IJPSR (2012), Vol. 3, Issue 11 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 07 July, 2012; received in revised form 15 October, 2012; accepted 28 October, 2012

MYELOPEROXIDASE AS A POTENTIAL BIOMARKER IN DIFFERENT STAGES OF KNEE OSTEOARTHRITIS

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Keywords:
Myeloperoxidase,
Kellgren-Laurence (K.L.) Score,
Osteoarthritis,
Biomarker

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QUICK RESPONSE CODE

IJPSR:
ICV (2011)- 5.07

Website:
www.ijpsr.com

ABSTRACT

Osteoarthritis (OA) is disabling condition leading to loss of articular cartilage. Etiology of OA is not clearly understood and diagnosis of early stages of OA is difficult due to lack of specific markers. Recent literature suggest that elevation of Myeloperoxidase (MPO) levels in synovial fluid in early stages of OA as compared to controls and late stage OA. Presence of MPO in blood and Urine samples of OA patients is still not studied. Present study is designed to explore the role of plasma and urine MPO levels in different stages of osteoarthritis. Based on Kellgren-Laurence (K.L.) Score, study subjects were divided into 4 groups as per 4 grades of severity of OA. These assignments were made by experienced joint surgeons and were based on radiographic & clinical findings. MPO levels in plasma and urine is estimated by ELISA technique. Levels of Plasma and Urine MPO are found to be significantly higher in case Grade 1, grade 2(Early Knee OA) & MPO level goes on decreasing in Grade 3 & 4 (Late Knee OA). Therefore Early detection of knee OA (Grade 1 & 2) through estimation of rise in serum and urine MPO level is possible and is very convenient as compared to synovial MPO levels. This will help Orthopedic Surgeons to check the disease progression by therapeutic intervention at early stages and to prevent further debilitating complications.

INTRODUCTION: OA is a condition that leads to destruction of articular cartilage resulting in complete loss of joint space. The definitive treatment for end-stage OA is total joint arthroplasty. This procedure, when performed at the appropriate time, will provide pain relief and restore function for the lifetime of the prosthetic joint.

Joint arthroplasty, however, has a finite life and particularly in the younger patients is likely to fail, necessitating the need for revision surgery. Late OA can easily be discerned on radiographs. Early OA, on the other hand, is difficult to diagnose with the routine imaging modalities.

It is plausible that diagnosis of this disease at an earlier stage may allow administration of therapeutic and preventative modalities that could halt or retard the progression of the disease.

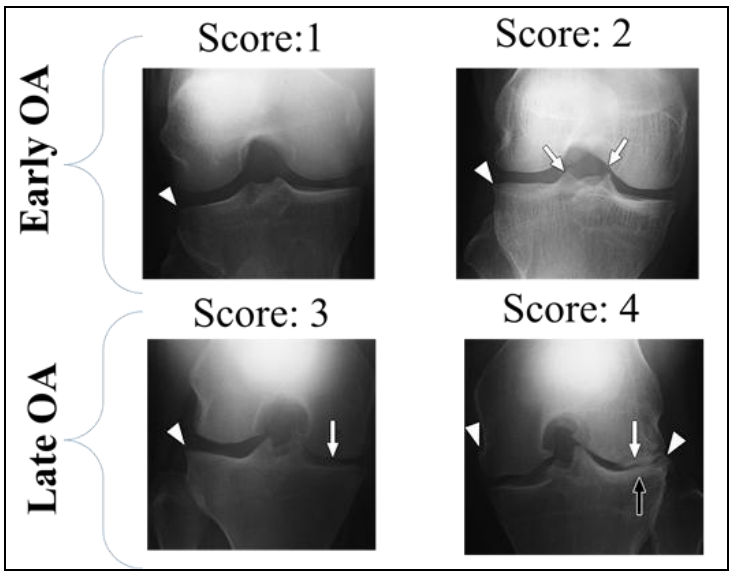
As such, a biomarker with sufficient sensitivity and specificity to identify early OA would be an invaluable tool. From a diagnostic viewpoint, it is also important that OA be distinguished from other arthropathies.

Recently in a study Patients with early OA demonstrated significantly elevated levels of MPO in synovial fluid [1]. Patients in the control and advanced OA groups demonstrated little elevation in MPO levels.

These results indicate that MPO may serve as diagnostic markers for the detection of early OA. This study was carried out to compare MPO levels in plasma and urine in early knee osteoarthritis & in late osteoarthritis.

MATERIAL AND METHODS: This study was carried out in the Department of Biochemistry in collaboration with Department of Orthopaedics. The study protocol was approved by the Institutional Ethics Committee. Before enrollment in the study, informed written consent was obtained from each subject

Study design: A total number of 90 subjects above the age of 20 years were participated in the present study. Detailed medical history and relevant clinical examination data and written consent were obtained from all subjects by explaining the study procedure. Based on Kellgren-Laurence (K.L.) Score on X ray findings, study subjects were divided into 2 groups i.e. Early OA (Grade 1 & Grade 2) and Late OA (Grade 3 & Grade 4) as per severity of OA. Each group consist of 30 patients. 30 age and sex matched controls were selected for comparison. These assignments were made by experienced joint surgeons and were based on radiographic & clinical findings.



Classification: Kellgren Lawrence Score [4]:

Selection of study subjects –

Inclusion and Exclusion criteria:

Inclusion criteria:

- **Cases:** All newly diagnosed & untreated cases of OA of both sexes of age group above 20, who are willing to give consent, were enrolled in study as cases.
- **Controls:** Age and sex matched people not having complain of knee pain, non-smoker individuals and those who given consent were selected as controls.

Exclusion criteria:

- Those who had not given consent.
- Patients suffering from Rheumatic arthritis, Gout, Tuberculosis arthritis, obesity, tuberculosis, leprosy other chronic systemic illness, liver disorders, renal disorders, congestive cardiac failure.
- Alcoholics and chronic smokers were excluded.
- Patients on long term treatment for OA were also excluded from the study

Collection of blood sample:

- About 4-5 ml of blood samples were collected from cubital vein in an EDTA vaccutainer for Myeloperoxidase (MPO) estimation.
- 5 ml of freshly voided, midstream urine sample taken in plane bulb.
- Hemolytic, icteric or lipemic specimens were rejected.
- Plasma was separated by centrifugation at 3000 rpm for 10 minutes.

Specimen Storage:

- If samples cannot be processed immediately then specimens should be capped and may be stored for up to 5 days at 2-8°C prior to assaying.
- Specimens held for a longer time should be frozen only once at -20°C prior to assay.
- Thawed samples should be inverted several times prior to testing.

Methods:

MPO Analysis [1]: To measure the level of MPO in the hyaluronidase-treated samples, we used an enzyme-linked immunosorbent assay (MPO; Oxis International; Portland, OR) and followed the manufacturer's instructions. In brief, antigen was captured in this "sandwich" ELISA by a solid phase monoclonal antibody and detected with a biotin-labelled goat polyclonal anti-MPO. An avidin alkaline phosphatase conjugate was then bound to the biotinylated antibody and p-nitrophenyl phosphate (pNPP) substrate was added. The release of p-nitrophenol was detected spectrophotometrically at 405 nm. The sensitivity threshold detection limit of this assay is 1.5 ng/mL and cross-reactivity with eosinophil peroxidase is <2%.

Facilities and Equipment: All the required facilities and equipment are available in the department of biochemistry. The study does not involve any harm to the any patient involved.

Statistical analysis: All the calculations were done using Microsoft Office Excel 2008. The association between the various parameters in different groups was evaluated using Pearson's correlation coefficient. P<0.05 was considered statistically significant. P-value of less than 0.001 (P < 0.001) was considered to be statistically highly significant. A two-way ANOVA analysis for correlations between age and MPO levels showed that although the development of OA was age related, the amount of MPO was unrelated to the age of the individual.

RESULTS: We found that MPO levels in plasma sample of Early OA patients is around 3 times greater than Late OA group and controls(p < 0.005). MPO levels in urine sample also shows around 2 fold increase in early OA patients as compared to Late OA as well as controls (p < 0.005). Difference in MPO levels in plasma and urine of Late OA and controls is insignificant as shown by **chart 1** and **table 1**.

TABLE 1: MPO LEVELS IN PLASMA AND URINE SAMPLE OF EARLY AND LATE OA

	Control (30)	Early OA (30)	Late OA (30)	p value
Plasma	76±4	240±8	80±7	p < 0.005
Urine	198±10	376±10	206±12	p < 0.005

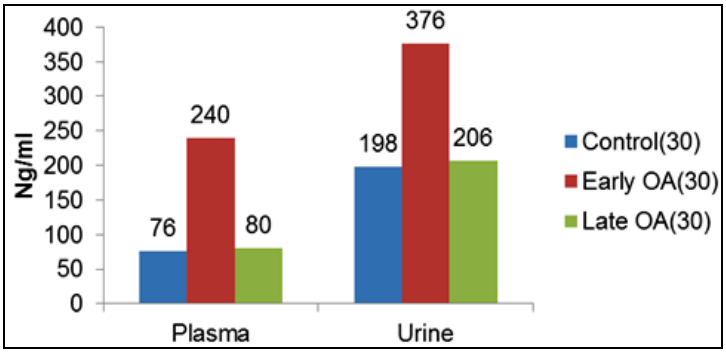


CHART 1: SHOWING DIFFERENT LEVELS OF MPO IN CONTROLS AND EARLY AND LATE OA

DISCUSSION: Our results demonstrate the presence of statistically significant elevation of Plasma and Urine MPO levels in Early OA patients as compared to Late OA and control group. Our present findings are consistent with MPO levels in synovial fluid published by a recent study by Marla J. Steinbeck *et al* [1]. The data presented here provide evidence that the presence of MPO in plasma and urine is indicative of an inflammatory involvement in the development of OA. It indicates the overexpression of mediators of inflammation within the synovial membrane (synovitis) of patients with early, but not late OA [3, 4, 5].

Biochemistry of OA: In the early stages, there is infiltration and activation of neutrophils and macrophages. This leads to production of ROS that include, $O_2^{\cdot-}$ H_2O_2, HOCl, and Cl_2. These products cause oxidative modification of articular cartilage. The two principle enzymes that neutrophils and macrophages employ to produce ROS are NADPH oxidase [7–10] and MPO [11–14] (**see figure 1**). NADPH oxidase catalyzes the formation of $O_2^{\cdot-}$ by reducing oxygen via cytochrome b_{558}. $O_2^{\cdot-}$ rapidly converts to H_2O_2 either spontaneously or catalytically through superoxide dismutase.

Neither $O_2^{\cdot-}$ nor H_2O_2 exhibit significant reactivity with biological compounds; however, MPO converts H_2O_2in the presence of Cl^- to HOCl and under acidic conditions to Cl_2. Of these, HOCl and Cl_2 are very reactive and have been shown to oxidize type II collagen (Col-II), one of the major extracellular matrix (ECM) components of articular cartilage, and increase their susceptibility to proteolytic degradation [6]. Of the known mammalian peroxidases, MPO is the only enzyme that can oxidize Cl^- to Cl^+ at physiologic pH and generate chlorinated products that were detected in the synovial fluids, plasma and urine of patients with early OA.

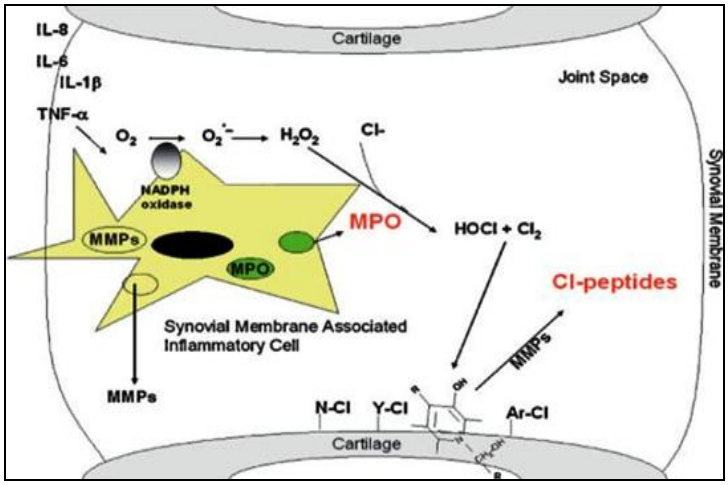


FIGURE 1: BIOCHEMICAL BASIS OF ARTICULAR CARTILAGE DEGRADATION

Recently, Marla J. Steinbeck *et al* [1] showed that HOCl and Cl_2 oxidize pyridinoline (PYD) compounds to form chlorinated moieties [6]. PYD crosslinks are integral members in the structural composition of articular cartilage that serve to covalently link helical regions of Col-II to one another, type IX collagen (Col-IX) to the surface of Col-II, and Col-IX to other molecules of Col-IX. This high degree of cross linking functionally stabilizes the collagen fibrillar superstructure, thus making it more resistant to proteolytic degradation.

In addition to its importance in the maintenance of the collagen superstructure, PYD also contains amine and phenol groups that are sensitive to HOCl and Cl_2 modification. Specifically, HOCl and Cl_2 are capable of displacing the phenol group on an aromatic ring of PYD to produce chlorinated 3-chloro-products. They also react with primary amines to generate long-lived N-chloramines, which have a lower oxidizing potential than HOCl and Cl_2, but they have longer lifetime (~18 h) and as such may be responsible for distant damage.

MPO is marker of active ongoing inflammation. In controls; the articular cartilage is in perfectly healthy state without any inflammation, so MPO levels are normal. In Late OA, even though there is active inflammation but there is no cartilage remaining for destruction, so MPO levels are within normal limit. But in Early OA, active inflammation is there and articular cartilage is also present for destruction so MPO levels are significantly high as compared to late OA and controls. This can be properly illustrated by **figure 2** given below.

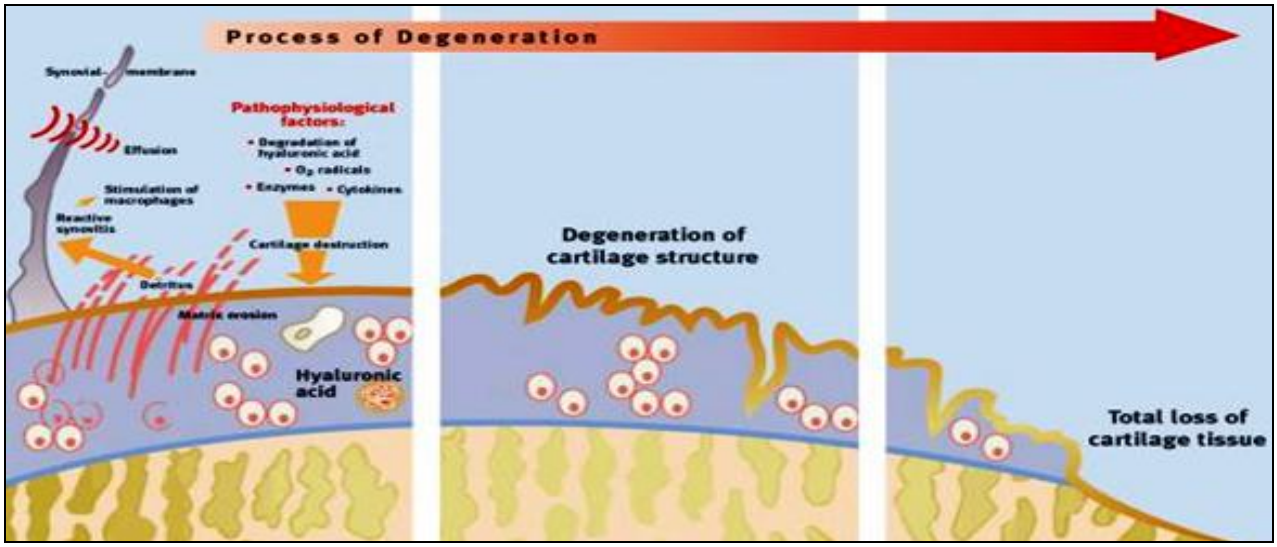


FIGURE 2: DIFFERENT STAGES OF CARTILAGE DEGENERATION

Current diagnosis of OA is based upon radiographic and clinical data. New tools and criteria for diagnosing and measuring the progression of OA are currently under investigation, including the development of biomarkers.

We have shown that the presence MPO in plasma and urine is unique to subjects with active cartilage degradation. It is very specific marker for early OA. As such, the presence MPO in plasma and urine should serve as important biomarkers for the early diagnosis of OA, and may be useful in determining disease progression. Availability of such "markers" would aid clinicians in the diagnosis of this condition at an early stage with potential for better delivery of care to preserve the joint.

ACKNOWLEDGMENTS: The authors would like to thank the Dept. of Biochemistry & Dept. of Orthopaedic, B.J. Medical College and Sassoon General Hospital, Pune, Maharashtra, India, for their help and support for this work. None of the listed authors has any professional and/or financial affiliations that may be perceived to have biased this presentation.

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How to cite this article:
Girish S, Chandanwale A and Adhau Shyam: Myeloperoxidase as a potential Biomarker in different stages of Osteoarthritis. *Int J Pharm Sci Res*. 3(11); 4477-4481.