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Lipid peroxidation and antioxidant enzymes in synovial fluid of patients with primary and secondary osteoarthritis of the knee joint

A. Ostalowska M.D., Ph.D.†, E. Birkner Assist. Prof.†, M. Wiecha M.A.†, S. Kasperczyk M.D., Ph.D.†*, A. Kasperczyk Ph.D.†, D. Kapolka M.D., Ph.D.‡ and A. Zon-Giebel M.D.‡

† *Medical University of Silesia, Department of Biochemistry, Jordana 19, 41-808 Zabrze, Poland*

‡ *Silesian Hospital of Rheumatology and Rehabilitation, Department of Rheumatology, Szpitalna 11, 43-450 Ustron, Poland*

Summary

Objective: Osteoarthritis of the knee (KOA) is a common, age-related, joint disorder associated with loss of articular cartilage, osteophyte formation, subchodral bone change and synovitis. Recent studies have shown that reactive oxygen species (ROS) may participate in the initiation and progression of KOA. This study examines potential changes in the activities of antioxidant enzymes (superoxide dismutase, both isoenzymes zinc-copper superoxide dismutase and manganese superoxide dismutase) and glutathione transformation enzymes (glutathione peroxidase, glutathione reductase and glutathione-S-transferase) in synovial fluid of KOA patients, and estimates their relationship to the degree of lipid peroxidation in synovial fluid evaluated by malondialdehyde concentration, synovial fluid viscosity, type and duration of KOA.

Design: Synovial fluid samples obtained by transdermal arthrocentesis from 41 patients with KOA (23 had primary KOA and 18 had secondary KOA) and 22 control subjects were analyzed. Activities of antioxidant enzymes were analysed with the use of kinetic method, MDA concentration was measured fluorometrically by the Ohkawa method, and synovial fluid viscosity was measured using a cone-late viscometer Brookfield DV-II+ and a test by Ropes.

Results: Patients with KOA had significantly increased activities of all enzymes when compared to the control subjects for both KOA subgroups. The synovial fluid viscosity was significantly decreased and the synovial fluid test by Ropes was abnormal in KOA patients, mainly in the secondary KOA subgroup. The activities of all antioxidant enzymes were significantly negatively correlated with synovial fluid viscosity and duration of KOA.

Conclusions: Patients with KOA display abnormal antioxidant status of synovial fluid with increased activities of antioxidant enzymes and decreased synovial fluid viscosity. Furthermore, synovial fluid viscosity, and activity of GR can be used to distinguish the primary from the secondary type of KOA.

Key words: Degenerative joint disease, Antioxidant enzymes, Malondialdehyde, Synovial fluid viscosity.

Abbreviations: CAT catalase, GPX glutathione peroxidase, GR glutathione reductase, GST glutathione-*S*-transferase, HA hyaluronic acid, KOA osteoarthritis of the knee joint, MDA malondialdehyde, MnSOD manganese superoxide dismutase, ROS reactive oxygen species, SF synovial fluid, SOD superoxide dismutase, ZnCuSOD isoenzymes zinc–copper superoxide dismutase.

Introduction

Osteoarthritis, also known as degenerative joint disease, is found more commonly in the knee (osteoarthritis of the knee joint, KOA) than in any other weightbearing joint in the human body[1,2]. The principal pathologic features of this disease include progressive focal degradation of the articular cartilage, which is associated with chronic pain and loss of knee function[3,4]. The underlying mechanism of cartilage matrix degradation in KOA is poorly understood but the reactive oxygen species (ROS) are implicated as the main causative factors[5–8]. The initially formed radical is generally superoxide radical ($O_2^{\cdot-}$); however, it may be converted to more harmful species, hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) by interaction with intracellular free metals. These reactive oxygen species are capable of oxidising and, subsequently, damaging numerous components of the joint, including collagen, proteoglycans and hyaluronan[9–11].

Fortunately, several lines of antioxidant defence exist both intra- and extracellulary to protect tissues against damage from ROS and other prooxidants. For example, there exists a complicated system of defence against ROS which is provided by antioxidant enzymes: superoxide dismutase (SOD), both isoenzymes zinc–copper superoxide dismutase (ZnCuSOD) and manganese superoxide dismutase (MnSOD), catalase (CAT) and glutathione transformation enzymes, including glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-*S*-transferase (GST)[12,13].

It was our hypothesis that if ROS are increased in involved joints of KOA patients, then products resulting from oxidative modification of synovial fluid (SF) components would increase and antioxidant status of the SF would

*Address correspondence and reprint requests to: Dr Slawomir Kasperczyk, M.D., Ph.D., Medical University of Silesia, Department of Biochemistry, ul. Jordana 19, 41-808 Zabrze, Poland. Tel/Fax: 48-32-272-23-18; E-mail: skasperczyk@slam.katowice.pl
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decrease. This study was designed with the following objectives: (1) to examine potential changes in antioxidant enzymes activities – SOD, both isoenzymes ZnCuSOD and MnSOD, and glutathione transformation enzymes GPX, GR and GST in osteoarthritic SF of the knee joints, and (2) to estimate their relationships to degree of lipid peroxidation in SF evaluated by malondialdehyde (MDA) concentration, SF viscosity, type and duration of KOA.

Materials and methods

PATIENT CHARACTERISTICS

SF samples were obtained from 64 patients with the knee joints exudates treated in the Department of Rheumatology, Silesian Hospital of Rheumatology and Rehabilitation in Ustron and in the Department of Orthopaedic Surgery, Special Hospital No 4 in Bytom.

Forty-two of these patients had been diagnosed as having KOA based on clinical, laboratory, and radiologic findings (mean age, 54 years; mean disease duration, 4 years). Twenty-four patients had the primary KOA, whereas 18 patients had the secondary KOA. All patients from the secondary KOA subgroup had a history of knee joint injuries. Twenty-two patients selected from these who had atraumatic and asymptomatic normal knees (mean age, 40 years) were classified as controls. In addition, this group consisted of patients without obesity (body mass index (BMI) less than 30), who did not work in professions related to excessive load of the knee joints (e.g., drivers of trucks), and did not practise injurious sports (e.g., soccer, skiing), and so far not diagnosed and not treated for osteoarthritis, post-traumatic, inflammatory or another knee joint pathology. The final verification of the study groups was carried out after preliminary analysis of SF, including a visual examination of color, turbidity, viscosity, test by Ropes, volume and biochemical parameters. SF samples collected from the control subjects demonstrated characteristic features of physiological SF. Patients with signs of rheumatoid arthritis, malignant tumours, diabetes, serious liver, kidney or heart insufficiency or other systemic diseases that might cause an increase in oxidations were also excluded.

The study was approved by Medical Ethics Committee of the Medical University of Silesia (NN-013-283/03).

SAMPLE PREPARATION

SF samples were obtained with needle aspiration or during knee arthroscopy, and next 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 into a test tube containing tripotassium ethylenediamine tetra-acetate (K_3EDTA) as an anticoagulant, immediately placed on ice and centrifuged at 3000 *g* for 30 min. Supernatant was separated and stored at −76°C until analysis but no longer than 4 weeks. Activities of antioxidant enzymes, MDA concentration, and SF viscosity in the study groups were determined.

ASSAY

In the test tube without an anticoagulant, volume, clarity and colour of SF were examined before centrifugation.

Execution test by ropes (the mucin clot test)

Concentration of hyaluronic acid (HA) in SF was determined indirectly by measurement of precipitation knocked out by acidification with acetic acid (addition of five drops of 5% acetic acid into 3 ml of SF). Data are shown as: 0 – compact reaction, 1 – compact/floccular reaction, 2 – floccular reaction, 3 – floccular/turbidity reaction, 4 – turbidity reaction.

Assay for SF viscosity

In the test tube containing K_3EDTA – before centrifugation – the SF viscosity was measured using a cone-late viscometer Brookfield DV-II$^+$ at 37°C. Data are shown as cP ($N{\cdot}s{\cdot}m^{-2}$).

Determination of SOD activity

The activity of SOD was indicated by the Oyanagui[14] method. Superoxide anion radical ($O_2^{\cdot-}$), produced in the reaction of xanthine with O_2 catalysed by xanthine oxidase, reacts with hydroxylamine producing nitric ion. Nitric ion combines with naphthalene diamine and sulfaniline acid producing a coloured product; concentration of this mixture is proportional to the amount of $O_2^{\cdot-}$ produced. Enzymatic activity is expressed in nitric unit (NU) in each millilitre of SF (NU/ml). One NU means 50% of inhibition by SOD of nitric ion production in this method. SOD activity was indicated in SF. In synovial fluid SOD isoenzymes, MnSOD and ZnCuSOD, were also indicated using potassium cyanide (KCN) as the inhibitor of the ZnCuSOD by the Oyanagui method.

Determination of CAT activity

CAT was analysed with the use of Aebi[15] kinetic method. Before CAT was marked, the SF was diluted 100 times with Tris/HCl buffer, pH 7.4. Kinetic designation was carried out in a quartz tank. 2.5 ml of substrate was mixed consisting of 50 mM Tris/HCl buffer with pH = 7.4 and perhydrol with 50 ml of SF. After 10 s, absorbance was measured at 240 nm and the kinetic changes of absorbance were marked every 30 s for 2 min. Enzymatic activity was not present in SF.

Determination of GPX activity

GPX activity in SF was assayed by the Paglia and Valentine[16] kinetic method. GPX catalyses reaction between reduced glutathione (GSH) and H_2O_2. The product of this reaction – oxidized glutathione (GSSG) – is recovered back to GST using nicotinamide adenine dinucleotide phosphate ($NADPH + H^+$) catalysed by GR. Decrease in absorbance is measured at 340 nm. Activity of GPX was determined as the quantity of micromoles of $NADPH+H^+$ used to recover GSH in 1 min converted to 1 l of SF (IU/l).

Determination of GR activity

GR activity was also assayed by the kinetic method[17]. The decrease of the concentration of $NADPH+H^+$ after reduction of GSSG back to GSH was measured. Activity of GR was determined as the quantity of micromoles of $NADPH+H^+$ used to recover GSH in 1 min converted to 1 l of SF (IU/l).

Determination of GST activity

GST was analysed by the Habig and Jakoby[18] kinetic method using 1-chloro-2,3-dinitrobenzene. GST reacts with 1-chloro-2,3-dinitrobenzene producing thioether. Increase in absorbance is measured at 340 nm. Activity of GST was determined as the quantity of micromoles of thioether produced in 1 min in 1 l of SF (IU/l).

Table I
Age, sex, duration of osteoarthritis of the knee (KOA), and degree of lipid peroxidation in synovial fluid evaluated by malondialdehyde (MDA) concentration in the KOA group, and separately in the primary KOA subgroup and the secondary KOA subgroup

	Control group	KOA group	*P* level when compared to control	Primary KOA subgroup	*P* level when compared to control	Secondary KOA subgroup	*P* level when compared to control	*P* level when compared primary to secondary KOA
	Mean ± S.E.M.	Mean ± S.E.M.		Mean ± S.E.M.		Mean ± S.E.M.		
Age (years)	41.0 ± 3.5	53.4 ± 2.3	<0.001	58.2 ± 2.5	<0.001	46.9 ± 3.8	0.091	0.015
Number of men/women	19/3	29/11	0.351	16/7	0.319	13/4	0.350	0.454
Duration of KOA (years)	–	4.04 ± 0.81	–	5.48 ± 1.12	–	1.75 ± 0.80	–	0.007
MDA concentration (μmol/l)	1.33 ± 0.09	1.49 ± 0.08	0.264	1.48 ± 0.10	0.233	1.50 ± 0.12	0.497	0.773

Determination of MDA concentration

MDA concentration was measured fluorometrically as 2-thiobarbituric acid-reactive substance (TBARS) in SF by the Ohkawa[19] method. SF sample was mixed with 8.1% sodium dodecyl sulfate, 20% acetic acid and 0.8% 2-thiobarbituric acid. The method was modified by adding sodium sulphate (100 mmol/l) and 3,5-diisobutylo-4-hydroxytoluen (2.5 μmol/l). After vortexing, SF sample was incubated for 1 h in 95°C and butanol–pyridine 15:1 (v/v) was added. The mixture was shaken 10 min and then centrifuged. Butanol–pyridine layer was measured fluorometrically at 552 nm (515 nm excitation). TBARS value is expressed as malonyldialdehyde equivalent. Tetraethoxypropane was used as the standard. Data are shown as micromole MDA/l SF (μmol/l).

STATISTICAL ANALYSIS

Statistical analysis was performed with Statistica 6.0 PL software. Statistical methods included mean and standard error of mean (S.E.M.). Shapiro–Wilk's test was used to verify normality and Levene's test to verify homogeneity of variances. Statistical comparisons were made by *t*-test, *t*-test with separate variance estimates or Mann–Whitney *U* test. Chi-square or Fisher test was used to analyse sex. 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.

Results

Table I depicts characteristics of the study population. The study population did not differ in sex. The primary KOA subgroup was the oldest and the control subjects were the youngest. The average duration of KOA was higher in the primary than in the secondary type of KOA ($P = 0.007$).

The SF activities of total SOD and both isoenzymes ZnCuSOD and MnSOD were significantly higher in KOA patients (+103%, +249% and +63%, respectively, $P < 0.001$) as well as for both the primary (+101%, +275% and +57%, respectively, $P < 0.001$), and the secondary (+105%, +216% and +71%, respectively, $P < 0.001$) type of KOA than in the control subjects (Fig. 1). The SF activities of total SOD, ZnCuSOD and MnSOD did not differ between the primary and the secondary KOA subgroup.

Also activities of GPX, GST and GR in SF were significantly higher in KOA patients than in the control subjects for both the primary and the secondary type of KOA (Fig. 2). In patients with KOA, the SF activities of above enzymes were significantly higher (+722%, +404% and +154%, respectively, $P < 0.001$), in the primary KOA subgroup about +659%, +349% and +105%, respectively ($P < 0.001$), and in the secondary KOA subgroup about +795%, +469% and +212%, respectively ($P < 0.001$). The mean synovial fluid GR activity was significantly higher in the secondary than in the primary type of KOA (22.6 ± 2.66 IU/l vs 17.6 ± 2.60 IU/l, $P < 0.001$), and the mean SF activities of GPX and GST were higher in the secondary than in the primary type of KOA but not statistically significant.

Also the mean concentration of synovial fluid MDA in KOA patients was higher than that in the control subjects but not statistically significant (1.49 ± 0.08 μmol/l vs 1.33 ± 0.09 μmol/l, $P = 0.264$) (Table I).

The SF viscosity was significantly lower in KOA group and the secondary KOA subgroup compared with the control subjects (−39% in KOA group, $P = 0.003$, and −62% in the secondary KOA subgroup, $P < 0.001$) (Fig. 3). The SF viscosity was lower by about −52% in the secondary than in the primary type of KOA (14.4 ± 2.65 cP vs 9.0 ± 1.89 cP, $P = 0.040$). The SF test by Ropes was incorrect in KOA patients, mainly the most incorrect in the

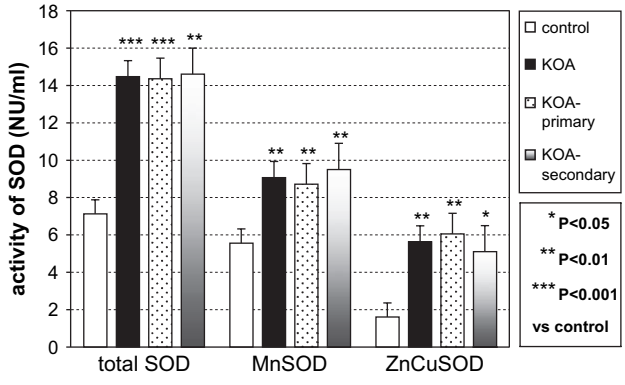

Fig. 1. SF total SOD, MnSOD and ZnCuSOD activities in KOA group, primary and secondary KOA subgroup and control subjects. Values are expressed as mean NU of enzyme equivalents per millilitre of SF. Error bars represent S.E.M.

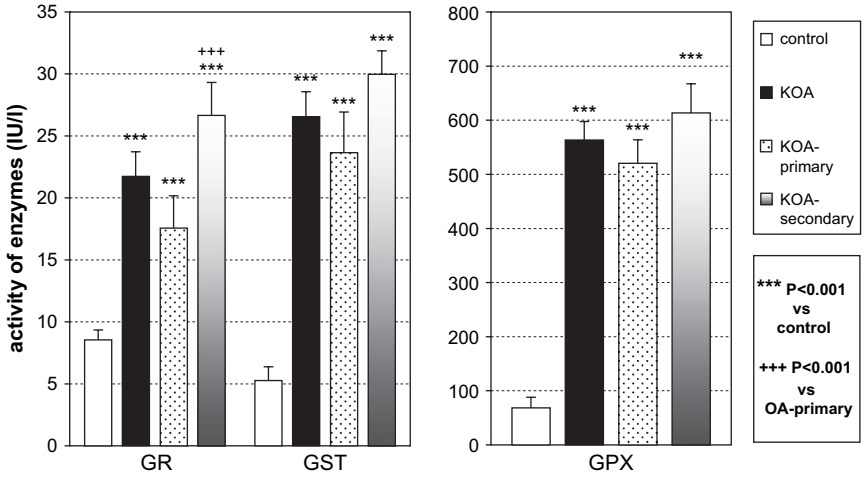

Fig. 2. Synovial fluid GR, GST and GPX activities in KOA group, primary and secondary KOA subgroup and control subjects. Values are expressed as mean IU of enzyme equivalents per litre of SF. Error bars represent S.E.M.

secondary KOA subgroup (1.70 $\pm$ 0.20 in the primary, and 2.47 $\pm$ 0.30 in the secondary type of KOA vs 0.62 $\pm$ 0.13, $P < 0.001$) (Fig. 3).

The SF activities of all antioxidant enzymes significantly positively correlated with test by Ropes ($R = 0.37$ with SOD, 0.28 with ZnCuSOD, 0.55 with GPX, 0.59 with GR, and 0.47 with GST) and negatively correlated with the SF viscosity ($R = -0.46$ with SOD, -0.37 with MnSOD, -0.57 with GPX, -0.43 with GR, and -0.43 with GST) and duration of KOA ($R = -0.36$ with GPX and -0.44 with GR). No relationships between patients age and all antioxidant enzymes activities, MDA concentration or SF viscosity level were observed in KOA patients in the study population. (Table II).

Discussion

KOA is a slowly progressing chronic disease, primary or secondary, which causes disturbance in cartilage metabolism, leading to cartilage destruction, and subsequently knee damage. ROS, including superoxide anion, hydrogen peroxide and hydroxyl radical, mediate articular cartilage and joint damage in patients with KOA and these patients often exhibit much higher levels of oxidants in SF[20,21].

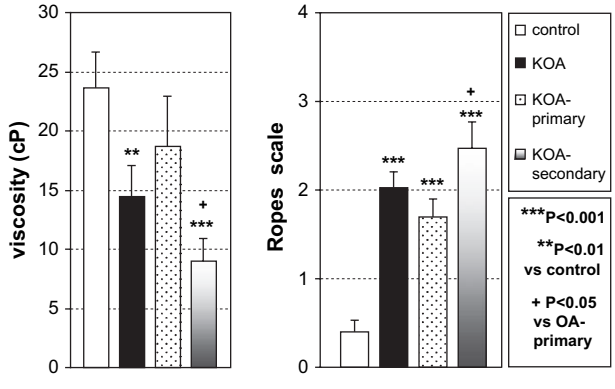

Fig. 3. SF viscosity and test by Ropes in KOA group, primary and secondary KOA subgroup and control subjects. Values are expressed as mean N s m^{-2} (cP) for viscosity and for Ropes test as a mean value of the scale: 0 – compact reaction, 1 – compact/floccular reaction, 2 – floccular reaction, 3 – floccular/turbidity reaction, 4 – turbidity reaction. Error bars represent S.E.M.

In the current study, patients with KOA were grouped according to their osteoarthritis types as patients with the primary KOA and the secondary KOA. All patients with KOA had significantly increased all study antioxidant enzymes activities and MDA concentration in SF, and decreased SF viscosity when compared to the control subjects. However, patients with the secondary type of KOA had significantly increased activity of GR in SF and significantly decreased SF viscosity when compared to the primary type of KOA.

The decrease of SF viscosity level present in the osteoarthritic knee joints as compared to control joints supports our hypothesis that O_2^- and the rest of ROS are elevated in involved joints. For the production of O_2^- in SF several theories have been proposed. Edmonds *et al.*[22] and Grisham[23] found that movement of the osteoarthritic joint with exudates, generates sufficient pressure to cause transient ischaemia of the superficial synovial membrane. This raises possibility that the joint is subjected to ischaemia and reperfusion injury that involves O_2^- products by the xanthine dehydrogenase enzyme[24]. Studies by Wientjes and Segal[25], and other researchers[7,8,26,27] using *in vitro* models on cell cultures revealed that under unstressed conditions articular cartilage cells produce O_2^- in SF, probably through the activation of NADPH oxidase[28,29]. Dahlgren and Karlsson[30] and Borsiczky *et al.*[31] found that phagocytosis is a source of O_2^- in involved joints containing neutrophils, monocytes, and macrophages as activated phagocytes. Disruption of components of joint through knee injury can lead to an increase of O_2^- production, partially due to release and oxidation of haemoglobin from erythrocytes with activation of NADPH oxidase[32–34].

Formed O_2^- can be converted to H_2O_2 or $^{\cdot}OH$. *In vivo* hydrogen peroxide is detoxified and metabolized by the antioxidant enzymes CAT and GPX. However, in the presence of transition metals, hydrogen peroxide can be further degraded to powerful oxidant hydroxyl radical.

Damage caused by ROS has been suggested as the cause of the decrease of SF viscosity. Whilst it is unknown, which mechanisms are responsible for this change, it might result in fragmentation of link proteins, loss of ability of

Table II
Correlations between age, duration of KOA, test by Ropes, SF viscosity and antioxidant enzymes – total SOD, MnSOD and ZnCuSOD, GPX, GR and GST activities and degree of lipid peroxidation in SF evaluated by MDA concentration in the study population (Spearman correlation – R, $P < 0.05$)

	MDA	SOD	MnSOD	ZnCuSOD	GPX	GR	GST
Age	NS	NS	NS	NS	NS	NS	NS
Duration of KOA	NS	NS	NS	NS	−0.36	−0.44	NS
Test by Ropes	NS	0.37	NS	0.28	0.55	0.59	0.47
Viscosity	NS	−0.46	−0.37	NS	−0.57	−0.43	−0.43

NS – no statistical significance.

proteoglycan monomers to associate with HA, fragmentation of HA[35], chemical modification of link proteins, and other changes by excessive ROS[36–38]. Takahasi *et al.*[39] and Bates *et al.*[40] found that exposition of hyaluronan on ROS, especially $^{\cdot}OH$, potentially results in decreased high molecular weight hyaluronan. Indeed, $^{\cdot}OH$ may inhibit cartilage proteoglycan synthesis, e.g., by interfering with adenosine triphosphate synthesis aggravating the effects of free-radical-mediated cartilage degradation[8,40,41].

In addition, high molecular weight hyaluronan can inhibit phagocytosis by polymorphonuclear lymphocytes. This function results in the inhibition of superoxide anion and hypochlorous acid production by phagocytes[42]. When hyaluronan is broken down into chain of lower molecular weight by ROS, it can no longer inhibit phagocytosis or the associated free-radical production; and even more free radicals will be produced within the joint.

However, we also hypothesised that the lipid peroxidation would significantly increase in association with an increase of ROS, which was not the case in this study. Lipid peroxidation, measured by MDA concentration, was found to be higher, although not significantly, in the SF from patients with KOA in this study. Grigolo *et al.*[43] found that chondrocytes, from osteoarthritic knees, activated *in vitro* produced ROS and significantly accelerated lipid peroxidation compared to those activated *in vivo*. This would suggest that SF with hyaluronan effectively protects from oxidative damage. Grigolo *et al.*[43]. found that intraarticular use of hyaluronan in the treatment of KOA, significantly decreased concentration of TBARS in SF. This would suggest that membrane lipids are only one of the possible targets of oxidative damage, and SF compounds such as HA would be more susceptible to oxidative stress.

Under normal circumstances, ROS are eliminated by scavengers and detoxifying reactions, catalysed mainly by antioxidant enzymes: SOD, CAT and glutathione transformation enzymes, including GPX, GR and GST. The first line of defence against ROS is SOD, which removes $O_2^{\cdot-}$ by catalysing the dismutation reaction. CAT protects cells and tissues by directly decomposing hydrogen peroxide. The glutathione transformation enzymes eliminate H_2O_2 in reaction catalysed by GPX. In SF, these antioxidant enzymes often coexist[44].

The absence or dysfunction of some of these defence systems make the cells and tissues vulnerable to oxidative damage[45].

We also hypothesised that the antioxidant status would decrease in association with an increase in ROS. Antioxidant status, measured by activities of antioxidant enzymes, was found to be significantly higher in SF from patients with KOA compared to control subjects. In addition to being in contrast to our hypothesis, it is also in contrast to many reports of human osteoarthritis, which have found decreased antioxidant enzyme activities in SF from involved joints. Ivanova and Ivanova[46] found that there was no SOD, no or low CAT in SF from osteoarthritic joints. Schumacher[47] found similar results as well as decreased SOD, CAT and GPX activities in SF from KOA patients, as compared to normal SF. The results reported in these papers are not surprising because these antioxidant enzymes are rarely present in extracellular fluids, which contain little or no CAT activity, and only low activities of SOD and GPX. There is also very little GR and GST[45]. In contrast, Terčič and Božič[48] found that high levels of ROS in SF can induce high activity of SOD locally to protect articular cartilage from the harmful effects of the ROS[24,33,49,50].

It is possible that difference between our study and studies by other investigators, regarding antioxidant status, is due to differences in the stage of the disease. Chronic joint disease may deplete antioxidant defences, whereas acute inflammation may upregulate them[44]. The samples used in the present study represent KOA with inflammatory exudates in the knee joints, especially in the secondary type of KOA. Besides, the negative correlation of SF antioxidant enzymes activities with duration of KOA may suggest that with longer disease duration, induction of antioxidant enzymes and consequently their activities in SF progressively decrease.

In conclusion, the SF viscosity level was decreased, and lipid peroxidation measured by MDA concentration was increased in osteoarthritic joints as compared to control joints, indicating greater oxidative modification of SF components by ROS in association with KOA. Concurrent with the increased presence of ROS in the osteoarthritic joints, was a tendency for changed antioxidant status with increased antioxidant enzymes activities, suggesting a potential adaptation to the increased ROS in SF from osteoarthritic knee joints.

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