Upregulation of Inducible Nitric Oxide Synthase and Nitrotyrosine Expression in Primary Knee Osteoarthritis

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Background: To investigate nitrite and inducible nitric oxide synthase (iNOS) levels in the plasma and synovial fluid of patients with primary knee osteoarthritis (OA) and to determine protein nitrotyrosine in synovial tissue of OA patients.

Material and Method: Thirty patients and 30 healthy controls were recruited into the present study. Plasma and synovial fluid nitrite levels were measured using Griess reaction. Plasma and synovial fluid iNOS concentrations were analyzed by enzyme-linked immunosorbent assay. Nitrotyrosine was detected immunohistochemically in synovial tissue of OA patients.

Results: Plasma and synovial fluid nitrite concentration in the OA group were significantly higher than those in the healthy control group were (p = 0.007 and p = 0.012). Furthermore, plasma iNOS levels were significantly higher in the OA group than those in healthy control group were (p = 0.04). Moreover, nitrotyrosine was detected immunohistochemically in macrophages, synovial lining layer and synoviocytes of synovial tissue in the OA group.

Conclusion: These findings indicate that reactive nitrogen species and nitrotyrosine-containing proteins may be involved in the joint destruction process, and play a potential role in the pathogenesis of knee osteoarthritis.

Keywords: Inducible nitric oxide synthase, Knee osteoarthritis, Nitrite, Nitrotyrosine

Osteoarthritis (OA) is a common form of chronic progressive degenerative joint disease resulting from several risk factors including obesity, genetics, and joint injuries(1,2). The knee is the most clinically significant site of primary osteoarthritis. The hallmark of the disease is progressive destruction of the articular cartilage with joint-space narrowing, subchondral bone sclerosis, osteophyte formation, and synovitis. The diagnosis of OA is normally based on clinical and radiographic changes that reflect disease severity by grading joint destruction. OA leads to pain, stiffness, swelling, reduced motion, and physical disability resulting in substantial morbidity, especially among the elderly and imposes an enormous economic burden(3). At present, the etiology and pathogenesis of OA remain incompletely understood(4,5). However, its biochemical factors have now been recognized as they play important roles in OA development.

Increasing evidence suggests that oxidative stress and inflammatory changes play the main causative factor in the pathogenesis of OA. The inflamed tissues localize proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α...
(TNF-α), and inflammatory cells such as macrophages and neutrophils which are major sources of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in knee osteoarthritis(6,7). Nitric oxide (NO) is a short-lived gaseous free radical that mediates key physiological processes in host defense mechanism, immunity, and inflammation. NO is synthesized through the oxidation of one of the guanidino nitrogens associated with L-arginine to NOH-arginine and ultimately to L-citrulline plus NO in the present of requisite cofactors(8). Like nitrite (NO₂), decomposition products of nitric oxide, stable metabolites and used as marker molecules to determine indirectly the presence of nitric oxide, have been reported in plasma and synovial fluid of patients with OA(9). NO production by activated macrophages is catalyzed by an inducible nitric oxide synthase (iNOS), 1 of 3 isoforms of nitric oxide synthase (NOS) enzyme. The primarily formed radicals are nitric oxide and superoxide radical which may be converted to more harmful species, hydroxyl radical, hydrogen peroxide (H₂O₂) and peroxynitrite(10). Peroxynitrite, a proficient nitrating and oxidizing chemical, can react with tyrosine to form nitrotyrosine and has been widely used as a marker of peroxynitrite. It can cause oxidative damage to lipid, protein, DNA including collagen, proteoglycans and hyaluronan(11-13). In recent years, reactive nitrogen species has been explored and implicated in OA(14,15). To our knowledge, there have been no reported data regarding the series of reactive nitrogen species and harmful molecules in knee osteoarthritis. Therefore, the purpose of the present study was to evaluate the reactive nitrogen species and harmful molecules in plasma, synovial fluid and synovial tissues from patients with primary knee osteoarthritis.

Material and Method

Study population

The present study has been approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University, Thailand and was conducted in compliance with the guidelines of the Declaration of Helsinki. All subjects gave their written informed consent prior to their participation in the present study.

Thirty patients aged 51 to 79 years with primary knee OA (21 females and 9 males; the mean age of 64.23±1.18 years) diagnosed with moderate to severe osteoarthritis (Ahlback grade 3-5 on physical examination and radiographic findings) based on the criteria of the American College of Rheumatology were recruited into the present study. The authors also enrolled thirty gender-and-age matched subjects (19 females and 11 males; the mean age of 63.57±1.25 years) with normal knee radiographs as the controls. Body mass index (BMI) was calculated as weight in kilograms divided by height squared in meters (kg/m²). None of the participants had underlying diseases such as other forms of arthritis, diabetes mellitus, histories of corticosteroids medication and antioxidant supplementation, cancer, or other chronic inflammatory diseases.

Laboratory methods

Sample collection

Blood samples of all participants were collected in ethylenediamine tetraacetic acid (EDTA) tubes, and were separated by centrifugation at 3,000 rounds per minute (rpm) for 10 minutes followed by immediate storage at -80°C until analysis. Synovial fluid and synovial tissues were taken from OA patients undergoing total knee replacement surgery at the Department of Orthopedics, King Chulalongkorn Memorial Hospital. The fluid was instantly centrifuged at 3,000 rpm for 10 minutes to remove cells and joint debris. The supernatant was separated and stored at -80°C until analysis. The synovial tissues were fixed for 24 hours in 10% neutral buffered formalin and embedded in paraffin.

Determination of nitrite

Nitrite levels in the plasma and synovial fluid were measured using a colorimetric microassay based on Griess reaction (Promega, Madison, USA), the simplest and most commonly used, which used sulfanilamide and N-1-napthylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. The concentration of azo compound was determined from the absorbance at 540 nm.

Determination of iNOS

Double-blind quantitative determination of plasma and synovial fluid iNOS was conducted using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cusabio Biotech, Wuhen, China) according to manufacturer’s instructions. Briefly, standards of recombinant human iNOS, plasma and synovial fluid samples were pipetted into 96-well microtiter plates precoated with specific antibody against human iNOS. After incubating for 2 hours at 37°C, the liquid in every well was thoroughly removed and incubated for 1 hour at 37°C with biotin-antibody
against iNOS. The wells were then washed three times with washing buffer, decanted any remaining washing buffer, and further incubated for 1 hour at 37°C with horseradish peroxidase-avidin. After five times washing with washing buffer, tetramethylbenzidine substrate solution was added to each well and incubated for 30 minutes at 37°C in the dark. Finally, the reaction was halted with the stop solution and absorbance was measured at 450 nm using a microplate reader. The iNOS concentration was determined by a standard optical density-concentration curve (0-60 IU/mL). The manufacture’s reported precision was <8% (intra-assay) and <10% (inter-assay). The sensitivity of this assay was <0.225 IU/mL.

Nitrotyrosine (NT) immunohistochemistry

Tissue sections for NT determination were prepared from paraffin-embedded synovial tissue that was cut into 4 μm sections. Paraffin from each section was removed by toluene and rehydrated in ethanol and water. Sections were then incubated with Tris-buffered saline (TBS; 50 mM Tris-HCl, 130 mM NaCl, pH 7.6) containing 3% H2O2 for 10 minutes at room temperature to inactivate endogenous peroxidase activity. Excess liquid was purged and sections were incubated with 0.5 μg/mL rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid) at 4°C for 16 hours. Sections were washed with TBS and incubated with Dako Envision peroxidase conjugated to goat anti-rabbit immunoglobins (Dako Envision System, Dako, Denmark) for 10 minutes at room temperature. Immunolabelling was detected using diaminobenzidine (DAB 0.02%) and H2O2 (0.006%) for 10 minutes at room temperature.

Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics 19 (IBM® SPSS® Statistics for Social Statistics and Research Methods). Demographic data between patients and healthy controls were compared by Chi-square tests and unpaired student’s t-tests. Comparisons of all groups were performed using one-way analysis of variance (ANOVA) with Tukey post hoc test if ANOVA showed significance. The data were expressed as means ± standard error of the mean (SEM). A p-values <0.05 were considered to be statistically significant for differences.

Results

The baseline demographic data of the subjects are shown in Table 1. There was no significant difference in age, gender, and BMI between the OA group and healthy control group. Thirty plasma and synovial fluid samples from the OA group and 30 plasma samples from the control group were recruited for nitrite concentration measurement. As demonstrated in Fig. 1, the OA group had significantly higher plasma nitrite concentrations compared to the control group (5.17±0.56 μM vs. 2.87±0.28 μM, p = 0.007). Although nitrite concentrations in the synovial fluid were lower than the paired plasma samples, the difference was not statistically significant (5.04±0.66 M vs. 5.17±0.56 M, p = 0.98).

Furthermore, iNOS concentrations were determined in the control and the OA group. The plasma and synovial fluid concentrations of iNOS are shown in Fig. 2. In the OA group, plasma iNOS concentrations were found to be significantly higher than that of the control group (2.38±0.17 IU/mL vs. 1.85±0.11 IU/mL, p = 0.04). The iNOS levels in the synovial fluid of the OA group were significantly lower than paired plasma samples (1.00±0.17 IU/mL vs. 2.38±0.17 IU/mL, p<0.001).

In addition, synovial tissues of knee OA and anterior cruciate ligament (ACL) were examined immunohistochemically using an antibody for nitrotyrosine (Fig. 3). Synovial tissues of the anterior cruciate ligament were taken as controls. Immunohistochemical staining revealed that the

Table 1. Baseline clinical characteristics of osteoarthritis patients and healthy controls. Data presented as mean ± standard error of the mean

<table>
<thead>
<tr>
<th></th>
<th>Knee OA</th>
<th>Healthy controls</th>
<th>p-value</th>
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<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64.23±1.18</td>
<td>63.57±1.25</td>
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</tr>
<tr>
<td>Gender (female/male)</td>
<td>21/9</td>
<td>19/11</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>26.07±3.71</td>
<td>24.90±2.02</td>
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BMI = body mass index; OA = osteoarthritis
Oxidative damage from the chronic production of endogenous reactive oxygen species and reactive nitrogen species have been associated with aging and obesity\(^{16,17}\). Increased production of ROS and RNS from inflammatory cells leads to oxidative stress, the condition within cells where the amounts of free radicals exceed the anti-oxidant capacity of the cells. Nitric oxide is a gaseous free radical, synthesized from L-arginine by nitric oxide synthases in various cell types. Proinflammatory cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)) activate iNOS during inflammatory process. In certain cases, conflicting evidences suggest damaging and protective effects in the same organ\(^{13,18}\).

In the present study, the authors found that nitrite levels, the stable end products of nitric oxide in plasma were higher in patients with primary knee OA. Additionally, the levels of nitrite were observed in synovial fluid of OA patients. In accord with previous studies, the authors reported that NO levels in the synovial fluid were increased in OA patients\(^{9,19,20}\). The elevation of iNOS in OA patients could be attributed to inflammatory process, indicating that oxidative stress is involved in the pathogenesis of OA.

Discussion

OA is the most common degenerative joint disorder leading to pain, stiffness, reduced motion, and disability. However, the exact mechanisms behind the pathophysiology of OA remain unclear but they have been associated with several risk factors such as aging, obesity, and traumatic injury. Biochemical factors have now been recognized as playing an important role in OA development especially oxidative stress/damage.

Nitrotyrosine, a marker of tissue destruction derived from oxidative stress, was detected in the synovial tissues of primary knee OA. This protein was also present in macrophages, synovial lining cells, and synoviocytes.

Fig. 1  Nitrite concentrations in plasma and synovial fluid of patients with OA and healthy controls. The data were expressed as the mean ± standard error of the mean (n = 30).

Fig. 2  Inducible nitric oxide synthase (iNOS) concentrations in plasma and synovial fluid of patients with osteoarthritis and healthy controls. The data are expressed as the mean ± standard error of the mean (n = 30).

Fig. 3  Immunohistochemical staining for nitrotyrosine: A) Positive control, kidney tissue; B) Isotypic control, anterior cruciate ligament (ACL); C) Controls, anterior cruciate ligament; D) Osteoarthritis patient, knee joint; A-D) Original magnification 10X (● represents subsynovium, ▲ represents synovial lining cells).

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In the present study, the authors found that nitrite levels, the stable end products of nitric oxide in plasma were higher in patients with primary knee OA. Additionally, the levels of nitrite were observed in synovial fluid of OA patients. In accord with previous studies, the authors reported that NO levels in the synovial fluid were increased in OA patients\(^{9,19,20}\). In parallel with the higher plasma concentrations of nitrite, iNOS concentrations also demonstrated significantly higher in OA patients. Moreover, the levels of iNOS were observed in the synovial fluid of OA patients. In agreement with our findings, Cedergren et al found increased level of iNOS in plasma and synovial fluid in the knees of osteoarthritis patients\(^{21}\). The elevation of iNOS in OA patients could be attributed to inflammatory process, indicating that oxidative stress is involved in the pathogenesis of OA.

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radical and nitric oxide; they may be converted to more harmful species, hydroxyl radical, hydrogen peroxide and peroxynitrite, which can cause oxidative damage to lipid, protein, and DNA. Peroxynitrite is a strong nitrating and oxidizing chemical that can react with tyrosine to form nitrotyrosine. The present of protein nitrotyrosine in tissues has been used as a marker for peroxynitrite. Our findings also revealed that there was an increased protein nitrotyrosine level in the synovial tissues of OA patients including macrophages, synovial lining cells, and synoviocytes. These data suggest that oxidative stress from ROS and RNS in synovial tissues may be involved in the joint destruction and inflammation. In addition to tyrosine nitration, peroxynitrite mediated the formation of other oxidation products such as oxidized thiols, which could act as transduction mechanisms that up-regulate inflammatory cells.

In fact, oxidative stress is a key mechanism contributing to the pathogenesis and pathophysiology of degenerative diseases including diabetes mellitus, Alzheimer’s disease, cardiovascular disease and OA. In addition, the oxidant-antioxidant imbalance and inflammation lead to pathophysiological effects associated with OA such as joint inflammation, cartilage degradation, and synovitis. Therefore, the development of preventive and therapeutic approaches for OA should be considered for balance redox reactions by decreasing oxidative stress and increasing antioxidant in blood circulation and local tissues of the knees of OA patients. It should be mentioned, however, that this research has some limitations. The present study was administered as a single-center trial with a relatively small sample size. Therefore, a prospective study conducted on random sampling of multiple centers with larger sample sizes is necessary to validate our data. Additionally, synovial fluid samples from healthy controls were not taken for ethical reasons.

In conclusion, the present study demonstrates that nitrite and iNOS levels increased in primary knee osteoarthritis. Furthermore, there was increased protein nitrotyrosine in the synovial tissues of the knees of OA patients. The elevated levels of nitrogen species may be key players in the pathophysiology of OA. However, further studies are needed to examine the effect of nitric oxide-derived species on the severity of OA.

Acknowledgment

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Potential conflicts of interests

None.

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การแสดงออกของ inducible nitric oxide synthase และ nitrotyrosine ตัวเข็มในผู้ป่วยของสำหรับ

ฉันยั่ว สำนัก, กอวัณี ศรีสวัสดิ์, ศิริชัย อิคิสแม็ง, อานภพ ศิริ, อินอรม, พิวพี เช็กคุ้ม, วิโรจน์ วิ่งartment, บรรณาธิการ สาระศาสตร์, วิทยาศาสตร์ นวัตกรรม

วัตถุประสงค์: เพื่อศึกษาระดับ nitric oxide synthase (iNOS) ในผู้ป่วยและมีไข้ของผู้ป่วยของเจลเลียม รวมทั้งศึกษา

ผลการศึกษา: ระดับ nitric oxide synthase (iNOS) ในผู้ป่วยมีไข้ของผู้ป่วยของเจลเลียมสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ระดับ iNOS ในผู้ป่วยมีไข้ของผู้ป่วย

สรุป: การศึกษาพบว่าระดับสาระสารอยู่ระหว่างและไปด้วย nitrotyrosine อาจมีส่วนเกี่ยวของกับเจลเลียมและมีบทบาทสำคัญต่อการกักเม็ด

ของโรคของเจลเลียม