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Biochemistry

# NITRIC OXIDE AND HSCRP IN STAGES OF KNEE OSTEOARTHRITIS

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| **Dr. Nilima J. Gupta** | Assistant Professor, Department of Biochemistry, B. J. Govt. Medical College and Sassoon General Hospital, Pune -411001. - Corresponding Author |
| **Dr. S.G.Varma** | Professor and Head, Department of Biochemistry, B. J. Govt. Medical College and Sassoon General Hospital, Pune -411001. |
| **Dr. Sunita Girish** | Associate Professor, Department of Biochemistry, Grant Medical College and Sir JJ Group of Hospitals, Mumbai-400008. |

**ABSTRACT** **Background:** Osteoarthritis is a leading cause of physical disability and impaired quality of life. There is increasing evidence of production of proinflammatory cytokines and mediators as early features of Osteoarthritis. Nitric oxide is formed by the conversion of arginine to citrulline. Acute phase reactant, C-reactive Protein (CRP) is important in nonspecific host defence against inflammation. Highly Sensitive C-reactive protein (hsCRP) is the most widely used biochemical marker of systemic inflammation. Aim and **Objectives:** The aim of the study is to measure serum hsCRP and Nitric oxide levels and to correlate between NO and hsCRP. Material and **Methods:** 60 newly diagnosed, untreated patients of knee osteoarthritis were selected, 30 each from early and late disease. 30 age and sex matched controls were selected. Levels of hsCRP and NO were estimated by standard methods.
**Results:** There was highly significant rise in hsCRP and NO value in early and late knee OA patients as compared to controls. The rise in hsCRP value was significantly high in late knee OA patients, while the rise in NO value was significantly high in early knee OA. There is mild positive correlation between hsCRP and NO in early knee OA while negative correlation in late knee OA. Conclusion: Higher serum hsCRP concentrations in late knee OA indicate association with severity and progression of disease. Higher NO levels in early OA suggesting its role in disease pathogenesis. High NO levels in early OA may help in early detection and timely treatment.

**KEYWORDS :** hsCRP, Inflammation, NO and Osteoarthritis

**Introduction:**
Osteoarthritis (OA) is a chronic degenerative joint disease which progressively causes loss of joint function and is the leading source of physical disability and impaired quality of life. Knee OA has higher prevalence rate as compared with other types of OA and also its presentation is at earlier age groups particularly in younger age groups of obese women[1,2,3].

Osteoarthritis is characterized by progressive destruction of articular cartilage, thickening and fibrosis of joint capsule and changes in subchondral bone within synovial joint. These changes are thought to be due to different biochemical changes in the articular cartilage [4,5]. Studies have shown association of systemic inflammatory markers with severity or clinical course of OA. Serum C-reactive Protein (CRP) is the most widely used biochemical marker of systemic inflammation. It has been shown that CRP in synovial fluid of OA patients is well correlated with the degree of inflammation[6].

Nitric oxide (NO) is formed by the conversion of L-arginine to citrulline which is catalyzed by the enzyme nitric oxide synthase (NOS). Nitrite, nitrate are used as biomarkers of NO formation[7,8]. NO has been studied in synovial fluid, serum of knee OA patients and has shown increase in NO level in OA patients[9,10]. Though these two markers are well studied no one tried to find correlation between these two markers at different stages of disease. Hence the present study is an attempt to estimate and correlate high sensitive C-reactive protein (hsCRP) and NO in early and late knee osteoarthritis.

**Material and Methods:**
The present study is descriptive study with cross sectional design and has been carried out in the Department of Biochemistry in collaboration with Departments of Orthopaedics of B.J. Medical College and Sassoon General hospital, Pune from May 2012 to August 2014. The study protocol was approved by the Ethics Committee of the Institute. Informed written consent was obtained from all the study subjects enrolled in the study.

**Selection of Study Subjects:**
Sixty newly diagnosed, untreated patients of knee osteoarthritis were selected for the study. 30 patients each from early (grade 1 and 2) and late (grade 3&4) knee OA from the orthopaedic OPD and ward of this institute and who were willing to participate in the study were selected based on Kellgren-Lawrence grading score. Patients were diagnosed by experienced orthopaedic surgeons based on clinical features and radiography. 30 age and sex matched apparently healthy subjects were selected as controls for comparison. Levels of hsCRP and NO were estimated by standard methods in both groups and controls. So, three groups in this study were,

Group A = Early Osteoarthritis patients (K-L grade 1, 2)
Gryoup B = Late Osteoarthritis patients (K-L grade 3, 4)
Group C = Controls

We excluded patients suffering from other arthropathies such as rheumatic diseases and having chronic systemic illness, other inflammatory diseases. We also excluded alcoholic and chronic smokers from the study.

**Collection of Blood Samples and Storage:**
Random blood samples were collected from the Cubital vein. About 3ml blood was taken in plain vacutainer for estimation of serum hsCRP & NO. Serum was separated by centrifugation at 3000 rpm for 10 minutes and stored at -200C in the deep freezer until further analysis.

**Facilities and Equipment:**
**High sensitive C Reactive Protein (hsCRP):**
To estimate serum hsCRP levels, we used quantitative turbidimetry (Spinreact; S.A./S.A.U. Clra.Santa Coloma, Spain) and followed manufacturer's instruction. Latex particles coated with specific anti-human CRP were agglutinated when mixed with samples containing CRP. The agglutination causes an absorbance change measured at 540nm in ERBA CHEM 5 plus V2 semi autoanalyzer, depending upon the CRP contents of the patient sample that can be quantified by comparison with a calibrator of known CRP concentration. Values less than 0.05 mg/L give non-reproducible results with this method. Sample with higher concentration diluted 1/3 by NaCl 9g/l and retested. Undetectable CRP values were recorded as 0.025 mg/L. detection limit is 0.05 to 5mg/l [11,12].

**Estimation of Serum NO end products as nitrite and nitrate[13,14]**
To measure the level of NO in the samples, we used a colorimetric stain (azo dye) for clear 96 well plates format and followed the manufacturer's instructions. In brief, enzyme nitrate reductase was used to convert nitrate to nitrite. Nitrite is then detected as coloured azo dye product of Griess reaction that absorbs visible light at 540nm. The

interaction of NO in a system is measured by determination of both nitrate & nitrite concentration in sample. The relative levels of nitrite and nitrate can vary substantially, therefore the most accurate determination of total NO production requires quantization of both nitrate and nitrite.

**Statistical Analysis:**

Data analysis was done using the SPSS (Statistical Package for the Social Science) Version 11 for window. The ANOVA test was used to find out significant difference in average values between 3 groups; 'Z' test was used to find significant difference between correlation values of 2 groups. Chi-square test was used to show association between variables in 3 groups. Scatter diagram is also used to show correlation between two variables. A probability value of 0.05 was accepted as the level of statistical significance. P-value less than 0.05 & 0.01 are considered to be statistically significant (S) & highly significant (HS) respectively. P-value more than 0.05 is considered to be statistically non-significant (NS).

**Results:**

The general demographic features of study groups are shown in table 1 of group A has 8 males and 22 females with mean age 48.87years. Group B has 10 males and 20 females with mean age 50.17 years and in Group C has 10 males and 20 females with mean age 48.50. Age and sex wise difference between group A, B and C is statistically not significant (P>0.05) so groups are comparable. (Table 1)

In this study, we observed a highly significant rise in hsCRP value in early and late knee OA patients as compared to controls (1.81 ± 0.93) (P value <0.01). The rise in hsCRP value is highly significant in late knee OA patients (5.96 ± 2.84) as compared to early knee OA patients (3.59 ± 2.09) (P value <0.01). (Table2)

We observed highly significant rise in NO value in group A and group B as compared to group C (55.84 ± 20.47) (P value <0.01). The rise in NO value is highly significant in group A (104.93 ± 28.37) as compared to group B (96.46 ± 31.49) (P value <0.01). (Table 2)

**Table 1 : Age and sex wise distribution of study subjects**

| Parameters | Group A (n=30) | Group B (n=30) | Group C (n =30) | P value |
|---|---|---|---|---|
| Age (years) | 48.87 ± 8.35 | 50.17 ± 4.83 | 48.50 ± 5.83 | >0.05 |
| Sex (M/F) | 8/22 | 10/20 | 10/20 | >0.05 |

M – male, F – female

**Table 2: Comparison of Serum hsCRP and NO Values in Study Groups**

| Parameters | Group A | Group B | Group C | F test | P value |
|---|---|---|---|---|---|
| | Mean ± SD (n=30) | Mean ± SD (n=30) | Mean ± SD (n=30) | | |
| hsCRP (mg/L) | 3.59 ± 2.09 | 5.96 ± 2.84 | 1.81 ± 0.93 | 29.34 | P<0.01 |
| NO (µM/L) | 104.93±28.37 | 96.46± 31.49 | 55.84± 20.47 | 27.97 | P<0.01 |

**Gr. A Vs Gr. B: P<0.01 Gr. A Vs Gr. C: P<0.01 Gr. B Vs Gr. C: P<0.01**

In our study, there is statistically significant difference observed in correlation value of hsCRP and NO in early and late osteoarthritis patients. (Table 3)

**Table 3: Comparison of Correlation Values between hsCRP Values and NO Values in Early and Late Osteoarthritis subjects**

| Correlation coefficient | Group A | Group B | Z test | P value |
|---|---|---|---|---|
| HSCRP vs NO | 0.20 | -0.27 | 1.76 | P < 0.05 |

**Fig. 1: Scattered Diagram showing Correlation between Serum hsCRP and NO in Study Subjects of Group A**

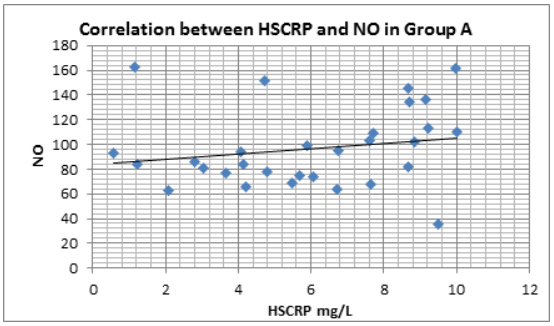
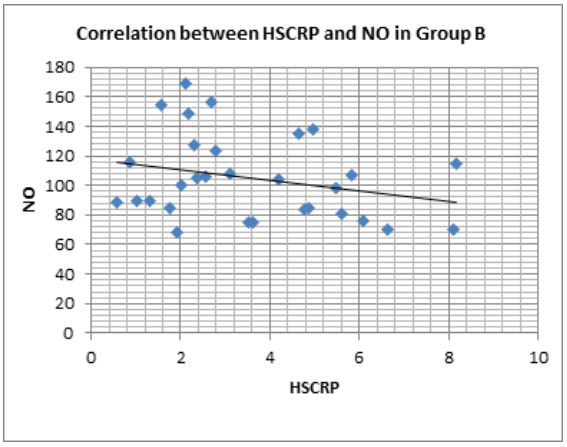



**Fig. 2: Scattered Diagram showing Correlation between Serum hsCRP and Plasma NO in Study Subjects of Group B**




**Discussion:**

In this study, we have observed highly significant rise in hsCRP value in early and late knee OA as compared to controls (1.81 ± 0.93)(P value <0.01). The rise in hsCRP value is also highly significant in late knee OA (5.96 ± 2.84) as compared to early knee OA (3.59 ± 2.09) (P value <0.01). (Table 2)

These findings are in accordance with the findings of that of Sharif *et al* (2000) which show CRP level at one time point are related to radiographic progression over a distant and prolonged period . Raised serum CRP also reflects events that precede a period of cartilage loss and later radiographic progression in knee OA[15]. According to Stu¨rmer et al (2004), there is most strong association of intensity of pain with low level rises in the serum hsCRP levels in advanced OA patients, whereas radiographic severity or extent is associated with serum hsCRP levels[6].

In response to inflammation tissue necrosis factor α (TNFα) and interleukin1 (IL1) are released; they stimulates interleukin6 (IL6) which stimulates synthesis of CRP from liver [6,16].

Serum hsCRP concentrations are higher in individuals with OA and are associated with OA severity as well as progression of disease. Thus, indicating role of inflammation in pathogenesis of OA.

We observed highly significant rise in NO value in group A and group B as compared to group C (55.84 ± 20.47) (P value <0.01). The rise in NO value is also highly significant in group A (104.93 ± 28.37) as compared to group B (96.46 ± 31.49) (P value <0.01). **(Table 2)**

Farrel et al (1992) proved that serum nitrite concentrations were increased in patients with rhuematoid arthritis (RA) and osteoarthritis (OA) compared with controls; Sakurai et al (1995), Ersoy et al (2002) observed increased concentrations of nitrite in synovial fluids and sera from RA and OA patients which is consistent with our results showing increase in NO in group A and group B and proposed deep tissue involvement in joint. [17,9,10]

Nitric oxide synthase (NOS) exists in three isoforms, neuronal (nNOS), inducible (iNOS) and endothelial (eNOS), and it is iNOS isoform, which is predominantly responsible for NO production in

articular cartilage. Neuronal constitutive form of NOS along with iNOS was also demonstrated in OA affected cartilage. nNOS activity has been found to be increased in human chondrocytes with OA as compared to normal chondrocytes.

NO is produced in large amounts by osteoarthritic chondrocytes in response to proinflammatory cytokine stimulation. Many studies have proved that NO is expressed in human osteoarthritis cartilage as demonstrated by immunostaining with anti-nitrotyrosine antibodies. The inducible nitric oxide synthase (iNOS) enzyme is also upregulated in OA chondrocytes, resulting in an excess of NO production and facilitating the release of inflammatory cytokines and other catabolic processes.[7,8,10,15,17,18,19,20]

NO is also implicated in chemotaxis of polymorphonuclear leucocytes indicating its role in inflammation. NO reacts with superoxide (O2•–) lead to the formation of peroxynitrite (ONOO–), a potent pro-oxidant with cell damaging effects. In cartilage, it activates metallo proteinases, promotes chondrocyte inflammatory responses and mediates chondrocyte apoptosis. NO inhibits both proteoglycan and collagen synthesis. All of these activities contribute to the catabolic consequences of NO in cartilage leading to cartilage damage. In widespread synovial inflammation, serum nitrite might increase when synovial fluid cleared by the lymphatic system enters the systemic circulation and by equilibration reach into the vascular compartment within the synovium.[8,10,15,17,18]

In our study, we observed positive correlation of HSCRP vs NO in Group A (Fig. 1) with Correlation coefficient of 0.20 and slight negative correlation Group B (Fig. 2) with Correlation coefficient of -0.27 (minus). In early OA, active inflammation suggested by raised hsCRP and availability of sufficient articular cartilage for destruction are responsible for significantly high levels of NO in blood as compared to late OA and controls. But in Late OA, even though there is active inflammation as indicated by high hsCRP levels, very little cartilage is remaining for destruction. As disease progresses; there is thickening of capsule and synovium and also decrease in cellular activity. There is marked fibrosis of the capsular tissues in late stages decreasing escape of NO molecules into the blood.

Though studies have shown inhibition of eNOS by CRP; we can't rule out inhibition of other isoforms of NOS by CRP. Combine effect of these factors may cause less increase in NO levels in late stages as compared to early.[21]

To the best of our knowledge, though previous studies have proved presence of hsCRP and NO in the serum, there is paucity of study of these markers in the stages of disease along with correlation between them.

We believe that the results of our present work combined with previous studies provide compelling evidence of presence of inflammatory markers in knee osteoarthritis, and further high levels of them in early stages. These findings may have important clinical relevance.

**Limitations :**

Study is done in small number of subjects which may not reflect entire population. Despite some limitations, the associations we found were highly significant and consistent with other studies addressing the above mentioned associations. Further Longitudinal studies with repeated assessments of these biochemical markers are required; as there is scarcity of data available on pathogenesis and mechanisms osteoarthritis development and progress. Further studies are required to find out the molecular and genetic basis of osteoarthritis.

**Conclusion :**

We can conclude that, detection of hsCRP supports inflammatory mechanisms contributing to the OA progression; and increase in hsCRP levels are suggesting association with radiographic progression as there is highly significant rise in hsCRP levels in late knee OA as compared to early knee OA. There is highly significant rise in levels of NO in early and late knee OA; suggesting that this inflammatory mediator have role in OA pathogenesis and development. There is high levels of NO in early knee OA as compared to late knee OA, which is suggestive of NO can detect disease in early stages.

Correlation study between these two parameters in early and late stage of disease is suggestive of complex role of NO and CRP in OA pathogenesis. Combination of these parameters can be used to assess progression of knee OA. This can help in timely treatment and to delay in disease progression. In future, these molecules can be target for therapy as treatment available today is only analgesics and in late stages surgery.

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