



## Oxidative stress and enzymatic antioxidant status of blood and synovial fluid in rheumatoid arthritis patients

Stres oksydacyjny i enzymatyczny układ entyoksydacyjny we krwi  
i płynie stawowym pacjentów z reumatoidalnym zapaleniem stawów

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### ABSTRACT

**INTRODUCTION:** Although the exact etiology of rheumatoid arthritis (RA) remains unknown, there is increasing evidence that reactive oxygen species (ROS) and oxidant/antioxidant imbalance are an important part of the pathogenesis of joint tissue injury.

**MATERIAL AND METHODS:** The activities of: manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (CuZnSOD) isoenzymes, catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST), and malondialdehyde (MDA) levels were determined in blood and synovial fluid samples from 178 RA patients and from 27 healthy controls.

**RESULTS:** The RA patients showed increased antioxidant enzyme activities and MDA levels. Decreased synovial fluid viscosity was associated with a tendency for a changed antioxidant system with increased antioxidant enzyme activities, thereby suggesting a possible adaptation to ROS production in the blood and synovial fluid in RA patients.

**CONCLUSIONS:** Correlating antioxidant enzyme activities and MDA levels to disease activity might provide further information about oxidative stress in RA pathogenesis.

### KEY WORDS

rheumatoid arthritis, antioxidant enzymes, synovial fluid

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## STRESZCZENIE

**WSTĘP:** W patogenezie reumatoidalnego zapalenia stawów jednym z mechanizmów uszkadzających strukturę stawu są reakcje z udziałem reaktywnych form tlenu (RFT).

**MATERIAŁ I METODY:** U 178 pacjentów z RZS oraz 27 zdrowych ochotników oznaczono we krwi i płynie stawowym aktywność enzymów antyoksydacyjnych: izoenzymów dysmutazy ponadtlenkowej manganowej (MnSOD) i cynkowo-miedziowej (CuZnSOD), katalazy (CAT), peroksydazy glutationowej (GPX), reduktazy glutationowej (GR) i transferazy-S-glutationowej (GST) oraz stężenie dialdehydu malonowego (MDA).

**WYNIKI:** We krwi i płynie stawowym pacjentów z RZS dochodzi do pobudzenia układu antyoksydacyjnego ze wzrostem aktywności enzymów antyoksydacyjnych, zwiększonym stężeniem MDA oraz spadkiem lepkości płynu stawowego.

**WNIOSKI:** Zależność między nasileniem zmian w układzie antyoksydacyjnym a aktywnością zapalenia stawów potwierdza udział RFT w patogenezie i przebiegu RZS.

## SŁOWA KLUCZOWE

reumatoidalne zapalenie stawów, enzymy antyoksydacyjne, płyn stawowy

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, autoimmune syndrome which produces degradation of articular cartilage and bone erosion. The long-term outcome of this disease is characterized by significant morbidity, loss of functional capacity and increased mortality. Although the pathophysiological basis of RA is not yet fully understood, reactive oxygen species (ROS) have been implicated in its pathogenesis [1].

Arthritic joints contain large amounts of fibroblasts, chondrocytes, macrophages, and especially neutrophils, which produce ROS [2]. The superoxide anion ( $O_2^{\cdot-}$ ) plays a pivotal role in inflammation, particularly in patients with inflammatory joint disease [3]. Superoxide damages endothelial cells, increasing the permeability of the microvasculature and promoting the migration of neutrophils to foci of inflammation. It can be converted to other, more aggressive ROS such as hydroxyl radical ( $OH^{\cdot}$ ) and hydrogen peroxide ( $H_2O_2$ ) [4]. These highly reactive oxygen species are able to attack all biological molecules including DNA, protein, lipids, lipoproteins and are also known as lipid peroxidation-inducing agents (5). One of the end-products of this process is malondialdehyde (MDA), whose elevated levels have been reported in the serum (or plasma) and synovial fluid of RA patients [1,6].

The destructive chain reactions initiated by ROS can be broken by antioxidant enzymes, which are able to convert them into harmless derivatives [7]. Superoxide dismutase (SOD) is the first line of defense against ROS catalyzing the dismutation of  $O_2^{\cdot-}$  into  $O_2$  and  $H_2O$ . Catalase (CAT) detoxifies  $OH^{\cdot}$  [8]. Glutathione peroxidase (GPX) is a selenoprotein that reduces lipidic or nonlipidic hydroperoxides as well as  $H_2O_2$  during glutathione oxidation. Glutathione reductase (GR) and glutathione-S-transferase (GST) facilitate this process by

maintaining an adequate level of reduced glutathione (GSH) [9]. It has been suggested that the pro-oxidant/antioxidant imbalance in RA may be due to the acceleration of some cellular reactions or insufficiency of the antioxidant defense systems. Several studies have been published to investigate the oxidant and antioxidant status in RA [10].

We hypothesized that RA patients would display altered oxidant and antioxidant systems in their blood and synovial fluid. This study was designed with the following objectives:

1. To examine potential changes in the activities of copper-zinc (CuZnSOD) and manganese (MnSOD) superoxide dismutase isoenzymes and CAT, and in the activities of glutathione transformation enzymes: GPX, GR and GST in blood and synovial fluid and compare these parameters with age-matched healthy subjects.
2. To estimate the relationship between the activities of antioxidant enzymes with the degree of lipid peroxidation evaluated by MDA levels in blood and synovial fluid, synovial fluid viscosity, disease activity and RA duration.

## MATERIALS AND METHODS

### Patients and Controls

The Medical Ethics Committee of the Medical University of Silesia approved the study protocol followed in this investigation (NN-013-283/03). All the subjects were enrolled voluntarily after being informed of the scope and goals of the trial.

The study included 55 male and 123 female RA patients aged 39 to 58 years (mean age  $49.8 \pm 8.1$ ). All the RA patients fulfilled the 1987 criteria for RA by the American Rheumatism Association and were in functional



classes I, II, III or IV (Tab. I), according to the revised criteria of the American College of Rheumatology [11]. Patients with an active disease duration of at least 6 months, as manifested in at least three joints that were swollen and six joints that were tender at the time of sample donation, were accepted for the study (Tab. II). In addition, the RA patients had an erythrocyte sedimentation rate  $\geq 24$ , a CRP  $\geq 1,3$ , or morning stiffness of at least 45 min in duration. All standard RA therapy

including nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs (DMARD<sub>s</sub>), and combinations of DMARD<sub>s</sub> were allowed as long as the doses were stable for 2 months prior to participation in the study. The specimens were randomized blindly for the different analyses, and not all the collected specimens were used for all the analyses; no analyses were excluded.

**Table I.** Age, sex, duration of rheumatic arthritis (RA), body mass index (BMI), Ropes test and synovial fluid parameters : viscosity, malondialdehyde (MDA) concentration, superoxide dismutase isoenzymes Mn-SOD and CuZn-SOD, glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione S-transferase (GST) activity in study population

**Tabela I.** Wiek, płeć, czas trwania reumatoidalnego zapalenia stawów (RA), wskaźnik masy ciała (BMI), próba Ropesa i parametry lepkości płynu stawowego, stężenie dialdehydu malonowego (MDA), aktywność izoenzymów MnSOD i ZNCuSOD, peroksydazy glutationowej (GPX), reduktazy glutationowej (GR) i transferazy-S-glutationowej (GST) w grupie badanej

	CONTROL	RA-remission	RA-low activity	RA-moderate activity	RA-high activity	p-value
Number of men/women	13/14	12/32	14/31	18/30	11/30	0.324
Age (years)	44.7 ± 11.4	50.7 ± 13.6	52.5 ± 15.4	48.9 ± 12.8	48.7 ± 14.8	0.329
RA duration (years)	–	11.9 ± 11.2	9.42 ± 7.71	6.72 ± 5.95	8.73 ± 8.72	0.224
BMI (kg/m <sup>2</sup> )	26.8 ± 4.44	27,3 ± 3.91	28.8 ± 4.75	26.8 ± 5.48	24.8 ± 4.53	0.093
Ropes test	0.62 ± 0.59	2,06 ± 1.29	2.60 ± 1.30	3.07 ± 1.04	3.36 ± 0.99	< 0.001
Viscosity	61.7 ± 38.8	9.80 ± 3.62	5.84 ± 2.70	19.6 ± 31.4	19.1 ± 30.0	0.006
Synovial MDA concentration (μmol/l)	1.31 ± 0.41	2.42 ± 2.25	1.89 ± 1.35	2.33 ± 2.02	2.57 ± 2.36	0.151
Synovial Mn-SOD activity (NU/ml)	4.42 ± 3.52	2.73 ± 3.88	5.86 ± 6.83	8.90 ± 8.24	8.27 ± 8.83	0.046.
Synovial cuzn-SOD activity (NU/ml)	4.04 ± 5.55	14.3 ± 4.08	8.89 ± 4.10	7.74 ± 4.43	10.1 ± 6.27	< 0.001
Synovial GPX activity (IU/l)	187 ± 240	536 ± 247	660 ± 294	655 ± 363	746 ± 365	< 0.001
Synovial GR activity (IU/l)	10.5 ± 2.91	46.1 ± 24.9	43.2 ± 22.7	46.8 ± 21.2	56.0 ± 26.2	< 0.001
Synovial GST activity (IU/l)	7.18 ± 4.74	14.5 ± 10.7	16.7 ± 11.9	17.8 ± 15.0	22.7 ± 20.7	0.013

**Table II.** Malondialdehyde (MDA) concentration, superoxide dismutase isoenzymes Mn-SOD and CuZn-SOD, glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST) and catalase (CAT) activity in serum and erythrocytes in study population

**Tabela II.** Stężenie dialdehydu malonowego (MDA), aktywność izoenzymów MnSOD i ZNCuSOD, peroksydazy glutationowej (GPX), reduktazy glutationowej (GR) i transferazy-S-glutationowej (GST) i katalazy (CAT) w osoczu i erytrocytach w grupie badanej

	CONTROL	RA-remission	RA-low activity	RA-moderate activity	RA-high activity	p-value
Erythrocyte MDA concentration (nmol/g Hb)	421 ± 145	461 ± 224	459 ± 157	542 ± 152	689 ± 253	< 0.001
Serum MDA concentration (μmol/l)	1.15 ± 0.37	2.99 ± 2.55	2.93 ± 2.24	2.69 ± 1.76	2.58 ± 2.03	0.030
Serum Mn-SOD activity (NU/ml)	4.35 ± 3.29	9.20 ± 6.04	8.65 ± 6.36	10.0 ± 6.40	8.11 ± 8.63	0.060
Serum cuzn-SOD activity (NU/ml)	2.31 ± 1.97	6.16 ± 5.21	6.08 ± 4.89	7.49 ± 4.85	6.87 ± 5.15	0.008
Erythrocyte SOD activity (NU/mg Hb)	9.69 ± 6.37	12.5 ± 6.30	13.3 ± 5.80	14.1 ± 5.09	15.8 ± 5.88	0.006
Erythrocyte GPX activity (IU/g Hb)	42.3 ± 12.7	73.4 ± 46.6	65.2 ± 42.5	66.6 ± 34.0	84.8 ± 51.4	0.104
Erythrocyte GR activity (IU/g Hb)	3.87 ± 0.88	6.80 ± 2.86	6.74 ± 2.29	6.96 ± 2.73	8.06 ± 2.99	< 0.001
Erythrocyte GST activity (miu/g Hb)	236 ± 294	191 ± 175	198 ± 184	154 ± 161	200 ± 209	0.729
Erythrocyte CAT activity (ku/g Hb)	314 ± 124	547 ± 257	514 ± 174	527 ± 147	557 ± 231	< 0.001

The control group consisted of 27 healthy volunteers precisely matched for age and gender. In addition to

having no RA symptoms, the controls were not obese (BMI < 30), did not work in professions related to



excessive load of the knee joints (e.g. truck drivers), did not practice injurious sports (e.g. soccer, skiing), with no history of rheumatoid arthritis, osteoarthritis, post-traumatic inflammatory or another knee joint pathology. Final verification of the controls was carried out after preliminary analysis of knee-joint synovial fluid, including a visual examination of color, turbidity, viscosity, Ropes test, volume and biochemical parameters. The knee-joint synovial fluid samples collected from the control group exhibited normal synovial fluid [12]. The patients and controls with any history of smoking, alcohol abuse and signs of malignant tumors, diabetes, serious liver, kidney or heart insufficiency or other systemic diseases that can cause an increase in oxidations were not included in the study. The women who were included in the study had not been taking oral contraceptives for at least 6 months before blood sample collection.

### Preparation of Blood and Synovial Fluid Samples

Fasting blood samples ( $\approx 10$  ml whole blood) were taken in the morning from the antecubital vein, using a Monovette system of blood collection. For hematological analysis, one ml was treated with  $K_3EDTA$  to prevent coagulation. From the untreated blood, plasma was separated by centrifugation at 2500 G for 15 min at  $4^\circ C$  for preparation of the lysate. The erythrocyte-rich precipitate was washed three times with physiological saline (3:1 v/v) and lysed by an addition of doubly distilled water. The plasma and lysate samples were stored at  $-76^\circ C$  until needed for analysis but for no longer than 3 months. C-reactive protein (CRP) and the rheumatoid factor (RF) were also determined in sera obtained from the non-coagulated blood samples. Synovial fluid (SF) samples were obtained by needle aspiration or during knee arthroscopy and divided into two equal portions. The first SF sample was drawn into a test tube without an anticoagulant, and the second SF sample was collected in a test tube containing  $K_3EDTA$ . Both were immediately placed on ice and centrifuged at 3.000 G for 30 min. The supernatant was separated and stored in the same conditions as plasma at  $-76^\circ C$ .

### Analyses of Blood and Synovial Fluid

#### Mucin Clot Test

The mucin clot test is a semiquantitative indicator of the amount of hyaluronic acid determined by adding 5 drops of 5% acetic acid into 3 ml of SF. A precipitate is formed and graded according to the following scale: 0 – compact reaction (a tight ropy clot in a clear solution), 1 – compact/floccular reaction (a soft clot in a turbid solution), 2 – floccular reaction (a friable clot in a cloudy solution), 3 – floccular/turbidity reaction

(flocculent material in a cloudy solution), 4-turbidity reaction (turbid supernatant with no evidence of clot).

### Assay for Synovial Fluid Viscosity

The SF viscosity was measured using a Brookfield DV-II cone plate viscometer in a test tube containing EDTA-treated SV before centrifugation. The data were reported in Pa s ( $N\ s/m^2$ ).

Determination of CuZnSOD and MnSOD activities  
The activities of these SOD isoenzymes were determined in serum, erythrocytes and SF by the Oyanagui method using potassium cyanide as the CuZnSOD inhibitor [13]. The superoxide anion radical produced in the reaction of xantine with  $O_2$ -catalyzed by xanthine oxidase, reacts with hydroxylamine producing a nitric ion. The nitric ion combines with naphthalene diamine and sulfaniline acid producing a colored product; the concentration of this mixture is proportional to the amount of produced  $O_2^{\cdot -}$ . The enzymatic activity is expressed as nitric unit per ml (NU/ml) of serum, lysate or SF. In this method, one NU means 50% of inhibition by SOD of nitric ion production.

### Determining CAT Activity

The catalase activity in erythrocytes and SF was analyzed by the Aebi kinetic method [13]. Before CAT was assayed, the lysate and SF were diluted 100-fold with a tris-HCl buffer, pH 7.4. The kinetics of the reaction were determined in a quartz tank: 2.5 ml of substrate were mixed with 50 mM tris-HCl buffer and perhydrol with 50 ml of SF. After 10 s, absorbance was measured at 240 nm and then every 30 s for 2 min to follow the kinetics of the reaction. The CAT activity was expressed as IU/mg Hb for lysate. No enzymatic activity was present in SF.

### Determining GPX Activity

The Paglia and Valentine kinetic method was used to determine the GPX activity in erythrocytes and SF [13]. GPX catalyzed the reaction between reduced glutathione and  $H_2O_2$ . The resulting oxidized glutathione (GSSG) was recovered back to GSH using nicotinamide adenine dinucleotide phosphate ( $NADPH^+H^+$ ) catalyzed by GR. The decrease in absorbance was measured at 340 nm. The GPX activity was determined as  $\mu mol$  of  $NADPH^+H^+$  needed to recover GSH in 1 min converted to 1 l of lysate or SF (IU/l).

### Determining GR Activity

The glutathione reductase activity in erythrocytes and SF was also assayed by the kinetic method [13]



measuring the decrease in  $\text{NADPH}^+\text{H}^+$  concentration after reduction of GSSG back to GSH. The GR activity was determined as  $\mu\text{mol}$  of  $\text{NADPH}^+\text{H}^+$  used to recover GSH in 1 min converted to 1 l of lysate or SF (IU/l).

### Determining GST Activity

The GST activity in erythrocytes and synovial fluid was analyzed by the Habig and Jakoby kinetic method using 1-chloro-2,3-dinitrobenzene [13]. GST reacted with 1-chloro-2,3-dinitrobenzene producing thioether. The increase in absorbance was measured at 340 nm. GST activity was determined as  $\mu\text{mol}$  of thioether produced in 1 min in 1 l of lysate or SF (IU/l).

### Lipid peroxidation (MDA) assay

Lipid peroxidation (as MDA level) in serum, erythrocytes and SF was measured fluorometrically as 2-thiobarbituric acid-reactive substance (TBARS) by the Ohkawa method [13]. Each sample was mixed with 8.1% sodium dodecyl sulfate, 20% acetic acid and 0.8% 2-thiobarbituric acid. The method was modified by adding 100 mmol/l sodium sulphate and 2.5  $\mu\text{mol/l}$  3,5-diisobutyl-4-hydroxytoluene (BHT). After vortexing, the serum, lysate or synovial fluid sample was incubated for 1 h at 95°C and butanol-pyridine 15:1 (v/v) was added. The mixture was shaken for 10 min and then centrifuged. The butanol-pyridine layer was measured fluorometrically at 552 nm (515 nm excitation). The TBARS value was expressed as an MDA equivalent. Tetraethoxypropane was used as the standard. The MDA value was expressed as  $\mu\text{mol/l}$  for serum, lysate and SF.

### Statistical Analysis

The statistical analysis was performed with Statistica 6.0 PL software. The statistical methods included mean and standard error of mean (SEM). An analysis of variance or the Kruskal-Wallis ANOVA test was used for multiple comparisons of data. Shapiro-Wilk's test was used to verify normality and Levene's test to verify the homogeneity of variances. Statistical comparisons were made by t-test, t-test with separate variance estimates or the Mann-Whitney U test. The chi-square or Fisher's test was used to analyse gender. Yates' correction for continuity was used if needed. Spearman non-parametric correlation was calculated. A value of  $p < 0.05$  was considered to be significant.

Table II also shows the age, gender, RA duration and BMI in the controls and RA patients, separately in functional classes I, II, III and IV.

The Ropes test gave different results among the study groups. A bigger difference was seen in functional Class IV ( $p < 0.001$ ; Tab. II, Fig. 3). The SF viscosity was significantly lower in the study group ( $-76\%$ ,  $p < 0.001$ ) as well as in functional class I ( $-84\%$ ,  $p = 0.042$ ), Class II ( $-91\%$ ,  $p = 0.006$ ), Class III ( $-68\%$ ,  $p = 0.026$ ), and Class IV ( $-69\%$ ,  $p = 0.032$ ) as compared to the controls (Tab. II, Fig. 3).

Compared to controls, the MnSOD activity in serum was significantly higher in the study group ( $+108\%$ ,  $p = 0.008$ ) as well as in functional Class I ( $+111\%$ ,  $p = 0.004$ ), Class II ( $+99\%$ ,  $p = 0.013$ ), and Class III ( $+131\%$ ,  $p = 0.001$ ) (Tab. II, Fig. 2). The MnSOD activity in SF was higher in the study group but the difference was not statistically significant ( $+59\%$ ,  $p = 0.131$ ), except for functional Class III ( $+102\%$ ,  $p = 0.023$ ) (Tab. II, Fig. 1).

The CuZnSOD activity in serum, erythrocytes and SF was significantly higher in the study groups ( $+187\%$ ,  $p = 0.001$ ,  $+44\%$ ,  $p = 0.007$ , and  $+141\%$ ,  $p < 0.001$ , respectively) as well as in all the functional Classes: I ( $+166\%$ ,  $p = 0.006$ ,  $+29\%$ ,  $p = 0.13$ , and  $+253\%$ ,  $p < 0.001$ , respectively), II ( $+163\%$ ,  $p = 0.004$ ,  $+37\%$ ,  $p = 0.045$ , and  $+120\%$ ,  $p = 0.005$ , respectively), III ( $+224\%$ ,  $p < 0.001$ ,  $+46\%$ ,  $p = 0.007$ , and  $+91\%$ ,  $p = 0.016$ , respectively), and IV ( $+197\%$ ,  $p = 0.001$ ,  $+63\%$ ,  $p = 0.001$ , and  $+150\%$ ,  $p = 0.002$ , respectively) as compared with the controls (Tab. II and III; Fig. 1 and 2).

The GPX activity in erythrocytes was higher in the study group but not statistically significant when compared to the controls ( $+70\%$ ,  $p = 0.1$ ) (Tab. III, Fig. 2). The GPX activity in SF was significantly higher in the study group ( $+254\%$ ,  $p < 0.001$ ) as well as in all the subgroups: remission ( $+186\%$ ,  $p < 0.001$ ), low ( $+253\%$ ,  $p < 0.001$ ), the moderate ( $+250\%$ ,  $p < 0.001$ ), and high disease activity subgroup ( $+298\%$ ,  $p < 0.001$ ), compared to controls (Tab. I, Fig. 1).

As seen in Tables I and II, Figures 1 and 2 the GR activity in erythrocytes and SF was significantly higher than the controls in the study group ( $+84\%$ ,  $p < 0.001$ ;  $+361\%$ ,  $p < 0.001$ , respectively) as well as in all the subgroups: remission ( $+76\%$ ,  $p < 0.001$ ;  $+339\%$ ,  $p < 0.001$ , respectively), low ( $+74\%$ ,  $p < 0.001$ ;  $+311\%$ ,  $p < 0.001$ , respectively), moderate ( $+80\%$ ,  $p < 0.001$ ;  $+346\%$ ,  $p < 0.001$ , respectively), and high disease activity subgroup ( $+108\%$ ,  $p < 0.001$ ;  $+433\%$ ,  $p < 0.001$ , respectively).

## RESULTS

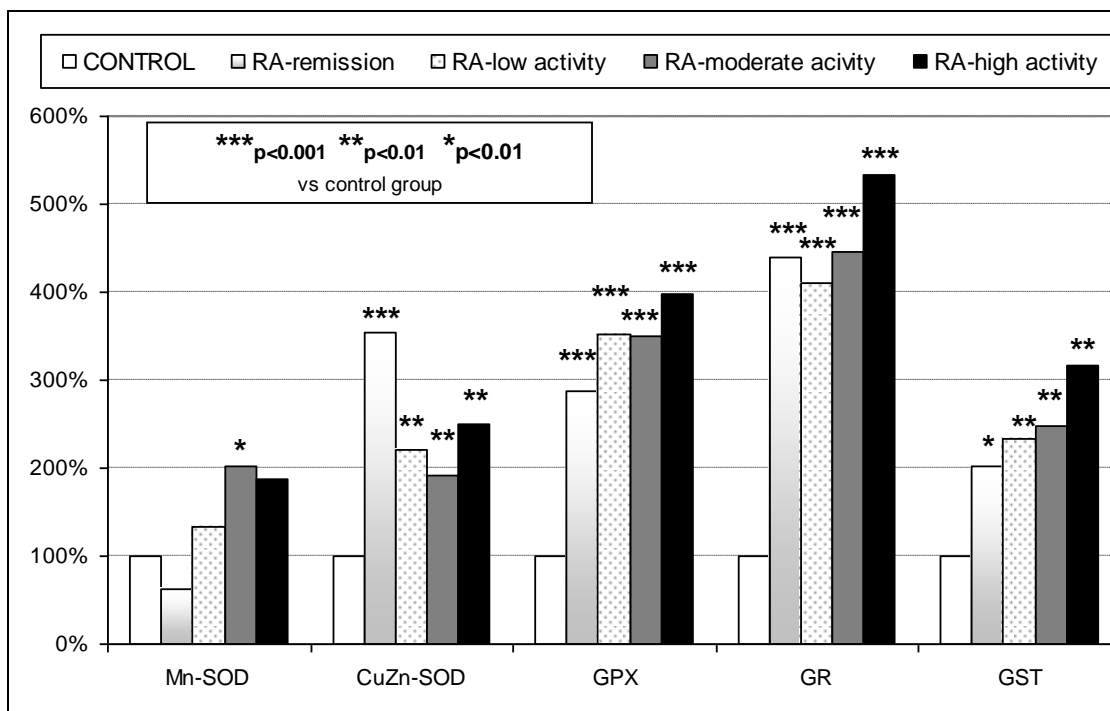


Fig. 1. Synovial fluid isoenzymes MnSOD and CuZnSOD, GPX, GR and GST activities in control group and RA patients, separately in remission, low, moderate and high disease activity subgroup. Data presented in % in comparison to controls as 10.

Ryc. 1. Aktywność izoenzymów MnSOD i CuZnSOD oraz aktywność GPX, GR i GST w płynie stawowym w grupie kontrolnej i grupie RA, oddzielnie w podgrupach: remisja, niska, średnia i wysoka aktywność choroby. Wyniki przedstawione w % w odniesieniu do grupy kontrolnej, która stanowi 100%.

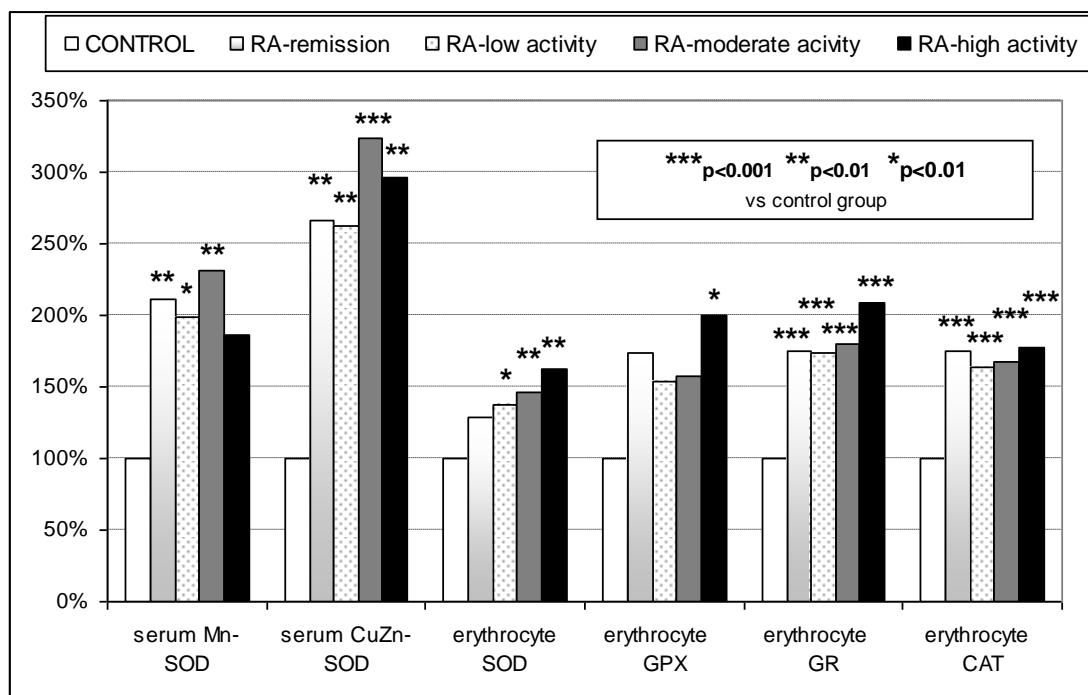


Fig. 2. Serum isoenzymes MnSOD and CuZnSOD and erythrocyte SOD, GPX, GR and CAT activities in control group and RA patients, separately in remission, low, moderate, and high disease activity subgroups. Data presented in % in comparison to controls as 100%.

Ryc. 2. Aktywność izoenzymów MnSOD i CuZnSOD w osoczu oraz aktywność SOD, GPX, GR i CAT w erytrocytach w grupie kontrolnej i grupie RA, oddzielnie w podgrupach: remisja, niska, średnia i wysoka aktywność choroby. Wyniki przedstawione w % w odniesieniu do grupy kontrolnej, która stanowi 100%.

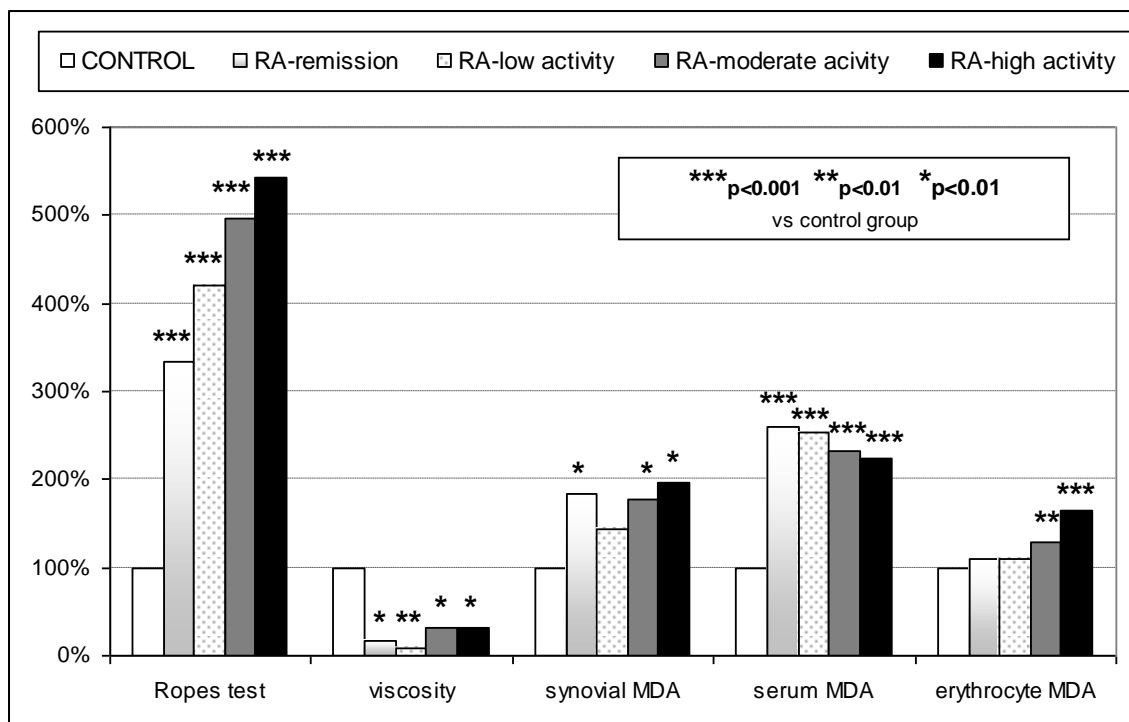


Fig. 3. Ropes test, synovial fluid viscosity and MDA level in synovial fluid in controls and RA patients, separately in the remission, low, moderate, and high disease activity subgroups. Data presented in % in comparison to control as 100%.

Ryc. 3. Próba Ropesa, lepkość płynu stawowego oraz stężenie MDA w płynie stawowym w grupie kontrolnej i grupie RA, oddzielnie w podgrupach: remisja, niska, średnia i wysoka aktywność choroby. Wyniki przedstawione w % w odniesieniu do grupy kontrolnej, która stanowi 100%.

The GST activity in erythrocytes was lower in the study group but not statistically significant than that of the controls (+21%,  $p = 0.5$ ) (Tab. II, Fig. 2). In SF, the GST activity was higher than that of controls in the study group (+158%,  $p = 0.001$ ) and in all the subgroups: remission (+102%,  $p = 0.011$ ), low (+132%,  $p = 0.002$ ), moderate (+148%,  $p = 0.003$ ), and high disease activity subgroup (+216%,  $p = 0.002$ ) (Tab. I, Fig. 1).

The MDA concentration in serum and SF was significantly higher in the study group (+143%,  $p = 0.002$  and +76%,  $p = 0.002$ , respectively) and in all the subgroups: the remission (+159%,  $p = 0.005$  and +84%,  $p = 0.027$ , respectively), the low (+154%,  $p = 0.002$  and +44%,  $p = 0.059$ , respectively), the moderate (+133%,  $p = 0.001$  and +77%,  $p = 0.022$ , respectively), and the high disease activity subgroup (+124%,  $p = 0.006$  and +96%,  $p = 0.016$ , respectively). In erythrocytes, MDA was significantly higher in the study group (27+%,  $p = 0.035$ ) and in the moderate (+29%,  $p = 0.006$ ), and high disease activity subgroups (+64%,  $p < 0.001$ ) (Tab. I and II, Fig. 3).

The activities of all the antioxidant enzymes, MDA concentration in blood and SF, and the Ropes test values significantly positively correlated with disease activity ( $r =$  from 0.18 to 0.64,  $p < 0.05$ , except for MnSOD). There were no relationships between the

measured parameters in blood and synovial fluid and the age of patients and duration of disease.

## DISCUSSION

Rheumatoid arthritis is a chronic immunoinflammatory multisystem disease with predominant synovial proliferation and destruction of the articular cartilage and bone. Although the characteristic feature is persistent inflammation, many mediators, eicosanoids, cytokines and elevated generation of ROS in the affected joints and impaired antioxidant systems have been associated to RA.

Several researchers have proposed different theories for the production of ROS in synovial fluid [1,2,3, 4,5,14]. In Figure 4 we present a simple scheme of individual ROS generation.

One source of oxidants generated at the site of inflammation in the rheumatoid arthritic knee is phagocytosis. Inflammation induces the influx of macrophages and neutrophils into the synovium and SF, which produce nearly 3 times more  $O_2^{\bullet-}$  than in healthy controls [15,16].

For ROS production, it has been proposed that movement of an inflamed joint generates sufficient pressure to cause transient ischemia of the superficial synovial



membrane [17]. Hypoxia/reoxygenation cycles can lead to articular cartilage injury, becoming serious complications in RA. Xanthine dehydrogenase (XOD), which normally utilizes  $\text{NAD}^+$  as the electron acceptor, is converted by proteolytic cleavage under the conditions of ischemia/reperfusion into xanthine oxidase (XO) [1]. XO generates  $\text{O}_2^{\bullet-}$  by converting hypoxanthine into xanthine, and xanthine into uric acid. Under normal conditions, XO accounts for only a minor proportion of total ROS production. During the ischemic period, excessive ATP consumption leads to the accumulation of purine metabolites, which upon subsequent reperfusion and influx of oxygen are metabolized by XO to yield massive amounts of  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$ .

Reoxygenation also stimulates NADPH oxidase activity in chondrocytes [2,18]. Studies by Tiku et al. [19], and others [20,21] using *in vitro* models on chondrocyte cultures revealed that under unstressed conditions, articular cartilage cells produce both  $\text{O}_2^{\bullet-}$  and  $\text{H}_2\text{O}_2$  in SF, probably through the activation of NADPH oxidase.

Hemoglobin liberated from disrupted erythrocytes into SF can accelerate lipid peroxidation in the presence of  $\text{H}_2\text{O}_2$  [22]. On the other hand, excess  $\text{H}_2\text{O}_2$  can cause degradation of the heme rings of hemoglobin, releasing iron (II) ions ( $\text{Fe}^{2+}$ ) that are capable of stimulating ROS formation [23]. In the presence of  $\text{Fe}^{2+}$  and other transition metals,  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\bullet-}$  are converted via the Fenton reaction to highly reactive hydroxyl radicals. Additionally, the neutrophil-associated enzyme myeloperoxidase (MPO) can oxidize halides, such as chloride ( $\text{Cl}^-$ ), and convert  $\text{H}_2\text{O}_2$  into hypochlorous acid ( $\text{HOCl}$ ), another highly reactive and damaging chemical species [16].

Because of the highly reactive nature of ROS, it is difficult to directly demonstrate their presence *in vivo*. It is considerably more practical to measure the ROS 'footprints', such as their effects on various lipids, proteins, and nucleic acids. Several studies of RA synovial fluid and tissue have demonstrated oxidative damage to hyaluronic acid, the presence of lipid peroxidation products, oxidized low-density-lipid proteins (LDL) and increased carbonyl groups reflecting oxidation damage to proteins [14]. Takahasi et al. [24] found that the SF concentration of hyaluronic acid can be used as a diagnostic marker for RA activity. Exposing hyaluronan to ROS, notably  $\bullet\text{OH}$ , potentially results in decreased high molecular weight hyaluronan. Indeed,  $\bullet\text{OH}$  may inhibit cartilage proteoglycan synthesis, e.g. by interfering with ATP synthesis, in part by inhibiting the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase in chondrocytes [17,19]. Alpaslan et al. [25] found that the intraarticular use of hyaluronan significantly decreased the TBARS concentration in SF. This would suggest that membrane lipids are only one of the possible targets of oxidative damage, and SF

compounds such as hyaluronic acid are more susceptible to oxidative stress.

Our results indicate that the antioxidant enzyme activities in the blood and SF of RA patients were higher than in the healthy controls. Furthermore, the synovial fluid viscosity was lower and the MDA level was higher in the blood and SF of the patients.

Human red blood cells and other mammalian cells have developed antioxidant defense systems to prevent oxidative damage, allowing their survival in an aerobic environment [26]. SOD activity, a catalyst for dismutation of  $\text{O}_2^{\bullet-}$  into  $\text{H}_2\text{O}_2$  and molecular oxygen, protects cells and tissues from superoxide radicals and other peroxides. Increased SOD activity results in an increase in  $\text{H}_2\text{O}_2$ ; if so CAT and glutathione transformation enzymes, including GPX, GR and GST, detoxify hydrogen peroxide and convert lipid hydroperoxides into nontoxic alcohols [6,7,8]. The statistically significant change in CAT and glutathione transformation enzymes may show that these enzymes play an important but not decisive role in the rheumatic event. It is possible that increased cellular  $\text{H}_2\text{O}_2$  levels cause the decreased SF viscosity and increased MDA levels observed in the patients.

Unlike its intracellular role, the extracellular presence of CAT is significantly less prominent in human fluids such as blood, tissue fluid, cerebrospinal fluid, synovial fluid and seminal plasma [10]. In the present study, we did not find any CAT activity in the investigated SF samples. However, it is possible that the lack of CAT activity may reflect errors in determining CAT in a complex biological system like synovial fluid. Alternatively, the lack of CAT activity might be due to the existence of another SF antioxidant defense mechanism, which decomposes  $\text{H}_2\text{O}_2$  without the participation of CAT, e.g. with GPX.

Under normal circumstances, human SF contains little SOD, CAT, GPX and GST. Thus, ROS generated in the inflamed rheumatoid joint would not be efficiently scavenged. Thus, increased MDA levels in SF from the knee joint of RA patients are an indication of increased lipid peroxidation *in vivo*. Circulating blood carries MDA from the SF to the blood, explaining the significantly higher MDA levels in the blood and SF of RA patients. Similar results have been previously reported [6,7,8].

There are conflicting reports about the activities of antioxidant enzymes in the blood and synovial fluid of RA patients [26,27]. In some of them, lower SOD levels were revealed [9], whereas Gambhir et al. [10] and Ozkan et al. [1] were not able to observe a significant change in SOD activities. Çimen et al. [7] reported that RA patients had higher MDA levels and lower CAT and GPX activities. Terčič et al. [12] found similar results as well as decreased SOD, CAT and GPX activities in SF from RA patients, as compared to normal synovial fluid. In another study [21], it was suggested that





these antioxidant enzymes are rarely present in extracellular fluids, such as SF, which contains little or no CAT activity, and only low activities of SOD isoenzymes and GPX. There is also very little GR and GST. In contrast, Afonso V et al. [28] found that high levels of ROS in SF can induce high SOD isoenzyme activity locally to protect articular cartilage from the harmful effects of ROS. Ostalowska et al. found [13,29] similar results as well as increased antioxidant enzyme activities in SF from patients with the secondary type of osteoarthritis of the knee joint (KOA). Some of them had a history of knee rheumatoid arthritis.

It is possible that these discrepancies are due to differences in the disease activity. Remission or a low disease activity status may deplete antioxidant defences, whilst moderate or high disease activity may upregulate them [30]. We expected a good correlation between the disease activity status and antioxidant enzyme activities. In conclusion, RA patients display altered oxidant and antioxidant systems in their blood and synovial fluid. The increased lipid peroxidation was associated with a tendency for alterations in the antioxidant system including increased activities of all of the antioxidant enzymes, thereby suggesting potential adaptation to the increased ROS in the blood and synovial fluid from RA patients.

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### Author's contribution

Study design – A. Ostalowska, S. Kasperczyk, E. Birkner  
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Data interpretation – A. Ostalowska, S. Kasperczyk, L. Słowińska  
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## Abbreviations

BMI – body mass index  
CAT – catalase  
GPX – glutathione peroxidase  
GR – glutathione reductase  
GST – glutathione-S-transferase  
MDA – malondialdehyde  
MnSOD – manganese superoxide dismutase  
RA – rheumatoid arthritis  
ROS – reactive oxygen species  
SF – synovial fluid  
CuZnSOD – copper-zinc superoxide dismutase  
Disclosures  
None

## REFERENCES

1. Ozkan Y., Yardım-Akadın S., Sepici A., Keskin E., Sepici V., Simsek B. Oxidative status in rheumatoid arthritis. *Clin. Rheumatol.* 2007; 26(1): 64–68.
2. Hitchon C.A., El-Gabalawy H.S. Oxidation in rheumatoid arthritis. *Arthritis. Res. Ther.* 2004; 6: 265–278.
3. Schiller J., Fuchs B., Arnold J., Arnold K. Contribution of reactive oxygen species to cartilage degradation in rheumatic diseases: molecular pathways, diagnosis and potential therapeutic strategies. *Curr. Med. Chem.* 2003; 10: 2123–2145.
4. Henrotin Y., Kurz B., Aigner T. Oxygen and reactive oxygen species in cartilage degradation: friends or foes? *Osteoarthritis Cartilage* 2005; 13: 643–654.
5. Henrotin Y.E., Bruckner P., Pujol J.P. The role of reactive oxygen species in homeostasis and degradation of cartilage. *Osteoarthritis Cartilage* 2003; 11: 747–755.
6. Kamanli A., Naziroglu M., Aydılekin N., Hacıevliyagil C. Plasma lipid peroxidation and antioxidant levels in patients with rheumatoid arthritis. *Cell. Biochem. Funct.* 2004; 22: 53–57.
7. Çimen M.Y.B., Çimen Ö.B., Kaçmaz M., Öztürk H.S., Yorgancıoğlu R., Durak İ. Oxidant/antioxidant status of the erythrocytes from patients with rheumatoid arthritis. *Clin. Rheumatol.* 2000; 19: 275–277.
8. Taysi S., Polat F., Gul M., Sari R.A., Bakan E. Lipid peroxidation, some extracellular antioxidants, and antioxidant enzymes in serum of patients with rheumatoid arthritis. *Rheumatol. Int.* 2002; 21: 200–204.
9. Afonso V., Champy R., Mitrovic D., Collin P., Lomri A. Reactive oxygen species and superoxide dismutases: Role in joint diseases. *Joint Bone Spine* 2007; 74: 324–329.
10. Gambhir J.K., Lali P., Jain A.K. Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis. *Clin. Biochem.* 1997; 30: 351–355.
11. Aletaha D., Ward M.M., Machold K.P., Nell V.P., Stamm T., Smolen J.S. Remission and active disease in rheumatoid arthritis: Defining criteria for disease activity states. *Arthritis Rheum* 2005; 52(9): 2625–2636.
12. Terčič D., Božič B. The basis of the synovial fluid analysis. *Clin. Chem. Lab. Med.* 2001; 39: 1221–1226.
13. Ostalowska A., Birkner E., Wiecha M., Kasperczyk S., Kasperczyk A., Kapoła D., Zon-Giebel A. Lipid peroxidation and antioxidant enzymes in synovial fluid of patients with primary and secondary osteoarthritis of the knee joint. *Osteoarthritis Cartilage* 2006; 14: 139–145.
14. Yamazaki K., Fukuda K., Matsukawa M., Hara F., Matsushita T., Yamamoto N., Yoshida K., Munakata H., Humanishi C. Cyclic tensile stretch loaded on bovine chondrocytes causes depolymerization of hyaluronan: involvement of reactive oxygen species. *Arthritis. Rheum.* 2003; 48: 3151–3158.
15. Cedergren J., Forslund T., Sundqvist T., Skogh T. Intracellular oxidative activation in synovial fluid neutrophils from patients with rheumatoid arthritis but not from other arthritis patients. *J. Rheumatol.* 2007; 34: 2162–2170.



16. Robinson J., Watson F., Bucknall R.C., Edwards S.W. Activation of neutrophil reactive-oxidant production by synovial fluid from patients with inflammatory joint disease. Soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells. *Biochem. J.* 1992; 286: 345–351.
17. Borsiczky B., Szabo Z., Jaberansari M.T., Mack P.P., Röth E. Activated PMNs lead to oxidative stress on chondrocytes: a study of swine knees. *Acta Orthop. Scand.* 2003; 74: 190–195.
18. Mazzetti I., Grigolo B., Pulsatelli L., Dolzani P., Silvestri T., Roseti L., Meliconi R., Facchini A. Differential roles of nitric oxide and oxygen radicals in chondrocytes affected by osteoarthritis and rheumatoid arthritis. *Clin. Sci.* 2001; 101: 593–599.
19. Tiku M.L., Yan Y.P., Chen K.Y. Hydroxyl radical formation in chondrocytes and cartilage as detected by electron paramagnetic resonance spectroscopy using spin trapping reagents. *Free Radic. Res.* 1998; 29: 177–187.
20. Tanabe T., Otani H., Mishima K., Ogawa R., Inagaki C. Phorbol 12-myristate 13-acetate (PMA)-induced oxyradical production in rheumatoid synovial cells. *Jpn. J. Pharmacol.* 1997; 73: 347–351.
21. Carlo M.D., Loeser R.F. Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. *Arthritis Rheum.* 2003; 48: 3419–3430.
22. Borsiczky B., Zadravec G., Röth E. Haemarthros induced articular cartilage degradation. *Acta Chir. Hung.* 1997; 36: 43–45.
23. Dabbagh A.J., Trenam C.W., Morris C.J., Blake D.R. Iron in joint inflammation. *Ann. Rheum. Dis.* 1993; 52: 67–73.
24. Takahashi T., Tominaga K., Takano H., Ariyoshi W., Habu M., Fukuda J., Maeda H. A decrease in the molecular weight of hyaluronic acid in synovial fluid from patients with temporomandibular disorders. *J. Oral. Pathol. Med.* 2004; 33: 224–229.
25. Alpaslan C., Bilgihan A., Alpaslan G.H., Güner B., Özgür Yis M., Erbaş D. Effect of arthrocentesis and sodium hyaluronate injection on nitrite, nitrate, and thiobarbituric acid-reactive substance levels in the synovial fluid. *Oral. Surg.* 2000; 89: 686–690.
26. Bae S.C., Kim S.J., Sung M.K. Inadequate antioxidant nutrient intake and altered plasma antioxidant status of rheumatoid arthritis patients. *J. Am. Coll. Nutr.* 2003; 22: 311–315.
27. Sarban S., Kocyigit A., Yazar M., Isikan U. Plasma total antioxidant capacity, lipid peroxidation, and erythrocyte antioxidant enzyme activities in patients with rheumatoid arthritis and osteoarthritis. *Clin. Biochem.* 2005; 38: 981–986.
28. Afonso V., Champy R., Mitrovic D., Collin P., Lomri A. Reactive oxygen species and superoxide dismutases: role in joint diseases. *Joint Bone Spine* 2007; 74: 324–329.
29. Ostalowska A., Kasperczyk S., Kasperczyk A., Słowińska L., Marzec M., Stoltny T. et al. Oxidant and anti-oxidant systems of synovial fluid from patients with knee post-traumatic arthritis. *J. Orthop. Res.* 2007; 25: 804–812.
30. Grisham M.B. Reactive oxygen species in immune responses. *Free Rad. Biol. Med.* 2004; 36: 1479–1480.