SCIENTIFIC ARTICLE

Effect of Advanced Oxidation Protein Products on Articular Cartilage and Synovium in a Rabbit Osteoarthritis Model

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Objective: Advanced oxidation protein products (AOPPs), a marker of oxidative stress, are prevalent in many kinds of disorders. Osteoarthritis (OA), mainly resulting from the regression of cartilage, chronic inflammation of the synovium and the subchondral bone remodeling. Although the inflammatory response of AOPPs on fibroblast-like synoviocytes (FLSs) were reported, the effect of AOPPs on cartilage and synovial in vivo remains unclear. Therefore, our study aims to investigate whether AOPPs have an effect on the articular cartilage and synovial in a rabbit model of OA.

Methods: OA model were created by anterior cruciate ligament transection and medial meniscus resection (ACLT + MMx). Forty-eight male New Zealand rabbits were randomly divided into 3 groups: sham-operated group, AOPPs/ACLT + MMx group, and phosphate buffered saline (PBS)/ACLT + MMx group. In sham-operated group, the anterior cruciate ligament was just exposed without transection, and then the incision was sutured. Then intra-articular injection of AOPPs or PBS was performed in the other two groups. Through four weeks and eight weeks of treatment, rabbits in each group were sacrificed. Both hind legs were removed. India ink staining and Safranin O and fast green staining were used to evaluate the macroscopic and microscopic cartilage morphology. The protein expression of matrix metalloproteinases (MMP)-3, MMP-13 in synovium was measured by Western blot.

Result: The India ink score and Mankin score of AOPPs/ACLT + MMx group were both higher than the other two groups at the two time points. Western blot have revealed that intra-articular injection of AOPPs upregulated the protein expression of MMP-3 and MMP-13 in synovium.

Conclusion: AOPPs participated in the occurrence and development of OA by upregulating the protein expression of MMP-3 and MMP-13 in synovium.

Key words: Cartilage; Osteoarthritis; Oxidation protein products; Synovium

Introduction

Osteoarthritis (OA) is characterized by degradation and loss of articular cartilage, subchondral bone remodeling, and inflammation of the synovial membrane at the clinical stage of the disease. The final common pathway of cartilage destruction results from a failure of chondrocytes to maintain a homeostatic balance between matrix synthesis and degrada-

tion¹. OA is thought to be the most prevalent chronic joint disease. The incidence of OA is rising because of the ageing of population and the epidemic of obesity².

The imbalance between peroxidation and antioxidant defense systems results in the accumulation of reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals. Oxidative stress (OS) happens

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afterword, and then OS may cause damage to proteins, lipids, and DNA and even lead to cell death. Advanced oxidation protein products (AOPPs) are considered a novel markers of oxidant-mediated protein damage and dityrosine-containing, cross-linking protein products mainly formed during OS by reaction of plasma albumin with chlorinated oxidants³. The accumulation of AOPPs has been found in many diseases, such as obesity, and inflammatory bowel diseases^{4,5}.

Although we have found that AOPPs have an effect on the inflammatory response of fibroblast-like synoviocytes (FLSs) *in vitro*⁶, whether AOPPs participate in the occurrence and development of OA remains unclear. Therefore, our study aims to investigate the effect of AOPPs on articular cartilage and synovial in a rabbit model of OA.

In this study, rabbit OA model was created by anterior cruciate ligament transection and medial meniscus resection in both knees (ACLT + MMx)⁷. A sham-operated group was established to eliminate the impact of pain after operation. Then intra-articular injections of AOPPs or phosphate buffered saline (PBS) were performed in the other two groups. Together, these experimental groups allowed us to certify intra-articular injections of AOPPs that do and do not create injury to the cartilage as well as synovium.

Materials and Methods

AOPPs Preparation and Determination

AOPPs were prepared *in vitro* as described previously³. In brief, rabbit serum albumin (RSA, Sigma, USA) solution (20 mg/ml) was incubated with 40 mmol/L hypochlorous acid (HCLO) in PBS (pH = 7.4) for 30 min at 37 °C. The reaction was then stopped via overnight dialysis against PBS to remove free HCLO. To remove contaminated endotoxin, all samples were passed through a Detoxi-Gel column (Millipore, USA). Endotoxin levels in AOPPs-modified rat serum albumin (AOPPs-RSA) and unmodified RSA were then measured using a Limulus Amoebocyte Lysate kit and were found to be below 0.025 EU/ml. AOPPs content was determined in the samples as described previously⁸. Briefly, 200 µl of sample or chloramine-T was placed in a 96-well plate, followed by 20 µl of acetic acid. The absorbance at 340 nm was obtained immediately in the microplate reader. AOPPs content in the AOPPs-RSA and unmodified RSA were 50.50 ± 2.80 µmol/g protein and 0.25 ± 0.06 µmol/g protein respectively.

Animals and Treatments

All animal procedures were performed at the Laboratory Animal Centre of Southern Medical University with approval from guidelines set by the Animal Experiment Committee of Southern Medical University. Forty-eight male New Zealand rabbits were randomly divided into 3 treatment groups of 16 rabbits each. After 1 week of acclimatization, in the first group (sham-operated group), anterior cruciate ligament was just exposed without transection, and then the incision was sutured. Rabbits in the other 2 groups (AOPPs/ACLT + MMx group and PBS/ACLT + MMx group) received intra-articular

TABLE 1 Scoring systems for assessment of macroscopic changes in articular cartilage using India ink

Score	Macroscopic changes
1	Intact surface: surface normal in appearance and does not retain India ink
2	0 mm < Fissures ≤ 4 mm
3	4 mm < Fissures ≤ 8 mm
4	8 mm ≤ Fissures
5	0 mm < Full depth erosion ≤ 2 mm
6	2 mm < Full depth erosion ≤ 5 mm
7	5 mm < Full depth erosion
The measurements represent the length of the lesion.	

injection (every other day) of 1 ml AOPPs (200 µg/ml) or PBS during both 4 weeks and 8 weeks, respectively. Half of the each group's rabbits were sacrificed through 4 weeks' treatment, then the other half were sacrificed through 8 weeks. Both hind legs were removed, and cartilage, synovium were processed as described below.

Assessment of Macroscopic Changes in Articular Cartilage

The cartilage surface is painted, for 15 s, with a 20% (v/v) dilution of blue Indian ink (Aladdin, USA) in PBS containing protease inhibitors. Any excess is blotted off with a moist cotton swab. For frozen joints, the specimens are thawed by immersion in PBS, pH 7.4 for 20 min prior to the application of Indian ink. Indian ink adheres to fissured cartilage and consequently enhances visualization of lesions by providing contrast with surrounding normal cartilage. It does not adhere to the subchondral bone and the latter appears white to yellow surrounded by blacker fissured cartilage when a deep lesion is present^{9,10}. At last, we assessed the macroscopic changes of cartilage via the scoring system (Table 1).

Histological Analysis

Following assessment of macroscopic changes, samples were decalcified for four weeks by using Ethylene Diamine Tetraacetic Acid (EDTA). Samples were then dehydrated, paraffin embedded, sectioned to 5 µm, and stained to assess cell morphology and matrix content via Safranin O and fast green. Slides were scored by using a Mankin score system (Table 2) by three blinded reviewers, and scores were averaged across reviewers¹¹.

Western Blot Analysis

For immunodetection of MMP-3 and MMP-13, Protein in the synovium was extracted by tissue homogenate method, and then lysed with RIPA lysis buffer. The protein containing supernatant was obtained by a centrifugation at 4°C, 12000 r/min for 30 min. The protein quantification was determined by BCA Protein Assay kit (KeyGEN BioTECH, China) and then heated in sodium dodecyl sulfate-polyacrylamide gels electro-

TABLE 2 Mankin score

Structure	
Normal	0
Irregular surface, including fissures into the radial layer	1
Pannus	2
Superficial cartilage layers absent	3
Slight disorganization (cellular rows absent, some small superficial clusters)	4
Fissures into calcified cartilage layer	5
Disorganization (chaotic structure, clusters, osteoclast activity)	6
Cellular abnormalities	
Normal	0
Hypercellularity, including small superficial clusters	1
Clusters	2
Hypocellularity	3
Matrix staining	
Normal/slight reduction	0
Staining reduced in radial layer	1
Reduced in interterritorial matrix	2
Only present in pericellular matrix	3
Absent	4
Tidemark	
Normal	0
Vessel in-growth	1

phoresis (SDS-PAGE) protein loading buffer at 95°C for 10 min. Proteins (30 µg) were loaded per lane onto a 10% SDS-PAGE for GADPH (36 kDa), MMP-3 (53 kDa) and MMP-13 (48 kDa), and electrotransferred subsequently to a PVDF membrane. After blocking with 5% BSA in TBST, the membrane were immunoblotted with chicken anti-GADPH, rat anti-MMP-3 and rat anti-MMP-13 antibodies (1:1000, Bioworld Technology, USA) overnight at 4 °C. The membrane

were washed 3 times with TBST and followed by incubation with anti-rat or anti-chicken horseradish peroxidase-conjugated secondary IgG (1:5000) at room temperature for 1 hour. The immunoreactive bands were visualized using an ECL kit (Thermo Scientific, USA). The relative levels of protein were determined by densitometry using Gel-Pro analyzer 4.0 software.

Statistical Analysis

Results were expressed as mean ± standard deviation (SD). Statistical differences between means of different groups were evaluated by using one-way ANOVA or Welch ANOVA (equal variances not assumed). Multiple comparisons were performed using the LSD method or Dunnett's T3 method. Two-tailed *P* value < 0.05 was considered as statistical significant. Statistical analyses were performed using SPSS 13.0 software.

Results

Effects of AOPPs on Macroscopic Changes of Articular Cartilage

In the sham-operation group, cartilages' surface was intact and did not retain India ink. However, in the PBS/ACLT + MMx group, there were some areas of fissures, therefore, India ink particles were absorbed to the cartilages' surface. Furthermore, full depth erosion of all cartilage zones were observed in the AOPPs/ACLT + MMx group. And the bones appeared as pale areas which were surrounded by India ink stained cartilage, highlighting fissures in cartilage contiguous to the erosion (Fig. 1).

Relative to sham-operation group, the other 2 groups had significantly higher scores of India ink staining. Through four weeks of treatment, with India ink score 2.50 ± 0.46 in the PBS/ACLT + MMx group to 4.19 ± 0.60 in the AOPPs/ACLT +

Fig. 1 Gross image of cartilage using India ink staining. (A, B) Surface normal in appearance and does not retain India ink. (C, D) Surface retains India ink as light gray patches and elongated specks. (E) Surface retains ink as intense black patches. (F) Loss of cartilage exposing the underlying bone.

Fig. 2 Quantification of cartilage damage via India ink staining.

MMx group ($P < 0.000$). Moreover, through eight weeks of treatment, with India ink score 3.06 ± 0.62 in the PBS/ACLT + MMx group to 5.75 ± 0.60 in the AOPPs/ACLT + MMx group ($P < 0.000$, Fig. 2).

Effects of AOPPs on Histological Changes of Articular Cartilage

We observed the histological changes of articular cartilage through Safranin O and fast green staining. Cartilages' surface of the sham-operation group was smooth and uniform staining throughout articular cartilage. In the PBS/ACLT + MMx group, we noticed a decrease in numbers of chondrocyte, and a loss of staining in full depth cartilage. In the AOPPs/ACLT + MMx group, we observed a serious erosion of

cartilage, cluster formation below the erosion, and vessel across the tidemark to calcified cartilage layer (Fig. 3). Cartilages of the sham-operation group showed little to no regression, while the other 2 groups showed clear evidence of regression and injury.

Quantitatively, the Mankin score for the PBS/ACLT + MMx group was higher than the sham-operation group through four weeks of treatment ($P < 0.000$), as well as eight weeks ($P < 0.000$). However, AOPPs/ACLT + MMx group was higher than the PBS/ACLT + MMx group through four weeks of treatment ($P < 0.000$), the same results happened through eight weeks of treatment ($P < 0.000$) (Fig. 4).

Effects of AOPPs on the Expression of MMP3 and MMP13 in Synovium

In order to explore the effect of AOPPs on synovium, Western blot was performed to examine if AOPPs upregulate the expression of MMP-3 and MMP-13 in synovium. Early studies have shown that MMP-3 and MMP-13 was two important matrix metalloproteinases (MMPs) to degrade the matrix of cartilage. According to the protein bands (Fig. 5, Table 3), compared with the sham-operation group, synovium in the PBS/ACLT + MMx group expressed more MMP-3 and MMP-13 through four weeks of treatment ($P < 0.000$) as well as eight weeks ($P < 0.000$). While MMP-3 and MMP-13 in the PBS/ACLT + MMx groups were significantly lower than AOPPs/ACLT + MMx groups through four weeks of treatment ($P < 0.000$) as well as eight weeks of treatment ($P < 0.000$). All of the above dates indicated that intra-articular injection of AOPPs upregulated the expression of MMP-3 and MMP-13 in synovium.

Fig. 3 Histological observation of full thickness cartilage and partial subchondral bone by Safranin O and fast green staining. (A, B) Uniform staining throughout cartilage. (C) Loss of staining in superficial zone of cartilage and a focal decrease in cells. (D) Loss of staining in all the cartilage and surface irregularities. (E, F) Loss of staining in all the cartilage, serious erosion in cartilage and cluster formation.

Fig. 4 Quantification of cartilage damage via Mankin score system. (A) Through four weeks of treatments, every each item of Mankin score of AOPPs/ACLT + MMx group were higher than the other two groups. (B) Through eight weeks of treatments, a similar result to four weeks was got.

Discussion

Previous studies have showed that AOPPs were able to trigger respiratory burst and inflammation through the activation of immune cells such as monocyte, increased the generation of Reactive oxygen species (ROS), and stimulated the synthesis and release of inflammatory factor, such as tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), IL-1⁸. AOPPs also can enhances oxidative stress (OS) and inflammation, then accelerate OX-LDL deposition and atherosclerosis¹². In addition, recent studies have confirmed that RAGE is a member of the immunoglobulin superfamily of cell surface molecules, which interacts with multiple ligands, including

AOPPs and AGEs^{13,14}. Furthermore AOPPs-albumin, either prepared in vitro or formed in vivo, bound to endothelial RAGE, induced ROS generation by activate the endothelial cells NAD(P)H oxidase, and triggered a downstream signaling transduction involving ERK 1/2, p38 MAPK, and nuclear translocation of NF- κ B. The cascade triggered by AOPPs resulted in overexpression of adhesion molecules such as ICAM-1 and VCAM-1¹⁵. Therefore, AOPPs was not only the result of oxidative stress, but also the cause of ROS. As a mediator to pathogenesis, it has a wide range of pathological effect and participates in the occurrence and development of human disease.

Fig. 5 Results of Western blot. Expression of MMP3 and MMP13 in synovium.

Ageing changes in the musculoskeletal system increase the propensity to OA but the joints affected and the severity of disease are most closely related to other OA risk factors such as joint injury, obesity, genetics and anatomical factors that affect joint mechanics. In OA, continuous oxidative stress to cells and matrix is one major mechanism underlying pathogenesis¹⁶. As the consequences of oxidative stress, the level of oxidative modification damage of protein that exists in the blood, synovial fluid and articular cartilage of OA patient has rose^{17–19}. Our study and some other investigator have demonstrated that as an origin of ROS, the oxidative modification of protein participated in a lot of pathological process^{12,20,21}. Therefore, we have reasons to believe that, as an endogenous disease-causing agent, the oxidative modification of protein have participated in the pathological process of OA through oxidative stress approach.

In this study, we developed an OA model in the knee joint of male New Zealand rabbit and quantitatively assessed the subsequent treatment on the cartilage. Interestingly, the cartilage damage occurred when an OA model was created, but not when a sham-operation was made. The results suggested that cartilage damage wasn't due to the pain of incision. However, in the AOPPs/ACLT + MMx groups, both India ink score and Mankin score were substantially higher than the PBS/ACLT + MMx groups at the two time points, respectively. This suggested the intra-articular injection of AOPPs was able to accelerate the regression of cartilage in rabbit OA model.

Previous studies have indicated that non-invasively localized synovitis commonly exist in the joint of OA patient,

particularly the synovium adjacent articular cartilage²². It's generally agreed that synovitis was not only the secondary pathological changes of OA, but also accelerated the progress of OA²³. During synovitis, immune cells diffusing into the synovial fluid (SF) and synovium produce inflammatory mediators that are able to stimulate cartilage degradation by synoviocytes and chondrocytes and that cause the hyperplasia of the synovial tissue. For example, macrophages produce MMPs themselves, but are also an important source of interleukin 1 β (IL-1 β) and TNF- α , cytokines that stimulate matrix metalloproteinase production by chondrocytes and synoviocytes²⁴.

MMP-3 and MMP-13, which degrade the extracellular matrix of cartilage. MMP-3, also known as a stromelysin-1, has broader substrate specificity, with activity against type II, III, IV, IX, X and XI collagens, proteoglycans, fibronectin and laminin²⁵. MMP-13, which preferentially degrades type II collagen and aggrecan²⁶. In the current experiment, we could see AOPPs challenge leads to increase of protein expression in both MMP-3 and MMP-13. From these lines of evidence, it is very likely that elevated concentration of AOPPs could result in the release of MMPs in synovial tissues, which could accelerate the disease progression in OA model by destroying the synovium tissue and articular cartilage of the joints.

In conclusion, this study indicates that AOPPs are able to accelerate the progression of the rabbit OA model. The main reason may be an abnormal expression of MMP-3 and MMP-13 of synovium tissue stimulated by AOPPs. The

TABLE 3 Semi-quantitative analysis of MMP-3 and MMP-13 expression in synovium (mean $\pm$ SD)

Groups	MMP-3/GADPH		MMP-13/GADPH	
	4 week	8 week	4 week	8 week
Sham	0.065 $\pm$ 0.006	0.053 $\pm$ 0.011	0.101 $\pm$ 0.022	0.367 $\pm$ 0.033
PBS/ACLT + MMx	0.552 $\pm$ 0.024	0.839 $\pm$ 0.084	0.564 $\pm$ 0.030	1.322 $\pm$ 0.085
AOPPs/ACLT + MMx	1.006 $\pm$ 0.080	1.098 $\pm$ 0.088	0.966 $\pm$ 0.080	1.621 $\pm$ 0.041
Statistic value	$F = 565.669, P = 0.000$		$F = 414.095, P = 0.000$	
	$F = 357.380, P = 0.000$		$F = 776.334, P = 0.000$	

findings will give us a new insight to explore OA and provide a better understanding about the pathogenetic role of oxidation protein products.

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