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Original Research Article

A Case Control Study Evaluating Pro-Inflammatory Cytokines and Degeneration Biomarkers in Various Stages of Knee Osteoarthritis

Ankita Dwivedi[1]*, Dr. Umesh K.More[2], Dr. Sanjay Deo[3],Dr.Sarita A. Shinde[4]

[1]*Research Scholar, Dr. D. Y. Patil Medical College, Hospital and & Research Centre, Dr.D.Y. Patil Vidyapeeth (DPU) Pimpri, Pune – 411018.

[2]Professor, Dept. of Biochemistry, Dr. D. Y. Patil Medical College, Hospital and & Research Centre, Dr. D.Y.Patil Vidyapeeth (DPU) Pimpri, Pune – 411018.

[3]Professor, Dept. of Orthopaedics, Dr. D. Y. Patil Medical College, Hospital and & Research Centre, Dr. D.Y.Patil Vidyapeeth (DPU) Pimpri, Pune – 411018

[4]Professor, Dept. of Biochemistry, Dr. D. Y. Patil Medical College, Hospital and & Research Centre, Dr.D.Y.Patil Vidyapeeth (DPU) Pimpri, Pune – 411018.

***Corresponding Author: -** Ankita Dwivedi

*Dr. D. Y. Patil Medical College, Hospital and & Research Centre, Pimpri, Pune – 411018.

ABSTRACT:

Objective: This study was conducted to evaluates the levels of pro-inflammatory cytokines and biomarkers in serum in various stages of osteoarthritis and establish their correlation with stages of Knee Osteoarthritis (OA).

Methods: This case control study was conducted in the Department of Orthopaedics and Biochemistry in Dr. D.Y. Patil Medical College & Hospital, Pune. a total of 95 subjects between the age of 40 and 75 years were included out of which there were 63 cases of knee osteoarthritis, and 32 normal healthy subjects were included in control group. Pain at rest was assessed using visual analogue scale (VAS) and patients were assessed on the American College of Rheumatology (ACR) criteria to determine the severity. Serum samples were collected from all the recruited patients and serum levels of IL-1β, TNF-α, IL-6 , CTX-I and CTX-II were measured using ELISA.

Results: In our study cohort, 63 patients were osteoarthritis cases based on Kellegren & Lawrence grading of OA Knee. The most prevalent K/L grade was Grade 2 cases followed by grade 1. Average weight and average BMI of the cases was significantly higher than average weight and average BMI of the controls. Both cytokines TNF-α and IL-6 were significantly higher in the patients of Knee OA. Correlations of Bio markers with OA grades revealed a significant positive correlation between OA grades and IL-6 as well as TNF-α. Increased levels of CTX-I were also found indicating bone degradation in the Knee OA group.

Conclusion: The present study demonstrated that TNFα and IL-6 had a significant correlation with the grading of Knee OA. TNFα and IL-6 were found to be correlated with pain and therefore could serve as biomarkers of disease progression.

INTRODUCTION:

Osteoarthritis (OA), especially Knee osteoarthritis is a well-studied chronic joint degenerative disorder which is characterized by the loss of components of articular cartilage and this loss is result

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of imbalance between destruction of extracellular matrix and repair (1). Most of the structure of joint is affected in this disorder along with the effect on synovial membrane and subchondral bone (2). The exact mechanism for the pain in the knee in case of OA is not clear, however it is believed that it is due to the local chronic inflammation of the knee joints, which is mediated by the production of various inflammatory cytokines in the synovial membrane like tumour necrosis factor alpha (TNFα), interleukin (IL)-6, IL-1β and TNF-alpha (3). Moreover, some specific cytokines also provoke the damage of the extracellular matrix of the joint tissues. Few cytokines have also been found as the biomarkers of the severity of OA and pain associated with it. Findings of some recent studies have shown that pain mechanism in OA might also be related to these biomarkers associated with the degradation of bone and cartilage (11).

These cytokines contribute towards the pathogenesis of OA by increasing the degradation of cartilage and induction of hyperalgesia through various number of direct and indirect actions. It has been well-explored that IL-1β leads to destruction of cartilage, and TNFα is responsible for the process of inflammation. TNFα activates sensory neurons directly via its receptors and initiates a cascade of inflammatory reactions via the production of ILs. Sensory neurons are activated by TNFα through its receptors, and this initiates a cascade of inflammatory reactions via ILs production (6). These mediators further induce synovial cells and chondrocytes to produce other pro-inflammatory cytokines such as IL-6 (4,5). Number of inflammatory cells are increased in the synovial tissue due to the increased production of IL-6 as it a pro-inflammatory cytokine. This stimulates the proliferation of chondrocytes and effect of IL-1β is amplified. Moreover, it has also been reported that IL-6 plays a complex role in pathogenesis of OA by initiation of inflammatory responses like production of metalloproteinase tissue inhibitors and limit the damage of cartilage via negative feedback (6). In case of long-term radiographic progression in persistent chronic arthritis, collagen type I (CTX-1) and type II (CTX-II) degradation markers reflect breakdown of bone and cartilage and are good predictors of long-term radiographic progression in chronic persistent arthritis (7). It has been reported by Streich et al., that increased level of CTX-1 level is correlated with increased local production of inflammatory cytokines which further results in uncoupling of bone turnover and finally degradation of cartilage (8). Correlation between levels of CTX-I with K-L grading of knee arthritis and joint space width has been well-established which reflects the general pathological changes in the knee (9).
It has been recently shown that that both the degradation products viz. CTX- I and CTX- II measured in the urine of patients at baseline, highly predicted the five year radiographic progression, especially in patients who did not have any visible damage.

It was also found that changes in CTX- II induced by the therapy very well predicted the radiographic progression of disease independent of the disease activity markers (10). As the degradation of collagen could be reflective of cartilage destruction in the joints of patients, this serves as a potentially useful and specific biomarker for evaluating radiographic progression. Both CTX- I and CTX- II are like process variables, these biomarkers are appropriate to evaluate the relationship between the inflammation, bone and cartilage destruction than the radiographic damage
All this suggests that the study should be conducted to explore the dynamic state of cytokines in OA. Under the hypothesis that relationships between these cytokines and clinical evaluations in OA patients are possible, the present study evaluated the association between proinflammatory cytokines and biomarkers from the patients with early stage (Kellegren-Lawrence grade1-2) and late stage (grade 3-4) to those in healthy donors. This classification of OA has been widely used for radiological classification to identify and grade OA where 5 grades have been used (0, normal to 4, severe). The severity is defined by the radiological signs found to be evidence for OA.

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MATERIALS AND METHODS

Study design:

A case- control study was conducted in the Department of Orthopaedics and Biochemistry in Dr. D.Y. Patil Medical College & Hospital, Pune, Maharashtra. The study was approved by Institutional research and ethics committee. (Ref. No. DYPV\EC\388\2019).

Study population:

In this study, a total of 95 subjects between the age of 40 and 75 years were included. A total of 63 cases of knee osteoarthritis of all grades and 32 normal healthy age and gender matched controls were enrolled during the period January 2020 till June 2021. All patients with signs and symptoms pertaining to primary knee osteoarthritis reporting to Orthopedic OPD were screened and those who fit into the clinical criteria of primary knee osteoarthritis as per American college of rheumatology were included in this study. Healthy subjects falling into this age group with no signs and symptoms of osteoarthritis and preferably first-degree relatives of the cases were taken. Patients who received Steroids or any other intraarticular injections, had past knee surgery, Secondary OA owing to fracture, Rheumatoid arthritis, SpA (seronegative spondyloarthropathies), having gout, pregnant and lactating mothers were excluded. A convenient sampling method was used to select the cases and controls.

Informed written consent was obtained from all the participants prior to enrolment. All the patients were assessed clinically, and diagnosis was confirmed using radiographs. Grading of osteoarthritis was done using kellgren and lawrence classification.

Data collection:

Demographic data like age, gender, weight, height, body mass index(BMI), occupation and history of performing strenuous activities were taken.

Tests and examinations conducted:

Systemic and local examination was performed. Pain at rest was assessed using visual analogue scale (VAS) and patients were assessed on the American College of Rheumatology (ACR) criteria to determine the severity.

5 cc of venous blood was collected from the recruited cases and controls and the samples were centrifuged immediately to separate serum. The serum samples were stored at -80℃ until further analysis.

Serum cytokine ELISA:

The serum samples were analysed for the concentration of 3 Pro-Inflammatory markers IL-1 β, IL-6, TNF-α and 2 cartilage derived markers CTX-I and CTX-II. The concentration was measured by ELISA (Enzyme-Linked Immuno-sorbent Assay) (Elabscience; catalog no:E-EL-H0835) according to the manufacturer's protocols.

100 µl standard or sample was added to the wells and incubated for 90 min at 37℃. Further the liquid was discarded. Immediately 100µl Biotinylated Detection antibody working solution was added to each well and incubated for 60 min at 37℃. The liquid was aspirated and plates were washed 3 times. Following this,100µl HRP conjugate working solution was added and incubated for 30 min at 37℃. The liquid was aspirated and plates were washed 5 times. Further 90µl Substrate Reagent was added and incubated for 15 min at 37℃. Finally, 50µl Stop solution was added and the readings from the plates at 450nm were taken immediately. All assays were performed using the ELISA kits from the same manufacturer with the above method.

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Statistical analysis:
Primary data was collected in paper based proforma and the data was then entered in Microsoft Excel spreadsheets 2016. Statistical analysis was done on IBM SPSS STATISTICS VERSION 20. Categorical variables were taken in the form of frequencies and proportions and cross tabulations were done for the chosen parameters and column proportions were compared using Chi square test. Continuous variables were expressed in the descriptive statistics tables as means, standard deviation, maximum and minimum value and compared using Mann Whitney μ test (for 2 independent samples) and Kruskal – Wallis test (for > 2 independent samples) since the data distribution was not normal. Correlation between variables was performed using Spearman's Rank correlation.

RESULTS

Demographic characteristics of the enrolled patients:
A total of 95 participants (53 males and 43 females) were enrolled in this case control study. 63 osteoarthritis cases based on Kellegren & Lawrence grading of OA Knee were included. Grade 2 cases were the most prevalent (47.6%) followed by grade 1(30.2%). There were 12.7% and 9.5% cases in Grade 3 and 4 respectively. Age and gender matched 32 selected controls without osteoarthritis had similar gender ratio and average age compared to the osteoarthritis cases. Average weight (68.7±5.7 kg) and average BMI (25.3±1.5 kg/m2) of the cases was significantly higher than average weight (64.8±4.9 kg) and average BMI (22.6±6.1 kg/m2) of the controls ($p<0.05$).

Clinical characteristics of the enrolled patients:
41.3 % of the patients were found to have bilateral knee osteoarthritis and left knee was most (58.7%) affected. Early Morning Stiffness was reported by majority (88.9%) of the patients and 100% of the cases reported pain in the affected knee joint. Based on the American College of Rheumatology Clinical criteria for osteoarthritis of the Knee. viz. Age > 50 Years (81.3%), Stiffness < 30 min (40.6%), Crepitus (79.7%), Bony Tenderness (98.4%) ,Bony Enlargement (32.8%) and No palpable Warmth (29.7%) were observed in varying proportions among the cases.

The average duration of complaints related to knee osteoarthritis was 2.43±2.2 years whereas the average pain assessed using visual analogue scale was 6.8 ± 1.

Biomarkers in Osteoarthritis:
Median IL-1Beta in controls (10.97) was similar to median value in cases (10.45). Median IL-6 value (1.9) in cases was significantly higher than controls (0.81) ($p<0.001$). Median CTX-I value in cases (0.03) was significantly higher than controls (0.02) ($p<0.05$) whereas median CTX-II values were comparable (0.11 vs 0.15, $p>0.05$). Median TNF-alfa value in cases (43.96) was significantly higher than controls (12.7) ($p<0.001$).

Correlations of Bio markers with OA grades:
Correlations of Bio markers with OA grades was studied which revealed significant positive correlation between OA grades and IL-6 (Spearman-s rho=0.293, $p<0.05$) as well as OA grades and TNF-alfa (Spearman-s rho=0.263, $p<0.05$). Other biomarkers too showed positive correlations however the correlations were not significant.

The median values of all 5 markers studied were comparable between the cases of age < 50 and age ≥ 50 years, males and females and between the cases with unilateral and bilateral OA. ($p>0.05$) This indicates that the serum concentrations of any of these markers correlated with age, gender or laterality.

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DISCUSSION:

High levels of proinflammatory cytokines have been strongly correlated with the development and progression of OA. This is because of the alteration in expression of few genes, stimulation to produce reactive oxygen species, alteration in the metabolism of articular components and resorption of bones (11, 12). Loss of this functioning has been related to high serum levels of the proinflammatory cytokines (13, 14). A study wherein muscle biopsy was performed to evaluate the strength of muscles in individuals suffering from knee OA and control group which showed that knee OA group had less muscle strength and high level of protein/receptor levels of IL-6 as compared to healthy subjects. IL-1 and IL-8 were also slightly elevated, but this difference was not statistically significant.

In the present study, we examined the level of various pro-inflammatory cytokines and biomarkers in knee OA patients and compared them with the healthy controls. We further compared the levels of pro-inflammatory cytokines and biomarkers in early stage with late stage of osteoarthritis i.e., within grades. This was done to confirm if inflammation plays a substantial role in the development of pain in OA. There was total 95 participants recruited for this case-controlled study of which 53 were males and 43 females. In our study cohort, 63 patients were osteoarthritis cases based on Kellegren & Lawrence grading of OA Knee. The most prevalent K/L grade was Grade 2 cases followed by grade 1 which was similar to study conducted by Shimura et al (14). On the contrary, a study conducted to evaluate the prevalence of OA knee in Indian population of older than 40 years using K-L scale showed that grade 1 is the most prevalent grade among OA patients (15).

Average weight and average BMI of the cases was significantly higher than average weight and average BMI of the controls. This was in coherence with a previous study conducted by Shimuru et al., who also showed that BMI of the patients with advanced-stage knee OA group was significantly higher (16).

IL-1 has been found to be one of the most important cytokines in early stages of OA. Chondrocytes are very sensitive to this cytokine and increase in IL-1 has been found to cause destruction of cartilage's extracellular matrix which further increases collagenolytic activity of metalloproteases further increasing the nitric oxide activity, which leads to induction of apoptosis of chondrocytes (17). However, to the surprise we did not find any increase in IL-1β in the case cohort as compared to the control group. On the contrary, many other studies conducted revealed increased inflammatory protein IL-1β in sera of OA patients with joint effusions compared to controls (18, 19, 20).

Regarding knee OA, one of the most measured cytokines in the literature is IL-6 (21, 22). Similar to various previously conducted studies (21, 23), we observed that serum levels of IL-6 had a nearly twofold increase in patients when compared to controls, comparable to a study that showed increased IL-6 in patients with more severe disease as measured by radiologic knee OA findings [21,23]. TNF is another cytokine which has been compared to other proinflammatory cytokines and shown to be elevated in elderly patients with knee OA (21). Median TNF-alfa value in cases was significantly higher than controls in this study as well (22). As mentioned previously also, bone resorption mediated by osteoclast is mediated by cysteine protease cathepsin K which causes degradation of major protein in bone, type I collage, releasing C-terminal telopeptide of CTX-I which is a measure of resorption of bones for various in vitro, preclinical and clinical studies (7,8,9). Median CTX-I value in cases was found to be significantly higher than controls indicating bone degradation. This result was at par with various previously reported studies wherein OA was

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associated with high levels of CTX-I (24) However, median CTX-II values were comparable in both the groups which was contrary to the previously reported data (24).

Correlations of Bio markers with OA grades revealed a significant positive correlation between OA grades and IL-6 as well as TNF-α. Other biomarkers too showed positive correlations however the correlations were not significant. A previous study similar to our study indicated that the levels of TNFα were positively correlated with OA grading in rheumatoid arthritis patients, whereas no correlation between the concentration of TNFα and OA grade was found (25). This was contrary to the previous study conducted by Orita et al., which showed TNFα was not correlated with the KL grade, whereas IL-6 had a significantly negative correlation (2).

Overall, the present study demonstrated that the concentrations of proinflammatory cytokines can be correlated with the KL grades knee OA patients. TNFα and IL-6 had a significant correlation with the grading. These 2 cytokines, therefore, play a role in the pathogenesis of in osteoarthritic knees in different ways. TNFα and IL-6 were found to be correlated with pain. Future studies with a larger cohort would provide more evidences supporting this data for further clinical use.

Tables:

Table 1: Demographic Characteristics of the Study Subjects

Parameters	Subgroups	Mean	Std. Deviation	P
Age(In years)	Cases (N=63)	60.13	11.22	0.236
	Controls(N=32)	57.22	9.99	
Height (In Meters):	Cases (N=63)	1.65	0.07	0.532
	Controls(N=32)	1.66	0.08	
Weight (In Kg)	Cases(N=63)	68.65	5.56	0.020
	Controls(N=32)	64.77	4.92	
BMI (Kg/m2)	Cases(N=63)	25.29	1.52	0.001
	Controls(N=32)	22.63	6.06	
Gender		Males(n=53)	Females(n=42)	0.275
	Cases(N=63)	38(60.3%)	25(39.7%)	
	Controls(N=32)	15(46.9%)	17(53.1)	

Table 2: Clinical Characteristics of OA cases.

Parameters	Cases(N=63)	
	Frequency	%
Most affected side		
Left	37	58.73
Right	26	41.27
Laterality		
Bi-Lateral	26	41.27
Uni-Lateral	37	58.73
Symptoms		
Early Morning Stiffness	56	88.89
Knee Pain present	63	100
Family history of OA Knee	19	30.16
American College of Rheumatology Clinical criteria for osteoarthritis of the Knee		
Age > 50 Years	52	81.3
Stiffness < 30 min	26	40.6
Crepitus	51	79.7
Bony Tenderness	63	98.4
Bony Enlargement	21	32.8
No palpable Warmth	19	29.7
	Mean	SD
Duration since pain present	2.43	2.23
Visual Analogue Scale Score	6.84	1.072
Kellegren & Lawrence grading of OA Knee		
Grade 1	19	30.2
Grade 2	30	47.6
Grade 3	8	12.7
Grade 4	6	9.5

Table 3 : Comparison of bio Markers between controls and cases.

	Controls (N=32)				Cases (N=63)				P
	Mean	Std. Deviation	Range	Median	Mean	Std. Deviation	Range	Median	
IL-1Beta	10.78	6.09	17.79	10.97	15.67	13.24	43.33	10.45	0.302
IL-6	1.27	1.15	3.77	0.81	3.9	3.53	13.85	1.9	<0.001
CTX-I	0.027	0.01	0.02	0.02	0.05	0.083	0.64	0.03	0.016
CTX-lI	0.22	0.3	1.06	0.11	0.23	0.2	0.83	0.15	0.106
TNF-alfa	51.95	97.8	496.09	12.69	69.24	42.69	117.14	43.96	<0.001

Table No 4 : Correlations of Bio markers with OA grades.

Correlations							
			IL-1Beta	IL-6	CTX-I	CTX-lI	TNF-Alfa
Spearman's rho	OA GRADE	Correlation Coefficient	0.223	0.293*	.149	.086	.263*
		P	.079	.020	.243	.506	.037
		N	63	63	63	62	63

Table 5 : Correlations of Bio markers with Age, Gender and OA laterality in Cases.

Parameters	Subgroups	Statistics	IL-1Beta	IL-6	CTX-I	CTX-II	TNF-Alfa
Age (In completed years)	Less than equal to 50	Mean	17.99	2.98	0.045	0.22	85.14
		N	14	14	14	13	14
		SD	14.54	3.28	0.029	0.22	42.84
		Median	15.23	1.87	0.035	0.17	118.73
	Above 50	Mean	15.01	4.17	0.07	0.24	64.71
		N	49	49	49	49	49
		SD	12.93	3.59	0.094	0.2	41.99
		Median	9.341	2.97	0.034	0.176	54.79
	P		0.591	0.155	0.649	0.61	0.124
Gender	Female	Mean	13.61	3.88	0.062	0.26	71.92
		N	38	38	38	37	38
		SD	12	3.7	0.11	0.22	41.64
		Median	7.13	2.3	0.03	0.18	66.54
	Male	Mean	18.81	3.95	0.04	0.2	65.18

		N	25	25	25	25	25
		SD	14.63	3.35	0.02	0.16	44.8
		Median	15.31	2.68	0.04	0.13	49.85
	P		0.18	0.679	0.617	0.301	0.465
Laterality	Uni-Lateral	Mean	17.8	4.06	0.04	0.26	68.1
		N	37	37	37	37	37
		SD	13.5	3.56	0.03	0.22	44.1
		Median	15.14	2.53	0.04	0.2	54
	Bi-Lateral	Mean	12.65	3.69	0.07	0.2	70.8
		N	26	26	26	25	26
		SD	12.5	3.56	0.12	0.17	41.3
		Median	6.91	2.82	0.03	0.13	66.9
	P		0.123	0.591	0.878	0.135	0.818

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