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Intra-articular injection of the cyclooxygenase-2 inhibitor parecoxib attenuates osteoarthritis progression in anterior cruciate ligament-transected knee in rats: role of excitatory amino acids

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Summary

Objective: Our present study examined the effect of intra-articular cyclooxygenase-2 (COX-2) inhibitor parecoxib on osteoarthritis (OA) progression and the concomitant changes in excitatory amino acids' (EAAs) levels of the anterior cruciate ligament-transected (ACLT) knee joint dialysates.

Methods: OA was induced in Wistar rats by anterior cruciate ligament transection of the knee of one hindlimb, the other was left unoperated and untreated. Rats were placed into four groups: Group ACLT/P received intra-articular parecoxib injection (100 μg) in the ACLT knee once a week for 5 consecutive weeks starting at 8 weeks after surgery. Group ACLT/S received the same procedure as group ACLT/P with saline injection instead. Naïve (Naïve/P) rats received only intra-articular parecoxib injection in one knee once a week for 5 consecutive weeks without surgery. The sham-operated rats underwent arthrotomy only without treatment. Twenty weeks after surgery, knee joint dialysates were collected and EAAs' concentration was assayed by high-performance liquid chromatography, and gross morphology and histopathology (Mankin and synovitis grading) were examined on the medial femoral condyles and synovia.

Results: Parecoxib alone had no effect on cartilage and synovium of normal knees in Naïve/P rats. In ACLT/P rats, parecoxib treatment showed a significant inhibition of cartilage degeneration of the medial femoral condyle at both the macroscopic level (1.15 ± 0.17 vs 2.55 ± 0.12, $P < 0.05$) and the Mankin scores (3.03 ± 0.28 vs 8.82 ± 0.43, $P < 0.05$). Intra-articular parecoxib injection also suppressed the synovial inflammation of ACLT joint compared to the ACLT/S group (3.92 ± 0.41 vs 9.25 ± 0.32, $P < 0.05$). Moreover, glutamate and aspartate levels were also significantly reduced in the ACLT/P group compared to the ACLT/S group by parecoxib treatment ($91.2 \pm 9.4\%$ vs $189.5 \pm 17.0\%$, $P < 0.05$ and $98.2 \pm 11.6\%$ vs $175.3 \pm 12.4\%$, $P < 0.05$, respectively).

Conclusion: This study shows that intra-articular injection of COX-2 inhibitor parecoxib inhibits the ACLT-induced OA progression; it was accompanied by a reduction of glutamate and aspartate concentration in the ACLT joint dialysates. From our present results, we suggested that intra-articular parecoxib injection, in addition to the anti-inflammatory effect, inhibiting the EAAs' release, may also play a role in inhibiting the traumatic knee injury induced OA progression.

Key words: Cyclooxygenase-2, Osteoarthritis, Parecoxib, Microdialysis, Glutamate.

Introduction

Osteoarthritis (OA), the most common cause of pain and disability in the elderly, is a complex disease characterized by bone remodeling, synovium inflammation, and cartilage loss. Although OA is classified as a noninflammatory arthropathy, inflammation has been shown to play a significant role in the disease progression[1]. Patients with rupture of the anterior cruciate ligament (ACL) develop post-traumatic OA of the knee[1]. Restoration of knee stability provides symptomatic relief, but does not reduce the degenerative changes in the ACL-injured knee[2]. This suggests that the development of post-traumatic OA may not only a purely biomechanical origin but biochemical changes have may also involve.

Prostaglandins (PGs) are known to play a role in the pathogenesis of inflammation and inflammatory pain[3]. PGs play peripheral and central roles in inflammatory processes, nociceptor sensitization, and pain generation.

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PGs are the targets for cyclooxygenase (COX) inhibitors, they are used to provide analgesia and inhibit inflammatory process[4]. COX is the rate-limiting step in PGs' production and increased COX activity is considered the critical factor in driving PGs' synthesis at inflammatory sites[4]. Two isoforms of COX have been identified; the constitutive COX-1 is distributed in most tissues and is responsible for physiological PGs' production[5], while COX-2 is induced in various cell types, including chondrocytes, when exposed to cytokines, mitogens, and endotoxins[6]. Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both COX-1 and COX-2 at standard anti-inflammatory dose and this dual inhibition leads to a number of side effects, in particular gastrointestinal ulceration[7]. Cartilage, the target for extracellular matrix destruction in inflammatory arthropathies, is the primary site for pathogenic processes in OA. Cultured chondrocytes from OA joints show increased prostaglandin E2 (PGE_2) production and COX activity[8]. Unstimulated human chondrocytes do not contain detectable COX-2 mRNA, but it is induced by interleukin 1β (IL-1β)[5]. Animal data show that inhibition of COX-2 provides analgesic and anti-inflammatory effects[4].

Glutamate is the main excitatory neurotransmitter in the synapses of the central nervous system (CNS). Several studies have shown that excitatory amino acids (EAAs), such as glutamate and aspartate, actively contribute to the local regulation of bone metabolism[9,10]. Animal and clinical studies have shown an increase in glutamate and aspartate levels in the joint fluid in active arthropathies[11,12], and intra-articular injection of glutamate results in increased joint blood flow and inflammation in joints[13]. The involvement of EAAs in peripheral nociceptive signal transduction had been postulated in animal models of acute arthritis[14]. A recent study indicated a critical involvement of EAA receptors in the pathophysiology of arthritic pain[15]. We previously reported that glutamate and aspartate levels are significantly increased in the dialysates of ACL-transected (ACLT) knees, and suggested a role of EAAs in early OA development[16].

Parecoxib, a water-soluble prodrug of the oral formulation valdecoxib, is approximately 28,000-fold more potent against COX-2 than against COX-1[17,18]. It is the first injectable COX-2 inhibitor available[18]. COX-2 inhibitors are known therapeutic agents for OA. However, the physiological importance of COX-2 and its interaction with glutamate receptors is still not clearly understood. In this study, by using a microdialysis technique, we examined the effects of intra-articular injection of the COX-2 inhibitor parecoxib on the progression of OA and EAA changes. In this ACLT-OA animal model, we found that parecoxib reduced the progression of OA and it was associated with a significant decrease in glutamate and aspartate levels in the dialysates of ACLT knee joints compared to saline-treated ACLT knees.

Methods

ANIMAL MODEL

The experimental protocol was approved by the Animal Care and Use Committee of our Institute and conformed to the guidelines for the care and use of animals in research of National Defense Medical Center, Taiwan. Thirty-two 3-month-old-male Wistar rats (body weight 270–325 g) were used. The surgical procedure was modified from that described by Stoop *et al.*[19] and our previous study[16]. Cefazolin (20 mg/kg) was given intraperitoneally preoperatively and every 12 h for 3 days after operation for prophylactic infection control. Wound healing, infection, and any other complications were monitored continuously during the 20-week observation period.

EXPERIMENTAL DESIGN AND COX-2 INHIBITOR PARECOXIB INJECTION

Eight weeks after surgery, the ACLT rats were divided into two groups. Rats in the ACLT/P group ($n = 9$) were intra-articularly injected with parecoxib (100 μg in 0.1 ml; Dynastat, Pfizer) in the ACLT knee once a week for 5 weeks, while rats in the ACLT/S group ($n = 8$) were injected on the same schedule with 0.1 ml saline only. The sham-operated group ($n = 9$) received arthrotomy only with no injection. Another six naïve rats received intra-articular parecoxib injection as parecoxib control (Naïve/P group). Twenty weeks after surgery, microdialysis was performed, then the rats were sacrificed and the knees examined histologically. The experimental protocol is summarized in Fig. 1.

CONSTRUCTION AND PLACEMENT OF THE MICRODIALYSIS PROBE

Twenty weeks after ACLT or sham operation, a microdialysis probe was implanted in each knee joint under isoflurane anesthesia as described above. A 27-gauge needle attached to a tuberculin syringe was passed through the joint capsule lateral to the patellar ligament, then the microdialysis probe was inserted through the needle into the knee joint as described in our previous report[16], and its position was confirmed by X-ray examination. The microdialysis probe[16] was constructed using two 5 cm polyethylene tubes (0.008 inch inner diameter, 0.014 inch outer diameter) and a 1.5 cm cuprophan hollow fiber (300 μm outer diameter, 200 μm inner diameter, 50 kDa molecular weight cut-off; Filtral, AN 69-HF, Eicom Co., Kyoto, Japan). A 12 cm Nichrome-Formvar wire (0.0026 inch diameter) was passed through two 1 cm polycarb tubes (194 μm outer diameter, 102 μm inner diameter) and the cuprophan hollow fiber. The ends of the external silastic tubes were connected to a syringe pump (CMA-100, CMA/Microdialysis Inc., Solna, Sweden) for sample collection. The dialysis probe was perfused with modified Ringer's solution (8.60 mg/ml of NaCl, 0.30 mg/ml of KCl, and 0.33 mg/ml of $CaCl_2$, pH 7.4) at a flow rate of 5 μl/min. After a 30-min equilibration period, the dialysate was collected over the next 3 h. All samples were collected in polypropylene tubes on ice and then frozen at −80°C until assayed.

MEASUREMENT OF EAAs

High-performance liquid chromatography (HPLC) with a fluorescence detector (model 126, Beckman Instruments Inc., Fullerton, CA, USA) was used for EAA measurement, as described in our previous report[16,20]. In brief, a reverse-phase C-18 column with *o*-phthaldialdehyde pre-column derivatization and fluorescence detector was used. Each sample was injected onto the column and eluted for 20 min at a flow rate of 0.45 ml/min with a linear gradient from 100% mobile phase A (20 mM sodium acetate, pH 7.2, containing 0.18% triethylamine, and 0.3% tetrahydrofuran) to 75% mobile phase A, 25% mobile phase B (100 mM sodium acetate/acetonitrile/methanol 1/2/2) in 30 min, then for 5 min with 100% mobile phase B. Using this protocol, glutamate, aspartate, serine, taurine and glutamine were successfully separated. External standards containing 0,

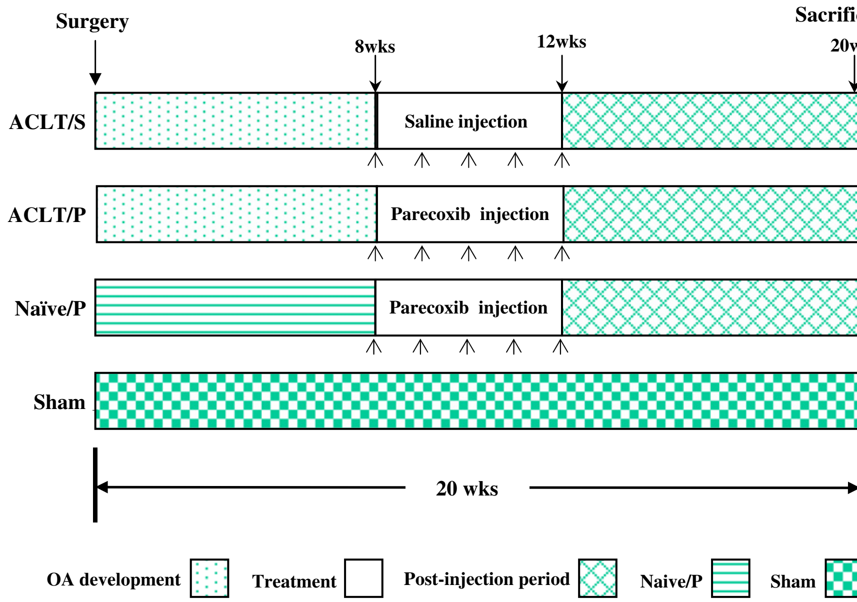

Fig. 1. Experimental course. ACLT/S ($n = 8$); ACLT/P ($n = 9$); Naïve/P ($n = 6$); and sham (arthrotomy without ACLT, $n = 9$). Saline injection: intra-articular injection of 0.1 ml of saline once a week for 5 consecutive weeks starting at week 8 after surgery. Parecoxib injection: as for the saline group, but using parecoxib (100 μg/0.1 ml).

10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M standard amino acids were run before and after each sample group. The detection sensitivity was 10^{-8} M. The recovery rate of the dialysis probe was 25% at an infusion rate of 1 μl/min. The contralateral knee of rats did not receive any surgery and the concentration of EAAs of the contralateral knee joint dialysates was defined as 100% as control. All standards and samples were analyzed in duplicate.

GROSS MORPHOLOGY AND HISTOPATHOLOGICAL EXAMINATIONS OF KNEE JOINTS

The width of the bilateral hindlimb knee joints was measured from medial to lateral aspect of joint line by calipers (AA847R, Aesculap, AG&CO, KG, Germany) before operation (baseline) and at 4, 8, 12, 16, and 20 weeks after operation. At week 20 after surgery and microdialysis sample collection, rats were sacrificed under sodium pentobarbital (50 mg/kg) anesthesia, then perfused intracardially with heparinized saline (400 ml) followed by freshly prepared 4% paraformaldehyde in 0.1 M phosphate-buffered saline, pH 7.4. The knees were disarticulated aseptically, then the joints were cut 0.5 cm above and below the joint line, stripped of muscle, fixed in 10% neutral buffered formalin for 2 days, and then decalcified for 7 days in 10% formic acid, which was changed daily. After X-ray confirmation of decalcification, the joints were cut in the midsagittal plane, washed in running tap water, and paraffin-embedded in an automatic processor (Autotechnicon mono 2, Technicon Co, Chauncey, NY). Previous studies have demonstrated that the medial femoral condyles show the most advanced change following ACLT[16,21], so these and associated synovia were selected for histological preparation and assessment. Serial articular cartilage sections (5 μm) were cut on a Leica 2065 rotatory microtome (Leica Instruments, Wetzlar, Germany) from the central weight-bearing surface of the medial femoral condyles of the ACLT and sham-operated knees. Synovial membrane specimens were carefully dissected from the suprapatellar pouch and the medial tibiofemoral compartments and 10 slides were prepared from each knee. Cartilage was stained with hematoxylin/eosin (H&E) and Safranin-O/fast green stain to assess general morphology and matrix proteoglycans. All samples were numbered randomly and evaluated by two blinded observers to prevent bias. Randomization and numbering were performed by one assistant who did not participate in any of the operations, injections, or evaluations. Immediately after sacrifice, each knee was examined for gross morphologic changes of cartilage lesions of the medial femoral condyle using previously described methods[22,23]. Briefly, cartilage erosion was graded on a scale of 0–4 with 0 = surface appears normal; 1 = minimal fibrillation, or a slight yellowish discoloration of the surface; 2 = erosion extended to the superficial or middle layers; 3 = erosion extended to the deep layer; and 4 = erosion extended to the subchondral bone. Microscopic examination of the articular cartilage in the medial femoral condyles was graded according to the Mankin's grading system[24]; this score assesses structure (0–6 points), cellularity (0–3 points), matrix staining (0–4 points), and tidemark integrity (0–1 points), and has a maximum of 14 points. The final score for each cartilage was based on the most severe histologic changes observed in multiple sections from each specimen. The Mankin score was divided into three stages: stage I (mild degenerative change, 0–6 points), stage II (moderate degenerative change, 7–9 points), and stage III (severe degenerative change, 10 or more points, i.e., cartilage disorganization or complete cartilage loss with extensive exposure of subchondral bone). The synovium from ACLT and sham-operated knees of each group was also processed, embedded in paraffin, and stained with H&E for cellular assessment by histology[25]; this score assessed (1) synovial lining layer: hyperplasia of synovial lining cells (0–3 points), hypertrophy of synovial lining layer (0–3 points), and infiltration of inflammatory cells (0–3 points); (2) subsynovial tissue: proliferation of granulation tissue

(0–3 points), vascularization (0–3 points), and infiltration of inflammatory cells (0–3 points), with a maximum of 18 points. The total scores were divided into three stages: 0–6 points (mild synovitis), 7–12 points (moderate synovitis), and 13 or more points (severe synovitis).

DATA AND STATISTICAL ANALYSIS

All data are presented as the mean ± S.E.M. and analyzed by one-way ANOVA with Fisher's *post hoc* tests for multiple comparisons. A P value less than 0.05 was considered significant.

Results

One rat showed mild serous discharge from the wound the third day after ACLT, however, the symptom subsided after dressing was changed and after 3 more days of antibiotics (cefazoline) treatment. No sepsis or hemarthrosis was observed in the present study. At sacrifice, all ACLT knees showed complete transection of the ACL. The level of daily activity was similar in the rats of all four groups, and there were no significant differences in body weight between the groups during the whole study period.

KNEE JOINT INFLAMMATION AND MACROSCOPIC EVALUATION

Severity of knee joint inflammation, reflected by an increase in hindpaw knee joint width, was observed at week 20 after ACLT. Figure 2 shows the change in knee joint width at 20 weeks after surgery compared to before surgery; the increase in joint widths of ACLT/S, ACLT/P, Naïve/P and sham-operated groups were 2.31 ± 0.78 mm, 1.09 ± 0.45 mm, 0.10 ± 0.04 mm and 0.12 ± 0.03 mm, respectively. ACLT resulted in a significant increase ($P < 0.05$) in the knee joint width compared to that of sham-operated and Naïve/P rats. When compared to saline injection, parecoxib significantly reduced joint swelling in ACLT rats ($P < 0.05$). On gross examination of the cartilage, the medial femoral condyles showed cartilage degeneration with fibrillation, erosion, and ulcer formation and the macroscopic score was 2.55 ± 0.12 in the ACLT/S group (Table I). In the ACLT/P rats, minor irregularity and fibrillated lesions were observed on the medial femoral condyles and the macroscopic score was 1.15 ± 0.17 (Table I). In the Naïve/P and sham-operated rats, the cartilage on the medial femoral condyles was normal, with a glistening translucent smooth surface, and no cartilage defects or osteophytes were noted, the macroscopic scores were 0.25 ± 0.04 and 0.34 ± 0.12, respectively (Table I). Significant differences on the macroscopic scores were found between Naïve/P and both ACLT/S and ACLT/P groups ($P < 0.05$). Similar results were also found between sham-operated and both ACLT/S and ACLT/P groups (Table I). Moreover, the grade of cartilage damage in the ACLT/P group was significantly lower than that in the ACLT/S group ($P < 0.05$). In OA rats, the synovia from the ACLT/S group were hypertrophic and showed a reddish yellow discoloration, while, in the ACLT/P rats, the synovia were thinner and the discoloration less intense. The synovia from the Naïve/P and sham-operated rats had a white luster and transparent appearance and showed neither hyperemia nor evidence of synovitis.

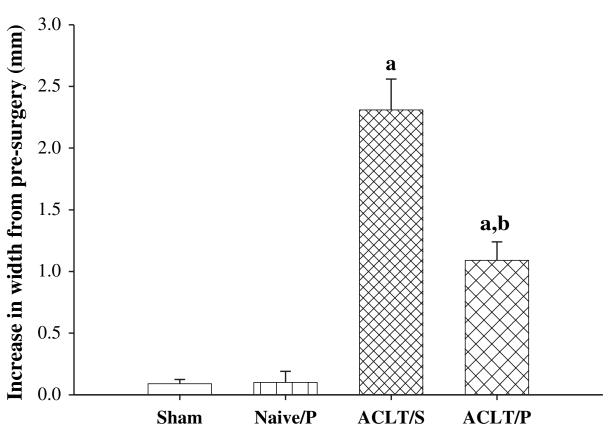

Fig. 2. Time-course of joint width changes after surgery. The widths of the bilateral hindlimb knee joints were measured in each rat before, and at 4, 8, 12, 16, and 20 weeks after surgery. The data (mean ± S.E.M.) are expressed as the difference in knee width between the values at week 20 after surgery and before surgery in each group. **a**, $P < 0.05$ compared to the Naïve/P and sham-operated groups; **b**, $P < 0.05$ compared to the ACLT/S group.

MICROSCOPIC FINDINGS

Specimens from the ACLT/S rats showed fibrillation and fissures on the cartilage surface, and loss of Safranin-O/fast green staining and depletion of chondrocytes in cartilage layer, and hyperplasia of chondrocytes and decrease of Safranin-O/fast green staining in the calcified cartilage layer, and cleft and irregular bony trabeculum between the junction of calcific cartilage and subchondral layers [Fig. 3(A)]. In the ACLT/P knee, there was a marked reduction in the severity of lesions of the medial femoral condyles, only superficial irregularity and mild local fibrillation extending to the superficial layer of cartilage were observed, and the pericellular Safranin-O/fast green staining persisted with milder cell loss. Recovery of proteoglycan and relative normality of chondrocytes in the calcified cartilage layer and smooth bony trabeculum in the subchondral layer was observed [Fig. 3(B)]. The medial femoral condyles of the Naïve/P and sham-operated rats showed a normal histological appearance, with no disruption of cartilage surface integrity. Preservation of Safranin-O/fast green staining and chondrocytes in calcified cartilage layer and smooth bony trabeculum in subchondral layer was also observed [Fig. 3(C,D)]. Moreover, significant differences in the histological OA score were found between the Naïve/P group (1.04 ± 0.15) and both the ACLT/S (8.82 ± 0.43) and ACLT/P groups (3.03 ± 0.28) ($P < 0.05$) (Table II). Similar results were also observed between the sham-operated group (1.33 ± 0.26) and the ACLT/S and ACLT/P groups

Table I
Macroscopic evaluation of the articular cartilage of the medial femoral condyles of the knee joint

Group	Score
ACLT/S ($n = 8$)	2.55 ± 0.12[a]
ACLT/P ($n = 9$)	1.15 ± 0.17[a,b]
Naïve/P ($n = 6$)	0.25 ± 0.04
Sham ($n = 9$)	0.34 ± 0.12

Data are expressed as mean ± S.E.M. **a**, $P < 0.05$ compared to the Naïve/P and sham-operated groups; **b**, $P < 0.05$ compared to the ACLT/S group. The maximum score is 4.

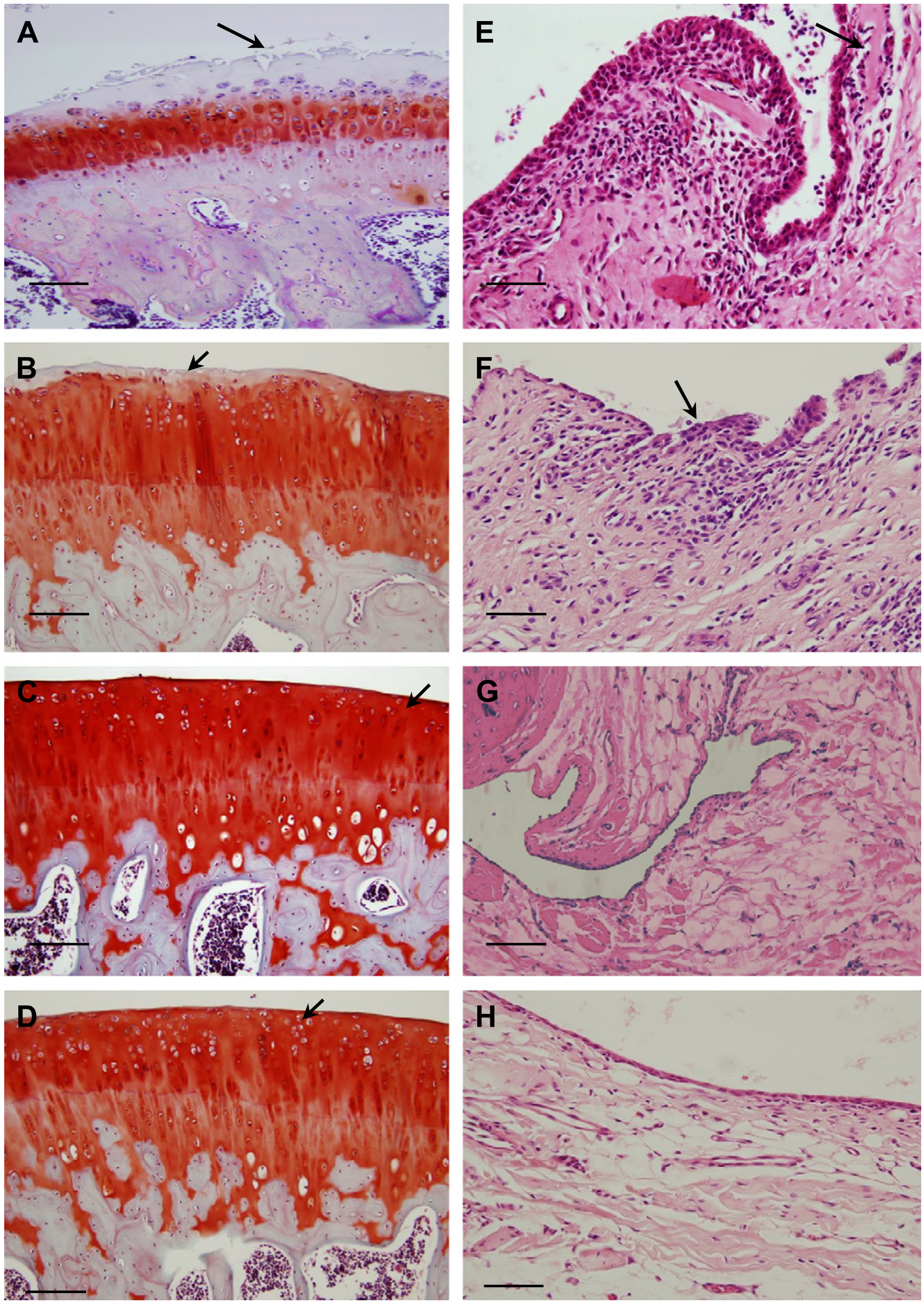

Fig. 3. Histopathological evaluation of cartilage of the medial femoral condyles (stain, Safranin-O/fast green; original magnification, 200×) and synovium (H/E stain, original magnification, 400×) of the knee at week 20 after surgery. (A) ACLT/S group showing surface fibrillation (arrow), chondrocyte loss, and a decrease in Safranin-O/fast green staining intensity, which extends into the radial zone. Cloning of chondrocytes is apparent in the transitional and radial zones. Decrease of Safranin-O/fast green staining in matrix and hyperplasia of chondrocytes in the calcified cartilage layer, and cleft and irregular bony trabeculum between junction of calcified cartilage and subchondral layer are observed. (B) ACLT/P group showing irregularity of the superficial layer of the cartilage (arrow), and mild loss of Safranin-O/fast green staining of the superficial layer of the cartilage. Recovery of proteoglycan and relative normality of chondrocytes in the calcified cartilage layer and smooth bony trabeculum in the subchondral layer are observed. In (C) Naïve/P and (D) sham groups, the superficial layer of cartilage is smooth and no disruption of surface integrity is observed (arrow). The cartilage matrix is well stained with Safranin-O/fast green. Preservation of Safranin-O/fast green staining and chondrocytes in calcified cartilage layer and smooth bony trabeculum is observed in subchondral layer. (E) Synovial membrane from the ACLT/S group, showing hyperplasia of lining cells and hypertrophy of the synovial and subsynovial tissue. Mononuclear cell infiltration of the underlying tissues and a fibrinoid exudate (arrow) are also seen. (F) Synovial membrane from the ACLT/P group shows focal synovitis (arrow) with mild mononuclear cell infiltration. Synovial membrane from the (G) Naïve/P and (H) sham groups shows no synovial lining cell hyperplasia (scale bar = 100 μm).

Table II
Histological evaluation of the articular cartilage of the medial femoral condyles and synovial tissue of the knee

Group	Osteoarthritic score	Synovitis score
ACLT/S ($n = 8$)	8.82 ± 0.43[a]	9.25 ± 0.32[a]
ACLT/P ($n = 9$)	3.03 ± 0.28[a,b]	3.92 ± 0.41[a,b]
Naïve/P ($n = 6$)	1.04 ± 0.15	1.38 ± 0.09
Sham ($n = 9$)	1.33 ± 0.26	1.82 ± 0.33

Data are expressed as mean ± S.E.M. **a**, $P < 0.05$ compared to the Naïve/P and sham-operated groups; **b**, $P < 0.05$ compared to the saline group. Osteoarthritic score (Mankin score) = 0–14; synovitis score = 0–18.

($P < 0.05$; Table II). The Mankin score of the ACLT/P group was significantly lower than that of the saline group ($P < 0.05$). The synovia from the ACLT/S group rats were thick, had focal villi, and showed hyperplasia of the lining cells, with moderate mononuclear cell infiltration [Fig. 3(E)], while the synovia from the ACLT/P group rats showed focal synovitis (arrow) with mild mononuclear cell infiltration [Fig. 3(F)]. The histology of the synovia from the Naïve/P and sham-operated rats was normal [Fig. 3(G,H)]. The synovitis scores are shown in Table II; the score was significantly lower in the Naïve/P group (1.38 ± 0.09, $P < 0.05$) than that in the ACLT/S group (9.25 ± 0.32) or in the ACLT/P group (3.92 ± 0.41) (Table II). Similar results were found between the sham-operated group (1.82 ± 0.33) and both the ACLT/S and ACLT/P groups ($P < 0.05$) (Table II).

EAA LEVELS IN KNEE JOINT DIALYSATES

The mean resting concentrations of glutamate, aspartate, serine, taurine and glutamine in joint dialysates of the contralateral control rats were 2.01 ± 0.15, 0.33 ± 0.02, 6.12 ± 0.47, 17.52 ± 1.54 and 23.5 ± 1.98 μM (mean ± S.E.M.), respectively, and they were defined as 100% for the control basal level. EAA levels in knee joint dialysates were measured at 20 weeks after surgery in all four groups taking the levels in the contralateral control knee as the 100% value. No significant difference of glutamate and aspartate in the knee joint dialysates was observed between bilateral knees in the Naïve/P group (data not shown). A significant increase in glutamate level was found in the ACLT/S group (189.5 ± 17.0%) ($P < 0.05$) compared to the Naïve/P (89.8 ± 8.4%), sham-operated (109.3 ± 2.3%) or ACLT/P (91.2 ± 9.4%) group. No difference was observed between ACLT/P, Naïve/P ($P = 0.565$) and sham-operated groups ($P = 0.487$) [Fig. 4(A)]. The differences of aspartate concentrations were similar to the changes of glutamate; 175.3 ± 12.4% for ACLT/S, 102.5 ± 9.9% for Naïve/P, 104.2 ± 4.1% for sham-operated, and 98.2 ± 11.6% for the ACLT/P groups [Fig. 4(B)]. Other amino acid levels such as serine, taurine, or glutamine had no difference among four groups.

Discussions

This study provides evidence, for the first time, that intra-articular injection of the COX-2 inhibitor parecoxib attenuates the progression of OA in ACLT rats. This is also the first study showing a statistic correlation between the reduction of glutamate and aspartate levels in ACLT joint dialysates after parecoxib treatment of the severity of OA.

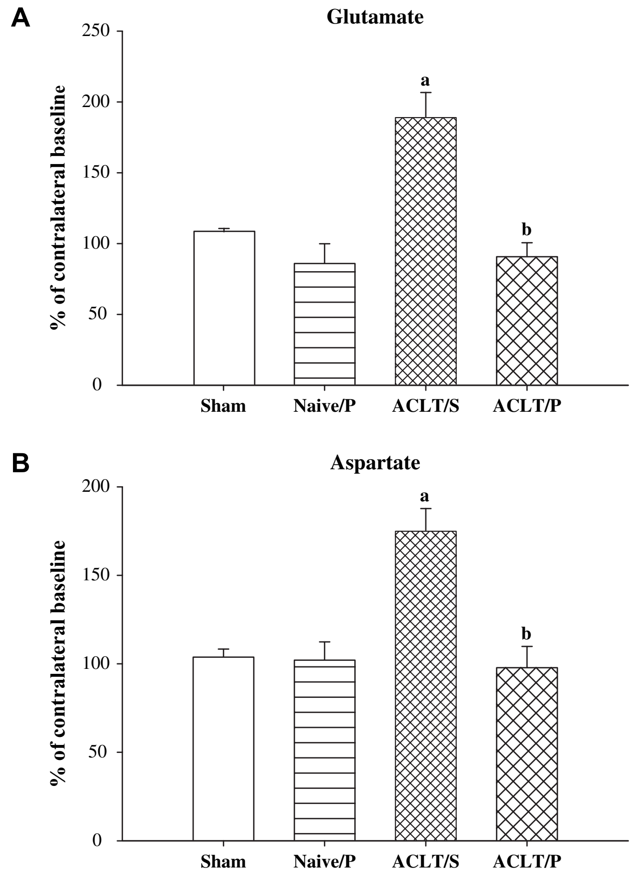

Fig. 4. Glutamate and aspartate levels in joint dialysates of ACLT knees from the four groups. EAA levels in the knee joint dialysates were measured at week 20 after surgery. The dialysates were collected for 3 h. The mean resting concentration of glutamate and aspartate in synovial fluid dialysates of the contralateral control rats were 2.01 ± 0.15 and 0.33 ± 0.02 μM, respectively. The EAA concentrations in the contralateral knee, at week 20, were taken as the basal value (100%) and the glutamate (A) or aspartate (B) levels in the ACLT knee, Naïve/P or sham-operated knee are expressed as the % differences in EAA concentration from the basal 100%. Data are expressed as mean ± S.E.M. **a**, $P < 0.05$ compared to the Naïve/P and sham-operated groups; **b**, $P < 0.05$ compared to the ACLT/S group. ACLT/S ($n = 8$); ACLT/P ($n = 9$); Naïve/P ($n = 6$); and sham ($n = 9$).

Breakdown of the cartilage matrix leads to fibrillation, fissures, gross ulcerations, and even full thickness loss of the joint surface[19,26]. Knee instability following a complete tear of the ACL often induces OA accompanied by degradation of the articular cartilage matrix[2,27]. In our present study, both the macroscopic and Mankin scores were significantly lower in the ACLT/P group than in the ACLT/S group. Moreover, reduction in the severity of the structural changes and loss of Safranin-O/fast green staining were observed in the ACLT/P group. For OA progression, synovial inflammation was found to play an important role[1,28]. In the synovium, the efficacy of NSAIDs is attributed to suppression of neutrophil activation and inhibition of inflammatory PGs' synthesis[3,29]. Amin *et al.* [29] found up-regulation of COX-2 protein expression and PGE2 level in the chondrocytes from OA and rheumatoid arthritis cartilage. Moreover, COX-2 induction was observed in the chondrocytes of OA joints, which produces PGs and induces local nociceptors' sensitization and thus modulates inflammatory processes[29], and PGE_2 was demonstrated to accelerate cartilage degeneration by inhibiting proteoglycan biosynthesis[30]. Selective COX-2 inhibitors are effective for OA treatment by attenuating synovial inflammation and cartilage destruction[4,5].

Parecoxib is a highly selective COX-2 inhibitor and is used for perioperative analgesia/antiinflammation[17,18]. Parecoxib, the prodrug of valdecoxib, has been developed as the only COX-2-selective inhibitor available in a parenteral formulation; it is rapidly metabolized into the active form, valdecoxib, in the liver after intravenous or intramuscular injection with peak plasma valdecoxib concentration occurring between 30 min and 3.5 h and an elimination half-life of approximately 8 h[31,32]. Parecoxib is stable in human plasma suggesting that nonenzymatic hydrolysis and plasma esterases or amidases are not involved in amide hydrolysis to valdecoxib[33]. However, the exact mechanism and the pharmacokinetics of parecoxib in the joint still need further investigation. In our present study, intra-articular parecoxib injection inhibited the progressive cartilage destruction and the associated synovia inflammation. This parecoxib treatment only stopped the continuous destruction on the cartilage, but did not reverse the developed cartilage damage.

An important finding of our present study was the significant reduction of glutamate and aspartate levels in the joint dialysate by the parecoxib injection compared to the saline group. Mechanical regulation of glutamate transporter expression in bone has recently been described[34], it suggests a role of EAAs in paracrine intercellular communication in bone[10]. Free radicals are intermediate products in COX-mediated PGs' synthesis and are known to be involved in *N*-methyl-D-aspartate (NMDA) receptor-mediated glutamate excitotoxicity[35], and PGs produced in the COX pathway were demonstrated to stimulate Ca^{2+}-dependent glutamate release in cultured astrocytes[36]. Moreover, intra-articular injection of EAA results in thermal hyperalgesia and mechanical allodynia, which are attenuated by local injection of NMDA or non-NMDA receptor antagonists[11]. Pain in the OA knee might result not only from mechanical stimuli, but also from neurogenic inflammation[37]. The OA-associated pain may also result from glutamate release from axons innervating the inflamed region[38]. Injection of kaolin/carrageenan mixture into the knee joint induced an immediate increase in glutamate and aspartate levels in the joint and persisted for hours[11]. Moreover, in inflamed arthritic knees, an increase of glutamate concentration was observed not only in the axons in the inflamed region[14], but also in the synovial fluid[12]. Endogenous glutamate was found in intracellular vesicular constituents associated with vesicular glutamate transporter isoenzyme-1 (VGLUT-1), and activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, which were expressed in cultured rat costal chondrocytes, will release glutamate[39]. In our laboratory, we have demonstrated VGLUT-1 and -2 protein expression in cartilage, synovium and meniscus tissues in Wistar rat knee. It suggests that cartilage, synovium and meniscus may release glutamate by ACLT of knee (unpublished observation). Functional NMDA receptors have been found in a number of bone cells, including rat and human osteoblasts and osteoclasts, MG-63 osteosarcoma cells, and bone marrow megakaryocytes[9,10]. Moreover, glutamate has been reported to mediate intercellular communication in bone cells and contributes to bone matrix regulation[40]. It is important to evaluate the effects of drugs for treating OA on the pathophysiological processes that occur in this disease, such as bone changes, synovial inflammation, and, in particular, cartilage destruction. The present study is the first to demonstrate that intra-articular injection of parecoxib inhibits the OA progression in association with a reduction of glutamate and aspartate concentration in the dialysates of the ACLT knee. However, our present results do not exclude the contribution of other biological substances in the OA knee joint.

In conclusion, our results show that COX-2 inhibitor parecoxib reduces the ACLT-induced OA progression as demonstrated by the reduction in synovitis and cartilage damage, and the reduction of glutamate and aspartate levels in ACLT joint dialysate after parecoxib treatment. The inhibition of the inflammatory process during the early phase of OA can be enough to account for a further improvement of cartilage lesions. These findings provide a valuable contribution to the development of new therapeutic strategies for OA. The role of glutamate in OA development remains a mystery and further investigation is needed to better understand the effect of selective COX-2 inhibitors on OA progression and the role of EAAs in OA development.

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