	4.309×10^{-2}	3.773×10^{-3}

Graph- 2. MPO in leukocytes/ml blood-Interactive graphical representation

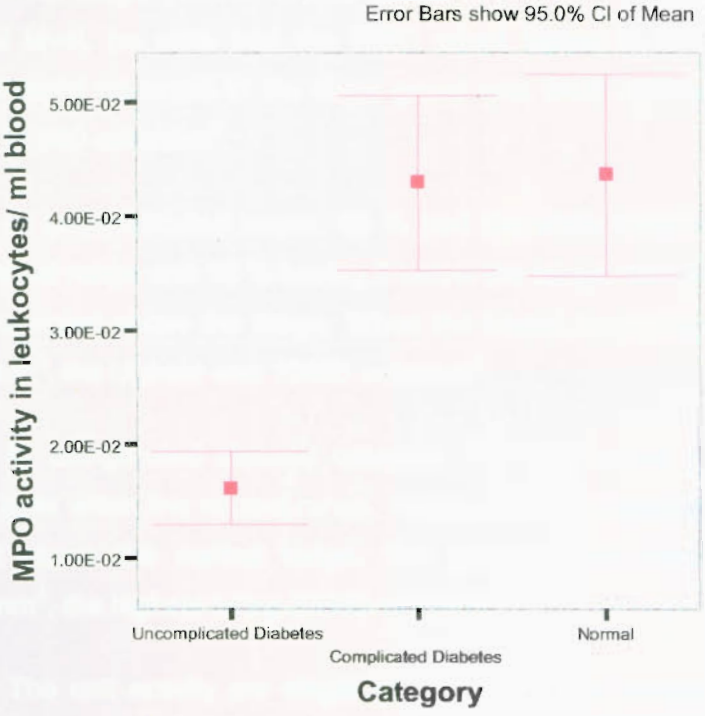


Table-8 ANOVA-MPO activity in leukocytes/ml blood

Category	Category	Mean difference	Std. Error	p-value
Normal	Uncomplicated diabetics	2.740×10^{-2}*	4.87×10^{-3}	0.001
	Complicated diabetics	6.726×10^{-4}	4.92×10^{-3}	0.892
Complicated diabetics	Uncomplicated diabetics	2.673×10^{-2}*	4.92×10^{-3}	0.001

* denotes high significance.

5.2. Enumeration of total white blood cells

Since the unit MPO activity of leukocytes per ml of blood was observed as different for each individual, it became necessary to consider the WBC count in each blood sample, to come at the MPO activity at single leukocyte level and also to study the MPO activity in relation to the varied WBC count/status observed in the study categories. The count expressed per mm^3 is given in Tables-1, 2 and 3.

Majority of normal cases (94 %) had their white blood cell count within the normal range (4,000- 11,000 cells/mm^3). When 6 % had it below the normal level, no case had the WBC count higher to normal range. The lowest count observed in the study group was 3400 cells/mm^3, the highest being 11,000 cells/mm^3.

In uncomplicated cases, 88 % had the white blood cell count within the normal range and 12 % having it below the range. No case was obtained with the count above the range. When the lowest count observed was 2400 cells/mm^3, the highest was 10800 cells/mm^3.

In complicated diabetic category, 72 % of cases possessed white blood cells within the normal range. No one had the count below, while 28 % had it above the normal range. The lowest count observed was 4000 cells/mm^3, the highest being 27400 cells/mm^3.

The unit activity per single cell (leukocyte) in normal, uncomplicated and complicated diabetics was calculated and it is shown in Tables-9, 10 and 11 respectively. It could be calculated from total WBC count present in one ml of blood and the unit MPO activity per ml of blood in each case.

Table – 9. Unit MPO/Cell of Normal Category (NC)

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
1	NC1	7.8×10^{6}	5.2605×10^{-9}
2	NC2	8.2×10^{6}	2.8343×10^{-9}
3	NC3	6.6×10^{6}	5.9988×10^{-9}
4	NC4	7.6×10^{6}	5.5884×10^{-9}
5	NC5	5.8×10^{6}	5.5496×10^{-9}
6	NC6	4.2×10^{6}	3.7707×10^{-9}
7	NC7	3.8×10^{6}	5.4395×10^{-9}
8	NC8	1.1×10^{7}	6.3665×10^{-9}
9	NC9	9.8×10^{6}	6.6949×10^{-9}
10	NC10	9.0×10^{6}	9.3010×10^{-9}
11	NC11	5.8×10^{6}	3.9894×10^{-9}
12	NC12	6.8×10^{6}	9.2251×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
13	NC13	6.6×10^{6}	7.6349×10^{-9}
14	NC14	9.6×10^{6}	5.1633×10^{-9}
15	NC15	8.2×10^{6}	3.8752×10^{-9}
16	NC16	8.2×10^{6}	5.2798×10^{-9}
17	NC17	5.2×10^{6}	9.3344×10^{-9}
18	NC18	1.1×10^{7}	1.7122×10^{-8}
19	NC19	8.6×10^{6}	5.8354×10^{-9}
20	NC20	6.4×10^{6}	6.6362×10^{-9}
21	NC21	8.2×10^{6}	5.0541×10^{-9}
22	NC22	3.4×10^{6}	5.8678×10^{-9}
23	NC23	4.8×10^{6}	8.0770×10^{-9}
24	NC24	7.6×10^{6}	5.6831×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
25	NC25	5.4×10^{6}	5.5418×10^{-9}
26	NC26	8.0×10^{6}	5.4632×10^{-9}
27	NC27	5.6×10^{6}	6.0600×10^{-9}
28	NC28	8.6×10^{6}	2.4537×10^{-9}
29	NC29	6.8×10^{6}	5.0209×10^{-9}
30	NC30	7.6×10^{6}	1.3883×10^{-8}
31	NC31	4.0×10^{6}	6.4016×10^{-9}
32	NC32	5.6×10^{6}	4.8480×10^{-9}
33	NC33	5.2×10^{6}	5.0232×10^{-9}
34	NC34	6.0×10^{6}	4.6448×10^{-9}
35	NC35	6.8×10^{6}	8.6050×10^{-9}
36	NC36	7.0×10^{6}	1.0328×10^{-8}
37	NC37	5.8×10^{6}	6.5426×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
38	NC38	4.8×10^{6}	2.1519×10^{-8}
39	NC39	8.2×10^{6}	7.8131×10^{-9}
40	NC40	7.4×10^{6}	3.9050×10^{-9}
41	NC41	5.0×10^{6}	6.2730×10^{-9}
42	NC42	7.4×10^{6}	2.0345×10^{-9}
43	NC43	4.0×10^{6}	2.7509×10^{-9}
44	NC44	6.4×10^{6}	4.0331×10^{-9}
45	NC45	3.4×10^{6}	7.3801×10^{-9}
46	NC46	4.0×10^{6}	3.9592×10^{-9}
47	NC47	7.2×10^{6}	5.0847×10^{-9}
48	NC48	5.2×10^{6}	8.0094×10^{-9}
49	NC49	5.0×10^{6}	8.1858×10^{-9}
50	NC50	6.8×10^{6}	2.8431×10^{-9}

Table – 10. Unit MPO /Cell of Uncomplicated Diabetic (UD) Category

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
1	UD1	5.35×10^{6}	2.8833×10^{-11}
2	UD2	5.15×10^{6}	1.8211×10^{-9}
3	UD3	4.85×10^{6}	3.1381×10^{-9}
4	UD4	5.0×10^{6}	3.4965×10^{-9}
5	UD5	7.4×10^{6}	1.3619×10^{-9}
6	UD6	3.0×10^{6}	3.0165×10^{-9}
7	UD7	4.8×10^{6}	2.0567×10^{-9}
8	UD8	5.4×10^{6}	3.3327×10^{-9}
9	UD9	5.0×10^{6}	2.5709×10^{-9}
10	UD10	8.8×10^{6}	1.0377×10^{-9}
11	UD11	4.8×10^{6}	2.1853×10^{-9}
12	UD12	3.2×10^{6}	2.2656×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
13	UD13	6.2×10^{6}	1.5923×10^{-9}
14	UD14	5.0×10^{6}	1.0695×10^{-9}
15	UD15	5.0×10^{6}	8.0213×10^{-10}
16	UD16	2.6×10^{6}	4.7463×10^{-10}
17	UD17	5.6×10^{6}	1.6344×10^{-9}
18	UD18	4.8×10^{6}	1.3712×10^{-9}
19	UD19	1.08×10^{7}	3.0375×10^{-9}
20	UD20	6.6×10^{6}	1.9009×10^{-9}
21	UD21	4.6×10^{6}	1.8332×10^{-9}
22	UD22	6.2×10^{6}	7.5967×10^{-9}
23	UD23	7.0×10^{6}	4.0400×10^{-9}
24	UD24	9.8×10^{6}	3.1481×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
25	UD25	4.0×10^{6}	3.8307×10^{-9}
26	UD26	6.4×10^{6}	1.3337×10^{-9}
27	UD27	5.8×10^{6}	5.1773×10^{-9}
28	UD28	3.8×10^{6}	2.1650×10^{-9}
29	UD29	4.2×10^{6}	7.9576×10^{-9}
30	UD30	6.6×10^{6}	3.6149×10^{-9}
31	UD31	7.6×10^{6}	3.9782×10^{-9}
32	UD32	4.8×10^{6}	6.4916×10^{-9}
33	UD33	2.4×10^{6}	6.3416×10^{-9}
34	UD34	4.6×10^{6}	2.2132×10^{-9}
35	UD35	4.8×10^{6}	3.4386×10^{-9}
36	UD36	6.4×10^{6}	4.2260×10^{-9}
37	UD37	5.8×10^{6}	3.1383×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
38	UD38	7.0×10^{6}	4.2751×10^{-9}
39	UD39	6.4×10^{6}	1.5265×10^{-9}
40	UD40	2.8×10^{6}	5.1418×10^{-10}
41	UD41	5.4×10^{6}	1.7139×10^{-9}
42	UD42	5.0×10^{6}	1.3163×10^{-9}
43	UD43	6.4×10^{6}	7.3271×10^{-9}
44	UD44	7.0×10^{6}	3.8931×10^{-9}
45	UD45	9.6×10^{6}	3.2137×10^{-9}
46	UD46	5.0×10^{6}	1.9004×10^{-9}
47	UD47	4.85×10^{6}	3.1593×10^{-9}
48	UD48	9.0×10^{6}	1.0284×10^{-9}
49	UD49	5.0×10^{6}	2.1390×10^{-9}
50	UD50	5.0×10^{6}	3.5993×10^{-9}

Table – 11. Unit MPO/Cell of Complicated Diabetic (CD) Category

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
1	CD1	2.08×10^{7}	4.880×10^{-9}
2	CD2	2.18×10^{7}	3.623×10^{-9}
3	CD3	1.06×10^{7}	6.549×10^{-9}
4	CD4	6.2×10^{6}	4.064×10^{-9}
5	CD5	6.6×10^{6}	5.656×10^{-9}
6	CD6	1.22×10^{7}	4.468×10^{-9}
7	CD7	5.0×10^{6}	8.659×10^{-9}
8	CD8	8.0×10^{6}	3.599×10^{-9}
9	CD9	6.4×10^{6}	9.480×10^{-9}
10	CD10	5.6×10^{6}	5.124×10^{-9}
11	CD11	5.2×10^{6}	3.738×10^{-9}
12	CD12	1.16×10^{7}	6.206×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
13	CD13	2.02×10^{7}	4.429×10^{-9}
14	CD14	9.4×10^{6}	2.615×10^{-9}
15	CD15	5.6×10^{6}	6.464×10^{-9}
16	CD16	7.6×10^{6}	3.342×10^{-9}
17	CD17	9.4×10^{6}	4.485×10^{-9}
18	CD18	1.46×10^{7}	2.177×10^{-9}
19	CD19	1.06×10^{7}	8.537×10^{-10}
20	CD20	1.0×10^{7}	6.129×10^{-9}
21	CD21	5.6×10^{6}	4.481×10^{-9}
22	CD22	1.0×10^{7}	2.005×10^{-9}
23	CD23	6.0×10^{6}	4.096×10^{-9}
24	CD24	6.2×10^{6}	9.521×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
25	CD25	6.0×10^{6}	5.279×10^{-9}
26	CD26	7.4×10^{6}	9.019×10^{-9}
27	CD27	9.4×10^{6}	$3.982.x\ 10^{-9}$
28	CD28	1.0×10^{7}	3.085×10^{-9}
29	CD29	5.6×10^{6}	5.068×10^{-9}
30	CD30	5.4×10^{6}	8.284×10^{-9}
31	CD31	4.0×10^{6}	1.018×10^{-8}
32	CD32	1.04×10^{7}	8.405×10^{-10}
33	CD33	1.0×10^{7}	6.263×10^{-9}
34	CD34	1.0×10^{7}	3.065×10^{-9}
35	CD35	1.14×10^{7}	1.389×10^{-9}
36	CD36	7.6×10^{6}	6.116×10^{-9}
37	CD37	9.4×10^{6}	5.820×10^{-9}

No	ID	Total WBC in 1 ml blood	Unit MPO/cell
38	CD38	1.6×10^{7}	3.310×10^{-9}
39	CD39	2.14×10^{7}	1.644×10^{-9}
40	CD40	2.64×10^{7}	5.231×10^{-9}
41	CD41	1.46×10^{7}	3.557×10^{-9}
42	CD42	2.74×10^{7}	2.943×10^{-9}
43	CD43	5.0×10^{6}	7.487×10^{-9}
44	CD44	1.22×10^{7}	1.770×10^{-9}
45	CD45	6.8×10^{6}	7.410×10^{-10}
46	CD46	4.0×10^{6}	2.674×10^{-9}
47	CD47	1.0×10^{7}	5.070×10^{-9}
48	CD48	6.4×10^{6}	2.908×10^{-9}
49	CD49	1.46×10^{7}	3.525×10^{-9}
50	CD50	9.4×10^{6}	4.468×10^{-9}

5.3. Estimation of myeloperoxidase per single leukocyte

The normal cases had the unit MPO activity per single cell in a range of 3.771×10^{-9} to 2.152×10^{-8} (Table-9). The mean unit MPO activity /cell of this category was $6.484 \times 10^{-9} \pm$ SE 4.908×10^{-10}.

The unit MPO activity per single leukocyte of uncomplicated diabetic cases was calculated for each case and shown in the Table-10. In this category, the unit activity per cell ranged from 2.883×10^{-11} to 7.958×10^{-9}. The mean unit MPO/cell of this category was $2.887 \times 10^{-9} \pm$ SE 2.601×10^{-10}

In complicated diabetic cases, the unit activity per cell was found to be ranging between 7.41×10^{-10} and 1.018×10^{-8} (Table -11). Here, the mean unit MPO activity/cell was $4.633 \times 10^{-9} \pm$ SE 3.442×10^{-10}.

The mean unit MPO activity in a single leukocyte of the three categories are presented in Table-12. Graph –3 is a representation of the comparative MPO in a single leukocyte level of the 3 categories. (Error bars show 95 % confidence interval of mean). The mean differences between the categories in relation to each other and their p-values are shown in Table-13.

Table: 12 Mean MPO in single leukocyte

Category	Mean	Std.Error
Normal	6.484×10^{-9}	4.907×10^{-10}
Uncomplicated diabetics	2.887×10^{-9}	2.601×10^{-10}
Complicated diabetics	4.633×10^{-9}	3.441×10^{-10}

Graph-3 MPO in single leukocyte -Interactive graphical representation

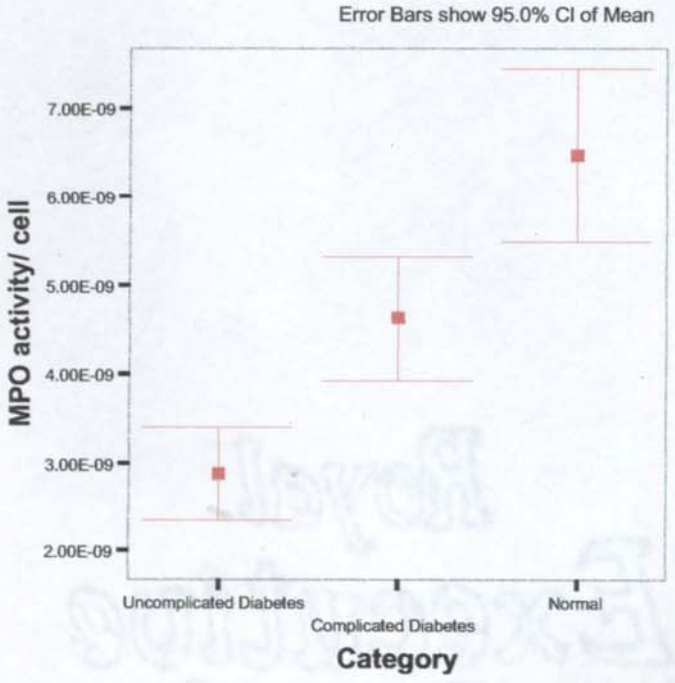


Table: 13 ANOVA- MPO activity in single leukocyte

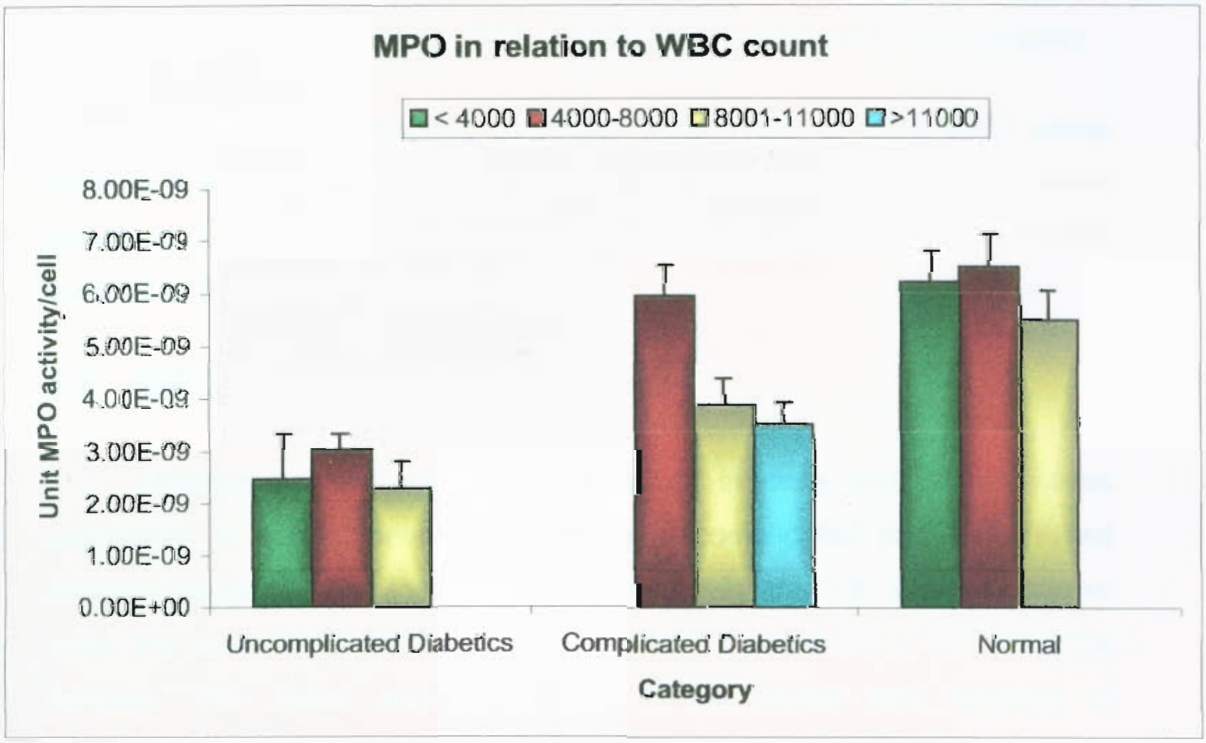
Category	Category	Mean difference	Std. Error	p-value
Normal	Uncomplicated diabetics	3.597×10^{-9} *	5.312×10^{-10}	0.001
	Complicated diabetics	1.851×10^{-9} *	5.367×10^{-10}	0.001
Complicated diabetics	Uncomplicated diabetics	1.746×10^{-9} *	5.367×10^{-10}	0.001

* denotes high significance.

5.4. MPO activity in relation to White blood cell count

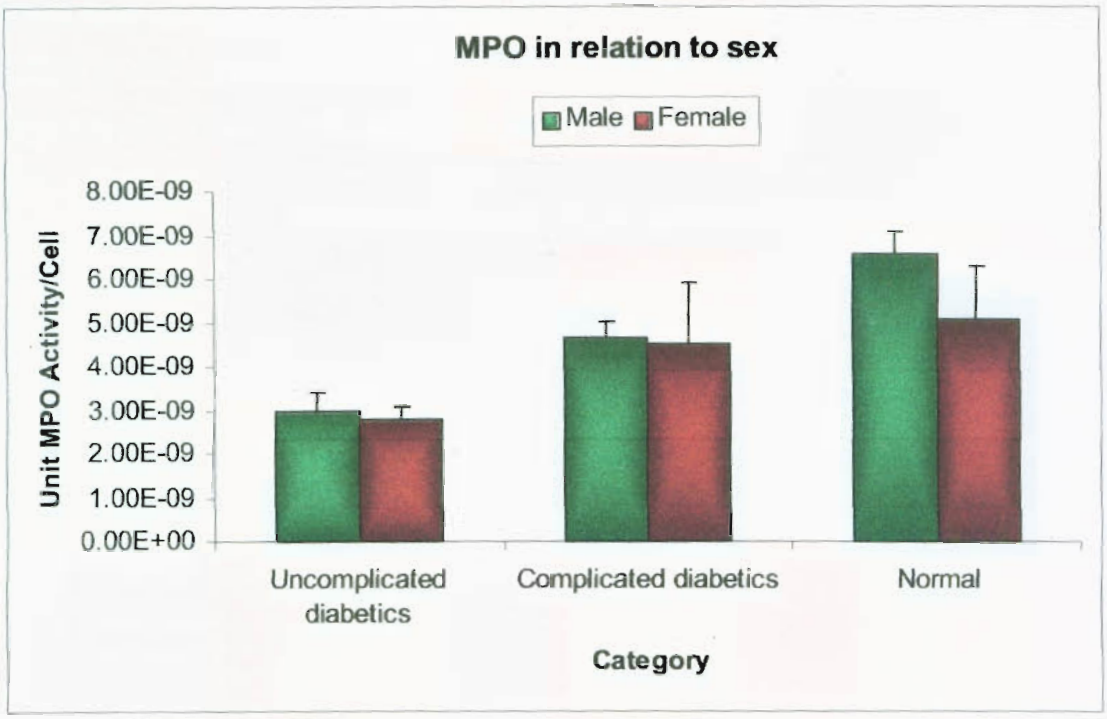
To study the MPO activity in relation to the WBC status, the unit myeloperoxidase activity per single cell was considered in different groups having WBC count/mm^3 below normal range (<4,000), normal range (4,000-8,000 and 8001-11,000) and above normal range (>11,000). It is presented in graph-4.

Graph-4



Category		MPO activity in per cell level			
		WBC Groups			
			Normal range		
		< 4000	4000-8000	8001-11000	>11000
Normal	Mean	6.229×10^{-9}	6.536×10^{-9}	5.511×10^{-9}	
	SE	5.885×10^{-10}	6.131×10^{-10}	5.605×10^{-10}	
Uncomplicated diabetics	Mean	2.463×10^{-9}	3.027×10^{-9}	2.293×10^{-9}	
	SE	8.799×10^{-10}	3×10^{-10}	5.151×10^{-10}	
Complicated diabetics	Mean		5.985×10^{-9}	3.882×10^{-9}	3.509×10^{-9}
	SE		5.785×10^{-10}	5.151×10^{-10}	4.162×10^{-10}

Graph-5

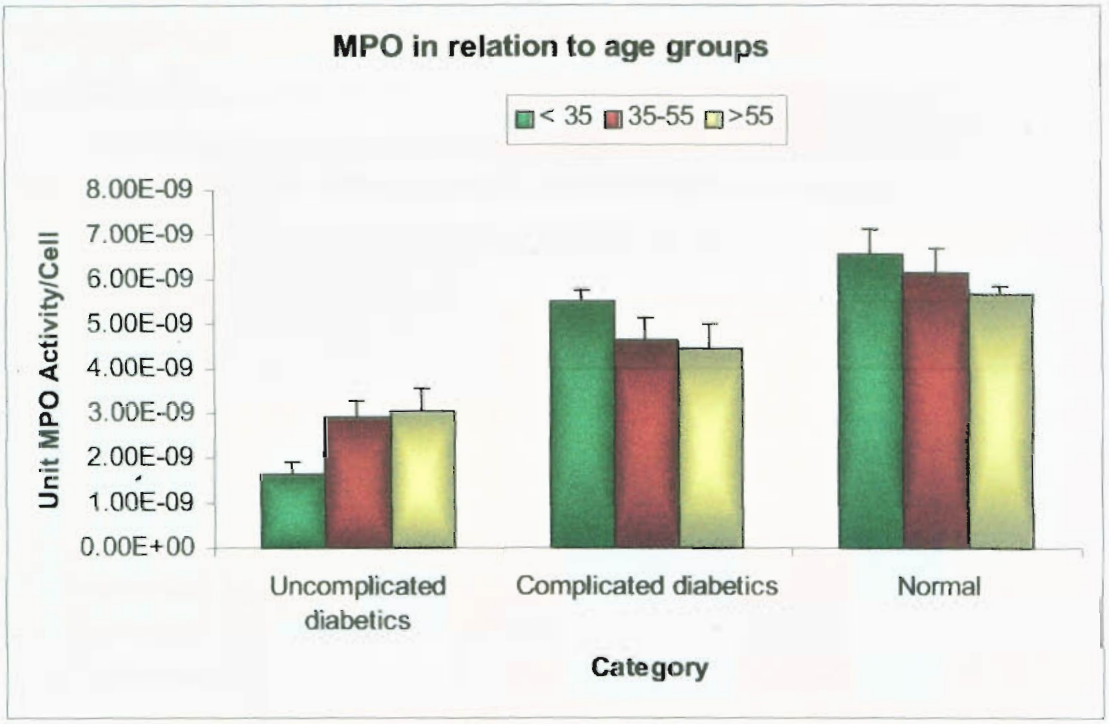


Category		Unit activity per cell	
		Sex	
		Male	Female
Normal	Mean	6.542×10^{-9}	5.089×10^{-9}
	SE	5.084×10^{-10}	1.184×10^{-9}
Uncomplicated diabetics	Mean	2.957×10^{-9}	2.803×10^{-9}
	SE	4.375×10^{-10}	2.478×10^{-10}
Complicated diabetics	Mean	4.644×10^{-9}	4.531×10^{-9}
	SE	3.564×10^{-10}	1.373×10^{-9}

5.6. MPO activity in different age groups

The Unit MPO activity per single cell was analyzed in different age groups (> 35 years, 35-55 years and >55 years) of all the 3 study categories (Graph-6). In control category, group of >35 years showed greater MPO activity (6.561×10^{-9}) compared to the 35-55 years (6.134×10^{-9}) and the >55 years group (5.695×10^{-9}). In uncomplicated diabetic category, while >35 years group showed least activity (1.617×10^{-9}), higher unit MPO/cell was observed in 35-55 years age group (2.922×10^{-9}) and > 55 years age group (3.065×10^{-9}). In complicated diabetics, as in normal people, cases under >35 years showed greater activity (5.517×10^{-9}) than 35-55 age group (4.651×10^{-9}) and >55 years age group (4.468×10^{-9}).

Graph-6

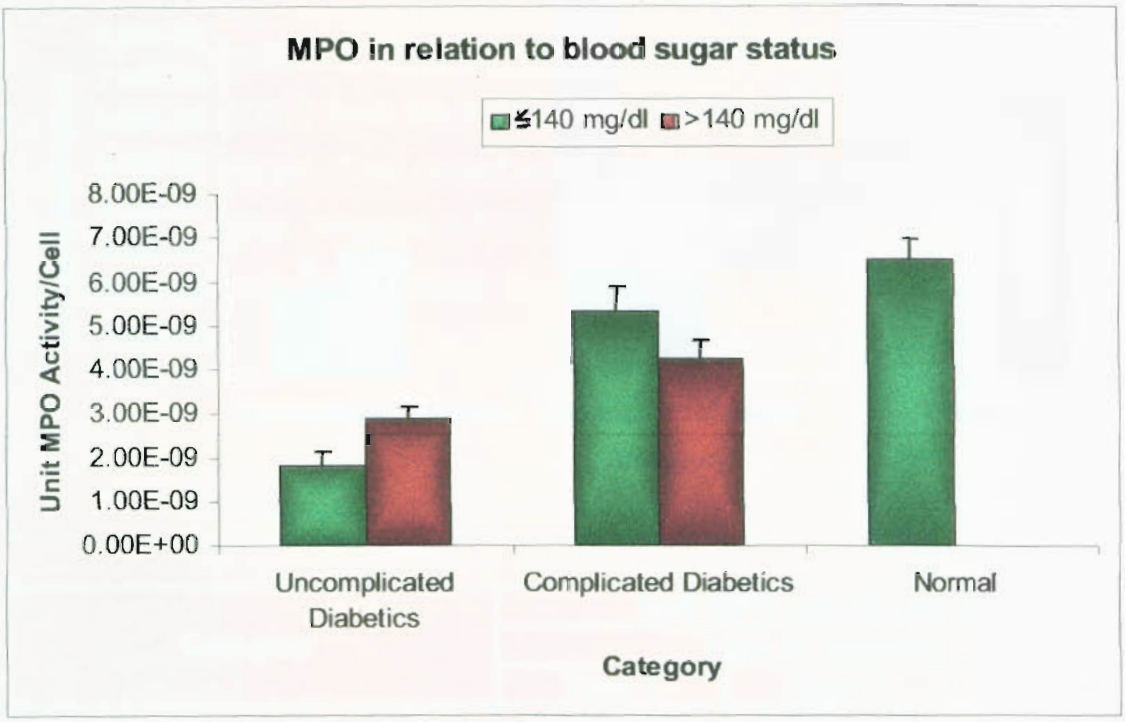


Category		MPO activity in per cell level		
		Age Group		
		< 35	35-55	>55
Normal	Mean	6.561×10^{-9}	6.134×10^{-9}	5.696×10^{-9}
	SE	5.887×10^{-10}	5.321×10^{-10}	1.871×10^{-10}
Uncomplicated diabetics	Mean	1.617×10^{-9}	2.922×10^{-9}	3.065×10^{-9}
	SE	2.742×10^{-10}	3.287×10^{-10}	5.013×10^{-10}
Complicated diabetics	Mean	5.517×10^{-9}	4.651×10^{-9}	4.468×10^{-9}
	SE	1.797×10^{-9}	4.708×10^{-10}	5.432×10^{-10}

5.7. MPO activity in relation to the blood sugar status

The individuals were grouped based on their random blood sugar, into controlled (≤140mg/dL) and uncontrolled (>140mg/dL) sugar groups, according to the guidelines for glycemic control published in the American College of Endocrinology Consensus Statement. The consensus panel recommends a treatment-targeted blood glucose level of <140 mg/dL.[124] The unit MPO activity per cell was analyzed for both these sugar groups of each study category. The normal people who naturally came under the controlled (≤140mg/dL) sugar group, showed greatest MPO activity (6.484×10^{-9}). The sugar controlled uncomplicated diabetics had their mean MPO activity at a

lower level of 1.788 x 10^{-9} while the uncontrolled group showed a higher MPO rate of 2.886 x 10^{-9}. In complicated diabetics it was 5.329 x 10^{-9} and 4.256 x 10^{-9} unit activity in the controlled and uncontrolled sugar groups respectively. Data is presented in graph-7.

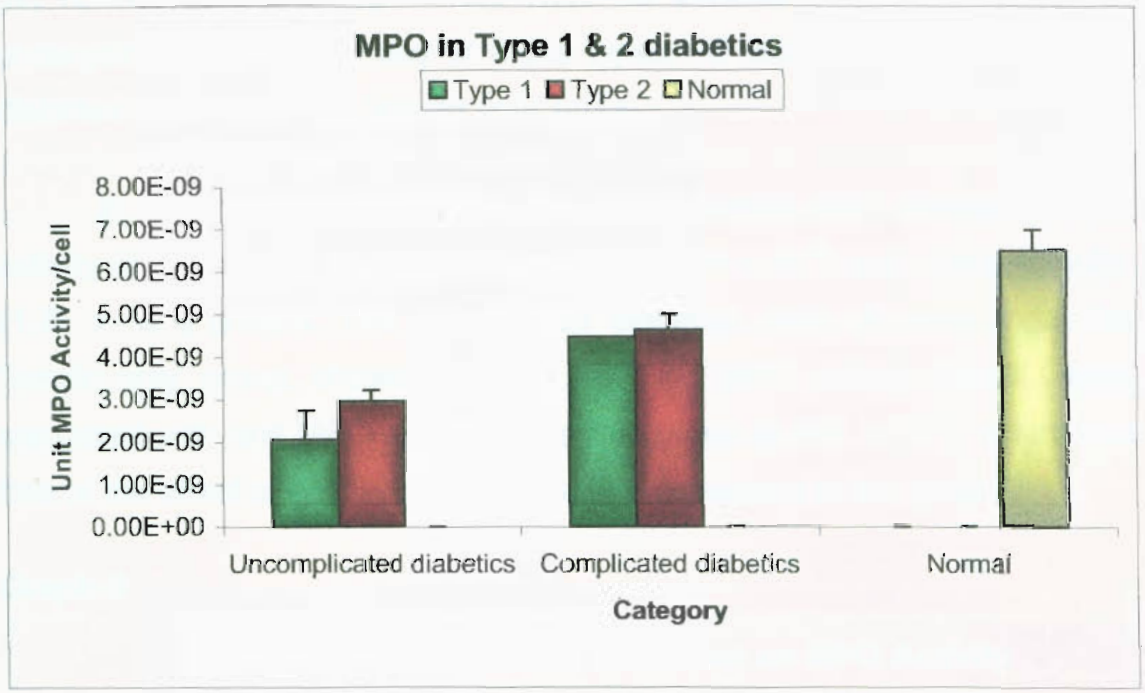


Category		Unit activity per cell	
		Blood sugar status	
		≤140	>140
Uncomplicated diabetics	Mean	1.788 x 10^{-9}	2.886 x 10^{-9}
	SE	3.333 x 10^{-10}	2.601 x 10^{-10}
Complicated diabetics	Mean	5.329 x 10^{-9}	4.256 x 10^{-9}
	SE	5.488 x 10^{-10}	4.389 x 10^{-10}
Normal	Mean	6.484 x 10^{-9}	
	SE	4.908 x 10^{-10}	

5.8. MPO activity in IDDM and NIDDM types

The diabetic people of this study came under both the diabetic types- Insulin Dependent (Type 1) and Non-Insulin Dependent (Type 2) Diabetes Mellitus. Both in uncomplicated and complicated diabetes, Type 1 was very few and less represented. The level of MPO activity was studied in these two groups and the result is presented in graph-8.

Graph-8



Category		Unit MPO activity per cell		
		Type of diabetes		
		Type 1	Type 2	Normal
Uncomplicated diabetics	Mean	2.042×10^{-9}	2.940×10^{-9}	
	SE	6.985×10^{-10}	2.725×10^{-10}	
Complicated diabetics	Mean	4.468×10^{-9}	4.636×10^{-9}	
	SE		3.515×10^{-10}	
Normal	Mean			6.484×10^{-9}
	SE			4.908×10^{-10}

The type 1 group of uncomplicated diabetes showed 2.042×10^{-9} unit MPO activity while type 2 had 2.940×10^{-9}. In complicated diabetic cases also, type 1 group exhibited a lower MPO activity of 4.468×10^{-9} compared to type 2 group, which showed 4.636×10^{-9} unit of MPO activity.

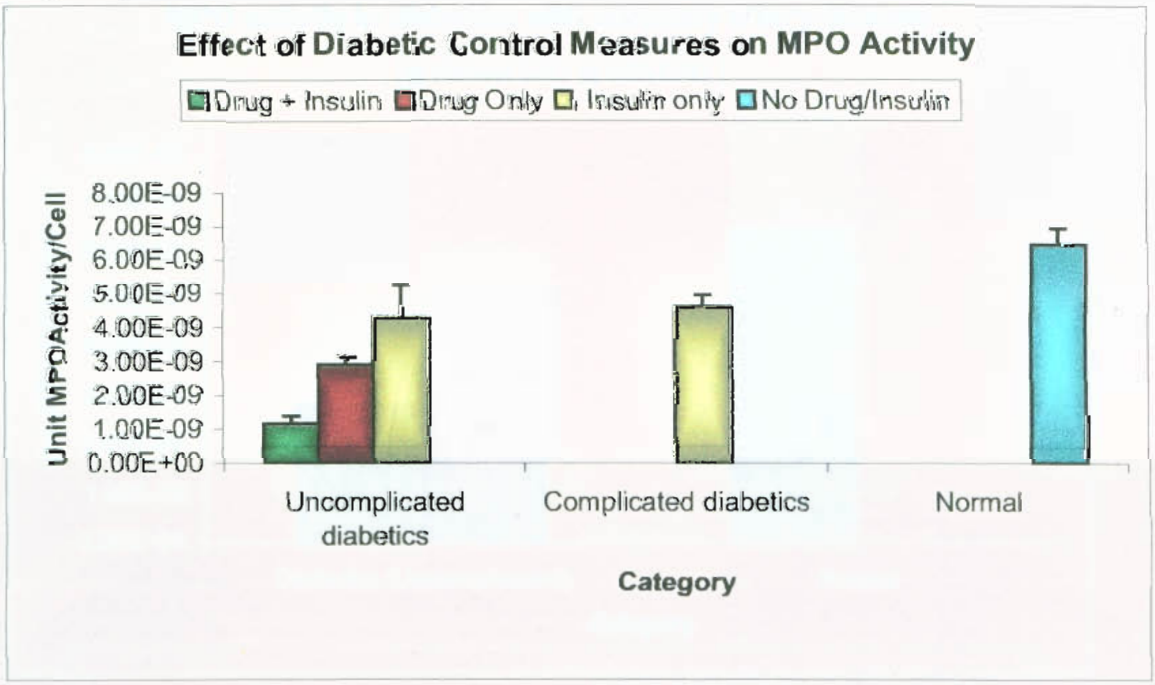
5.9. Effect of diabetic management on MPO activity

Based on the drug /insulin administration for diabetic management, the study population was grouped into 4: (i) cases in which blood sugar was regulated with both hypoglycemic drug and insulin, denoted as group 'Drug + insulin', (ii) cases regulated with hypoglycemic drugs only, denoted as group

'Drug only' and (iii) cases taking insulin alone, denoted as group 'insulin only' (iv) normal control group. There were no diabetic cases that were not undergoing treatment of any of these types. All cases of the complicated diabetes were under the 'insulin only' group.

In uncomplicated diabetics, the group 'insulin only' showed greater activity (4.294×10^{-9}) than the 'drug only' (2.899×10^{-9}) and 'drug +insulin' (1.71×10^{-9}) groups. The complicated diabetics, who all were under the 'insulin only' group, showed 4.633×10^{-9} unit MPO activity. Results were compared with unit MPO activity of normal group (6.484×10^{-9}), which was free of the effect of all these factors. Data is presented in the graph-9.

Graph-9

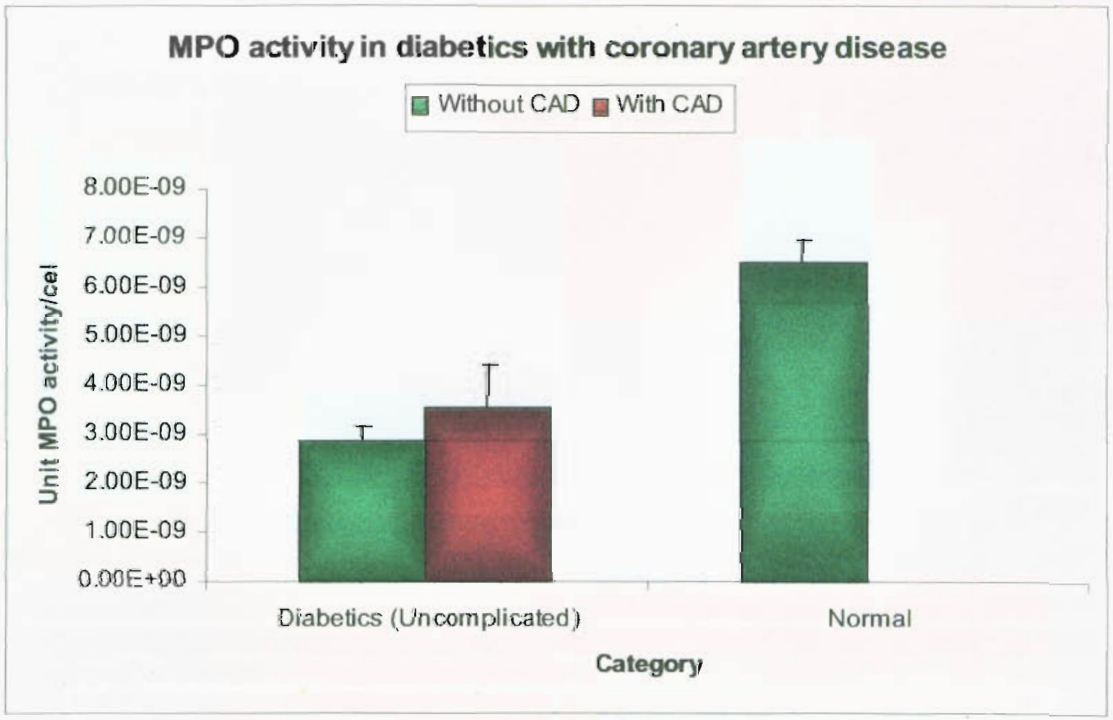


Category		Unit MPO activity per cell			
		Modes of diabetic management			
		Drug + Insulin	Drug Only	Insulin only	No Drug/Insulin
Uncomplicated diabetics	Mean	1.171×10^{-9}	2.899×10^{-9}	4.294×10^{-9}	
	SE	2.226×10^{-10}	2.687×10^{-10}	9.328×10^{-10}	
Complicated diabetics	Mean			4.633×10^{-9}	
	SE			3.442×10^{-10}	
Normal	Mean				6.484×10^{-9}
	SE				4.908×10^{-10}

5.10 MPO activity and coronary artery disease (CAD)

Coronary artery disease (CAD) is not uncommon in diabetic patients. In this study, myeloperoxidase activity was analyzed in 11.1% of diabetic patients suffering from this problem. The effect of CAD was studied in uncomplicated diabetic group, since the group was free of infection related complications. A level of 3.522 x 10^{-9} units MPO was observed in diabetic CAD patients. The diabetic patients who have no risk of CAD were showing MPO of only 2.875 x 10^{-9} units. It is compared with unit MPO (6.484 x 10^{-9}) observed in normal people and is shown in graph-10.

Graph-10

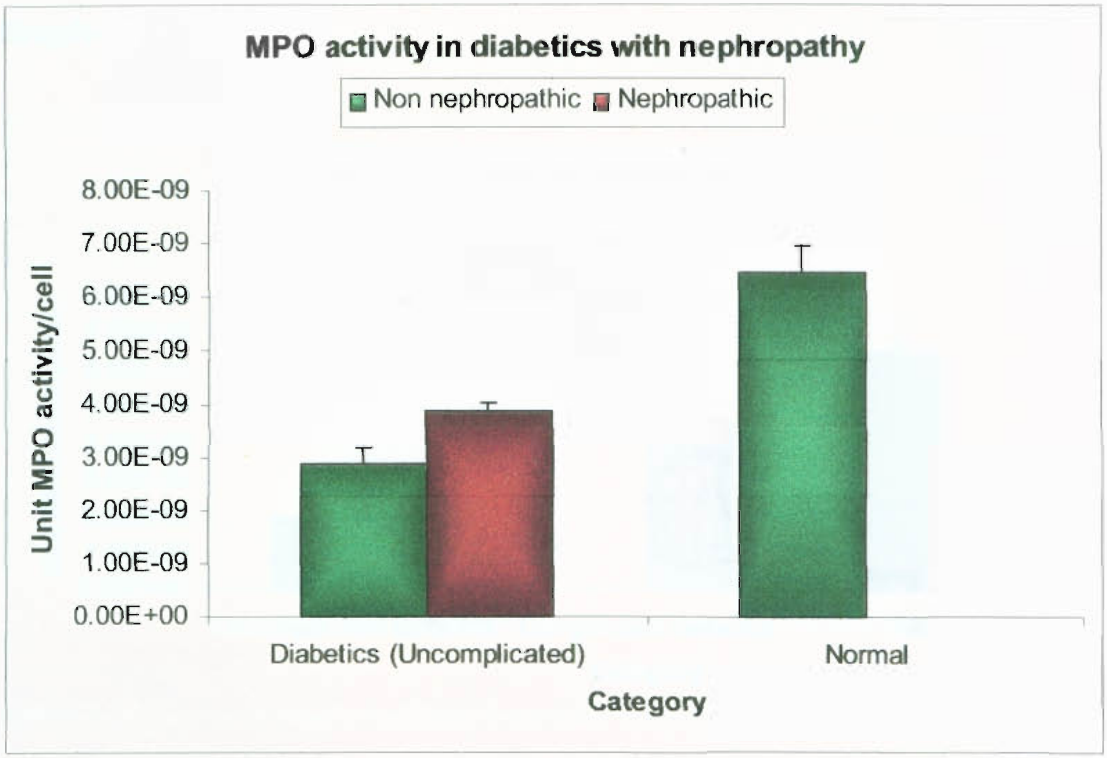


Category		MPO activity in per cell level	
		With CAD	Without CAD
Uncomplicated diabetics	Mean	3.522 x10^{-9}	2.875 x10^{-9}
	SE	9.014 x 10^{-10}	2.907 x 10^{-10}
Normal	Mean		6.484 x 10^{-9}
	SE		4.908 x 10^{-10}

5.11. MPO activity and nephropathy

In this study group, 10% of the diabetic cases (uncomplicated) were suffering from diabetic nephropathy. Their unit myeloperoxidase activity was compared with patients who do not have this complication (graph -11). The activity shown by the nephropathic group was 3.868×10^{-9} and nonnephropathic group was 2.875×10^{-9}

Graph-11

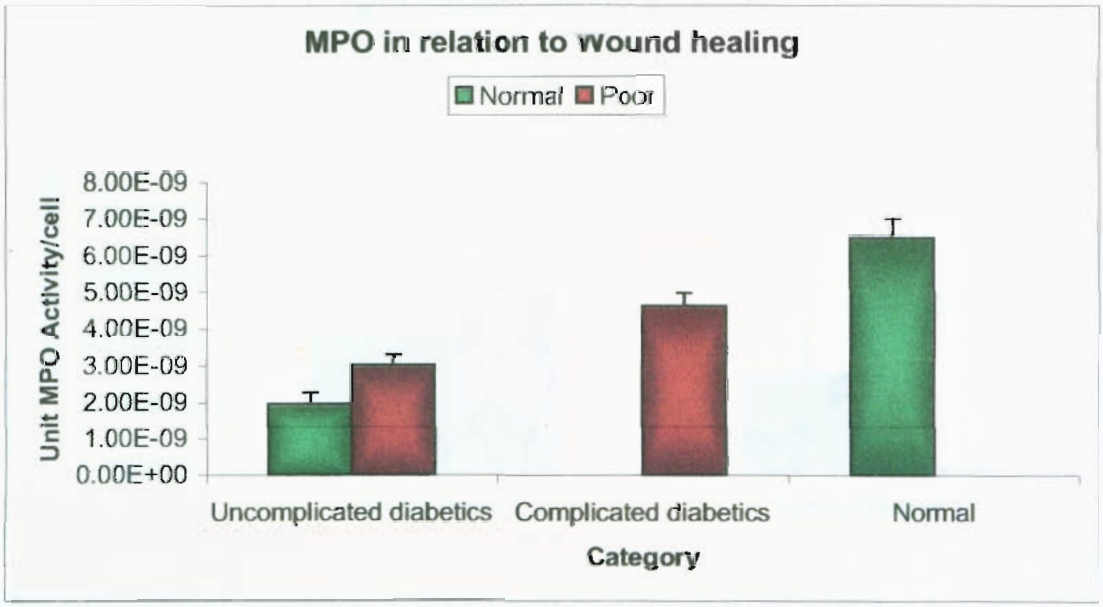


Category		MPO activity in per cell level	
		Non nephropathic	Nephropathic
Uncomplicated diabetics	Mean	2.875×10^{-9}	3.868×10^{-9}
	SE	2.907×10^{-10}	1.471×10^{-10}
Normal	Mean	6.484×10^{-9}	
	SE	4.908×10^{-10}	

5.12. MPO activity and impaired wound healing

The wound healing capacity of diabetic cases also was considered in relation to MPO activity. Even though uncomplicated cases were free of all infection, 12% of the patients had impaired healing of injuries. They showed MPO activity of 3.015 x 10^{-9}, which was greater, compared to the activity, 1.941 x 10^{-9} observed in those who had normal wound healing. In the complicated diabetic category, all were suffering from chronic foot ulcer due to difficulty in wound healing. Data is presented in the graph-12.

Graph-12

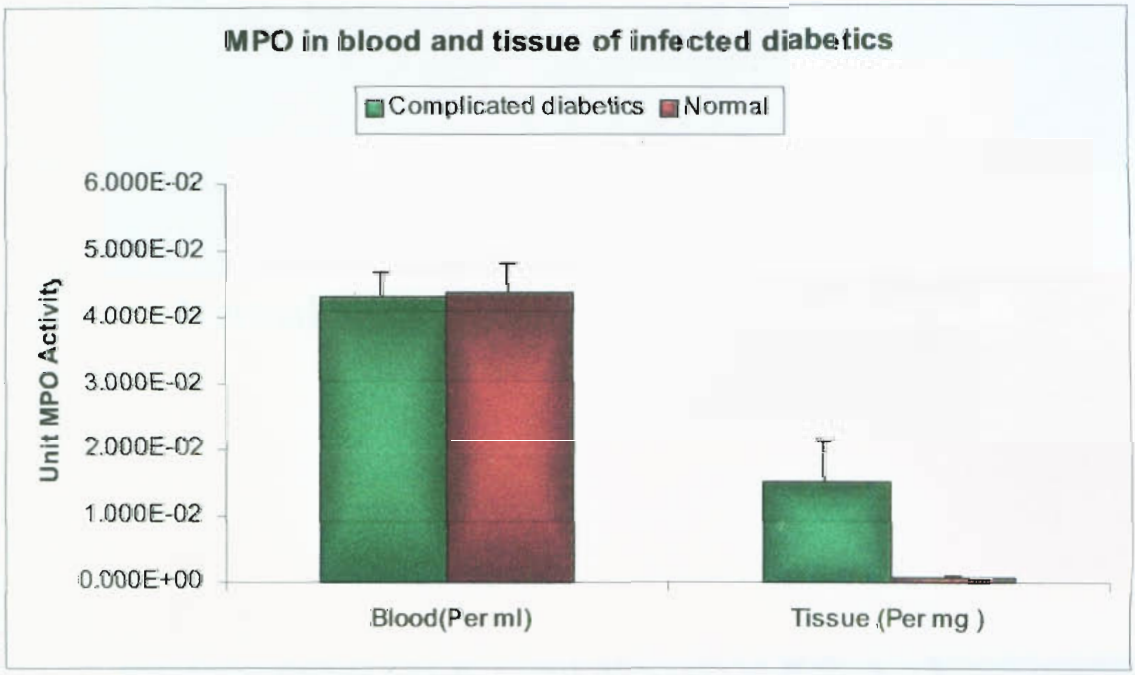


Category		MPO activity in per cell level	
		Wound healing	
		Poor	Normal
Uncomplicated diabetics	Mean	3.015 x 10^{-9}	1.941 x 10^{-9}
	SE	2.874 x 10^{-10}	3.35 x 10^{-10}
Complicated diabetics	Mean	4.633 x 10^{-9}	
	SE	3.441 x 10^{-10}	
Normal	Mean		6.484 x10^{-9}
	SE		4.908 x 10^{-10}

5.13 Estimation of tissue myeloperoxidase

Tissue myeloperoxidase was estimated in complicated (infected) diabetics in comparison with the non-diabetic normal, to get a comparative account. Also MPO level in tissue was considered as an index of neutrophil infiltration into the tissue. It was expressed as unit MPO activity per milligram tissue (Graph -13). The tissue MPO (units/mg) observed in normal in this study was 6.92×10^{-4}, while in infected diabetics it was 1.51×10^{-2}.

Graph -13

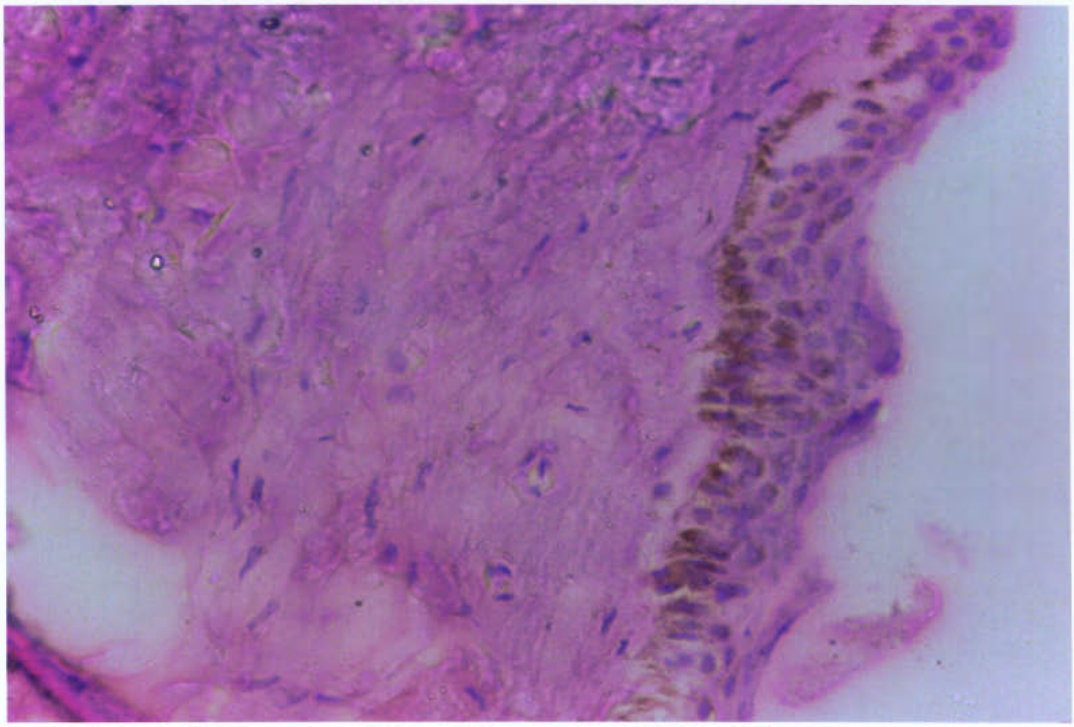


MPO Activity in blood (Units/ml) and in Tissue (units/mg)

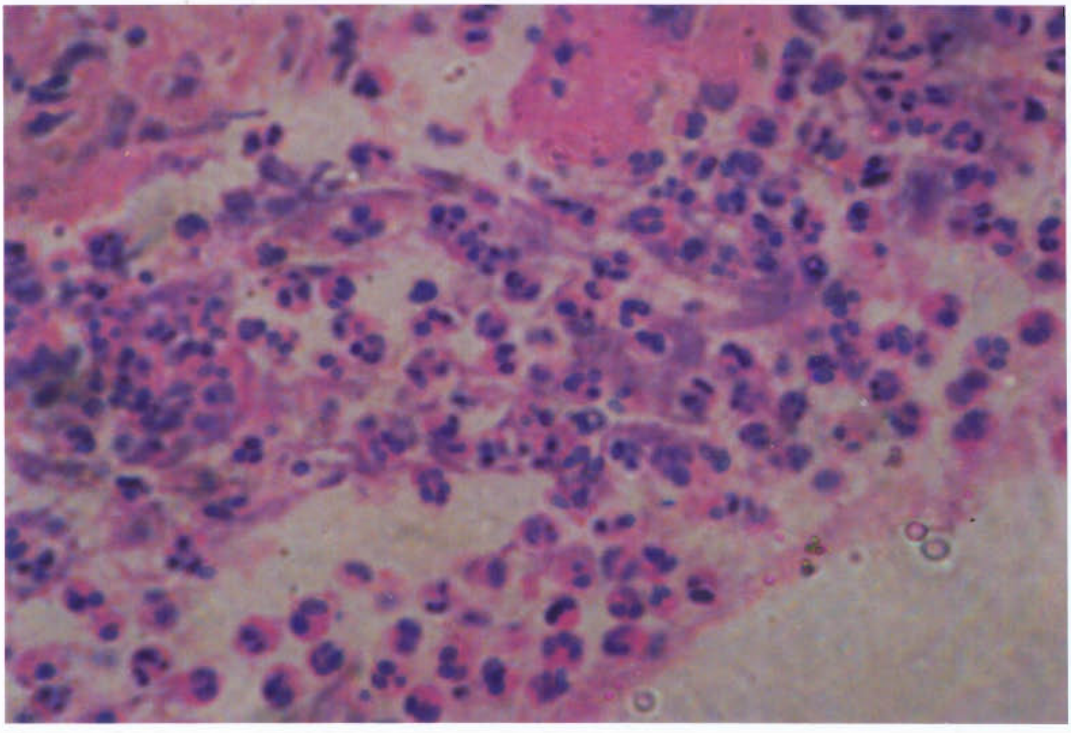
Category		Blood (Per ml)	Per leukocyte in blood	Tissue (Per mg)
Normal	Mean	4.376×10^{-2}	6.484×10^{-9}	6.92×10^{-4}
	SE	4.415×10^{-3}	4.907×10^{-10}	1.310×10^{-4}
Complicated diabetics	Mean	4.309×10^{-2}	4.629×10^{-9}	1.51×10^{-2}
	SE	3.773×10^{-3}	3.230×10^{-10}	6.25×10^{-3}

The histological study was also conducted both in chronic diabetic foot ulcer tissue and normal tissue to observe the specific pathological changes in ulcer tissue (Plate-1). The histology of sections showed regions

HISTOLOGY



Normal tissue without neutrophil infiltration



Tissue of Diabetic foot ulcer showing neutrophil infiltration

with dense neutrophil infiltration associated with edema and apparent, marked matrix dissociation. In the deeper regions there was an increased density of blood vessels, and many contained rounded endothelial cells surrounded by migrating neutrophils. Also there were greater than 95% neutrophils with occasional large macrophages actively phagocytosing depleted neutrophils. A significant elevation in infiltration of neutrophils was observed in complicated diabetic tissue when compared to normal control ($p>0.001$).

5.14 Electrophoretic analysis for MPO isozyme.

Native poly acrylamide gel electrophoresis was done to detect the isozymes of myeloperoxidase. After staining the gel, using specific peroxidase staining protocol, no clear cut band was obtained, which may be due to the low volume of blood samples that were used for enzyme extraction. The observation was the same in case of native protein analysis also, for the same sample volume. Though the enzyme extract of each sample was concentrated by lyophilization and repeated the procedures, no bands could clearly be seen.

6

Discussion

This study on myeloperoxidase (MPO), the major oxidative microbicidal enzyme of innate immune mechanism, was conducted in three different categories of people. They consisted of 50 diabetes mellitus cases without complications of infection (uncomplicated diabetics), 50 diabetic patients whose disease was complicated with infections (complicated diabetics) like ulcer foot syndrome and 50 normal healthy individuals as controls. The level of MPO enzyme was studied in total leukocytes of one milliliter blood and also at single leukocyte level in each individual. This enzyme is largely present in the neutrophil fraction of leukocytes. The purification procedures adopted here could give a leukocyte fraction, 90 % of which were neutrophils.

MPO estimation was carried out in each individual with a purpose of finding whether difference existed in MPO level between the three categories under study, whether the MPO level observed in diabetic patients is any way related to that disease and whether it contributes to the infection and related complications seen in association with this disease in some patients. This study also aimed at finding whether the MPO level has any correlation to parameters like total white blood cell count, sex, age, blood sugar status, types of diabetes, diabetic management measures, incidence of coronary artery disease, nephropathy, wound healing ability etc. of these patients. Besides these, the MPO level and histological features of the infected tissue of diabetic people were studied and compared to normal. Even though MPO is present in serum also, that was not considered here, since the target of this particular study was the MPO at cellular (leukocytic) level.

The MPO observed in the total leukocytes present in one ml of blood was considered as the index of the MPO level in an individual in this study. Since the number of leukocytes were found to be varying highly from individual to individual, the total cellular MPO of a person would naturally depend on their leukocyte count and also on the MPO produced by single leukocyte. The WBC count present in one ml of blood and unit MPO per ml blood helped to come at the enzymatic activity at single leukocyte level in each individual.

This study revealed that the level of myeloperoxidase enzyme is higher in normal people compared to the two diabetic groups under study. The mean of the cellular MPO in one ml blood of the 50 normal individuals was 4.376×10^{-2}. The MPO level at single leukocyte was also highest in normal (6.484×10^{-9}) compared to the other groups. Since these normal people were healthy and free of infection at the time of this study, their MPO level can be taken as the normal level, which may boost up when some infection occurs. Hansen *et al.*,[76] had observed that in normal individuals, the plasma myeloperoxidase is high at start of an infection and is quickly reduced towards the normal values. But what is the standard, optimum level/range of MPO required for a host to be safely protected from infections, is yet to be known. Nauseef [53] had observed that, though MPO activity is critical for optimal microbicidal activity of normal neutrophils, in the absence of MPO, auxiliary mechanisms protect most MPO deficient hosts from clinically significant sequence. But Parry *et al.*,[92] and Kitahara *et al.*,[93] are of the opinion that the intracellular killing is impaired in MPO deficient normal individuals. In the present work, only the MPO status was studied and not it's functional efficiencies, like intracellular killing etc. However, whatever MPO observed in these normal individuals were the level present in them when they were free of infection and normal in condition.

Of the three study categories, the enzyme level was lowest in the uncomplicated diabetic group, the mean value of MPO in 50 patients being as low as 1.636×10^{-2}. That is, a difference of 2.740×10^{-2} per ml blood existed in

the mean activity, between the normal and the uncomplicated diabetics. Variation in MPO existed at single leukocytic level also, between the uncomplicated and normal, with a difference of 3.597×10^{-9}.

This reduction in the MPO activity, compared to normal, in these uncomplicated or non-infected diabetic cases is found statistically highly significant ($p<0.001$), both in one ml blood and at single leukocyte level. Sato *et al.*, [84,99] and Uchimura *et al.*,[85] also had reported a reduced MPO activity in diabetic patients. These 50 uncomplicated diabetics, free of infection, were specifically selected from among the diabetic patients. The fact that this group included cases of even more than 10 years of disease duration, reveals that despite having a very low level of MPO for long, they were not affected by infections. This may be because diabetic patients, though have some degree of susceptibility to infection due to the low MPO activity as per previous reports, as part of their disease management, take utmost care against the chances of injuries and infections, following the instructions of the physicians for such stringent precautionary measures. Foot care is an important chapter in diabetic management with many dos and don'ts. It is to be believed that, to some extent, the care taken help survive this threat of infection that haunt a diabetic patient throughout his life. Those who cannot afford such care-demanding life style may get infected at some stage of their disease. This study, though hadn't included the socio economic status of patients as a parameter, it has been noticed during the course that the diabetic patients, suffered from infection and related complications like ulcer foot syndrome, were usually of low socio economic group.

The uncomplicated diabetic patients may remain so life long, or become a complicated case once they get infected at some stage. Wykretowicz *et al.*,[96] had reported of normal phagocytosis but diminished intracellular killing in diabetic patients with low MPO activity. According to Todar,[3] in phagocytosis, when the engulfed organism is not killed, the intracellular environment may guard the phagocytosed organism against the activities of antibiotics and other drugs. Hence they survive and multiply

inside phagocytic cells and get circulated throughout the body. He had further observed that some bacteria may damage the phagocytes in which they are trapped, by producing factors like aggressins, phopholipase etc. which, having deleterious effect on phagocytes and tissues, can bring out tissue destruction. As per this, once infected, the low level of MPO in uncomplicated diabetic cases observed in this study, may lead to diminished intracellular killing of phagocytosed organisms which can further lead to tissue destruction and infection.

MPO estimation was also carried out in diabetic patients who had complications of infections. Diabetic ulcer foot was the common infection observed in them. Joshi *et al.,*[125] had reported that ulcer foot infections in diabetic patients is caused mainly by pathogens coming under gram negative bacilli, anaerobes, or group A streptococci, with or without staphylococci etc. The MPO estimation in these infection-complicated diabetic patients showed that their enzyme level is distinct from the other two categories and the leukocytic MPO mean values, both in one ml blood and at single leukocyte levels, were found to be falling between those of normal and the uncomplicated group.

The mean cellular MPO per one ml blood in 50 patients of complicated diabetes was 4.309×10^{-2}, with a difference of only 0.0672×10^{-2} from that of normal (4.376×10^{-2}). That is, there is no significant difference existing in MPO level between the normal and complicated diabetics at this level. This, when compared to the corresponding difference (2.740×10^{-2}) between normal and uncomplicated diabetic cases, is considerably low. But coming to the enzyme at single cell level, the mean MPO activity of complicated diabetic group (4.633×10^{-9}) is almost the average of the cellular activity of a normal cell (6.484×10^{-9}) and an uncomplicated diabetic cell (2.887×10^{-9}). At this cellular level, the mean MPO activity of complicated diabetic group differs significantly from both that of normal ($p>0.001$) and also uncomplicated group ($p>0.001$). Dodds *et al.,*[100] also had

reported the occurrence of an increased MPO in diabetic patients with a high oral yeast count.

The above observations show that the total cellular MPO in one ml blood is reaching near that of normal in complicated diabetic group. A closer analysis of the data further shows that this high MPO is due to the increase in the quantity of the enzyme produced at single cell level and also by raise in the number of the leukocytes. In supportive of this, a hike in the production of leukocytes has been observed in these complicated diabetic patients, compared to the normal and non-infected (uncomplicated) cases. It is to be believed that, diabetic cases at the time of infection try to tide over the inherent deficit of this microbicidal enzyme, through the above stringent steps. But it shows that despite these measures, the MPO in the complicated diabetics could not reach the level of even the uninfected normal (when get infected, the MPO level of normal goes further up, as per previous reports[76]).This may be due to the strong influence of the underlying diabetic condition which predisposes an impairment in the production of MPO as observed in this study.

Cech *et al.*,[90,91] had noticed that in MPO deficient diabetic patients suffering from *Candida albicans* hepatic abscess, their intracellular killing is impaired, though the granulocytes have normal chemotactic and phagocytic ability. Nauseef[53], had specifically excepted diabetes mellitus patients suffered from severe candidial disease from the protection of auxillary mechanism, which he had proposed to be acting in the absence of MPO in normal individuals. As per these references, it seems that the infected diabetic cases of this study, which have a cellular MPO lower to normal, is not getting the protection of either the cellular MPO or the auxillary mechanism, and hence subjected to the worst effect of the infection.

On a close study of the enzyme status of the categories, at one ml blood level, it was found that 96 % of uncomplicated diabetics had their MPO below the mean activity of the normal (4.376×10^{-2}) in this study, while the

corresponding value in complicated diabetics was 62 %. At single leukocyte level, 92 % of uncomplicated diabetics and 84 % of complicated diabetics had MPO levels below the normal level. From this finding also it is evident that low MPO status is the order in diabetes, a comparatively better production happening in complicated or infected diabetes than in uncomplicated or uninfected group.

6.1 MPO activity in relation to white blood cell count

As mentioned earlier, WBC count was found as varying highly in individual cases of all the three study categories. In normal people, 94% had the WBC count within the normal range, while in uncomplicated diabetic cases it was 88 % and in complicated diabetics, 72 %. In uncomplicated cases, a general lowering in WBC count was noticed to the extent that no case with count above 11,000 cells /mm^3 has been obtained, while 28 % of the complicated had WBC above the range. The mean of the WBC ranges of the three categories also reflect this difference. When the complicated diabetics had a mean value as high as 10229 cells/mm^3 ± SE 827 it was 6648 cells/mm^3 ± SE 271 in normal and 5652 cells/mm^3 ± SE 254 in uncomplicated diabetics.

The unit MPO activity was also studied in relation to the fluctuations or differently observed WBC count in the individuals of three categories. Analysis of the data showed that even though majority of cases were falling within the normal range of WBC count (4,000-11,000/mm^3), the cases having their count in the lower strata of this normal range (ie, 4,000-8,000/ mm^3) showed a higher cellular MPO than those who had it in the upper strata of the range (ie, 8,001-11,000/mm^3). That is, the MPO production was not seen increased parallel to the increase of WBC in these cases. The differences in the mean activity between the groups falling in the lower and upper strata of normal range in normal, uncomplicated and complicated diabetics were 1.02 $x10^{-9}$, 7.37 $x10^{-10}$ and 2.10 $x10^{-9}$ respectively. This observation was further supported by the MPO status observed in the 28 %

of complicated cases, that had WBC count above the normal range. The level in this group was further lowered, though slightly, when the WBC count went high. But this pattern is seen as followed when the WBC count fell within and above the normal range, while those having < 4,000, had exemption from this.

6.2 MPO activity in relation to sex

Myeloperoxidase enzyme activity was found to be slightly higher in males, in all the three categories, than the females. A difference of 1.453 x 10^{-9} existed in the mean MPO activity between male and female in normal people. The corresponding differences in uncomplicated and complicated diabetic cases were as 1.54 x 10^{-10} and 1.13 x 10^{-10} respectively. That is, the difference between male and female among the normal group was a little higher compared to those in the diabetic categories. Also, the MPO differences in the two diabetic groups fall in a closer range. Kabutomori *et al.*,[89] in 1999, had reported a significantly higher myeloperoxidase index of neutrophils in females than males in their study on normal people. As per this study, it seems that the males enjoy the advantage of having a high MPO in the system, compared to females.

6.3 MPO activity in different age groups

The status of myeloperoxidase enzyme in various age groups of the study categories when considered, more MPO was observed in the <35 years group, in normal people, compared to the other age groups. Also, a general propensity of the unit MPO activity in the single cell level i.e., decreasing with increase in age (below 35 > 35-55 > above 55), has been noticed in normal category. A waning of the immune responses as the age progresses is quite natural. In uncomplicated diabetics, just the reverse of what observed in normal and complicated cases were noticed, i.e., activity being lesser in younger people and slightly increasing with age. In this group, which is characterized by a low MPO activity, complications like

coronary artery disease, nephropathy, uncontrolled sugar level etc. may precipitate the disease as age progresses, leading to a raise of MPO, compared to the level in the initial stage of the disease. The complicated diabetic cases, which is bound to produce a comparatively high MPO continuously due to the long prevailing infections (started in the beginning of diabetes in some or after remaining as uncomplicated for long in others) is found to be showing a fall in MPO as in normal cases, may be due to tiring of the system. Kaneshige *et al.*,[83] had observed that in patients with advanced stages of diabetes mellitus, the decreased intracellular killing of bacteria in granulocytes acts as one of the mechanisms of increased susceptibility to infection. In complicated cases of this study, a good number of patients were with disease duration of more than 10 years.

6.4 MPO activity in relation to the blood sugar status

The individuals were grouped based on their random blood sugar level, into controlled (≤140 mg/dL) and uncontrolled (>140 mg/dL) sugar groups. The unit MPO activity per cell was analyzed for both these groups under each category. The normal people, majority of whom naturally come under the controlled sugar group, showed the greatest MPO activity. Both the controlled and uncontrolled groups of uncomplicated diabetics had the MPO activity in the lowest, when compared to the corresponding groups of complicated and also to normal.

In complicated diabetic cases of this study, MPO activity was low (4.256×10^{-9}) in the uncontrolled sugar group while the controlled had a comparatively higher (5.329×10^{-9}) level of MPO. Complicated diabetics, whose leukocytes are infection stimulated, normally have an elevated level of MPO production compared to the unstimulated leukocytes of uncomplicated diabetics. There are reports[80, 81] that some microorganisms become more virulent in a high glucose environment. As per this, the uncontrolled sugar groups of complicated diabetic cases, that have a high glucose level, are supposed to have a high MPO also, to tackle the situation;

but the reverse was observed and better production of MPO was observed in cases having normal sugar level. That is, when the cells have an abnormal sugar status, the metabolism may go off track leading to lowering of MPO production. In supportive of our observation, Sato *et al.*,[84] also had reported a decreased MPO activity in poorly controlled diabetes mellitus patients. They had demonstrated that every step in leukocytic generation of reactive oxygen intermediates would be reduced in the leukocytes taken from poorly controlled diabetes patients. Kemona *et al.*,[98] also had observed the MPO activity decreased in leukocytes of uncontrolled diabetics and had opined that controlling of the disease does not change the MPO activity. But since MPO is seen as raised when the sugar was under control, the observation of Kemona *et al.*,[98] is only partly true here. According to Trivedi[80], hyperglycaemia reduces oxidative killing capacity because of increased glucose metabolism through polyol pathway depleting NADPH, which is necessary for the generation of superoxide free radicals and it will further account for the scarcity of substrates like hydrogen peroxide to which the MPO can act upon. Niethammer *et al.*,[95]also had reported that better control of diabetes lead to improvement of bactericidal capacity in juvenile diabetes.

In uncomplicated cases, in contrast to the above observation, this intracellular enzyme level was seen as decreased when sugar was under control. The low level of MPO at controlled sugar level, 1.788×10^{-9}, raised to 2.886×10^{-9}, when sugar level went high. But a close study reveals that a raise in MPO has not really occurred when sugar is raised in the system. This high level observed is almost the mean cellular activity observed in the uncomplicated group. But when the sugar level was lowered, a considerable reduction in the MPO had occurred. Anyway, in uncomplicated diabetes, the low cellular MPO is seen as improved in a high sugar environment. It is to be assumed that since a threat of infection exists in cases of increased sugar in the system, 'the high sugar groups' of the uncomplicated cases have their MPO boosted up, though not reaching the level of normal or complicated cases.

6.5 MPO activity in IDDM and NIDDM types

In this study, comparatively lesser number of type 1 cases have been present both in complicated and uncomplicated diabetes. Difference existed in the status of myeloperoxidase enzyme between Insulin Dependent (type1) and Non-Insulin Dependent (type 2) Diabetes Mellitus of both uncomplicated and complicated diabetes. A higher level of intracellular MPO existed in type 2 than type 1 patients in both the diabetic groups. Saeed *et al.*, [102] also had observed the same and put forth that MPO is inhibited by the abnormally elevated acetoacetate in that condition and it diminishes the neutrophil microbicidal activity which lead to the susceptibility to infections in type 1 patients. It may be a combined effect of both the reduced level, as has been observed in this study, and also the inhibition by acetoacetate that act in type 1 patients as was observed by Saeed *et al.*, [102]. Niethammer *et al.*,[95]also had reported instances of the impaired intracellular killing of normally phagocytosed *S. aureus* in type 1 diabetics.

Uchimura *et al.*,[85] observed a reduced MPO activity in type 2 patients, but it was when compared to normal people of their study. Irrespective of the diabetic types (I or II), we always had observed a low MPO activity in the diabetic patients, compared to normal. Delamaire *et al.*,[82] had opined that the types of uninfected diabetes have no effect on the PMN functions. But in the present study, since a lowering of myeloperoxidase, the significant bactericidal enzyme of PMNs, has been observed in type I than type 2 in both the diabetic groups and also based on the previous reports of intracellular killing impaired in type 1 diabetes[96]. It is suggested that MPO level can have some influence on PMN function. But to opine regarding the rate of phagocytosis, efficiency of intracellular killing etc., further study is to be carried out in these patients.

6.6 Effect of diabetic management on MPO activity

In diabetes management, the most important step is the control of blood sugar. Controlled diabetics are comparatively lesser susceptible to other diabetic complications. The common modes used for the control of blood sugar in diabetic cases also were considered here in relation to MPO level. In uncomplicated cases, the highest MPO level was observed in insulin treated (4.294×10^{-9}) than the drug treated (2.899×10^{-9}) or the combined therapy group (1.171×10^{-9}). Comparatively lesser MPO level was found in users of hypoglycaemic drug. All the complicated diabetic cases were under insulin treatment and hence there was no scope for it to be compared with the other modes in this category.

The work of Oldenborg, *et al.,*[105] have shown that insulin interferes with myeloperoxidase activity and subsequent myeloperoxidase –dependent generation of reactive oxygen metabolites in normal human neutrophils. But their observation was in an *in vitro* study. It is evident in our study that the MPO is not blocked in it's production stage in insulin treated patients, because in this group, myeloperoxidase level was seen as elevated compared to the drug treated groups. May be the enzyme is being inhibited by the hormone in the reaction stage, as observed by Oldenborg *et al.,*[105] thus becoming a reason for the complicated condition to prevail in spite of having comparatively high MPO in the system. In support of this, Kaneshige *et al.,*[83] had observed that although phagocytosis is normal in insulin treated subjects, unlike the diet treated subjects of their study, intracellular killing by granulocytes was significantly reduced in them compared to normal subjects.

Since the first therapeutic use of insulin in 1921, diabetes has been a controllable but chronic condition and the main risks to health are its characteristic long-term complications. These include cardiovascular disease, chronic renal failure due to diabetic nephropathy, diabetic retinopathy which can lead to blindness and diabetic neuropathy (nerve damage) which mainly leads to gangrene (necrosis and subsequent decay of

body tissues caused by infection or lack of blood flow) with risk of amputation of toes, feet, and even legs. The MPO status in diabetic patients having complications of coronary artery disease, nephropathy, impairment of wound healing was studied here.

6.7 MPO activity and Coronary Artery Disease (CAD)

The MPO status in the diabetics suffering from CAD was studied selectively in uncomplicated diabetic cases since the group was free of infections and related complications. About 11.1 % of the uncomplicated patients suffered from CAD in this study. A higher MPO activity has been observed in such patients compared to those who are free of CAD. Zhang *et al.*,[126] had observed elevated levels of leukocyte and blood MPO associated with the presence of CAD in non diabetic patients. An increased level of MPO in normal individuals with acute coronary syndrome has been reported by Baldus *et al.*,[107] also.

When 2.875×10^{-9} unit MPO was shown by the diabetic patients who are free of CAD, 3.522×10^{-9} units of MPO was observed in patients having CAD. Even though the level of MPO in the CAD patients does not reach the level of normal, the raise in the enzyme activity compared to the non-CAD group is to be considered as significant since it is in diabetic patients who normally have a decreased antioxidant status.[116] The oxidants produced by MPO is not properly scavenged, which may aggravate the coronary artery disease in these people. Zhang *et al.*,[108] had observed that vascular-bound MPO could use high-glucose-stimulated H_2O_2 and produce HOCl modified low density lipoprotein, (a specific biomarker for the MPO/HOCl/chlorinating species pathway) to amplify high-glucose-induced injury in the vascular wall. MPO/ H_2O_2/HOCl/chlorinating species may represent an important pathway in diabetes complications and a new mechanism in phagocyte- and systemic infection-induced exacerbation of diabetic vascular diseases. Anitra *et al.*,[106] had reported the property of MPO to catalyze the formation of a number of reactive oxygen species that modify LDL to a form that converts macrophages in 'lipid laiden' or 'foam' cells, the hallmark of atherosclerosis.

These show that MPO has the potential role of both as a marker and a mediator of coronary artery disease.

6.8 MPO activity and nephropathy

About 10 % of the uncomplicated diabetic cases were suffering from diabetic nephropathy. It is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli due to longstanding diabetes mellitus and characterized by nodular glomerulosclerosis. Diabetic nephropathy is the leading cause of end-stage renal disease. These patients were showing a high MPO activity than others. The mean of activity in the nephropathic group was 3.868×10^{-9} while that of non- nephropathic group was 2.875×10^{-9}. But Buraczynska *et al.*,[109] had observed a significant reduction in the intracellular myeloperoxidase level in their patients of chronic renal failure due to diabetic nephropathy, unlike observed here.

6.9 MPO activity and impaired wound healing

Impaired wound healing is a well-documented phenomenon both in experimental and clinical diabetes. Although the uncomplicated cases had no infection, 12 % of the patients were suffering from delayed healing of wound, which were not infected. Those with healing impairments showed comparatively higher mean unit MPO activity (3.015×10^{-9}) than those with proper wound healing (1.941×10^{-9}). As already observed, the complicated diabetic cases of this study having infection and poor wound healing also had the MPO raised (4.633×10^{-9}). Galeano *et al.*,[119] found delayed wound healing together with low collagen content, breaking strength, and increased malondialdehyde levels (which is an end product of lipid peroxidation due to MPO activity) in diabetic mice compared to healthy ones. They have suggested that an increased lipid peroxidation in diabetic mice might have a role in determining a defect of wound repair.

This study reveals that when infection occurs in diabetic patients, there is obvious rise in MPO production as part of neutrophil activation. Accardo-Palumbo *et al.*,[104] had noticed the occurrence of anti-MPO antibodies in some diabetic patients which can stimulate neutrophils to damage endothelial cells, eventually leading to diabetic angiopathy that in turn causes ischemic heart disease, stroke and also peripheral vascular disease, which contribute to the diabetic foot ulcers and the risk of amputation. Since the present study had not concentrated on the anti-MPO antibodies, their contribution to the diabetes related complications could not be discussed here, at the same time is not ruled out also.

6.10 MPO in tissues of infected diabetics

Estimation of myeloperoxidase in infected tissues was performed in complicated diabetics and also in non-diabetic normal people, as the index of neutrophil and macrophage infiltration into the tissues. It was expressed as unit MPO activity per milligram tissue. Comparison analysis of tissue MPO in test and control samples revealed that myeloperoxidase level was higher in tissues of complicated diabetic (1.51×10^{-2}) cases than that of controls (6.92×10^{-4}). But in the same complicated diabetics, single cell (leukocytic) MPO of blood was found as lesser compared to that of the same normal cases. This finding explains that even though the level of myeloperoxidase at cellular level (units/cell) in blood of diabetics is not as much of normal, the tissue myeloperoxidase (units/mg) at the infected area is far above to that of normal.

In this study, the histological study was also done in tissues of both chronic diabetics having infections of foot ulcer and normal people, to analyze the specific pathological changes happened in infected ulcer tissue. The histology sections of ulcer foot tissues showed regions that contained dense neutrophil infiltration associated with edema. An apparently marked matrix dissociation with greater than 95 % of neutrophils, and occasional large macrophages, also was observed. In the deeper regions there was an

increased density of blood vessels, and many contained rounded endothelial cells surrounded by migrating neutrophils. The boosted level of myeloperoxidase in tissue can be due to the large scale infiltration of neutrophils to the problematic infected area in diabetic patients as revealed here in the histological studies. The high myeloperoxidase activity in tissues is to be definitely correlated with the large neutrophil counts observed in the ulcer tissue sections. Diegelmann *et al.*,[120] had reported that chronic pressure ulcers contain the persistence of neutrophils and their destructive enzymes, which are responsible for the extensive matrix dissociation and thus contributes to the chronicity of these ulcers. The infiltration and accumulation of neutrophils may be contributing to the pathogenesis of chronic diabetic foot ulcers.

According to Stendahl *et al.*,[55], apart from being a potent antimicrobial system, the oxidizing activity of the MPO- H_2O_2-halide system could also elicit inflammatory reactions and tissue injury. The exposure to increasing amounts of powerful oxidant produced by the MPO system (HOCl) increased the susceptibility of fibronectin (a linker glycoprotein molecule in the extracellular matrix) to proteolytic attack by neutrophil elastase. Hence a loss of functional fibronectin would result in cell detachment and a distortion of normal tissue organization which leads to tissue injury by neutrophils in inflammation.[114] In addition, antioxidant status is impaired in diabetics compared to normals. According to some previous workers like Vijayalingam *et al.*,[116], Majchrzak *et al.*,[118] Muchova *et al.*,[117] the most important antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase were found less in diabetic patients. Due to this oxidative stress (imbalance between oxidants and antioxidants) and increased MPO activity, diabetic patients with foot ulcer fail to act against the pathogens and to heal the wounds, which may further lead to complications and amputation. Thus the myeloperoxidase act as one mechanistic link among the morbid conditions like persistent inflammation, oxidative stress etc. in diabetic patients.

7
Summary

This study on myeloperoxidase enzyme was conducted in three different categories of people - diabetes mellitus cases without complications of infection (uncomplicated diabetics), diabetic patients whose disease was complicated with infections like ulcer foot syndrome (complicated diabetics) and normal healthy individuals as controls.

The MPO level in individuals of the three categories were studied to find:

i. whether difference existed in the level of MPO between the three categories,

ii. whether the MPO level observed in diabetics is any way related to the disease, diabetes mellitus,

iii. whether the MPO level is related to the infections that is associated with diabetes in some patients,

iv. whether the MPO level can be correlated to parameters like total white blood cell count, sex, age, blood sugar status of diabetics, types of diabetes, diabetic management measures, incidence of coronary artery disease, nephropathy, wound healing ability etc. in diabetic patients.

v. whether difference existed in the MPO level and histological features in the tissues of infected diabetic people compared to normal.

The MPO level was estimated in the leukocytes present in one milliliter blood in each individual. Since the number of leukocytes was found as varying highly in each case, the total cellular MPO of one ml blood would

depend on the leukocyte status (count) of that person. The enzymatic activity per single leukocyte also was derived for each individual. Tissue MPO was estimated and expressed as unit MPO activity per milligram of tissue, both in normal and infected diabetics. The histological studies were also done on the infected tissues to see the specific pathological changes occurred due to infection. The MPO level observed was studied in relation to various significant parameters like white blood cell count, sex, age and blood sugar status of diabetics, types of diabetes, diabetic management measures, incidence of coronary artery disease, nephropathy, wound healing ability etc. in diabetic patients.

The results of the investigations carried out is summarized as follows:

- The unit MPO activity in leukocytes of one ml blood was found highest in normal ($4.376 \times 10^{-2} \pm$ SE 4.415×10^{-3}) individuals compared to the diabetics. Uncomplicated diabetic cases showed the least activity ($1.636 \times 10^{-2} \pm$ SE 1.589×10^{-3}). The mean activity in complicated diabetic cases was $4.309 \times 10^{-2} \pm$ SE 3.773×10^{-3}.
- The MPO activity when considered in a single leukocyte, that was also higher in the normal category while the uncomplicated diabetics showed the least. The mean enzymatic activity of complicated diabetic cases ($4.633 \times 10^{-9} \pm$ SE 3.445×10^{-10}) was almost the average of that observed in normal ($6.484 \times 10^{-9} \pm$ SE 4.908×10^{-10}) and uncomplicated diabetic ($2.887 \times 10^{-9} \pm$ SE 2.601×10^{-10}) cases.
- Compared to normal, the reduction observed in the MPO activity in uncomplicated cases was highly significant ($p<0.001$) both at one ml and at single cell level. Compared to the complicated diabetic cases also, the level of MPO in uncomplicated diabetics showed highly significant ($p>0.001$) reduction both at one ml blood and at single cell level. At the same time, the difference at one ml level between normal and complicated cases was found insignificant, since these values were close to each other, while their difference at single cell level was highly significant ($p<0.001$).

- In short, the MPO level of the study categories was following a general pattern i.e., Normal > Complicated diabetics > Uncomplicated diabetics. The uncomplicated diabetic patients since suffer from a very low MPO activity may have an impaired intracellular killing capacity as per previous reports. Though the extreme personal care and stringent precautionary measures taken against infection may protect these patients to a great extent, when once subjected to infection, it has been observed from the study carried out in complicated diabetics that, the system try to increase the level of MPO by overproducing the leukocytes and also by increasing enzyme at single cell level, to some extent. But despite these measures, the MPO in the complicated diabetics is not reaching the level of even the uninfected normal. When get infected, the MPO level goes further up[76]. This failure to reach the MPO level of normal may be due to the strong influence of the underlying diabetic condition that predisposes impairment in the production of MPO, as observed in this study.

- In all the three categories, total leukocyte count varied highly, falling either within or below/ above the normal range. The mean of the WBC ranges of the three categories also reflect the variation of the categories. When the complicated diabetics had a mean value as high as 10229 cells/mm^3 ± SE 827, it was 6648 cells/mm^3 ± SE 271 in normal and 5652 cells/mm^3 ± SE 254 in uncomplicated diabetics.

- The unit MPO activity, when studied in relation to varied leukocyte status observed in the diabetic patients and control, it was found that even though majority of cases were falling within the normal range of WBC count (4,000-11,000/mm^3), the cases having their count in the lower strata of this normal range (i.e., 4,000-8,000/mm^3) showed a higher cellular MPO than those had it in the upper strata of the range (i.e., 8,001-11,000/mm^3). That is, the MPO production was not seen as increased parallel to the increase of WBC in these cases. This could be further proved by the MPO status observed in the 28 % of complicated cases, that had WBC count above the normal range, in which the level

was further lowered, though slightly, when the WBC count went high. But this pattern is seen as followed when the WBC count fell within and above the normal range, while those having count < 4000 had taken exemption from this.

- Myeloperoxidase enzyme level was found to be slightly higher in males than females in all the three categories studied here. Females had a low representation in all the study categories.
- On studying the MPO-age relationship, a general propensity of the unit cellular MPO activity i.e., decreasing with increase in age (below 35 > 35-55 >above 55) has been noticed in normal category. In uncomplicated diabetics, just the reverse of the observations in normal was noticed. In complicated cases, the MPO-age relationship was observed as following the pattern of normal.
- The normal people who all naturally came under the controlled sugar group, showed the greatest MPO activity at cellular level. Both the controlled and uncontrolled groups of uncomplicated diabetics had the least MPO activity. In complicated diabetics, enzyme activity was high when the sugar was controlled, where as in uncomplicated group, MPO activity was comparatively higher when the sugar was uncontrolled.
- A higher level of intracellular MPO existed in type 2 than the type 1 patients in both the uncomplicated and complicated diabetic groups.
- In uncomplicated cases, the highest MPO level was observed in insulin treated than the drug treated or the combined therapy group. Comparatively lesser MPO level was found in users of hypoglycaemic drug. All the complicated diabetic cases were under insulin treatment and hence there was no room for its comparison with other modes of treatment in this category.
- About 11.1 % of uncomplicated diabetic cases were suffering from coronary artery disease. An elevated level of MPO was observed in

diabetic patients having coronary artery disease than those who were free of it.

- About 10 % of uncomplicated diabetic cases were suffering from diabetic nephropathy. These patients were showing a high level of MPO activity compared to others, free of it.
- About 12 % of uncomplicated diabetic cases had healing impairments. They showed higher unit MPO activity compared to those with proper wound healing. All the complicated diabetic cases of this study, that had infections and wound healing impairment, had comparatively high cellular MPO.
- Estimation of myeloperoxidase in tissues was performed in diabetics complicated with infection and non-diabetic normal people, as the index of neutrophil infiltration into the tissues. Comparison analysis of tissue MPO in test and control samples revealed that myeloperoxidase level was higher in infected tissues of diabetic cases than normal tissue. But in the same infection complicated diabetics, leukocytic MPO level (units/cell) was found as lesser compared to the same normal cases. That is, even though the level of leukocyte myeloperoxidase in blood of diabetics was not as much of normal, the tissue myeloperoxidase at the infected area was far above than that of control group.
- The reason for the boosted level of myeloperoxidase in infected tissue can be the large scale infiltration of neutrophils to the problematic, infected area in diabetic patients. The histopathological study supported this, revealing dense presence of neutrophils with occasional large macrophages actively phagocytosing depleted neutrophils. It is evident that the enhanced MPO/mg tissue in the complicated diabetics is not only due to the comparatively high cellular MPO production observed in the neutrophils of infected diabetics, but also due to the increased neutrophil infiltration in the area.

8

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