DNA Damage and Oxidative Stress in Patients with Osteoarthritis: a Pilot Study

Osteoartrit Hastalarında DNA Hasarı ve Oksidatif Stres: Pilot Çalışma

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Abstract

Objective: We aimed to investigate the effect of osteoarthritis on basal levels of DNA strand breaks and on the oxidative/anti-oxidative status of patients with osteoarthritis, and healthy subjects.

Patients and Methods: The study population contained 29 patients with knee osteoarthritis and 26 healthy controls. We used single-cell gel electrophoresis (also called comet assay) to measure DNA strand breaks in peripheral blood mononuclear leukocytes. Plasma levels of total antioxidant status (TAS) and total oxidative status (TOS) were determined, and oxidative stress index OSI was calculated using a novel automated measurement method.

Results: The mean values of DNA damage, plasma TOS and OSI were significantly higher in patients with osteoarthritis than in controls ($p < 0.001$, for all). Plasma TAS level was lower in patients than in healthy controls ($p < 0.001$). There was a significantly positive correlation between OSI and DNA damage ($r = 0.530$, $p < 0.001$).

Conclusion: The findings indicated that Reactive Oxygen Species produced in the progression of the disease may induce oxidative damage in normal cells. *(Rheumatism 2007; 60-3)*

Key words: Osteoarthritis, DNA damage, oxidative stress, total antioxidative status

Özet

Amaç: Bu çalışma osteoartritli hastalarda DNA hasarı ile serum oksidatif/antioksidatif durumun araştırılması amacıyla planlandı.

Hastalar ve Yöntem: Çalışmaya diz osteoartriti olan 29 hasta ve 26 sağlıklı kontrol grubu dahil edildi. Periferik lökosit DNA hasarı single-cell jel elektroforez (comet) yöntemi ile, plazma total oksidan, antioksidan miktarı ve oksidatif stres indeksi novel otomatik ölçüm yöntemi ile değerlendirildi.

Bulgular: Periferik lökosit DNA hasarı, plazma total oksidan (TOS) miktarı ve oksidatif stres indeksi (OSI) osteoartrit grubunda kontrol grubuna göre anlamlı ölçüde yüksek, total antioksidan miktarı ise düşük bulundu ($p < 0.001$). Ayrıca DNA hasarı ile oksidatif stres indeksi arasında pozitif korelasyon mevcuttu ($r = 0.530$, $p < 0.001$).

Sonuç: Çalışma sonuçlarımıza göre osteoartrit patojenezinde oksidatif stresin rolü olabileceğini ve buna bağlı gelişebilecek DNA hasarının osteoartritli hastalarda normal populasyona göre daha fazla olduğu düşünülebilir. *(Romatizma 2007; 22: 60-3)*

Anahtar kelimeler: Osteoartrit, DNA hasarı, oksidatif stres, total antioksidan kapasite

Introduction

Osteoarthritis (OA) is the most common form of arthritis and characterized by softening and disintegration of articular cartilage. The progressive deterioration and loss of articular cartilage leading to an irreversible impairment of joint structure are the final pathogenic events common to osteoarthritis (1).

In normal conditions, chondrocytes are living in an avascular environment with low oxygen supply as a consequence. Chondrocytes display a metabolism adapted to anaerobic conditions. In pathological conditions, oxygen tension in synovial fluid is subject to fluctuation as a consequence of ischemia-reperfusion phenomenon, p athological acceleration of tissue metabolism and sustained abnormal strains on the joint. In response to partial oxygen pressure variations, chondrocytes produced abnormal levels of Reactive Oxygen Species (ROS) that is generally produced by immune cells to assume host defense (2, 3). It has been found that ROS production is increased in joint diseases such as osteoarthritis and rheumatoid arthritis (4).

Reactive Oxygen Species are highly reactive transient chemical species, such as nitric oxide, superoxide and hydroxyl radical anion. Normal cellular metabolism appears to be a primary source for endogenous ROS, release of ROS from these cellular processes, and their evasion from

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antioxidant pathways (e.g. glutathione peroxidase, vitamin E), result in the background levels of modification of cellular molecules, including DNA, which can be detected in normal tissue. However, when the production of damaging ROS exceeds the capacity of the body's antioxidant defenses to detoxify them, a condition known as oxidative stress occurs (5, 6).

Deoxyribonucleic acid (DNA) is a particularly target for oxidation, as damage may lead to important alterations (6). ROS produced by activated neutrophils during the inflammatory response play an important role in the pathogenesis of inflammatory disease including hepatitis, gastritis, colitis, chronic renal failure, rheumatoid arthritis, and AS (7, 8).

It has been proposed that DNA damage induced by ROS may contribute to increased mutation rates, genome instability, apoptosis and associated tissue regeneration and cell proliferation (9). Therefore, we evaluated oxidative status, total antioxidant capacity and values of DNA damage in peripheral blood lymphocytes in patients with osteoarthritis and healthy controls.

Patients and Methods

Subjects

A consecutive sample of outpatients (n=166) with knee complaints was screened for knee osteoarthritis. Antero-posterior weight-bearing radiographs of both knees were taken. All patients' routine hematological and biochemical parameters were determined. Exclusion criteria included usage of supplemental vitamins, diabetes mellitus, coronary artery disease, malignancy, myoma uteri, systemic or local infection, inflammatory arthritis, hypertension, acute-chronic liver diseases, renal dysfunction, anemias, GI parasitic diseases, clinically unstable medical illnesses, neoplastic diseases, established deficiency of vitamin B12 or folate. In a 6-month period, out of 166 patients, 25 were undergoing treatment for OA, 34 had inflammatory rheumatic diseases or infectious or endocrine-related arthropathy, 59 were smoker, 11 were pregnant or nursing mother, 6 had used of any medication within 4 weeks before the initiation of the study, 2 did not wish to be interviewed. Finally, twenty-nine patients with knee OA based on clinical and radiological findings (20 females and 9 males, mean age 40.9 ± 2.5 years) included in the study.

Control group consisted of 26 healthy individuals (18 females, 8 males; mean age and 39.0 ± 4.7). The controls were recruited from the family of those in the patient group. Anteroposterior radiographs of both knees were used in controls. Routine haemathological and biochemical parameters were determined in controls. None of the controls was a smoker or alcohol consumer. All subjects were informed about the study.

Self-reported pain, stiffness and functional ability were measured by the Western Ontario McMaster Universities Osteoarthritis Index (WOMAC) (10). The WOMAC is a self-rating instrument designed for patients with lower extremity disease. WOMAC consists of three subscales: pain, stiffness and physical functioning. Global scores range from 0 (no disease) to 100 (worst disease).

Sample Preparation

After an overnight fasting, venous blood was withdrawn into heparinized tubes and citrated tubes. One mililiter of heparinized blood was pipetted into another tube immediately to measure lymphocyte DNA damage. Remaining blood was centrifuged at 3000 rpm for 10 min to separate plasma. The plasma samples were stored at -80 °C until analysis of total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI).

Lymphocyte separation

An amount of 1 ml heparinized blood was carefully layered over 1 ml Lympoprep (Oslo Norway) and centrifuged for 35 min at 500 x g and 25ºC. The interface band containing lymphocyte were washed with phosphate buffered saline (PBS) and then collected by 15 min centrifugation at 400 x g. The resulting pellets were resuspended in PBS. Membrane integrity was assessed by means of Trypan Blue exclusion method.

Measurement of lymphocyte DNA damage

The endogenous lymphocytes DNA damage was analyzed by alkaline comet assay according to Singh et al. (11) with minor modifications. Ten mL of fresh lymphocyte cell suspension (around 20.000 cells) were mixed with 80 mLof 0.7% low-melting-point agarose (LMA) (Sigma) in PBS at 37 °C. Subsequently, 80 μL of this mixture were layered onto slides that had previously been coated with 1.0 % hot (60 °C) normal melting point agarose (NMA), covered with a cover-slip at 4 °C for at least 5 min to allow the agarose to solidify. After removing the cover-slips, the slides were submersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris–HCl, pH 10–10.5; 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h. Slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH and 1 mmol/l Na2ETDA, pH > 13) at 4 °C for unwinding (40 min) and then electrophoresed (25 V/300 mA, 25 min). All of the above steps were conducted under red light or without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide (2 μ/ml in distilled; 70 μl/slide), covered with a coverslip and analyzed using a fluorescence microscope (Nikon, Japan) vided with epi-flourescence and equipped with rhodamine filter (excitation wavelenght, 546 nm; barrier fitler, 580 nm) The images of 100 randomly chosen nuclei (50 cells from each of two replicate slides) were analyzed visually from each subject, as described elsewhere (12). Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either of 0, 1, 2, 3, or 4 (from undamaged class 0 to maximally damaged class 4), so that the total scores of slide could be between 0 and 400 arbitrary units (AU). All procedures were completed by the same biochemistry staff and DNA damage was detected by a single observer who was not aware of subject's diagnosis.

Measurement of total antioxidant status

Plasma TAS levels were determined using a novel automated measurement method, developed by Erel (13). In this method, hydroxyl radical, which is the most potent radical, is produced via Fenton Reaction. In the classical

Fenton reaction, the hydroxyl radical is produced by mixing of ferrous ion solution and hydrogen peroxide solution. In the most recently developed assay by Erel, same reaction is used. In the assay, ferrous ion solution, which is present in the Reagent 1, is mixed by hydrogen peroxide, which is present in the Reagent 2. The sequential produced radicals such as brown colored dianisidinyl radical cation, produced by the hydroxyl radical, are also potent radicals. In this assay, antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, is measured. The assay has got excellent precision values, which are lower than 3%. The results are expressed mmolTroloxEquiv/L.

Measurement of total oxidant status

Plasma TOS levels were determined using a novel automated measurement method, developed by Erel (14). In this method, Oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmolH_2O_2Equiv /L).

Oxidative stress index

The percent ratio of the TOS to the TAS gave the oxidative stress index, an indicator of the degree of oxidative stress [14]. To perform the calculation, the result unit of TAS, mmol Trolox equivalent/l, was converted to μmol equivalent/l and the OSI value was calculated as below formula; OSI = [(TOS, μmol/L)/ (TAS, (mmolTroloxEquiv/L) x 100].

Statistical analysis

The values are expressed as mean ± S.D. for the patients and controls separately. The comparisons of parameters were performed with Student's t test and correlation analyses were performed using Pearson's correlation test. A p-value < 0.05 was accepted as significant. Data were analyzed using the Statistical Package for Social Sciences (SPSS 11.5, SPSS Inc, Chicago, IL).

Results

Demographic characteristics of the patients with osteoarthritis and controls are shown in Table 1. Mean ages of patients and controls were 40.9 ± 2.5 and 39.0 ± 4.7 years. There were no significant differences between two groups with respect to age, gender and body mass index (BMI). As can be seen in Table 2, mean value of DNA damage was significantly higher in patients than in healthy controls (21.5 ± 8.7 AU, 7.2 ± 3.9 AU; p < 0.001). Plasma TOS levels and OSI were higher in patients than in healthy controls (10.2 ± 0.7 vs 9.7 ± 0.6, p < 0.001; 1.3 ± 0.5 vs 0.7 ± 0.1, p < 0.001, respectively). Plasma TAS levels in patients were lower than in healthy controls (0.3 ± 0.01 vs 0.5 ± 0.1, p < 0.001). The value of DNA damage showed significantly positive correlations with OSI (r = 0.530, p = 0.001).

Discussion

Osteoarthritis is a progressive joint disease which affects the structural and functional integrity of articular cartilage. External mechanical loading is considered as an important factor for the development and progression of osteoarthritis (15). It is known that mechanical and chemical stresses may change the cellular adaptation to hypoxia, and leading to oxidative damage, resulting in downregulation of chondrocyte synthesis in osteoarthritis. Milam et al. (16) postulated that degenerative arthritis progresses due to the accumulation of free radicals induced by excessive mechanical stresses.

Oxidative stress, which often arises as a result of an imbalance in the human oxidative/antioxidative status, has been implicated in aging and a number of diseases such as cancer, atherosclerosis, rheumatoid arthritis, osteoarthritis, fibromyalgia and osteoporosis (17,18). Oxidative DNA damage has also been associated with a number of pathologies including neoplastic, neurodegenerative, cardiovascular and autoimmune diseases (19).

DNA is damaged by environmental genotoxic agents and by endogenous cellular reactions, free radicals are key triggers of DNA strand breakage, which is related to cell proliferation, cell differentiation, and cell death (20). Degradation of cartilaginous tissue slices by ROS which can directly damage cellular molecules including DNA in osteoarthritis (21, 22). The damage was supposed to be

Table 1. Demographic and clinical data of patients with osteoarthritis

	Patients (n = 29) mean ± SD	Controls (n = 26) mean ± SD	p
Age (years)	40.9 ± 2.5	39.0 ± 4.7	> 0.05
Gender (M / F)	20 / 9	18 / 8	> 0.05
BMI (kg/m²)	29.9 ± 3.3	27.6 ± 3.8	> 0.05
WOMAC	7.56 ± 1.02	2.3 ± 0.7	0.001

BMI: Body Mass Index, Western Ontario McMaster Universities Osteoarthritis Index

The values represent the mean ± SD, *Significance was defined as p < 0.05

Table 2. Comparative analysis of DNA damage and oxidative stress parameters in patients with osteoarthritis and healthy subjects

Parameters	Patients (n = 29) mean ± SD	Controls (n = 26) mean ± SD	p
DNA damage (ArbitraryUnit)	21.5 ± 8.7	7.2 ± 3.9	< 0.001
TOS (μmolH_2O_2/L)	10.2 ± 0.7	9.7 ± 0.6	< 0.006
TAS (mmolTroloxEquiv/L)	0.3 ± 0.01	0.5 ± 0.1	< 0.001
OSI (Arbitrary unit)	1.3 ± 0.5	0.7 ± 0.1	< 0.001

TOS- total oxidative status, TAS- total antioxidant status, OSI- oxidative stress index

The values represent the mean ± SD, *Significance was defined as p < 0.05

secondary to direct attack of proteoglycan and collagen molecules by free radicals. Free radicals degrade collagen and prevent formation of fibrils by this collagen (4).

Yamaza and coworkers (23) investigated free radicals and DNA damage occurred in synovial cells in patients with degenerative temporomandibular joint (TMJ) disease. They suggested that oxidative stress could lead to the synovial hyperplasia via DNA damage in the synovial cells in TMJ after mechanical loading. Firestein et al. (24) have been proposed that DNA strand breaks which may be related with free radicals was occur in synovium in patients with rheumatoid arthritis and osteoarthritis. The presence of apoptotic cells has been reported in the synovial membrane in RA as well as in osteoarthritis (25, 26).

Although originally characterized as a noninflammatory disease, current research indicates that pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1 β, interleukin-6 and others play a central role in the pathogenesis of osteoarthritis (27). Enhance production of inflammatory cytokines induce various enzymes such as NADPH oxidase, nitric oxide synthase, myeloperoxidase and eozinophil peroxidase. These enzymes which produce free radicals may contribute to oxidative DNA damage in inflammation (28).

We found that the mean values of DNA damage, plasma TOS and OSI values were higher, plasma TAS was lower in patients with osteoarthritis. There were no directly association between clinical findings and biochemical parameters. Further studies will be required to investigate radiological and clinical findings and their relation with oxidative stress parameters in patients with osteoarthritis. The limitation of our study is the relatively small sample size that could limit our ability to generalize the results to patients with osteoarthritis in general. In the light of our findings, it is possible to conclude that the patients with osteoarthrtritis may be exposed to a potent oxidative stress and the presence of DNA damage may be associated with the oxidative/antioxidative imbalance. However, the nature of this link, and whether it is direct or indirect, remains to be explored.

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