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Specificities of Oxidative Stress in the Blood and Synovial Fluid in Knee Osteoarthritis

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Abstract—Specific features of the regulation of free-radical oxidation in the blood and synovial fluid in knee osteoarthritis were studied. We examined 46 individuals with an average age of 63.26 ± 9.18 years with primary knee osteoarthritis at stages II–III and 27 almost healthy individuals. The enhanced superoxide eliminating activity of the blood plasma was accompanied by a tendency of an increase in nitrosative stress markers, the nitrites/nitrates. In the erythrocytes we observed an increased level of the secondary product of lipid peroxidation, malone dialdehyde, as well as the activation of the superoxide dismutase and glutathione *S*-transferase with decreased activity of the glutathione peroxidase and a reduced glutathione content. In knee osteoarthritis, the mononuclear cells of the peripheral blood were characterized by considerable activation of the superoxide dismutase and glutathione *S*-transferase, moderate activation of the catalase, and slight changes in the glutathione peroxidase activity, together with the enhanced activity of the xanthine oxidoreductase and myeloperoxidase. The redox balance disturbance in the mononuclear cells led, in the long run, to oxidative stress development, which contributed to an increased level of lymphocyte apoptosis.

Keywords: oxidative stress, knee osteoarthritis, enzymatic antioxidants, synovial fluid
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INTRODUCTION

Knee osteoarthritis, or the osteoarthrosis of the knee joint, is an age-associated disorder leading, together with other diseases, to chronic disablement. On the whole, the majority of people over 65 years old show X-ray and/or clinical signs of osteoarthritis, with the frequency of their occurrence increasing every 10 years, from 33% in 60- to 70-year-olds to 43.7% in 80-year-olds [11]. Risk factors such as constant overwork of the joint associated with excessive body weight, trauma, and other knee injuries can play an important role in the development of knee osteoarthritis in elderly people.

Cartilage aging is associated with an increased keratan sulfate content and decreased chondroitin sulfate content without any significant changes in the total content of the sulfated glycosaminoglycans. Moreover, the ability of the chondrocytes to synthesize native matrix proteins also becomes compromised. Chondrocytes aging is associated with increased production of proinflammatory mediators and matrix metaloproteases, the phenomenon known as the secretory phenotype of the aging cells [16]. Activated oxygen metabolites (AOMs) and oxidative stress are involved in aging processes associated with the development of knee osteoarthritis by way of oxidative protein modifications, telomere shortening in chondrocytes, and alterations of the signaling pathways regulating anabolic and catabolic activities in the cells [16].

Chondrocytes in human joints actively produce AOMs, including $NO^{\cdot}$, H_2O_2, and $O_2^{\cdot -}$, that are capable of inducing cartilage calcification and the apoptosis of chondrocytes. AOMs can damage cellular compartments and the extracellular matrix in the case of various synovial pathologies; for example, they can break proteoglycans and collagen into fragments and activate latent forms of collagenases and gelatinases. AOMs may also function as the signal messengers and activate the NF-κB and AP-1 transcription factors, thus inducing the expression of proinflammatory genes. Age-related weakening of the mitochondrial functions leads to the prevalence of glycolysis and to mitochondrial damage due to oxidative stress [20]. In this case, the synovial fluid may serve as something like an indicator of the joint's vital functions. The synovial fluid is the blood transudate and by its composition is rather similar to blood plasma, just slightly differing from it by the lower protein content and by the presence of the specific hyaluronic acid proteoglycan and other synoviocytes' secretion products [6].

The purpose of the current work was to study the specific features of the regulation of the free radical-associated oxidation in the blood plasma, the peripheral blood cells (mononuclear cells and erythrocytes),

and the synovial fluid in the knee osteoarthritis, as well as to study the apoptosis rate in the peripheral blood lymphocytes.

MATERIALS AND METHODS

Blood and synovial fluid were obtained from 46 individuals (average age of 63.26 ± 9.18 years, $M \pm SD$) with primary knee osteoarthritis at stage II–III according to the Kellgren–Lawrence scale. The group included 7 men and 39 women. Twenty-seven donors of the blood transfusion station of the corresponding age were examined as healthy individuals (the control group).

The blood (10 mL) was collected on an empty stomach in the morning from the elbow vein in sterile tubes with K2-EDTA as the anticoagulating agent; blood plasma and erythrocyte sediment were further obtained from it. The mononuclear fraction, including lymphocytes and monocytes, was obtained by centrifuging whole blood in the ficoll–verographine density gradient, $\rho = 1.077$, for 40 min at 1500 rpm [10]. The nonionic detergent Triton X-100 was used in the work. Synovial fluid was collected in sterile tubes with heparin by means of knee arthrocenthesis and was further centrifuged. The obtained supernatant was used for biochemical analysis.

The lipid peroxidation rate was assessed by the thiobarbituric acid-positive product content expressed as the malone dialdehyde (MDA) content [9]. To determine the total nitrite/nitrate content (NO_x^-), we used the colometric approach based on the Griess reaction [2]. The activity of the superoxide dismutase (SOD) and the superoxide eliminating activity (SEA) were assessed by inhibition of the reduction of tetrazolium nitroblue by the superoxide generated in the course of adrenaline autooxidation [8]. The catalase activity and the hydroperoxide utilization rate ($V_{H_2O_2}$) were determined by the decrease in the hydrogen peroxide content, which is able to form a colored complex with ammonium molybdate [4]. The glutathione peroxidase (GPx) activity was determined by the reduced glutathione oxidation rate in the presence of tretbutil hydroperoxide [1]. The glutation *S*-transferase (GST) activity was assessed by the rate of enzymatic production of the glutathione *S*-(2,4-dinitrobenzene) in the reaction of the reduced glutathione with 1-chloro-2,4-dinitrobenzene [1]. The reduced glutathione content (GSH) was assessed by the color reaction with 5,5-dithiobis-(2-nitrobenzoic acid), which results in formation of the compound with the absorption maximum at 412 nm [1]. The myeloperoxidase (MPO) activity was determined spectrophotometrically [7], and the NADPH-oxidase activity was assessed by the 2,6-dichlorophenyl indophenol reduction in the presence of NADPH [3]. Xanthine oxidoreductase (XOR) activity was assessed by the increase in the uric acid content to 295 nm [12]. The hemoglobin content was determined by the hemoglobin cyanide method with the standard Ekolab kit (Russia). Spectrophotometrical analyses were performed with a Beckman Coulter DU 800 spectrophotometer. The apoptosis rate in the peripheral blood lymphocytes was determined by laser flow cytofluorometry in a FACS Canto (Becton Dickinson) cytofluorometer with the commercially-available Annexin V-FITC apoptosis Detection Kit I (BD Biosciences).

Statistical processing of the obtained experimental data was performed with Statistica for Windows 6.1. To assess the normality of the variables distribution, the Kolmogorov–Smirnov and Lillieforce tests were used. The nonparametric Mann–Whitney U test was utilized to compare two independent groups, and we used the Spearman rank correlation, R, to assess the correlations. The differences were considered significant when $p < 0.05$; when $0.05 < p < 0.1$, we considered it a tendency for statistically significant changes.

RESULTS AND DISCUSSION

The performed investigation showed that the MDA content in the peripheral blood erythrocytes was 31% higher in the individuals with knee osteoarthritis than in the control group, while the MDA content in blood plasma was within the normal range (Table 1). Malone dialdehyde is the secondary product of lipid peroxidation and is able to react with proteins with the formation of adducts (ALE, advanced lipid peroxidation end products). This results in the intramolecular and intermolecular crosslinking and impairs the functions of proteins and other compounds containing amino groups [18]. MDA and 4-hydroxynonenal were found to be present in the cartilaginous tissue in osteoarthritis and to promote its degradation [23]. In [17] increased concentrations of malone dialdehyde in the synovial fluid in posttraumatic knee osteoarthritis as compared to normal conditions were reported.

The content of nitrosative stress markers, the nitrites/nitrates (NO_x^-), in the blood plasma of individuals with knee osteoarthritis shows a tendency for a statistically significant increase, $0.05 < p < 0.1$, but it does not correlate with the NO_x^- content in the synovial fluid (Table 1).

For redox homeostasis maintenance and protection from oxidative stress, a coordinated system of enzymatic and low molecular antioxidants works in the chondrocytes. The efficiency of this system largely depends on the activities of two associated enzymes, SOD and catalase, which eliminate superoxide anion radical and hydroperoxide, respectively. Hydroperoxide degradation also involves glutathion peroxidase, the cosubstrate of which is the antioxidant tripeptide glutathione GSH [15].

According to the obtained results, the superoxide eliminating activity in the blood plasma in individuals

Table 1. Redox indices of blood plasma and synovial fluid in the individuals with knee osteoarthritis

Index	Index values in blood plasma in groups, median (25–75% quartiles)		Index	Index values in the synovial fluid in groups, median (25–75% quartiles)	
	control group	individuals with knee osteoarthritis		control group	individuals with knee osteoarthritis
MDA, nM/mL	18.37 (18.06–20.83)	19.70 (17.95–23.69)	MDA, nM/mL	–	17.14 (13.55–19.20)
SEA, units/min mg	1.52 (1.42–1.60)	1.82 (1.51–2.19)*	SEA, units/min mg	–	4.35 (3.38–6.06)
$V_{H_2O_2}$, nM/mL	24.36 (18.20–33.66)	31.57 (21.17–38.44)	$V_{H_2O_2}$, nM/mL	–	6.33 (3.91–10.65)
GPx, MU/g	38.14 (30.89–40.58)	41.30 (36.46–51.71)	GPx, MU/g	–	40.1 (30.76–52.42)
NO_x^-, μM/L	15.81 (15.47–17.11)	17.35 (16.15–19.53)**	GSH, μM/g	–	1.06 (0.59–3.26)
			GST, MU/g	–	0.18 (0.12–0.52)
			NO_x^-, μM/L	–	19.55 (16.03–24.55)

* Statistically significant differences from the control group, U test, $p < 0.05$.

** Differences from the control group, U test, $0.05 < p < 0.1$; the tendency for statistically significant increase in NO_x^- content in knee ostearthritis.

Table 2. Correlative relationships between the redox indices in the individuals with knee osteoarthritis (Spearman rank correlation)

Index 1*	Index 2**	Correlation coefficient, R	p
SEA, blood plasm	SEA, synovial fluid	0.396	0.020
SEA, synovial fluid	GSH, synovial fluid	0.638	0.0005
SOD, mononuclear cells	NADPH-oxidase, mononuclear cells	0.495	0.007
SOD, mononuclear cells	MPO, mononuclear cells	0.709	<0.0001
SOD, mononuclear cells	XOR, mononuclear cells	0.786	0.001
MPO, mononuclear cells	NADPH-oxidase, mononuclear cells	0.690	<0.0001
MPO, mononuclear cells	XOR, mononuclear cells	0.618	0.011

* If $R > 0$, there is a positive correlation; if $R < 0$, there is a negative correlation.

** The higher R is in absolute value, the stronger is the correlation.

with knee osteoarthritis is 20% higher than in healthy individuals and positively correlates with the superoxide eliminating activity in the synovial fluid (R = 0.396, p = 0.02) (Tables 1, 2). At the same time, the rate of hydroperoxide utilization in the blood plasma of the individuals with knee osteoarthritis, as well as the glutathione peroxidase activity, do not differ from that in the control group. On the other hand, hydroperoxide, which is the product of the SOD, xanthine oxidoreductase, and NADPH-oxidase functioning, may give rise to the hydroxyl radical through the reactions of Fenton and Haber–Weiss, which in turn have pronounced cytotoxic properties [5]. Moreover, hydrogen peroxide, which is the substrate for the myeloperoxidase, may contribute to hypohaloid formation, which can damage biomolecules by means of oxidation or halogenation. In [17], an increased summary activity of SOD1 and SOD2 in the synovial fluid in the patients with posttraumatic knee osteoarthritis relative to the corresponding indices in the healthy individuals was demonstrated. It was also reported that there was a decreased level of SOD3 in the synovial fluid at the later stages of knee osteoarthritis as compared to the synovial fluid from injured joints without the signs of knee osteoarthritis; decreased levels of the antioxidants glutathione and ascorbate were also detected [21]. It was determined that the gene expression levels for all three SOD isoforms, especially

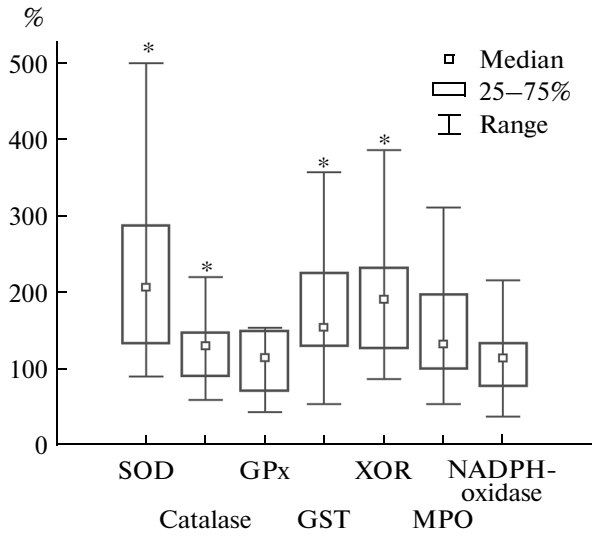

Fig. 1. Changes in the antioxidant and prooxidant enzyme activity in the blood mononuclear cell in individuals with knee osteoarthritis (percentage of normal activity). * $p < 0.05$—statistical significance of the differences.

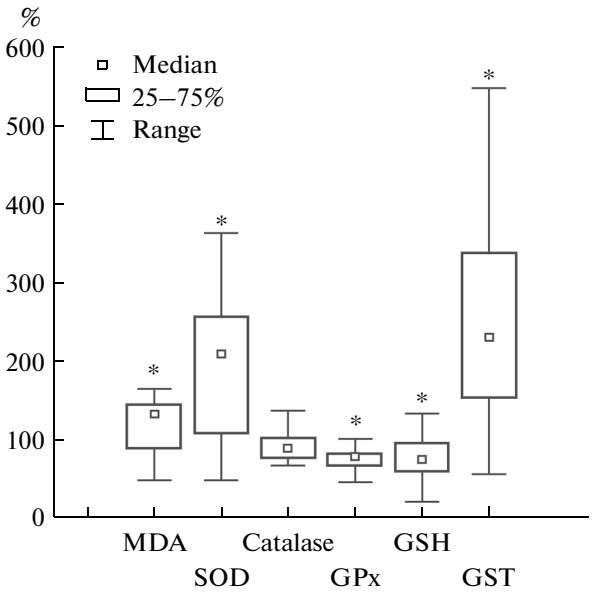

Fig. 2. Changes in the MDA levels and antioxidant enzyme activity in erythrocytes in individuals with knee osteoarthritis (percentage of normal activity). * $p < 0.05$—statistical significance of the differences.

SOD2, were decreased in the cartilaginous tissue in osteoarthritis, which is possibly caused by changes in the promoter DNA methylation pattern [22].

In the mononuclear cells of the peripheral blood, we observed increases in the activities of the enzymatic antioxidants, SOD and catalase, by 107 and 28%, respectively, relative to the corresponding indices in the healthy individuals, while the activity of the glutathione peroxidase was unchanged (Fig. 1). Moreover, the activity of the GST enzyme increased 86% in the mononuclears of the peripheral blood as compared to the control group. These data may indicate oxidative stress development in the mononuclear cells, which is associated with the intensive work of the antioxidant system components affected by their improper balance. It was detected previously in lymphocyte aging that the redox equilibrium in the cell shifts to prooxidative reactions and pronounced oxidative stress. It was demonstrated that the content of the oxidative stress markers, such as MDA, in human lymphocytes increases with age [13].

According to the results, the superoxide dismutase activity in the erythrocytes is two times increased with no statistical changes in the activity of its associated enzyme, catalase (Fig. 2). Moreover, a 21% decrease was detected in the activity of another enzyme able to utilize hydroperoxide, glutathion peroxidase, as well as a 16% decrease in the glutathione content. It should be noted that hydrogen peroxide produced by SOD degrades heme-containing proteins and releases ferrum ions from the hemoglobin, which enhances the cytotoxic effect of the peroxide. In addition, the enzymatic antioxidants catalase and glutathione peroxidase were observed to be inactivated in the presence of hydrogen peroxide [19, 21]. The GST activity appeared to be significantly increased in the erythrocytes of individuals with knee osteoarthritis, as was the case with blood mononuclear cells, which may be a compensatory response to the intensification of lipid peroxidation, in which the content of the secondary metabolite (MDA) was above the normal level in the erythrocytes. GSTs protect the cell from endogenous metabolites, which are produced under oxidative stress, and conjugates toxic products of lipid peroxidation, such as 4-hydroxynonenal, with GSH, thus furthering their excretion [14]. A considerable increase in the activity of glutathione peroxidase, GST, and glutathione reductase in the synovial fluid in knee osteoarthritis was reported earlier, especially in the case of hemarthrosis as compared to the normal state [17]. As was reported in [25], no differences were found in the reduced glutathione content in the synovial fluid between the patients with osteoarthritis and the individuals after knee trauma. However, the antioxidant vitamin E content in the synovial fluid was considerably lower in individuals with osteoarthritis. In our work, we also showed a statistically significant positive correlation between the superoxide eliminating synovial fluid activity and the glutathione content in it, $R = 0.638$, $p = 0.0005$ (Table 2).

The results show that the activity of myeloperoxidase, which participates in the hypochlorite production, is 32% higher than its normal level (Fig. 1). In osteoarthritis, leukocytes may penetrate into the synovial fluid and are able to produce hypochlorite, together with various AOMs, due to the presence of myeloperoxidase. According to [24], the myeloperox-

idase level in the synovial fluid, together with the modified Cl-peptides content, is a diagnostic marker of the inflammatory process at the early stages of osteoarthritis. It is well known that the oxidase isoform of xanthyne oxidoreductase is, on one hand, the source of $O_2^{\cdot -}$ and H_2O_2, but, on the other hand, it produces one of the most important antioxidants, uric acid [5]. In the mononuclear cells of the individuals with knee osteoarthritis, xanthine oxidoreductase activity is 91% higher than normal. According to the results, the SOD activity in mononuclear cells directly correlates with the activity of NADPH-oxidase, myeloperoxidase, and xanthine oxidoreductase, while the myeloreductase activity directly correlates with the activity of the NADPH-oxidase and xanthine oxidoreductase (Table 2). It is well known that the work of these enzymes is interrelated. The product of the reaction performed by one enzyme becomes the substrate in reactions performed by other enzymes. For example, the superoxide anion radical ($O_2^{\cdot -}$) is produced in reactions catalyzed by the NADPH-oxidase and xanthine oxidoreductase and appears as the substrate for the SOD that transforms $O_2^{\cdot -}$ into H_2O_2. Hydrogen peroxide may further serve as the substrate for the myeloperoxidase, with the formation of toxic products participating in the development of halogenizing stress [5].

The presented data on the redox balance disturbance in mononuclear cells of peripheral blood in individuals with primary knee osteoarthritis is supported by the results of the assessment of the apoptosis level of peripheral blood lymphocytes by means of laser flow cytofluorometry. Via cytofluorometric analysis with FITC-labeled annexins and propidium iodide, it appears to be possible to identify precisely cells at different stages of apoptosis and necrosis.

The results show that the portion of lymphocytes at the early apoptosis stage in the total lymphocytes pool is 5.54 (5.35–6.2)% in healthy individuals and 8.51 (6.15–10.08)% in patients with primary knee osteoarthritis (the results are presented as the median, 25–75% quartiles, $p = 0.013$), which is 53% more than in the normal state. This provides evidence of the considerable intensification of lymphocytes apoptosis in knee osteoarthritis.

CONCLUSIONS

The performed investigation allowed elucidation of some specific features of oxidative stress development in knee osteoarthritis. In the blood plasma, we detected enhanced superoxide eliminating activity and noted the tendency toward nitrosative nitrites/nitrates levels, which are markers of nitrosile stress. Profound disorders in the redox state were detected in the blood cells of individuals with knee osteoarthritis. In the erythrocytes of individuals with knee osteoarthritis, a twofold increase in the hydrogene peroxide-producing SOD activity was observed with insufficient activity of the enzymes performing peroxide utilization (the inhibition of the glutathione peroxidase, slight changes in the catalase activity, and reduced glutathione levels). This leads to the accumulation of the secondary product of lipid peroxidation, malone dialdehyde, despite the significant level of the glutathione-*S*-transferase activation. In mononuclear cells of peripheral blood, we also observed a strong imbalance in the functions of the enzymatic antioxidant system and the stimulation of prooxidant components associated with knee osteoarthritis. Significant activation of the enzymes producing reactive oxygen species (SOD and xanthine oxidoreductase) is accompanied by moderate activation of catalase and slight changes in the glutathione peroxidase activity with an increase in myeloperoxidase activity. This shifts the prooxidant–antioxidant system equilibrium to the prevalence of prooxidative processes and, finally, leads to oxidative stress development, which in its turn promotes lymphocyte apoptosis.

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