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Original scientific paper

CYTOGENETIC ALTERATIONS IN RHEUMATOID ARTHRITIS PATIENTS TREATED WITH METHOTREXATE AND DRY OLIVE LEAF EXTRACT

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Oxidative stress and inflammation are DNA instability factors for rheumatoid arthritis (RA) patients. The aims of this study were to evaluate cytogenetic alterations in Peripheral Blood Lymphocytes (PBL) in two groups of RA patients: the early and the long-term RA group; and to examine potential of concomitant treatment with Methotrexate (MTX) and Dry olive leaf extract (DOLE) against cytogenetic damage in RA patients after a 3-weeks treatment. A total of 32 RA patients and 10 healthy individuals were included. RA patients were equally divided into four groups: two groups with early phase RA (one treated with MTX alone, the other in combination with DOLE); and two long-term phase RA groups (group with active disease and group with low disease activity)-both treated with MTX and DOLE combination. PBL cultures were screened for chromosome aberrations and micronuclei frequencies. Significantly increased frequencies of micronuclei were shown in active phase RA disease (both early and long-term) but not in the group with low disease activity, as compared to controls. Chromosome aberrations were detected for all 4 RA groups. The highest frequencies of

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micronuclei and chromosome aberrations were found in the long-term active RA group. After 3 weeks-treatment, there were no significant decrease of the micronuclei frequencies compared to baseline, although they were reduced in all RA groups, except for the group with the long-term active disease. High level of cytogenetic damage in RA patients was concordant with duration and activity of the RA disease. At 3 weeks of therapy, neither the combined treatment (MTX+DOLE), nor MTX alone did not affect the frequency of micronuclei formation.

Keywords: Dry olive leaf extract, DNA damage, Micronuclei, Chromosome aberrations, Rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most prevalent inflammatory rheumatic disease, with the reported prevalence in adults of 0,5%-1%, being increased with age (ZLATKOVIC-SVENDA *et al.*, 2014). It is characterized by ongoing synovial joints inflammation and oxidative stress, leading to the affected cells genomic instability (EMERIT *et al.*, 1996). Genomic instability is defined as an increased genomic alterations tendency. Pathological consequences of genomic instability can accumulate during disease development, manifesting in the form of chromosome instability (YUROV *et al.*, 2009). Certain cytogenetic alterations in RA patients' lymphocytes and fibroblasts have already been observed, considered as associated with lymphocyte activation (KARAMAN *et al.*, 2011; KINNE *et al.*, 2001). Reactive oxygen species (ROS), released at the site of inflammation by phagocytic cells, may enter surrounding cells and react with nuclear DNA (BASHIR *et al.*, 1993). In the presence of activated phagocytes, *in vitro* cell cultures have displayed mutagenic and carcinogenic DNA lesions (REUTER *et al.*, 2010). As already reported, peripheral blood lymphocytes (PBL) and fibroblasts culture analyses in RA patients revealed the chromosomal aberrations presence and increased number of micronuclei (KARAMAN *et al.*, 2011; KINNE *et al.*, 2001). RA patients' lymphocytes have shown increased DNA fragmentation levels, resulting from unrepaired DNA damage (BASHIR *et al.*, 1993; KARAMAN *et al.*, 2011). Knowing the well-established link between genomic damage and neoplastic transformation, inflammation in RA patients could also promote carcinogenesis (CIOŁ KIEWICZ *et al.*, 2008).

Methotrexate (MTX) is a known clastogenic agent in mammalian cells, used as a standard therapeutic agent for RA patients. Combination therapies of MTX and bioactive natural compounds are being extensively explored nowadays, aiming to maintain positive effect of the inflammation reduction and at the same time reduce the long-term MTX usage adverse effects during the course of the RA disease (LAEV and SALAKHUTDINOV, 2015; MADHYASTHA *et al.*, 2008). Varieties of complementary and alternative medicines were explored so far as potential treatments for rheumatoid arthritis. Dry olive leaf extract (DOLE) is a polyphenol-rich natural product of olive tree (*Olea europaea* L.) with proved anti-inflammatory, antigenotoxic and antioxidant effect *in vivo* and DNA-protective abilities *in vitro* (SILVA *et al.*, 2006; MILJKOVIĆ *et al*., 2009; ÇOBAN *et al*., 2014; ČABARKAPA *et al*., 2014; ŽUKOVEC TOPALOVIĆ *et al*., 2015). When summarizing these actions, it is a promising agent as the RA supportive therapy.

We have previously demonstrated increased levels of primary DNA damage in both early phase and long-term phase RA patients (ČABARKAPA *et al*., 2016). It was also shown that a 3-weeks supplementation with DOLE combined with MTX could significantly reduce DNA

damage levels in early phase RA patients, but not in patients with the established, long-term disease (ČABARKAPA *et al*., 2016). Adding to our previous research, the aim of the present study was to determine whether a 3-week dry olive leaf extract supplementation combined with methotrexate could affect the peripheral blood lymphocytes frequencies of micronuclei formation in newly-diagnosed RA patients and long-term RA patients, both in active disease and with low disease activity. The second aim was to examine the association between cytogenetic alterations and both disease duration and disease activity RA patients, by comparing long-term and newly-diagnosed disease patients. Prior the combined treatment was introduced, background (baseline) levels of cytogenetic alterations were determined by micronucleus (MN) test and chromosome aberrations assay (CAs) in PBL cultures.

MATERIALS AND METHODS

Participants

Thirty-two patients (25 females and 7 males) diagnosed with RA, participated in this study with a mean (SD) age of 65.1 (11.2) years. Ten healthy individuals (7 females and 3 males) aged 54.22 (9.09) years, were enrolled as non-treated controls with no history of chronic illness. As part of a pilot study, patients were recruited on a voluntary basis at the Institute of Rheumatology, University of Belgrade, Faculty of Medicine, Serbia. This study was approved by the local Ethics Committee (Institute of Rheumatology, Belgrade, Serbia), prior to study initiation. The study was performed in agreement with the Declaration of Helsinki and participants' informed consents were obtained.

Blood sampling

Blood samples were collected from each donor in the morning, before the meal, by venipuncture from the *basilic* vein (at the beginning of the treatment and on the 21st day). The blood amount of 5 mL added with heparin as anticoagulant was taken to set up the whole peripheral blood cultures for cytogenetic analyses. Another 15 mL of blood was taken for routine clinical disease activity checking.

Investigated groups

The study comprised four experimental RA groups and one control group. Patients were recruited to experimental groups with regard to the activity of their RA disease, as assessed with disease activity of 28 joints (DAS28) score. DAS28 score is a commonly used score for disease activity evaluation in RA patients. It is calculated by designated physician, according to The American College of Rheumatology criteria, including the five following components: tender joint count out of 28 joints, swollen joint count out of 28 joints, Patient Global Health visual analogue scale (VAS), Physician Global Health visual analogue scale (VAS) and one laboratory parameter. Since we have used two laboratory parameters: the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), we have calculated DAS28-ESR and DAS28-CRP (HOCHBERG *et al.*, 1992; SOKKA, 2003). Disease activity was set with regard to the achieved DAS28-ESR score as generally agreed, as: DAS28 >5.1 - high disease activity, DAS28 <3.2 - low disease activity and DAS28 <2.6 -remission (HOCHBERG *et al.*, 1992).

Experimantal groups were:

EAc MTX – early active phase patients with RA, newly-diagnosed, started to be treated with MTX alone at the beginning of the study (*n*=8),

EAc MTX+DOLE – early active phase patients with RA, newly-diagnosed, started to be treated with MTX in combination with DOLE at the beginning of the study (*n*=8),

LtLa MTX+DOLE – long-term phase RA patients with low disease activity (DAS 28 ≤ 3.2), diagnosed with RA for more than six months, treated with MTX continuously for at least 6 months prior to this study initiation (*n*=8).

LtAc MTX+DOLE – long-term phase RA patients with active disease (DAS 28>3.2), diagnosed with RA at least six months before, continuously treated with MTX for at least 6 months prior to this study initiation (*n*=8).

Treatment

For the early active RA patients, Methotrexate was introduced at the beginning of the study according to disease activity and in accordance with the physicians' recommended guidelines (10-15 mg/week). Long-term RA patients were on a stabile MTX dose for at least 6 months before being included in the study. Dry olive leaf extract (DOLE) was introduced at the beginning of the study and patients were instructed to take two capsules twice daily (total 760 mg/day). Laboratory parameters collected at the clinic were: serum C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fibrinogen and rheumatoid factor (RF). All other parameters were determined as described in Methods.

Olive leaf extract

Dry olive leaf extract was purchased from Galenika a.d., Belgrade, Serbia and was used in this study in a form of capsule crude extract. Each capsule contained 190 mg of a dry powdered extract, manufactured by the Frutarom Switzerland Ltd. (Wadenswil, Switzerland). The extract was produced applying an ethanol (80% m/m) extraction procedure from the dried leaves of *Olea europaea* L. and standardized to 18-26% of oleuropein. HPLC analysis revealed a complex mixture of phenolic compounds: oleuropein (19.8%), luteolin-7-O-glucoside (0.04%), apigenine-7-O-glucoside (0.07 %), quercetin (0.04%) and 0.02% of caffeic acid (DEKANSKI *et al.*, 2009).

Chromosome aberrations analysis

Peripheral blood lymphocytes from whole blood were cultivated in 10 mL of RPMI 1640 medium with HEPES and L-glutamine (Capricorn Scientific, Ebsdorfergrund, Germany), 10% of fetal calf serum (Capricorn Scientific, Ebsdorfergrund, Germany), 10 mg/mL antibiotics (penicillin+streptomycin; Sigma Aldrich, United Kingdom) and 2% of phytohemagglutinin (PHA-M, Capricorn Scientific, Ebsdorfergrund, Germany). Duplicate cultures for each patient were grown for 72 hours at 37 ºC. Colchicine (Sigma Aldrich, United Kingdom) was added to a culture medium in concentration of 0.1 μg/mL, 2 hours before the end of cultivation period, to obtain metaphases. At the end of cultivation period of 72 hours cultures were centrifugated and cells were resuspended and exposed to hypotonic treatment (0.075 M KCl). After that, cells were fixed three times with methanol and acetic acid fixative (3:1) and each fixation was followed by

centrifugation. Cells were resuspended in 1 mL of fixative after the last centrifugation, and dropped using a pipette onto cooled wet microscopic slides. Slides were drie**d** by passage over the flame and stained with 4% Giemsa dye for 15 min. The procedure was done according to the method described by VERMA and BABU (1989). For each person 50 metaphases were analyzed. Metaphases were screened for numerical and structural aberrations. Acrocentric chromosomes associations (AA) and premature centromere division (PCD) frequencies were also noted as cytogenetic alterations. The associations between the short arms of acrocentric chromosomes, also reported in the literature as satellite associations, are known as cytogenetic endpoint to adverse effects in cells (CARADONNA *et al.*, 2015). Mitotic configurations consistent in split centromeres and splayed chromatids in all or most of the chromosomes or premature centromere division (PCD) have been described as evidence of a functional cell cycle impairment (CORONA-RIVERA *et al.*, 2005). Alteration of the sequence of centromere separation or premature centromere division has been found to be significantly higher in populations exposed to various xenobiotics, especially cytostatics that can alter the stability of chromosomes and lead to aneuploidy (BAJIC *et al.*, 2007). In order to investigate the chromosomal damage in RA patients, frequencies and types of chromosomal aberration were observed and expressed for all groups of patients.

Micronucleus assay

For micronuclei analysis, 0.5 mL of whole blood was added to 5 mL of culture medium. The duplicate cultures were set up for each patient and were incubated at 37˚ C for 72 h. A 50 μL of cytochalasin B from stock solution (6 mg/mL; Sigma Aldrich, United Kingdom) was added to each culture, after 44 h of cultivation to prevent cytokinesis and obtain binucleated cells. After 72 hours, the cells were separated from the medium by centrifugation, treated with hypotonic solution 0.075 M KCl for 3 min at 37°C and fixed three times in 3:1 methanol and acetic acid. Cells were dropped onto microscopic slides, air dried at room temperature and stained with 2% Giemsa dye for 10 min. Around 1000 binucleated cells (mean ± SD = 1002.73±8.45, range = 983–1021) from each subject were screened for the presence of MN according to the criteria for the identification described by FENECH (1993). Frequency of cells with micronuclei, nucleoplasmic bridges and nuclear buds was calculated and expressed for all patients in every of the RA patients groups.

Statistical analysis

Data were expressed as frequencies for chromosome aberration analysis and micronucleus test. The Man Whitney U test was used for differences between groups with regard to the examined parameters. The level of significance was set at $P<0.05$. Statistical analyses were performed by using the Graph Pad Prism 5.0 Software (California, USA).

RESULTS

Clinical and laboratory parameters for RA experimental groups are given in Table 1. All groups of patients had baseline DAS 28 of >3.2, indicating moderate to high disease activity, except for the LtLa group were disease activity was low. Laboratory parameters, CRP, ESR and fibrinogen indicated higher level of inflammation in LtAc MTX+DOLE as compared to both the

early phase groups (EAc MTX and EAc MTX+DOLE), and to LtLa MTX+DOLE group, the latter being presented as the group with the lowest inflammation. There were no observed significant differences between the two early phase RA groups with regard to DAS 28-ESR and DAS 28-CRP, whereas those parameters were significantly lower in both long-term RA groups, as compared to the early phase groups.

Table 1. Clinical characteristics of the rheumatoid arthritis patients groups

Groups	Early phase RA patients		Long term RA patients	
Clinical parameters	EAc MTX *n*=8	EAc MTX+DOLE *n*=8	LtLa MTX+DOLE *n*=8	LtAc MTX+DOLE *n*=8
Disease duration (years)	<1	<1	13.0 (11.3)	11.4 (10.2)
Symptom onset (years)	0.5 (0.2)	0.7 (0.6)	12.3 (11.9)	14.1 (11.1)
Positive RF, No (%)	6 (75)	7 (87.5)	8 (100)	9 (100)
DAS28- ESR score	5.6 (0.7)	5.6 (0.9)	2.8 (0.9)	5.2 (0.6)
DAS28- CRP score	4.7 (0.6)	5.1 (0.7)	2.1 (0.8)	4.3 (0.8)
Laboratory parameters				
Serum CRP (mg/L)	3.3 (3.7)	9.8 (8.0)	3.1 (2.2)	12.2 (5.6)
Fibrinogen (g/L)	4.1 (0.9)	4.5 (1.3)	3.9 (1.2)	5.4 (0.6)
ESR (mm/hr)	25. 0 (13.4)	31.1 (15.3)	18.9 (8.3)	38.9 (14.8)

Values are presented as mean (standard deviation, SD), unless otherwise specified. DAS 28, Disease Activity Score comprising of 28 tender and swollen joints count, based on current C-reactive protein (DAS 28-CRP) OR erythrocyte sedimentation rate (DAS 28-ESR); CRP, C-reactive protein; RF, rheumatoid factor; ESR, Erythrocyte sedimentation rate.

Baseline values of chromosome aberration types in PBL of the RA patients were assessed and presented in Table 2. Numeric chromosomal aberration frequencies of in PBL were higher in all RA patient groups when compared to controls, but the only significant difference was obtained for the long term active disease patients ($P< 0.05$). There were no structural aberrations detected, observing numerical aberrations which included endoreduplications, trisomies of chromosomes from the C group and monosomies of chromosomes from the D group.

The frequency of PCD and incidence of acrocentric chromosomes associations, as cytogenetic anomalies resulting from the cell's response to adverse effects were also measured. While PCD frequencies showed no difference when compared to controls, acrocentric associations were considerably higher in all RA patients groups. Acrocentric associations were higher in active disease groups (EAc MTX, EAc MTX+DOLE and LtAc MTX+DOLE) than in long-term patients with low disease activity (LtLa MTX+DOLE) (Table 2). The highest frequencies of all chromosome alterations were found in long-term active RA patients.

Table 2. Cytogenetic analysis of baseline chromosomal aberrations in human peripheral blood lymphocytes in RA patient groups and controls

Group	Frequency of numerical aberrations / 50 metaphases	AA	Partial PCD	Complete PCD
Controls *n*=10	0.8 (0.2)	4.5 (0.8)	1.7 (0.6)	0
EAc MTX *n*=8	2.2 (1.2)	18.8 (3.8)*	2.8 (0.7)	1.1 (0.1)
EAc MTX+DOLE *n*=8	1.7 (0.9)	17.9 (4.6)*	2.1 (0.9)	0
LtLa MTX+ DOLE *n*=8	2.0 (0.9)	11.7 (3.6)*	1.0 (0.9)	0
LtAc MTX + DOLE *n*=8	4.0 (1.4)*	30.1 (8.6)*	2.1 (0.2)	0.7 (0.0)

A 50 metaphases per donor were analyzed for the frequency of chromosomal aberrations (CA) including all types of numerical chromosome aberrations, associations of acrocentric chromosomes (AA), and partial and complete premature centromere division (PCD): partial PCD- in 1 or 2 individual chromosomes; compete PCD- with 50-100 % chromosomes with PCD per metaphase; Values are presented as mean (standard deviation, SD), unless otherwise specified. *$P<$ 0.05, differences between the control and experimental RA groups by Mann Whitney U test.

Micronuclei are observed in binuclear cells in form of small round shape nuclear bodies in cytoplasm not attached to the main nuclei and represent DNA material excess, not incorporated into the main nucleus after cell division (Fig. 1B, 2C). Evaluated by micronucleus assay, the measure of the chromosome stability changes is the frequency of micronuclei per 1000 examined binucleated cells. Examples of the observed changes in RA patients binucleated lymphocytes in this study are presented in Fig. 1. It is also possible to measure other forms of cytogenetic alterations in micronucleus assay, such as nuclear buds (NB) (Fig. 1D) and nucleoplasmic bridges (NPB) (Fig. 1E). Apoptotic and necrotic cells were excluded from the analysis (Fig. 1F). Frequencies of micronuclei and total aberrant changes (including nuclear buds and nucleoplasmic bridges) from cultured RA patients' human lymphocytes at baseline and at 3 weeks after treatment are presented in Table 3.

Results from binucleated cells in cytokinesis block micronucleus assay are shown in Table 3. Significantly higher frequencies of micronucleated cells were found at baseline in both groups of early active RA patients and in both groups of long-term RA patients (both active and low disease activity group) as compared to controls. Significant MN frequency differences were found in-between the RA patients' groups. After the treatment, at 3-week time point, frequencies of MN were reduced compared to baseline in the early phase active RA groups, but the difference was not significant.

Speaking about long-term phase RA groups, after 3 weeks of combined treatment, the MN frequencies in the long-term patients with low disease activity did not change, while the long-term active phase patients have shown the increased number of MN per 1000 binucleated cells as compared to their baseline values, thus being the highest among all RA groups (Table 3). Considering frequencies of total aberrant changes in binucleated cells, including nucleoplasmic bridges, buds and micronuclei, their values at 3 weeks after treatment were decreased as compared to baseline in all experimental groups except for the long-term active phase RA patients which remained unchanged. When total aberrant changes frequencies were compared at baseline and at 3 weeks after treatment, more pronounced changes were detected in long-term

patients with low disease activity than in early phase patients, but those differences were not significant due to high variations.

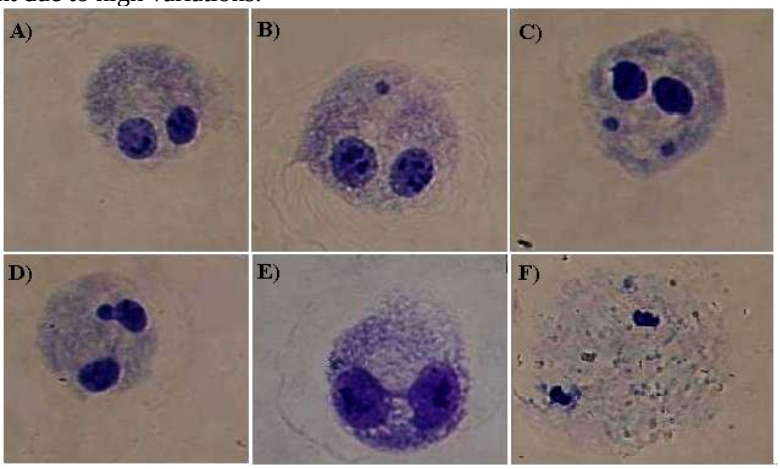

Figure 1. Examples of the lymphocytes scored in the micronucleus assay in rheumatoid arthritis patients: A) binucleated cell; B) binucleated cell containing one micronuclei; C) binucleated cell containing multiple micronuclei; D) binucleated cell containing nuclear bud; E) binucleated cell with nucleoplasmic bridge; F) necrotic cell;

Table 3. Frequencies of micronuclei (MN) in cultured human lymphocytes of rheumatoid arthritis patients at baseline and at 3 weeks after treatment, by using cytokinesis-blocked micronucleus assay

	Frequency MN (pooled)		MN range (pooled)		Frequency TA (pooled)		TA range (pooled)	
Groups	Baseline	3 weeks	Baseline	3 weeks	Baseline	3 weeks	Baseline	3 weeks
Controls *n*=10	5.7 (3.8)	7.2 (2.6)	1-10	5-11	6.7 (5.1)	8.2 (3.3)	1-13	6-13
EAc MTX *n*=8	35.3 (18.0)*	24.6 (11.1)*	18-54	16-38	40.3 (32.8)	38.3 (12.5)*	18-79	23-52
EAc MTX+DOLE *n*=8	33.0 (16.9)*	30.1 (4.9)*	21-45	24-35	42.0 (26.9)*	39.0 (17.0)*	23-74	29-56
LtLa MTX+ DOLE *n*=8	34.2 (7.9)*	32.6 (18.9)*	21-47	12-49	72.0 (9.0)*	65.6 (26.5)*	63-81	35-82
LtAc MTX+ DOLE *n*=8	34.3 (8.1)*	47.7 (15.9)*	25-39	34-70	71.0 (11.1)*	73.2 (21.4)*	61-83	52-95

A total of 1000 binucleate cells were scored for micronuclei (MN) per patient and results are pooled as a mean value (standard deviation, SD) from all participants in the groups; TA – total aberrations in binucleated cells including nucleoplasmic bridges, buds and micronuclei. *$P<0.05$, differences between control and experimental RA groups by Mann Whitney U test for MN and TA.

DISCUSSION

The effects of combined Methotrexate and Dry olive leaf extract treatment on chromosome instability in rheumatoid arthritis (RA) patients were analyzed with regard to disease duration (early and long-term disease RA patients), and to disease activity (active disease and low disease activity). Increase in micronuclei or chromosome aberration frequencies reflected genomic abnormalities in RA patients peripheral blood lymphocytes, which could be positively connected with the active disease phase inflammation process.

Previous research has shown that activated monocytes/macrophages could increase superoxide production in chronic inflammatory diseases, followed by release of long-lived metabolites that act as clastogenic factors (EMERIT *et al.*, 1996; FIRESTEIN, 2003). Oxidative damage in RA, caused by reduced glutathione levels, was found to induce T-cell hyporesponsiveness in synovial fibroblasts, which could be an important therapeutic fact (GRINGHUIS *et al.*, 2000).

Elevated reactive oxygen species (ROS) are established source for DNA damage and genomic instability. Increased comet-tail length and MN frequencies, as well as decreased antioxidant enzymes superoxide dismutase and glutathione peroxidase levels have already been demonstrated in RA patients, in both active and inactive disease period (KARAMAN *et al.*, 2011). Elevated DNA damage, related to increased oxidative stress levels, was also correlated with disease activity according to JIKIMOTO *et al.* (2002). Our previous finding has shown increased primary DNA damage by comet assay, both in early phase RA patients and in long-term patients, the highest damage being demonstrated in the early active stage of the disease (ČABARKAPA *et al*., 2016).

Increase in micronucleated cell number is suggestive for chromosome instability. Increased micronuclei frequencies in our study have demonstrated presence of genomic damage for all experimental RA groups, concordant with other studies, like RAMOS-REMUS *et al.* (2002) and KARAMAN *et al*. (2011). Our results of micronuclei frequencies indicated higher damage in long-term disease groups than in newly-diagnosed. Total aberrations in cytokinesis-block micronucleus assay, including nuclear buds and nucleoplasmic bridges, as well as micronuclei, were also significantly higher in long-term RA patients, possibly caused by longer disease duration. These aberrant genetic endpoints are positively associated with each other and their coincident presence within individual cell suggests common mechanism of generation, either by chromosomal damage or structural chromosome rearrangements (CHEONG *et al*., 2013).

Increased frequencies of micronuclei could also be explained by other mechanisms, apart from chromosome breaks. Micronuclei could also originate from amplified genome regions, excluded from the nucleus by a so-called nuclear budding process (IMLE *et al*., 2009). Nuclear budding frequencies were increased in our RA patient groups, indeed. Also, increased frequencies of nucleoplasmic bridges were observed, that usually originate from an exchange-type structural rearrangement (THOMAS *et al*., 2003). With regard to the above-mentioned micronuclei formation in RA patients in our study, it was probably originated from genome amplifications and chromosome exchanges, since we did not detect any chromosome breakage in lymphocyte cultures.

At three weeks after the introduction of experimental treatments, decrease in micronuclei frequencies were observed in both groups of early phase RA, but not in the long-

term RA patient groups. However, the changes in micronuclei frequencies were not significant, as compared to baseline values in corresponding groups.

Although DNA damage reduction was observed in our previous study, where all RA groups treated with combined DOLE and MTX treatment showed significant improvement in DNA damage levels detectable with the comet assay (ČABARKAPA *et al*., 2016), the current study showed no significant effect of combined treatment on DNA damage levels detectable by micronucleus test.

Differences in DNA damage reduction observed by comet assay in the previous study (ČABARKAPA *et al*., 2016) and observation by micronucleus test in the current results, could be explained by the fact that the comet assay detects DNA damage that originates mostly from the single strand breaks (SSB). SSB usually get repaired more quickly and efficiently than the double strand breaks (DSB), that are being involved in micronuclei formation (ILIAKIS *et al*., 2004; VON SONNTAG, 2006). It is plausible that high level of inflammation in active RA disease phase leads to increased oxidative damage levels, mostly presented in the form of DNA single strand breaks. If they are not repaired efficiently, two close-by SSBs lead to formation of DSBs, and this reaction is largely amplified by the presence of peroxyl radicals (PRISE *et al*., 1993). Treatment with DOLE may prevent oxidative stress induced formation of SSBs and such damage-amplification reactions are thus intercepted. Agents that induce reactive oxygen species were also shown to produce error-prone joining of broken DNA strands (BRYANT, 2007). If incomplete joining of chromatin ends occur, it would lead to chromosome aberrations. It could be suggested that early phase RA patients have more efficient removal of DNA damage than the long-term RA patients, concluding that longer disease duration compromise the repair capacity in RA patients. This could explain differences in micronuclei reduction between the early and the long-term phase patients.

Considering chromosome aberrations, RA groups have shown numerical aberrations, such as endoreduplications, trisomies of chromosomes from the C chromosome group and monosomies of the D chromosome group, which are in accordance with previous authors findings (KINNE *et al.*, 2001). Endoreduplication is a mitotic manifestation produced after the replication of the nuclear genome in the absence of cell division. KINNE *et al.* (2001) have found significant positive correlation between RA patients' disease duration and levels of polysomies in synovial fibroblasts for chromosomes 6 and 8, as well as positive correlation between RA collagenase digests levels of polysomy for chromosome 7 and serum CRP concentrations level. Together with the results from the present study, results of increased aneuploidies in long-term active RA patients indicate a positive link between RA inflammation duration and numerical aberration occurrence. Same authors suggested that Methotrexate treatment did not influence genomic stability in their study, considering the fact that no correlations were found between the polysomy level for any of the chromosomes in RA synovial fibroblasts and methotrexate treatment (KINNE *et al.*, 2001). In favor for this assumption, we have found low level of aberrations in long-term patients with low disease activity that has been on MTX therapy for many years, too.

Another cytogenetic alteration that should be mentioned is increased frequency of acrocentric chromosomes associations (AA) detected in our study, found to be considerably higher in all RA patient groups vs controls. Mutual aggregation of acrocentric chromosomes

short arms is called satellite association, known to be positively correlated with the degree of NOR (Nuclear Organizer Regions) staining (MILLER *et al.*, 1977). NOR are sites of ribosomal RNA genes, localized at the second constriction of the acrocentric chromosome. It was shown that cells with more rDNA activity and increase of the cell cycle duration would have the increased production of AA (REEVES *et al.*, 1982). When DNA damage is increased in the cell, damage signal elicits cell cycle arrest, inducing either DNA repair or apoptosis (HUEN and CHEN, 2008). It is possible that a prolonged cell cycle, due to response to DNA damage, leads to an increased occurrence of AA, since the RA groups with the most severe DNA damage have also shown the highest frequency of AA. This still remains to be studied in detail by examining of the link between DNA damage and cell cycle aberrations in RA patients.

CONCLUSION

Chromosome instability is increased in patients with early phase rheumatoid arthritis as well as with long-term disease, as assessed by the micronucleus test and chromosome aberrations evaluation. More pronounced genomic damage was observed in active RA, both in early phase and in long-term phase disease than in low disease activity. The results indicate a possible link between the cytogenetic alterations and disease duration as well as with disease activity. Aggressive inflammatory phenotype in the active RA could induce cytogenetic alterations, such as increased frequencies of micronuclei and acrocentric chromosome associations in lymphocytes of RA patients. Neither Methotrexate treatment alone nor Methotrexate and Dry olive leaf extract combination treatment did significantly affect the levels of cytogenetic alteration after three weeks of treatment according to our study; although patients in the early phases of the RA disease exhibited some potential for micronuclei reduction after 3 weeks, long-term RA pathology was much less influenced.

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CITOGENETSKE PROMENE KOD PACIJENATA SA REUMATOIDNIM ARTRITISOM NAKON TRETMANA METOTREKSATOM I SUVIM EKSTRAKTOM LISTA MASLINE

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Izvod

Oksidativni stres i inflamacija su faktori u vezi sa pojavom DNK nestabilnosti kod pacijenata sa reumatoidnim artritisom (RA). Cilj ove studije je evaluacija citogenetskih promena u limfocitima periferne krvi (PBL) kod dve grupe pacijenata sa RA: grupe sa ranim i dugotrajnim RA; kao i ispitivanje potencijala kotretmana sa metotreksatom (MTX) i suvim ekstraktom lista maslina (DOLE) protiv citogenetskih oštećenja kod pacijenata sa RA nakon tronedeljnog tretmana. U studiju je uključeno ukupno 32 pacijenta, podeljenih u četiri grupe: dve grupe sa ranom fazom RA (jedna tretirana samo sa MTX, druga u kombinaciji sa DOLE); i dve grupe pacijenata sa dugotrajnim RA (grupa u remisiji i grupa sa aktivnom bolešću) - obe tretirane kombinacijom MTX i DOLE. Kulture PBL su pregledane na prisustvo hromozomskih aberacija i određena je učestalost mikronukleusa. Značajno povišena učestalost mikronukleusa je pokazana kod pacijenata u aktivnoj fazi bolesti (ranoj i dugotrajnoj), ali ne i kod grupe pacijenata u fazi remisije, u poređenju sa kontrolnom. Hromozomske aberacije su zabelezene kod sve četiri grupe pacijenata. Najviša učestalost mikronukleusa i hromozomskih aberacija nađena je kod grupe sa dugotrajnom aktivnom fazom RA. Iako su učestalosti mikronukleusa smanjene u svim grupama sa RA nakon tronedeljnog tretmana u poređenju sa početnim vrednostima, grupama, smanjenje je bilo značajno samo u grupi sa dugotrajnom aktivnom bolešću. Pokazano je da je visok stepen citogenetskog oštećenja kod pacijenata sa RA u vezi sa duzinom trajanja i aktivnošću bolesti. Nakon 3 nedelje terapije, ni kombinovani tretman (MTX+ DOLE), niti samostalna primena MTX nisu uticali na učestalost pojave mikronukleusa.

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