The role of oxidative and nitrosative stress in the pathology of osteoarthritis: Novel candidate biomarkers for quantification of degenerative changes in the knee joint

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Abstract

Osteoarthritis (OA) is the most frequently diagnosed joint disorder worldwide with increasing prevalence and crucial impact on the quality of life of affected patients through chronic pain, decreasing mobility and invalidity. Although some risk factors, such as age, obesity and previous joint injury are well established, the exact pathogenesis of OA on a cellular and molecular level remains less understood. Today, the role of nitrosative and oxidative stress has not been investigated conclusive-ly in the pathogenesis of OA yet. Therefore, the objective of this study was to identify biological substances for oxidative and nitrosative stress, which mirror the degenerative processes in an osteoarthritic joint. 69 patients suffering from a diagnosed knee pain participated in this study. Based on the orthopedic diagnosis, patients were classified into an osteoarthritis group (OAG, n=24) or in one of two control groups (meniscopathy, CG1, n=11; anterior cruciate ligament rupture, CG2, n=34). Independently from the study protocol, all patients underwent an invasive surgical intervention which was used to collect samples from the synovial membrane, synovial fluid and human serum. Synovial biopsies were analyzed histopathologically for synovitis (Krenn-Score) and immunohistochemically for detection of end products of oxidative (8-isoprostane F2α) and nitrosative (3-nitrotyrosine) stress. Additionally, the fluid samples were analyzed for 8-isoprostane F2α and 3-nitrotyrosine by competitive ELISA method. The analysis of inflammation in synovial biopsies revealed a slight synovitis in all three investigated groups. Detectable concentrations of 3-nitrotyrosine were reported in all three investigated groups without showing any significant differences between the synovial biopsies, fluid or human serum. In contrast, significant increased concentrations of 8-isoprostane F2α were detected in OAG compared to both control groups. Furthermore, our data showed a significant correlation between the histopathological synovitis and oxidative stress in OAG (r=0.728, P<0.01). There were no significant differences between the concentrations of 8-isoprostane F2α in synovial fluid and human serum. The findings of the current study support the hypothesis that oxidative and nitrosative stress are components of the multi-factory pathophysiological formation of OA. It seems reasonable that an inflammatory process in the synovial membrane triggers the generation of oxidative and nitrosative acting substances which can lead to a further degradation of the articular cartilage. Based on correlations between the observed degree of inflammation and investigated biomarkers, especially 8-isoprostane F2α seems to be a novel candidate biomarker for OA. However, due to the finding that also both control groups showed increased concentrations of selected biomarkers, future studies have to validate the diagnostic potential of these biomarkers in OA and in related conditions of the knee joint.

Introduction

Osteoarthritis (OA) describes a heterogeneous group of joint diseases, which are characterized by a progressive loss of articular cartilage in association with the formation of subchondral sclerosis and inflammation in the synovial compartments. Through the successive impact of these degenerative changes on the physiological function of the joint, patients complain about prolonged pain, decreased mobility and therefore a consecutive decline in quality of life.¹ From epidemiological purposes, OA is one of the most frequently diagnosed diseases worldwide, which is especially pronounced in the elderly, reaching prevalence of about 30% in patients aged over 70 years.²³ Since OA is mostly diagnosed in the knee and hip joint, the resulting decline in functionality in association with the current demographic changes presupposes the importance of comprehensive early diagnostic tools and therapeutic interventions.⁵

Etiologically, OA can be subgrouped into primary or secondary forms. While the causes for the primary OA remain less understood, the pathophysiology of the secondary OA can be roughly classified into metabolic-endocrine (e.g. chondrocalcinosis, hemochromatosis), anatomical (e.g. malformations, instabilities), traumatic or inflammatory origins.³ Basically, the integrity of joints depends on a dynamic balance between assembling and dismantling of the cartilage components (e.g. chondrocytes, extracellular matrix, synovia).⁶ If this balance is disturbed, chondrocytes produce increased amounts of extracellular components to preserve the physiological function of the joint, which indeed reduces its resistance against physical and biochemical stress.⁷ Under these conditions, the cartilage gets successively damaged, which can be morphologically interrelated by the presence of tears in the tissue, inducing increased abrasion by asperities, which in association with inflammatory events induce the irreversible loss of the cartilage.⁸

Since the success of a non-surgical therapy is closely linked to the time point of the initial diagnosis, a lot of research tries to describe the underlying pathomechanisms of OA formation to facilitate novel diagnostic markers for early diagnosis and potential innovative therapeutic approaches. Several studies were able to prove that the degenera-
tive processes of the cartilage tissue, triggered by altered biomechanical patterns, are closely related to pro-inflammatory events which are associated with an increased impact of oxidative and nitrosative stress.6,12

Reactive oxygen species (ROS) are highly reactive chemical species which are generated physiologically during metabolism or even during aerobic ATP generation in the complexes of the mitochondria.13 In OA, the affected chondrocytes as well as the synoviocytes start to produce pro-inflammatory cytokines (e.g. interleukin-1β, tumor necrosis factor α)14-16 which were shown to induce an increased expression of prooxidant enzymes in the synovial membrane, like xanthin-oxidase, which in fact is one of the sources for increased amounts of ROS in OA.17 Additionally, reactive nitrogen species (RNS), which are generated by nitric oxide synthases, are able to react directly with ROS by generating highly reactive molecules18,19 which themselves are able to induce DNA fragmentation or even lipid peroxidation (Figure 1).20

Research in OA was able to show that the amount of RNS as well as the corresponding synthases are upregulated in chondrocytes during the inflammation phases of OA.21 In consideration of further confounders in the pathology of OA, ROS as well as RNS are able to decrease the synthesis capacity of collagen and proteoglycans which thereby supports the tissue breakdown by associated upregulation of matrix metalloproteinases and promotes the maintenance of the pro-inflammatory environment.24-16

Since this biochemical cascades are also evident in an early stage of OA, the quantification of end-products generated by oxidative and nitrosative stress could be an unexplored diagnostic marker for the determination of the extent of joint damage. Therefore, the aim of the present study was to identify novel biomarkers for degenerative changes of the knee joint, based on the pathophysiological impact of oxidative and nitrosative stress.

Materials and Methods

Study design

The present study focused on the quantification of oxidative and nitrosative stress in OA of the knee. For comparison, patients suffering from OA with consecutive total knee arthroplasty (TKA) surgery were compared to patients with acute knee pain diagnosis and symptom-free patients. The study was composed in a cross-sectional design, where samples from the synovial membrane, synovial fluid as well as human serum were collected during invasive surgery and subsequently analyzed for end products of oxidative and nitrosative stress.

Subjects

Sixty-nine patients who were treated for different knee conditions in our orthopedics department participated in this study. All recruited patients ran through a comprehensive anamnesis (inter alia: size, weight, pain intensity by a visual analog scale, VAS) and physical examination, followed by an X-ray of the affected knee joint. Based on the summarized diagnostic findings (evaluation of the symptomatology, calculation of the cartilage damage, X-ray classification after Kellgren and Lawrence),22 the patients were classified into three groups:

The first group (osteoarthritis group, OAG) consist of patients with a diagnosed OA of the knee joint, who reported chronic knee pain with symptoms lasting longer than six months (n=24). The second group (control group 1, CG1) contains patients suffering from acute knee pain, which consisted less than six months and were free of symptoms before. This particular group acts as a control group and mostly involved patients with meniscal lesions (medial and lateral meniscus) (n=11). The third group acts as a second control group (CG2) which contained patients who were mostly in clinical therapy for an anterior cruciate liga-

Table 1. Demographic characteristics of the three investigated groups.

| Subjects (n) | OA-Group | Control Group 1 | Control Group 2 | P-values |
|-------------|----------|----------------|----------------|----------|
| Age (years) | 48.58±2.94 | 52.91±5.01 | 29.74±0.79 | <0.001* |
| Sex (w/m in %) | 41.7/58.3 | 36.4/63.6 | 58.8/41.2 | 0.282 |
| Height (cm) | 171.80±2.06 | 172.89±2.81 | 172.46±1.65 | 0.945 |
| Weight (kg) | 84.25±3.87 | 78.89±5.80 | 85.11±3.73 | 0.679 |
| BMI (kg/m²) | 28.42±1.07 | 26.22±1.39 | 28.50±1.03 | 0.477 |
| Pain (VAS 0-100 mm) | 2.1±0.28 | 1.44±0.29 | 2.07±0.19 | 0.352 |

*significant group difference (P<0.05).
(fibrocytes, fibroblasts, endothelial cells) as well as the migrated leukocytes were evaluated. The histopathological examination was done according to Krenn et al., in which the morphological changes in the stroma and the covering cell layer are used for the calculation of a specific score which is conferrable to the stage of synovitis (0 points = no synovitis; 9 points = high-grade synovitis).

**Immunohistochemical detection of 3-nitrotyrosine und 8-isoprostane F2α in synovial biopsies**

Primary, frozen sections were incubated with 3% H2O2 in PBS for 20 minutes at room temperature. After three times washing with PBS, the primary antibody was transferred and sections were incubated overnight at 4°C. For quantification of nitrosative stress, a monoclonal mice 3-nitrotyrosine antibody (Millipore, Billerica, Massachusetts, USA) was used, whereas a polyclonal goat 8-isoprostane F2α antibody (Oxford Biomedical Research, Oxford, England) was applied for determination of oxidative stress. After overnight incubation and three times washing with PBS (1% Triton X 100), a corresponding secondary biotinylated antibody was transferred and samples were incubated for 45 minutes at room temperature. Subsequently after washing, samples were incubated with AB-complex (BioScience GmbH, Ransbach-Baumbach, Germany) for 20 minutes. For densitometry, peroxidase substrate 3,3’-diaminobenzidine (DAB, Sigma-Aldrich, Saint Louis, Missouri, USA) was applied. Examination of 3-nitrotyrosine was done with OxiSelect™ Nitrotyrosine ELISA kit (Cell Biolabs Inc., San Diego, California, USA). In the same manner, OxiSelect™ 8-iso-Prostaglandin F2α ELISA kit (Cell Biolabs Inc., San Diego, California, USA) was used for evaluation of 8-isoprostane F2α. Photospectrometric data were statistically analyzed by GraphPad Prism version 5 (GraphPad Software Inc., San Diego, California, USA).

**Statistical analysis**

Frequency distributions were assessed using χ²-test. Pearson’s correlation coefficients were calculated to estimate correlations between the assessed parameters. To compare biomarkers between two groups, t-tests were used. Assessment for normality of data was carried out by Kolmogorov-Smirnov test. As the normality condition was fulfilled, a one-way ANOVA was used to determine differences between all three groups. In case of rejected normality or a too small analyzable sample size (n < 10), a Kruskal-Wallis test was performed as a non-parametric alternative to the one-way ANOVA. When a significant group effect was found by ANOVA, Tukey’s post hoc tests were performed. Statistical significance was set at P < 0.05 for all analyses and means with respective standard deviations are used to present data (M ± SD), both in tables and running text. Vertical bars in figures represent standard error of means (SEM). All statistical analyses were performed using SPSS version 17 (SPSS Inc., Chicago, Illinois, USA).

**Results**

**Demographic characteristics and stage of OA**

Subjective pain intensity (VAS scale, 0-100mm) did not show a statistical significant correlation with the stage of OA according to Kellgren-Lawrence by Pearson’s correlation coefficient, in either medial (r = 0.039, P = 0.77), lateral (r = 0.063, P = 0.65) or femoropatellar compartment (r = 0.202, P = 0.14). Within the CG1, arthroscopically seen meniscal lesions of the medial meniscus showed a very large correlation to the perceived pain intensity of the patients (r = 0.756, P = 0.02), which was not reportable for lesions of the lateral meniscus (r = 0.478, P = 0.19).

Concerning the BMI of the patients and the stage of OA after Kellgren-Lawrence a moderate correlation for the medial (r = 0.315, P < 0.01), lateral (r = 0.267, P = 0.03) as well as for the femoropatellar compartment (r = 0.48, P = 0.001) existed. Application of this comparison to the CG1 showed no statistical correlation between the BMI of the patients and the degree of meniscal lesions (medial meniscus: r = 0.087, P = 0.83; lateral meniscus: r = 0.183, P = 0.64).

**Degree of synovial inflammation**

The degree of inflammation in the investigated synovial biopsies was quantified by synovitis-score after Krenn and colleagues. Based on the histopathologic changes in the HE-stained samples this score was applied for the synovial cover cell layer thickness and conformity, the cell density of the stroma and the amount of leukocyte infiltration. The total count has a range from 0 points (no synovitis) to a maximum of 9 points (high-grade synovitis). An inter-group comparison by ANOVA showed no significant differences between the three investigated groups OAG (0.96±0.22), CG1 (0.27±0.16) and CG2 (0.79±0.21) (P=0.28), representing a low grade synovitis in all three investigated groups.

No significant correlations between the calculated synovitis-score and baseline characteristics of the OAG were found for pain intensity (r = -0.057, P = 0.39), BMI (r = 0.357, P = 0.06) or extend of OA after Kellgren-Lawrence in all compartments (medial: r = -0.147, P = 0.24; lateral: r = 0.147, P = 0.24, femoropatellar: r = 0.35, P = 0.43).

**Immunohistochemical detection of 3-nitrotyrosine und 8-isoprostane F2α in synovial biopsies**

The quantitative detection of 3-nitrotyrosine by immunohistochemical staining showed comparable values in the three groups [OAG: n=4 (17%), 38.07±4.45; CG1: n=3 (27%), 45.48±3.08; CG2: n=5 (15%), 34.09±7.03, P=0.48].

The detection of 8-isoprostane F2α representing oxidative stress showed a trend of higher values in the OAG [n=4 (17%), 77.54±5.13] compared to both control groups [n=3 (27%), CG1: 63.99±2.43; CG2: n=5 (15%), 63.57±1.54], due to the low sample size this difference was not significant (P=0.12).
Quantification of 3-nitrotyrosine and 8-isoprostane F2α in synovial fluid and human serum

Due to the very small concentrations of 3-nitrotyrosine and 8-isoprostane F2α in both fluids of some patients, not all 69 synovial and serum samples were able to detect measurable values of the biomarkers. Especially in the diagnostics of the synovial membrane, only a small amount of samples were able to reach detectable concentrations of questioned biomarkers. Supposedly due to the sampling technique by arthroscopic lavage, the ELISA method was only able to detect valid 3-nitrotyrosine concentrations in 13 of 69 synovial samples. Within the analyzed samples, no significant differences between the OAG (n=37, 7332.23 ±5895.39 nM), CG1 (n=4 (36%), 2915.15 ±2451.85 nM) and CG2 (n=6 (18%), 539.07±340.39 nM) were found. Accordingly, the quantification of oxidative stress marked by 8-isoprostane F2α in synovial fluid was only successful in 29 of 69 synovial fluid samples. Similarly to the nitrosative stress indicator 3-nitrotyrosine, also the oxidative biomarker showed no significant differences between the groups (OAG: n=9 (38%), 30094.22±2334.35 pg/mL; CG1: n=5 (45%), 16450.29±5806.01 pg/mL; CG2: n=15 (44%), 16522.13±3542.21 pg/mL).

Detectable concentrations of 3-nitrotyrosine and 8-isoprostane F2α in human serum were recorded in 37 of 69 and 48 of 69 samples, respectively. For nitrosative stress, there was no significant difference between the OAG [n=14 (58%), 43.45±9.01 nM] and both control groups [CG1: n=7 (64%), 32.50±11.34 nM, P=0.24; CG2: n=16 (47%), 74.56 ±20.233 nM, P=0.1] in the 3-nitrotyrosine level. Additionally, the comparison between both control groups revealed no significant difference (P=0.1). In context of oxidative stress, the concentrations of 8-isoprostane F2α in the serum were descriptively higher in OAG [n=16 (67%), 3152.3±500.4 pg/mL] than in comparison to CG1 [n=5 (45%), 1723.04±367.46 pg/mL] and CG2 [n=27 (79%), 2875.44 ±281.99 pg/mL], but these differences were not significant (OAG vs. CG1, P=0.07; OAG vs. CG2, P=0.3; Table 2).

Discussion

The main goal of the present study was the identification and quantification of biomarkers for oxidative and nitrosative stress in the pathology of OA of the knee. In fact, we postulated that local damage of the synovial membrane presupposes the appearance of specific biomarkers in the synovial fluid, which could simultaneously be detectable in the blood system by mechanical compression/decompression of the cartilage or after transport through the lymphatic system. Furthermore, in order to classify the appearance of biomarkers, the findings were compared to two control groups, representing on the one hand an acute pain symptomatology (CG1, meniscal lesion) and on the other hand a roughly pain free knee pathology (CG2, ACL-reconstruction). Therefore, samples from synovial membrane, synovial fluid as well as blood samples were investigated to evaluate the level of inflammation and to provide evidences for end products of oxidative (8-isoprostane F2α) and nitrosative (3-nitrotyrosine) stress. Anamnestic data and their diagnostic potential

The success of clinical interventions in OA is highly depending on the early diagnosis. Therefore, researchers try to find easy collectable data (e.g. anamnestic parameters) or even minimal invasive parameters, which are able to reflect the severity and extent of the OA. Thus, we compared values of perceived pain and BMI with the stage of disease. In context of perceived pain, our findings agreed with former findings of the literature which show no significant correlation between the subjective pain intensity and the severity of the disease in all three investigated groups. Since the impact of metabolic disorders and overweight are also well established factors of influence in the pathology of OA formation, the correlation between size and weight (illustrated by the BMI) of our patients with their degree of arthrosis are of particular interest. Based on these findings we are able to support the hypothesis that an increased BMI is positively correlated with the degree of arthrosis after Kellgren-Lawrence in all three compartments (medial, lateral, femoropatellar). In addition, also the histological evaluation of synovitis after Krenn et al. showed a positive correlation between BMI and synovitis score. In fact, there is no valid pathophysiological statement about this interrelation to date.

Nitrosative stress in OA

Previous studies were able to show that increased concentrations of nitrotyrosine are detectable in patients suffering from OA which is supported by our present findings. However, our data showed that synovial biopsies of all three investigated groups indeed contained detectable concentrations of biomarkers for nitrosative stress, but without indicating significant differences. By comparison to previous literature, Sandhu and colleagues reported about crucial differences between nitrotyrosine con-

Table 2. Overview of calculated scores and sample analysis for 3-nitrotyrosine and 8-isoprostane of the three investigated groups. Number in parentheses indicates the analyzable sample size for the specific marker within the group. No significant differences between groups were found.

| Subjects (n) | 24 | 11 | 34 |
|-------------|----|----|----|
| Synovitis score | 0.96±0.22 | 0.27±0.16 | 0.79±0.21 |
| Immunohistochemical staining | | | |
| 3-nitrotyrosine | 38.07±4.45 (4) | 45.48±3.08 (3) | 34.09±7.03 (5) |
| 8-isoprostane | 77.54±5.13 (4) | 63.99±2.43 (3) | 63.57±1.54 (5) |
| Synovial concentrations | | | |
| 3-nitrotyrosine (nM) | 7332.23±5895.39 (3) | 2915.15±2451.85 (4) | 539.07±340.39 (6) |
| 8-isoprostane (pg/mL) | 30094.22±2334.35 (9) | 16450.29±5806.01 (5) | 16522.13±3542.21 (15) |
| Serum concentrations | | | |
| 3-nitrotyrosine (nM) | 43.45±9.01 (14) | 32.50±11.34 (7) | 74.56±20.233 (16) |
| 8-isoprostane (pg/mL) | 3152.3±500.4 (16) | 1723.04±367.46 (5) | 2875.44±281.99 (27) |
The increased amount of nitrotyrosine in RA patients was also associated with an increased degree of inflammation in comparison to the OA group. Since our study revealed no significant differences in inflammation between the OA group and both control groups, it can be suggested that our control groups were not applicable as such. Support for this assumption comes from Misko and colleagues, which were able to show increased concentrations of 3-nitrotyrosine in synovial fluid of patients with OA, meniscal lesions and ACL-ruptures in comparison to a healthy control group. Although, our data reports similar results for the investigated groups, the presented concentrations have to be seen as approximate values, because the sampling method by arthroscopic lavage presuppose false low concentrations which were mathematically extrapolated after BCA assay. In addition, we suppose that the sampling method was causative for the decreased analyzable samples size by ELISA for both, nitrosative and oxidative, biomarkers. Therefore, nitrotyrosine measurements of synovial biopsies have to be compared to a healthy control group in the future to illustrate their diagnostic potential. However, the detection of nitrotyrosine in synovial fluid seems to be an interesting biomarker for an inflammation associated joint disorder, without showing particular sensitivity for OA.

Similar results were obtained by measurements of nitrotyrosine in human serum. Although, several studies reported about significant increased concentrations of 3-nitrotyrosine (μM-range) in OA patients compared to healthy subjects, the present data were on the one hand much lower (nM-range) and on the other hand not different from our investigated groups. Within the background of different established methods and detection thresholds, our results are well in line with findings by Kaur & Halliwell, who were additionally able to report that the measurable concentrations of patients with RA were significantly higher than in patients with OA. Although, we were able to support our hypothesis that the biomarker for nitrosative stress is supposedly originated by joint degradation due to greater concentrations in the synovial fluid of a symptomatic knee compared to human serum, the investigation of 3-nitrotyrosine in human serum seems to be more reasonable in context of RA than OA.

**Oxidative stress in OA**

For identification of oxidative stress in OA the impact of ROS on lipid peroxidation was measured by 8-isoprostane F2α. The current findings show that the concentrations of 8-isoprostane F2α in synovial biopsies seem to be higher in OA patients compared to both control groups, but the analyzable sample size was too small to detect a significant difference. Since both control groups revealed detectable concentrations of 8-isoprostane F2α, we concluded that both groups are not equitable with a healthy patient population in the first place and in addition, that all three clinical indications (OA, meniscus lesion, ACL-lesion) are affected by an increased oxidative stress. These findings were supported by a detectable histopathological synovitis in all three investigated groups and by findings of Grigolo et al. who reported increased lipid oxidation in OA patients compared to a healthy control group and RA patients, independently from a rise in NO production as a source for nitrosative stress. Based on the significant correlation between the histopathological synovitis and oxidative stress (r = 0.728, P < 0.01), we suggest a considerable pathophysiological impact of the processes in the synovial membrane on the pro-inflammatory environment. Therefore, it seems reasonable that next to the cartilage and bone tissue also the synovial membrane plays a key role in the pathophysiological formation of OA (Figure 1).

![Figure 1. Pathological formation of reactive oxygen species (ROS) and reactive nitrosative species (RNS) in osteoarthritis (OA) of the knee. Additionally, this figure shows the influence of migrated leukocytes and affected synoviocytes in OA pathophysiology. Abbreviations: O2 = oxygen, O2− = superoxide anion, H2O2 = hydrogen peroxide, NO = nitric oxide, ONOO− = peroxynitrite anion, NADPH-Ox = NADPH-oxidase, XO = xanthine oxidase, SOD = superoxide dismutase, iNOS = inducible nitric oxide synthase, Arg = arginine, MMP = matrix metalloproteinases.](image-url)
of the knee joint (meniscus lesion, ACL-lesion).

Conclusions

The findings of the current study support the hypothesis that oxidative and nitrosative stress are components of the multi-factorial pathophysiological formation of OA, which has already been shown for other chronic-degenerative diseases such as diabetes mellitus, Alzheimer’s disease or several cardiovascular conditions. In context of OA, it seems reasonable that an inflammatory process in the synovial membrane triggers the generation of oxidative and nitrosative acting substances which may lead to a further degradation of the articular cartilage. Based on correlations between the degree of inflammation and the radiological scores for OA according to Kellgren-Lawrence, 3-nitrotyrosine and 8-isoprostan F₂α seem to be novel candidate biomarkers for OA. However, due to the finding that also both control groups showed increased concentrations of selected biomarkers, future studies have to validate the diagnostic potential of the biomarkers in OA and in other medical conditions of the knee joint (meniscus lesion, ACL-lesion).

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