ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE CYTOLOGY

FIRST ROUND OBSERVATION ON INCREASED MICRONUCLEI FREQUENCY AMONG RHEUMATOID ARTHRITIS PATIENTS

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ABSTRACT

This study was carried out to assess: i) The spontaneous frequency of cytogenetic damage in lymphocytes of rheumatoid arthritis patients using the Cytokinesis blocked micronucleus assay (CBMN) and ii) To know whether DNA damage occur in rheumatoid patients. Cytokinesis blocked micronucleus assay was performed on cultured peripheral blood lymphocytes of 25 rheumatoid arthritis patients and frequency of micronuclei were compared with 25 age/sex and socioeconomically matched control individuals. The results of the present study revealed that there was statistically significant ($P<0.05$) increase in total micronuclei of rheumatoid patients as compared to control. There was no significant change in the number of apoptotic and necrotic cells among RA patients as compared to the control. However, cells with nuclear buds and nucleoplasmic bridges predominated significantly ($P<0.05$) in rheumatoid patients as compared to controls. As assessed by CBMN assay DNA damage occurs in rheumatoid arthritis patients.

KEYWORDS

Cytokinesis Blocked Micronuclei frequency, Rheumatoid Arthritis, DNA damage

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease influenced by both genetic and environmental factors[1-2]. The disease is characterized by inflammation of the synovial tissue and local articular damage[3]. RA is a chronic systemic inflammatory illness with prevalence of approximately 0.75% in India[4]. Disability in this inflammatory polyarthritis primarily stems from progressive bone erosion and co-morbidity with coronary artery disease, infection and lymphoma[5-6]. As with many other autoimmune conditions, RA affects women more commonly than men. RA is the most common form of arthritis, affecting 0.3% to 1% of the adult population, mainly women after the age of fifty years[7-8]. RA is associated with severe disability and substantial morbidity[9-10] and there is a growing recognition of premature RA-related mortality[11-16].

Rheumatoid arthritis results in chronic pain, disability, fatigue, and loss of productivity both in the workplace and at home. Its impact extends beyond chronic pain and inability to function normally. In particular, there are significant economic burdens attributable to the disease, which affects society as a whole, as well as individual patients and their families. Work disability in patients with rheumatoid arthritis occurs early and increases over time, and is a major driver of its economic impact. Rheumatoid arthritis is also associated with cardiovascular disease and a range of other important co-morbidities which shortens the life expectancy[17].

Rheumatoid Factor (RF), a circulating antibody to immunoglobulin G, is a key serum analyzer used in diagnosis of RA as well as an aid for the prognosis of RA-severity[3]**.**Although the etiology of RA is presently unknown, studies of RA heritability in two Northern European regions have demonstrated that an average of 60% of the disease variance can be attributed to genetic factors[2].

The association of certain chromosomal aberrations with arthropathy has been previously described, but there are limited numbers of reports in the literature. Some uncertainties have arisen regarding the significance of two types of studies performed (those looking for genetic markers in the affected families and those examining family patterns in larger groups), the methods used to treat RA for which varied criteria are available, and the age of study subjects, i.e. the increase in prevalence of the disease over time. Studies of families and of twins have suggested that a genetic contribution to RA is low, with familial occurrence being as attributable to the common incidence of the disease as well as similar environments among family members. There are some families who have affected members much more frequently, though, and for these people, this conclusion may be false. Chronic arthritis has been associated with chromosome deletion 22q11.2[18]. According to Athreya *et al.*[19] particular chromosomal polymorphisms were seen in a proband and her grandmother with the coagulation abnormality (bleeder's disorder) and Rheumatoid arthritis.

Analysis of cytogenetic alterations, sister chromatid exchanges and cell proliferative abilities in cultured peripheral blood lymphocytes of patients with acute and chronic reactive arthritis as well as rheumatoid arthritis were studied earlier , whereby no increased risk of genetic alterations were found. However, two children, one with 18q deletion syndrome and another with supernumerary marker chromosome 15, both presented juvenile idiopathic arthritis type disease, aggressive

progression and moderate response to inflammatory, corticosteroid, and immunosuppressive treatments[20].

To date, few cytogenetic studies have been carried out on RA patients, yet the parameters taken into consideration are more commonly routine Chromosomal aberrations or Sister chromatid exchange frequency estimates. These parameters require more time and are cumbersome in addition to their sensitivity. Cytome assay is relatively more sensitive short term assay to monitor DNA damage. Earlier genotoxicity assessment using micronuclei assay from oral mucosa cells in rheumatoid arthritis patients was carried out[21]. These authors made a comparative assessment between RA patients receiving the drug treatment and the non receivers of drug and found that genotoxicity is associated with RA itself and not with the drug use.

Since 1985, Cytokinesis- block micronucleus test (CBMN) in human peripheral blood lymphocytes has been accepted by many laboratories as an optimal method for evaluation of genotoxic effect[22-23]. It is known that micronuclei (MN) increase is a consequence of chromosomal damage in dividing cells[22], and that there is a positive correlation between these two biological endpoints[24]. This test has been applied to the biological monitoring of human populations exposed to mutagenic and carcinogenic agents. Micronuclei are extra nuclear bodies composed of chromosomal fragments or entire chromosomes that were not incorporated into daughter nuclei at mitosis. They result from chromosome breakage or interference with the mitotic apparatus, events thought to be related to carcinogenesis[25]. The aim of our study was to determine Cytokinesis Blocked Micronuclei (CBMN) frequency in peripheral blood lymphocytes of patients diagnosed with RA factor, before any application of medication.

MATERIALS AND METHODS

Table I indicates the particulars of rheumatoid arthritis patients considered in the study group. For comparison 25 age/sex matched individuals with similar socioeconomic conditions and who had not any relative or history of familial Rheumatoid Arthritis were selected as control group. Blood samples of control and RA patients were processed simultaneously using the same batch of chemical reagents. Whole blood lymphocytes cultures of 25 control and 25 Rheumatoid Arthritis (RA) patients was set up in parallel following the standard protocols[26]. After 44 hour of initiation of cultures Cytochalasine-B was added in a final concentration of 6µg/ml of culture. These cultures were terminated after 72 hours of incubation. The cells were spun at low speed, supernatant removed and then treated briefly with chilled 0.075 M KCL, for about 5-9 minutes

.

Table I

Particulars of rheumatoid arthritis patients considered in the study group (Data pooled from 25 patients)

Mean Age	(M/F)	Age at onset	Family History	Common symptoms				Psycho/ social condition	Mean RA factor (Units/ml)
				Joint pain	Joint swelling	Joint stiffening	Any other		
44.36 ± 3.63	79% F 21% M	41.07 ± 3 years	84% Sporadic and 16% familial	50% whole body pain, 50% specific regions like knee, wrist, shoulder and ankle	58% had symptomatic joint swelling	47% had joint stiffening in morning	80% had gait and movement problem	90% mentally stressed	180 ± 20

The cells were again centrifuged, supernatant removed and fixed in Carnoy's fixative. After 2 to 3 washes of fixative, air dried preparations were made. Slides were stained with May Grundwald stain. For the CBMN analysis, 1000 binucleated cells were evaluated per subject under a microscope (100X) to identify MN keeping in mind the standard guidelines[27]. Observations were recorded on master tables and later transferred to a computer file. We applied Students -'t' test to compare the obtained results.

RESULTS AND DISCUSSION

The results of the present study **(Table II)** revealed that there was statistically significant increase in total micronuclei of RA patients as compared to control at $P<0.05$. During scoring of slides, both cytotoxicity score as well as DNA damage indices were considered. Cytotoxicity measurement parameters included number of binucleated, multinucleated, apoptotic and necrotic cells. While presence and frequency of micronuclei in either mononucleated, binucleated or multinucleated cells were considered as DNA damage indices. Further number of cells with nuclear bridges and nuclear buds were also recorded **(Plate I)**.

There was no significant change in the number of apoptotic and necrotic cells among RA patients as compared to the control. However, cells with nuclear buds and nucleoplasmic bridges predominated significantly ($P<0.05$) in RA patients as compared to controls.

The results of the present study are a part of a major project undertaken by the authors to appraise long term efficacy of therapies in rheumatoid arthritis patients. Multiple cytogenetic parameters namely CAs, SCE frequencies, CBMN assay, G2 assay as well as sensitive comet assay are being studied with a randomized, self controlled, case series, follow up approach. Initial results of CBMN were striking and therefore a move towards this study was initiated. In the present paper a comparison of MN frequency among RA patients has been made with control population. These RA patients have been diagnosed and confirmed with the disease condition by appropriate clinical tests. Blood samples had been taken from them before they started receiving any therapeutic medication so as to rule out the possibility of the effects of medication on the MN frequency.

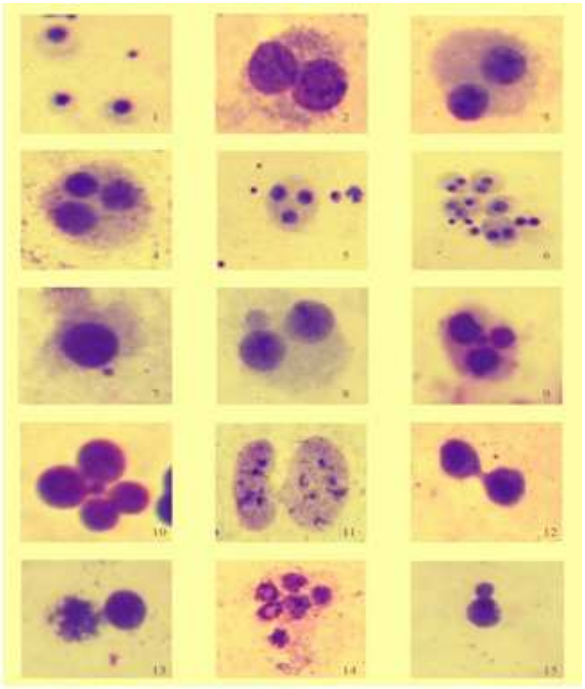

Plate I ***Fig 1: Mononucleated cells,***
Fig 2 and 3: Binucleated Cells,
Fig 4 and 5: Trinucleated Cells,
Fig 6: Cluster of mononucleated cells, Binucleated and Trinucleated cells
Fig 7: Mononucleated cell with single micronuclei,
Fig 8: Binucleated cell with single micronuclei,
Fig 9: Trinucleated cell with single micronuclei,
Fig 10: Tetranucleated cell with single micronuclei,
Fig 11 and 12: Nucleoplasmic Bridges,
Fig 13: Apoptosis,
Fig 14: Cell Necrosis,
Fig 15: Nuclear bud

Table II

Results of Cytokinesis Blocked Micronuclei assay from Rheumatoid Arthritis patients compared with control (Data pooled from 25 proband)

Proband	Cytostatic /cytotoxicity score					DNA damage indices in BN cells				
	Total no of cells scored	No of BN cells	No of MLN cells	No of apoptotic cells	No of necrotic cells	No of mono-nucleated cells with MN	No of BN cells with MN	Total no of MN	No of BN cells with NPB	No of BN cells with NBud
Pooled values control	23526	18534	4932	71	118	60	270	312	36	252
Percentile values for control	1000	788	210	03	05	03	11	13	02	11
Pooled values RA Patients	25050	20326	4557	100	150	167	943	1040	254	904
Percentile values for RA patients	1000	811	182	04	06	07	38*	42*	10*	36*

**** Significantly different at P< 0.05***
MN= Micronuclei, BN = Binucleated, MLN = Multinucleated, NPB=Nucleoplasmic Bridges, NBud= Nuclear bud

The findings clearly indicate that total frequency of MN was significantly higher than the control group. The frequency of MN observed in control group does match with background frequencies earlier reported[28]. The findings of the present study are supported by the finding of an earlier report[21], where genotoxicity assessment using micronuclei assay from oral mucosa cells in rheumatoid arthritis patients was carried out.
These authors made a comparative assessment between RA patients receiving the drug treatment and the non receivers of drug and found that genotoxicity is associated with RA itself and not with the drug use. The significance of CBMN assay in genotoxicity monitoring and DNA damage evaluation studies has been well documented in quite a number of reports[22-23, 29-33]. Therefore, no attempt here is made to emphasize the importance of CBMN test.

Increased frequency of MN observed in the present study is a clear indication of higher DNA damage occurring in RA patients. The findings, has a significance since a link between DNA damage and the probable onset of disease condition can be ruled out.

Earlier, approaches to study DNA damage among RA patients have been reported. Many findings have addressed that there is the role of therapeutic drugs to induce chromosomal changes. Dahlqvist[34] studied chromosomal changes in rheumatoid patients treated with CPH82. They found that the frequency of chromosomal aberrations and sister chromatid exchanges in peripheral blood lymphocytes increased significantly after 12 weeks of treatment and remained elevated even after 48 weeks. The number of CAs and SCE were significantly increased in CPH82 treated patients as compared with the RA patients treated with other disease modifying anti rheumatic drugs (sulphasalazine gold, D-penicillamine, azathioprine, methotrexate, cyclophosphamide). Only two patients treated with cyclophosphamide and azathioprine had changes of comparable level. Their results suggested a mutagenic potential of CPH82 similar to that described for other

immunosuppressive drugs and the newer podophyllotoxin derivatives etoposide and teniposide.

Ermis *et al.*[35] earlier studied clonal chromosomal aberrations in cell cultures of synovial tissue from RA patients. The only recurrent aberration, trisomy #7 was found in 6 of 7 cultures. In four cultures, trisomy #7 occurred as a clonal change in up to 20% of the analyzed cells with an increase of the proportion of +7cell with duration of the *in vitro* culture. Apart from this recurrent change, a variety of partly clonal, partly non-clonal numerical and structural chromosome aberrations were observed in all cases. These findings support the view that chromosomal aberrations may play a role in the pathogenesis of invasive growth (although is not a true neoplastic process) of the synovial tissue in RA patients.

Increase in Micronuclei is correlated directly with increase in chromosomal aberrations. The authors of present study are, therefore, investigating CAs and other cytogenetic parameters of RA patients along with CBMN assay. Interestingly positive correlations are being observed (unpublished records due to small sample size). Once a detailed analysis of all cytogenetic parameters is done more specific conclusions will be drawn. However, a preliminary observation on increased frequency of micronuclei in non treated RA patient's blood samples indicates that some level of DNA damage is taking place and probably that might have an influence on the pathogenesis of the disease.

CONCLUSION

As assessed by CBMN assay DNA damage occurs in rheumatoid arthritis patients.

ACKNOWLEDGEMENT

The authors acknowledge Department of Biotechnology, New Delhi for the financial support (RGYI Grant # BT/PR13156/GBD/27/208/2009), and Dr. Bankim Desai (Rheumatologist and consultant Physician) for helping out in blood collection of RA patients.

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