Osteoarthritis and Cartilage

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Glucosamine sulfate reduces experimental osteoarthritis and nociception in rats: association with changes of mitogen-activated protein kinase in chondrocytes

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SUMMARY

Objective: To study the effects of oral glucosamine sulfate on the development of osteoarthritis (OA) and to examine concomitant changes in the nociceptive behavior of rats.

Methods: OA was induced in Wistar rats by anterior cruciate ligament transection (ACLT) of the right knee; the left knee was untreated. The OA + glucosamine group received oral glucosamine sulfate (250 mg/kg/day) *in a 2-g wafer* once a day for 10 consecutive weeks starting at week 5 after ACLT. The OA group was treated as above with 2-g wafers (placebo). The control group of naïve rats received 2-g wafers only. The glucosamine alone group comprised naïve rats receiving glucosamine sulfate only. Nociceptive behavior (mechanical allodynia and weight-bearing distribution of hind paws) during OA development was analyzed pre- and 3, 6, 9, 12, 15, and 18 weeks post-ACLT. Macroscopic and histologic studies were then performed on the cartilage and synovia. Immunohistochemical analysis was performed to examine the effect of glucosamine on expression of mitogen-activated protein kinases (MAPKs) in the articular cartilage chondrocytes.

Results: OA rats receiving glucosamine showed a significantly lower degree of cartilage degeneration than the rats receiving placebo. Glucosamine treatment also suppressed synovitis. Mechanical allodynia and weight-bearing distribution studies showed significant improvement in the OA + glucosamine group as compared to the OA group. Moreover, glucosamine attenuated p38 and c-Jun N-terminal kinase (JNK) but increased extracellular signal-regulated kinase 1/2 (ERK) expression in OA-affected cartilage.

Conclusion: Our results indicate that treatment with oral glucosamine sulfate in a rat OA model (1) attenuates the development of OA, (2) concomitantly reduces nociception, and (3) modulates chondrocyte metabolism, possibly through inhibition of cell p38 and JNK and increase of ERK expression.

Introduction

Osteoarthritis (OA), characterized by the progressive degradation of articular cartilage, is a complex disorder that can affect the majority of joints in the body. Although OA is classically defined as a progressively degenerative disease of articular cartilage, the key role of inflammation in the pathogenesis of OA has been identified[1]. Although the pain associated with OA is primarily localized to the joint, a number of OA patients have exhibited increased nociception in adjacent or even remote body areas[2,3]. This phenomenon, referred to as secondary hyperalgesia or allodynia, is thought to be the result of nociceptive/central sensitization[4].

Glucosamine is an amino monosaccharide nutrient and precursor of the glycosaminoglycan disaccharide unit, which is the building block of the proteoglycan component of the cartilage matrix. In human OA patients, administration of 1500 mg of

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glucosamine/day for 3 years prevented joint-space loss and relieved symptoms when compared to placebo, suggesting that glucosamine possesses some anti-inflammatory activity that can alter disease progression[5]. Glucosamine has wide availability, relatively low cost, absence of known side effects, high oral absorption rate (>87% in dogs and cats), and is readily transferred into cartilage[6]. Glucosamine sulfate is the most frequently used form of glucosamine for the treatment of OA[5,7], but its efficiency in the treatment of OA is still controversial[8]. However, despite the widespread use of glucosamine by patients with OA in clinical practice, the role of glucosamine in the development of OA and OA-induced nociception has not been well elucidated.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that are part of the signal transduction pathways connecting inflammatory and other extracellular signals to intracellular responses[9]. The MAPK family consists of p38 kinase, c-Jun N-terminal kinase (JNK), and the extracellular signal-regulated kinases 1/2 (ERKs). The JNK and p38 kinases are activated in response to inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)[10,11], while ERK mediates signaling by cytokines and growth factors[12,13]. Inhibitors of p38, JNK, and ERK are under development for treatment of arthritis, and they have shown efficacy in experimentally induced arthritis and joint pain[9,13—16]. However, the *in vivo* effects of glucosamine sulfate on cartilage MAPK activity in the pathogenesis of OA are still unknown. In the present study, we investigated the effects of oral glucosamine sulfate on nociception of the knee joint as assessed by mechanical allodynia and hind paw weight distribution in an experimental rat OA model. Gross morphology and histopathology were examined in the cartilage and synovia. Immunohistochemical analysis was performed to examine the effect of glucosamine sulfate on MAPK expression in articular cartilage chondrocytes.

Methods

Animals

Two-month-old male Wistar rats (body weight = 275—315 g) were used and maintained on a 12-h light-dark cycle under climate-controlled conditions of 22—24°C with a relative humidity of 50—55%. Animals with pre-existing anatomical or gait abnormalities were excluded. The use of rats conformed to the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiology Society and was approved by the National Sun Yat-sen University Animal Care and Use Committee (approval number 96004).

Surgical technique for induction of OA

OA was induced in rats by anterior cruciate ligament transection (ACLT) of right knee; the left knee was untreated. The surgical procedure is modified from the protocol described by previous study[17]. The animals were not immobilized after surgery and were allowed daily unrestricted cage activity.

Experimental design

Five weeks after ACLT, the animals were divided into four groups. The OA + glucosamine group received oral glucosamine sulfate (Sigma, St. Louis, MO; 250 mg/kg/day) in a 2-g wafer once a day for 10 consecutive weeks starting at week 5 after ACLT. The OA group was treated as above except that they received 2-g wafers alone (placebo). The control group comprised naïve rats receiving 2-g wafer only. The glucosamine alone group was made up of naïve rats receiving glucosamine sulfate (250 mg/kg/day in wafer) once

a day for 10 consecutive weeks. Nociceptive behavior (mechanical allodynia and weight-bearing distribution on the hind paws) during OA development was analyzed, and changes of knee joint width were measured pre- and 3, 6, 9, 12, 15, and 18 weeks post-ACLT, following which the animals were sacrificed. Gross morphology and histopathology were examined in the femoral condyles, tibial plateau, and synovia. The sample sizes of the control, OA, OA + glucosamine, and glucosamine alone groups were 6, 12, 12, and 6, respectively.

Assessment of nociception

The effect on pain was assessed by mechanical allodynia and weight-bearing distribution of hind paws were measured pre- and 3, 6, 9, 12, 15, and 18 weeks after ACLT.

Mechanical allodynia

In the mechanical allodynia, paw withdrawal thresholds to mechanical stimulus were determined using the up and down method by applying calibrated von Frey filaments (North Coast Medical, Inc. Morgan Hill, CA, USA)[18]. The diameters of the filaments corresponded to a logarithmic scale of the force exerted, and thus the perceived intensity could be measured on a linear and interval scale. The withdrawal threshold was determined by Chaplan's "up-down" method involving the use of alternate large and small fibers to determine the 50% withdrawal threshold[18]. Each von Frey hair was applied to the plantar surface of the paw for 5 s. The von Frey filament was applied to each paw for five trials at approximately 3-min intervals. Any sign of discomfort (vocalization, flinching) or attempt of the animal to withdraw the paw was considered positive response.

Weight-bearing distribution test

The effect of joint damage on the weight distribution through the right and left knees was measured using a Dual Channel Weight Averager (Singa Technology Corporation, TW) which independently measures the weight bearing to each hind paw. Changes in hind paw weight distribution between the right and the left limbs were utilized as an index of joint discomfort in the OA knee[19,20]. The percent weight distributed onto the right hind paw was calculated by the following equation: [weight on right hind paw/(weight on right hind paw + weight on left hind paw)] × 100. The quantitative method was according to previous study[21].

Inflammation, gross morphology, and histopathological examination of the knee joints

The width of the bilateral hind limb knee joints was measured from the medial to the lateral aspect of the joint line by using calipers before (baseline) and 3, 6, 9, 12, 15, and 18 weeks after ACLT. The gross morphological changes in the cartilage were examined according to previously described methods[22]. The joints were sectioned 0.5 cm above and below the joint line, fixed in 10% neutral buffered formalin for 3 days, and then decalcified for 2 weeks in buffered 12.5% ethylenediaminetetraacetic acid (EDTA) and formalin solution. The cartilage was stained with hematoxylin—eosin (H & E) and Safranin-O/fast green stains to assess the general morphology and matrix proteoglycans. Microscopic examination of the articular cartilage of the medial and lateral femoral condyles and the tibial plateau was graded according to Mankin's grading system[23]. A representative specimen of the synovial membrane from the medial and lateral compartments of

the knee was dissected from the underlying tissues for histological examination, as previously described[24].

Immunohistochemistry for p38, JNK, and ERK MAPKS

Immunohistochemical analysis was performed to examine the effect of glucosamine sulfate on p38, JNK, and ERK expression in the articular cartilage chondrocytes. Cartilage specimens were processed for immunohistochemical analysis as described in previous studies[25,26]. Briefly, sections (2 μm) of the paraffin-embedded specimens were placed on slides, deparaffinized with xylene, and dehydrated in a graded series of ethanol, following which endogenous peroxidase activity was quenched by 30-min incubation in 0.3% hydrogen peroxide. The antigen was retrieved by enzymatic digestion with proteinase K (20 mM; Sigma) in phosphate-buffered saline (PBS) for 20 min. After washing in ice-cold PBS, slides were incubated overnight at 4°C with anti-phospho-p38 (Thr180/Tyr182, 1:100 dilution; catalog no. 9211), anti-ERK (Thr202/Tyr204, 1:100 dilution, catalog no. 9101), or anti-JNK (Thr183/Tyr185, 1:100 dilution, catalog no. 9258) in a humidified chamber. All the antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA). The sections were incubated for 90 min with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 200 times in 1% bovine serum albumin (BSA) in PBS.

The antigens present in the each cartilage specimen were quantified using our previously published method[26] and estimated by determining the number of chondrocytes that stained positive in the entire thickness of cartilage according to procedures in previous studies[14,27]. The cartilage was divided into six microscopic fields (three each in the superficial and deep zones, magnification 400×), and the results were averaged. For each OA specimen, it was ensured prior to evaluation that an intact cartilage surface could be detected and used as a marker for the validation of the morphometric analysis. The final results were expressed as the percentage of chondrocytes staining positive for the antigen (cell score), with the maximum score for each cartilage specimen being 100%. Each slide was reviewed by two independent readers (HSY and HSP) who were blinded to the treatment groups. The combined data obtained from the medial and lateral femoral condyle and tibial plateau were considered for statistical analysis (six rats per group).

Data and statistical analysis

All data were presented as mean (95% confidence interval). The data were analyzed by using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls *post hoc* test (SigmaStat 2.03, for Windows). Differences resulting in *P*-values of less than 0.05 were considered significant.

Results

No signs of drug toxicity were noted in the rats treated with glucosamine sulfate. The level of daily activity was similar in all three experimental groups, and there were no significant differences in body weight between the groups over the study period.

Nociceptive behavior (mechanical allodynia and weight-bearing distribution) in OA

Figure 1(A) shows the effect of oral glucosamine sulfate on mechanical allodynia compared with placebo in ACLT-induced OA rats. At 15 and 18 weeks after ACLT-induced OA, the force required for hind paw withdrawal in the OA + glucosamine group was significantly higher than that in the OA group [7.7 (6.4–9.0) vs 5.3 (3.5–7.1) g, *P* = 0.027 and 8.0 (6.5–9.5) vs 5.0 (4.1–5.9) g, *P* = 0.001,
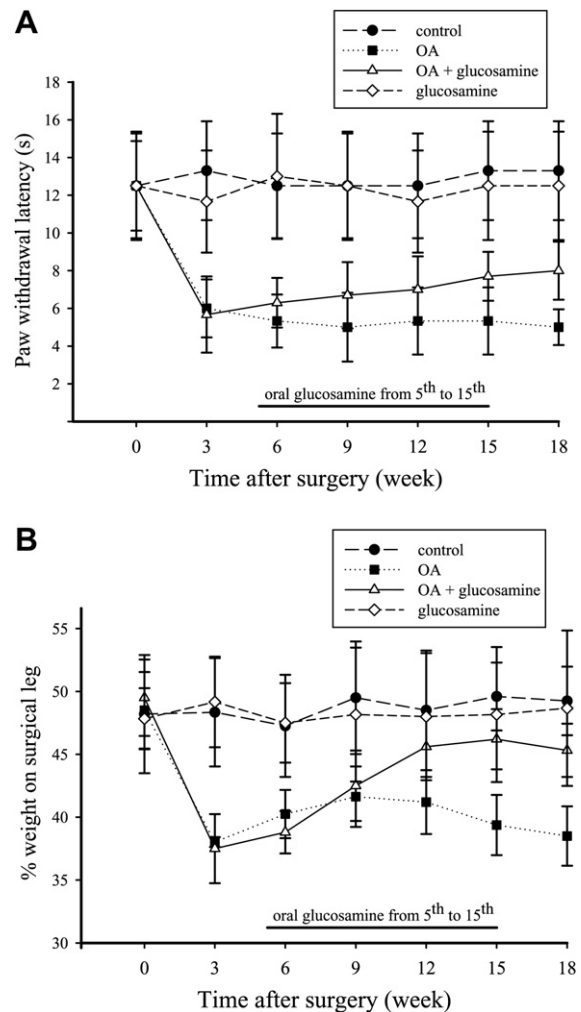

Fig. 1. Time course of the anti-allodynic effect of glucosamine sulfate in the ACLT-OA nociceptive model. (A) In mechanical allodynia, the time required to elicit hind paw withdrawal in the OA + glucosamine group was significantly increased compared with OA rats at 15 and 18 weeks after ACLT. (B) The time course of the % weight bearing of the right (ACLT) hind paw for 18 weeks after ACLT. A significant difference was noted between OA and OA + glucosamine groups at 15 and 18 weeks after surgery. In the glucosamine alone (naïve rats treated with glucosamine sulfate alone) group, similar changes are observed, as in the control group. Data shown represent the mean (95% confidence interval) of the ipsilateral hind paw of each group.

respectively] [Fig. 1(A)]. Eighteen weeks after the induction of OA, the mechanical threshold in the hind paw of the side contralateral to the side of ACLT was 10.8. (9.6–12.0), 11.7 (10.1–13.3) and 12.5 (9.9–15.1) g in the OA, OA + glucosamine, and control groups, respectively. Before ACLT (baseline values), the mechanical threshold in the hind paw on the side contralateral to the ACLT side was 11.7 (10.1–13.3), 11.7 (10.1–13.3), and 12.5 (9.9–15.1) g for the OA, OA + glucosamine, and control groups, respectively. Glucosamine alone did not change the mechanical allodynia behavior.

In the weight-bearing distribution test, the % weight on the right hind paw was reduced at 3, 6, 9, 12, 15, and 18 weeks after ACLT compared to that in the control group [Fig. 1(B); *P* < 0.001, *P* < 0.001, *P* < 0.001, *P* = 0.003, *P* < 0.001, and *P* < 0.001, respectively]. The OA + glucosamine group showed significantly higher % weight on the right hind paw than that of the OA group at 12, 15, and 18 weeks after surgery [45.6% (43.2–48.0) vs 41.2% (38.7–43.7), *P* = 0.011; 46.2% (43.8–48.6) vs 39.3% (37.0–41.8), *P* < 0.001; 45.3% (43.2–47.3) vs 38.2% (36.1–40.9), *P* < 0.001, respectively].

Administration of glucosamine significantly reversed alterations in hind limb weight bearing was noted of 12, 15, and 18 weeks after ACLT.

Knee joint width and gross morphologic changes

In knee joint width changes, as shown in Fig. 2, with a significant difference between the control group and both OA + glucosamine and OA groups at 6, 9, 12, 15, and 18 weeks after the ACLT. The width of the hind limb knee joint of the OA + glucosamine group was significantly lower than that of the OA group at 12, 15, and 18 weeks after surgery [0.5 (0.4–0.6) mm vs 1.2 (1.0–1.3) mm, $P < 0.001$; 0.6 (0.5–0.7) mm vs 1.1 (1.0–1.3) mm, $P < 0.001$ and 0.7 (0.5–0.8) mm vs 1.3 (1.1–1.4) mm, $P < 0.001$, respectively] (Fig. 2). In the OA group, gross characteristics of cartilage degeneration, such as fibrillation, erosion and ulcer formation, and osteophytes formation, were seen in the femoral condyle and tibial plateau. Markedly less severity of cartilage damage was seen in the OA + glucosamine group. In the control and glucosamine alone groups, the cartilage of the femoral condyle and tibial plateau was macroscopically normal, with a glistening, smooth surface, and no cartilage defects or osteophytes were observed. A significant difference in gross morphologic score was found between the OA group and both OA + glucosamine and control groups (Table I; $P = 0.005$ and $P < 0.001$, respectively), but not between the control group and glucosamine alone group ($P = 0.053$). The grade of cartilage damage in the OAPP + glucosamine group was significantly lower than that in the OA group (Table I; $P = 0.005$). Synovia from the OA group were hypertrophic and showed a reddish-yellow discoloration, whereas in the OA + glucosamine group, they were thinner and the discoloration was less intense. Synovia from the control group had a white luster and transparent appearance, with no hyperemia or evidence of synovitis.

Microscopic findings

The cartilage of the control and glucosamine alone groups had a normal histological appearance. A thin, glistening, smooth lamina filled with flattened chondrocytes was observed, and no loss of proteoglycan was seen in the matrix on Safranin-O staining [Fig. 3 (A)]. Specimens from the OA group showed obvious histological
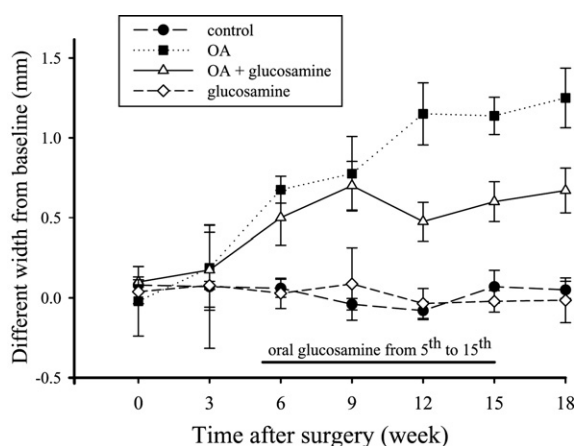
Table I
Macroscopic evaluation of the articular cartilage of the femoral condyles and tibial plateau

Groups	Macroscopic score	P-Value	
		Comparison vs control	Comparison vs OA
Control	0.4 (0.3–0.4)	–	<0.001
OA	2.6 (2.1–3.0)	<0.001	–
OA + glucosamine	1.6 (1.0–2.1)	0.005	0.005
Glucosamine alone	0.5 (0.4–0.5)	0.053	0.001

Data are expressed as mean (95% confidence interval). For the macroscopic score, refer to Methods. OA: ACLT-induced OA knee treated with wafer; OA + glucosamine: OA knee treated with glucosamine sulfate; control: naïve rats receiving wafer only; glucosamine alone group: naïve rats receiving glucosamine sulfate only.

changes, including complete disorganization, moderate-to-severe hypocellularity, proteoglycan reduction on Safranin-O/fast green staining, and denudation of articular surface and fissures extending into the deep zones [Fig. 3(B)]. Osteophytes were present at the medial margins of the femoral condyle and tibial plateau. In the OA + glucosamine group, there was marked reduction in the severity of the femoral condyle and tibial plateau lesions: only fibrillation and fissures extending into the superficial layer of cartilage were observed [Fig. 3(C)]. In the glucosamine alone group [Fig. 3(D)], similar changes are observed as in the control group. Mankin's score for the OA + glucosamine group was significantly lower than that for the OA group. Significant differences were found between the control and both OA and OA + glucosamine groups (Table II). Cartilage degeneration was more severe at the medial sides than at the lateral sides of the femoral condyle and tibial plateau in the OA group (Table II). Synovia from the OA group were thick, had focal villi, and showed hyperplasia of the lining cells and moderate infiltration of mononuclear inflammatory cells. The histology of the synovia from the control and glucosamine groups was within normal limits. The synovitis scores on microscopic evaluation are shown in Table II; significant differences were found between the control group and both OA and OA + glucosamine groups and between the OA + glucosamine group and the OA group (Table II). The synovitis score was lower for the OA + glucosamine group than the OA group ($P < 0.001$), suggesting that synovial inflammation was less severe in the OA + glucosamine group. The reductions resulted primarily from a less marked degree of synovial hyperplasia.

Immunohistochemistry of phospho-p38, phospho-JNK, and phospho-ERK MAPKS in articular cartilage

The immunolocalization of phosphorylated MAPK (phospho-p38, phospho-JNK, and phospho-ERK) protein expression in cartilage specimens from the control, OA, OA + glucosamine, and glucosamine alone groups was examined using the appropriate antibodies (Figs. 4–6). As shown in Fig. 4, fewer positive phospho-p38 chondrocytes were observed in the control group [Fig. 4(A)] than in the OA group [Fig. 4(B)]. In the OA group at 18 weeks after ACLT, positive phospho-p38 immunoreactive chondrocytes were apparently increased in the superficial and transitional cartilaginous zones [Fig. 4(B)]. Oral glucosamine inhibited OA-induced upregulation of phospho-p38 expression in chondrocytes of cartilage [Fig. 4(C)]. Glucosamine alone did not produce significant changes of phospho-p38 expression in cartilage specimens [Fig. 4 (D)]. Quantification of the number of immunoreactive phospho-p38 cells showed that glucosamine significantly inhibited OA-induced upregulation of phospho-38–positive chondrocytes [Fig. 4 (F); $P = 0.002$].

Fig. 2. Time course of joint width changes after ACLT. The widths of the bilateral hind limb knee joints were measured in each rat before and again at 3, 6, 9, 12, 15, and 18 weeks after ACLT. The data are expressed as the difference in knee widths between the values at each time point and at time zero (before surgery). In the glucosamine alone group, changes similar to those in the control group are observed. Data presented as mean (95% confidence interval).
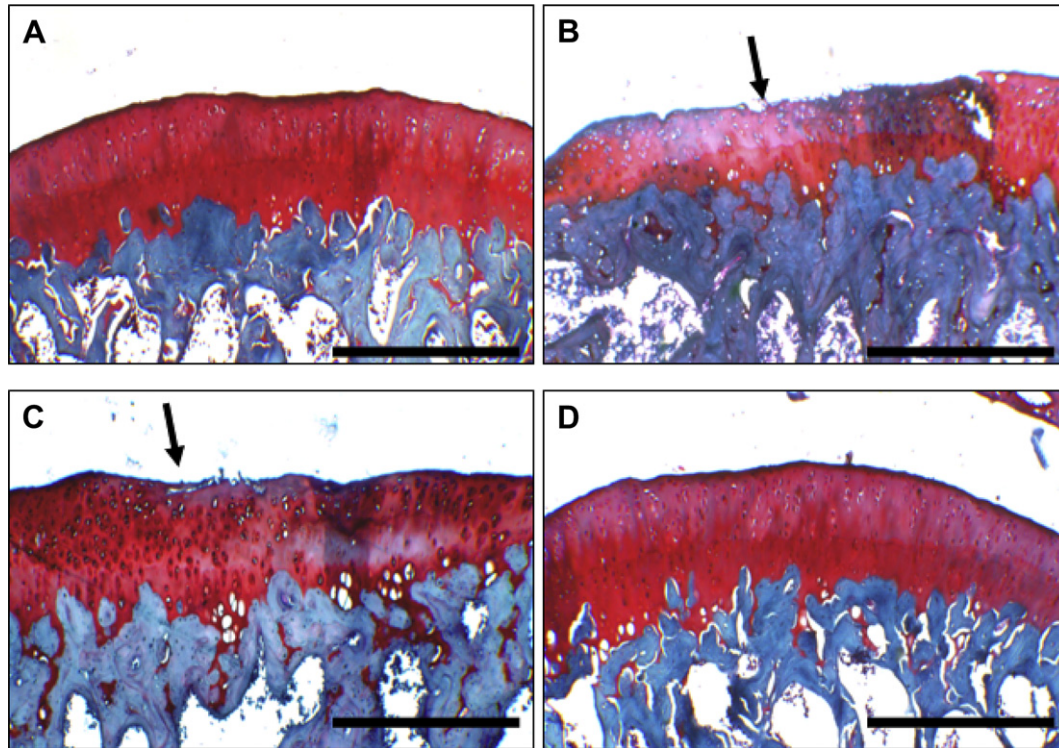
Fig. 3. Histopathological evaluation of the articular cartilage of the femoral condyles and tibial plateau. (A) In the control group, the surface of the superficial cartilaginous layer is smooth, and the cartilage matrix is consistently well stained with Safranin-O/fast green. (B) The specimen from the OA group shows a decrease in the cartilage thickness, disappearance of the surface layer cells (arrow), a fissure extending into the transitional and radial zones, and chondrocyte hypocellularity in the transitional and radial zones. (C) The specimen from the OA + glucosamine group shows mild irregularity of the surface layer, fibrillation of and fissures in the superficial cartilaginous layer (arrow), and slight diffuse hypercellularity in the transitional and radial zones. (D) In the glucosamine alone group, similar changes are observed, as in the control group. Scale bar = 500 μm.

As shown in Fig. 5, only a very small number of chondrocytes stained positive for phospho-JNK in cartilage specimens in the control and glucosamine alone groups [Fig. 5(A and D)]. In the OA specimens, phospho-JNK protein expression was clearly seen in chondrocytes within the superficial and transitional cartilage, similar to the findings in phospho-p38 expression [Fig. 5(B)]. The phospho-JNK protein expression was significantly increased in both the OA and OA + glucosamine groups compared with the control group (Fig. 5(F); $P < 0.001$ and $P = 0.007$, respectively). Glucosamine significantly inhibited OA-induced upregulation of phospho-JNK expression in chondrocytes of cartilage [Fig. 5(C and F), $P = 0.006$].

Table II
Histological evaluation scores of the articular cartilage and synovial membrane as obtained by light microscopy

Group	Control	OA	OA + glucosamine	Glucosamine alone
Location				
Femoral condyle				
Medial side	1.4 (0.4–2.4)	8.5 (6.0–11.0)	3.6 (2.8–4.4)	1.6 (0.8–2.4)
Comparison vs control: P-value	–	<0.001	0.002	0.710
Comparison vs OA: P-value	<0.001	–	<0.001	<0.001
Lateral side	1.2 (−0.1–2.5)	8.1 (6.1–10.1)	3.3 (2.8–3.8)	1.2 (0.7–1.7)
Comparison vs control: P-value	–	<0.001	<0.001	1.000
Comparison vs OA: P-value	<0.001	–	<0.001	<0.001
Tibial plateau				
Medial side	1.1 (−0.1–2.3)	7.8 (6.1–9.5)	2.7 (1.9–3.5)	1.5 (0.7–2.3)
Comparison vs control: P-value	–	<0.001	0.020	0.488
Comparison vs OA: P-value	<0.001	–	<0.001	<0.001
Lateral side	0.9 (0.0–1.8)	7.2 (6.2–8.2)	2.5 (2.0–3.0)	0.8 (0.3–1.3)
Comparison vs control: P-value	–	<0.001	0.001	0.817
Comparison vs OA: P-value	<0.001	–	<0.001	<0.001
Synovial membrane	1.5 (−0.1–3.1)	8.3 (6.3–10.3)	3.5 (2.7–4.3)	1.4 (0.6–2.2)
Comparison vs control: P-value	–	<0.001	0.010	0.888
Comparison vs OA: P-value	<0.001	–	<0.001	<0.001

Data are expressed as mean (95% confidence interval). For definitions of the osteoarthritic score (Mankin) and synovitis score, refer to Methods. OA: ACLT-induced OA knee treated with wafer; OA + glucosamine: ACLT-induced OA knee treated with glucosamine sulfate; control: naïve rats receiving wafer only; glucosamine alone group: naïve rats receiving glucosamine sulfate only.
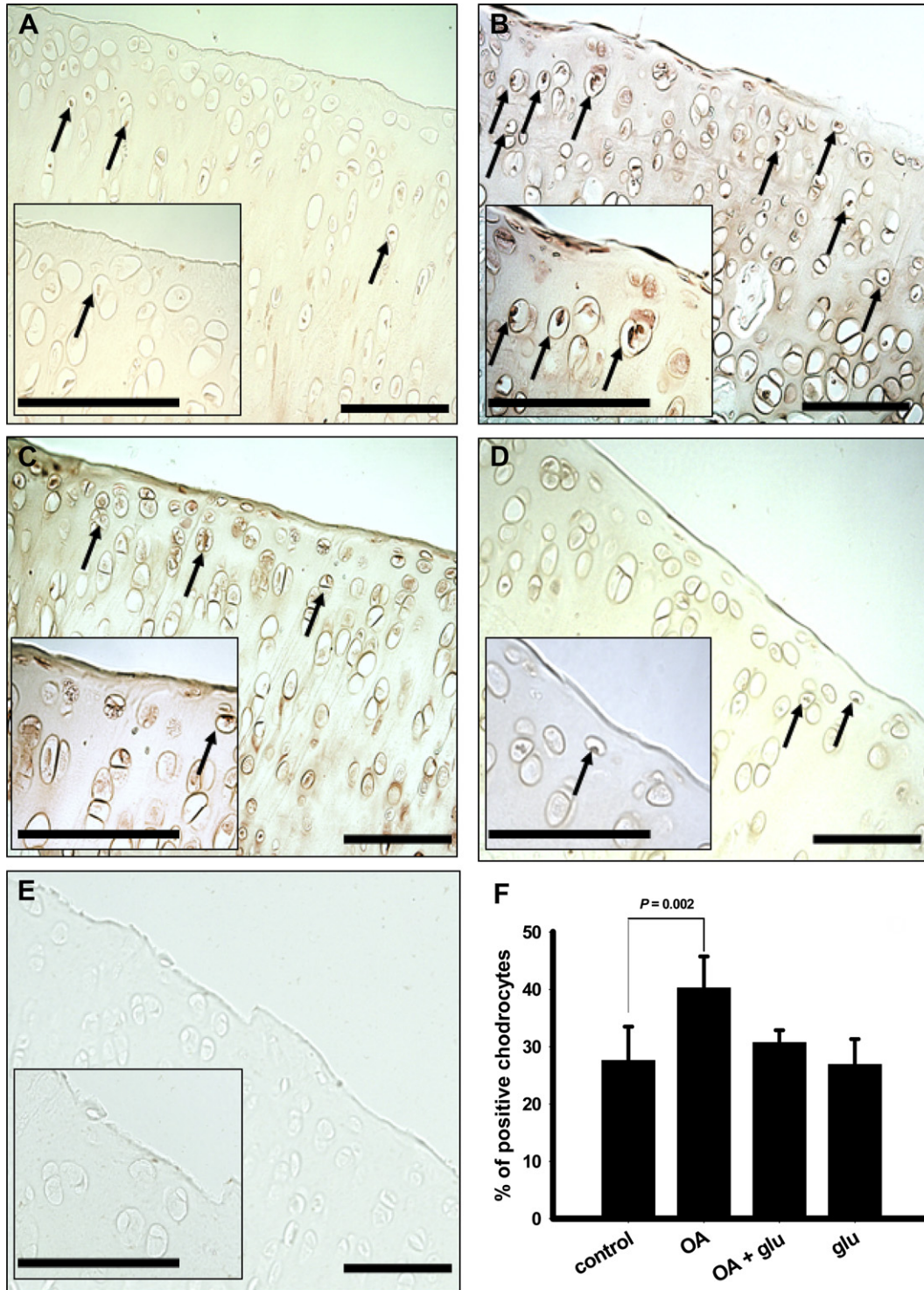
Fig. 4. Distribution of phospho-p38 protein immunoreactivity in the cartilage of the control, OA, OA + glucosamine, and glucosamine alone groups. Positive immunoreactivity of the phospho-p38 protein is indicated by the red-brown color (arrows). Distribution of anti-p38 immunoreactivity in the cartilage of the (A) control, (B) OA, (C) OA + glucosamine, and (D) glucosamine alone groups and (E) the negative control from OA. All were stained with antibodies against the phospho-p38 protein. (F) Quantitative analysis showed that glucosamine significantly reduced the ACLT-induced increase in the number of p38-positive chondrocytes in cartilage of the OA knee. The data were presented as mean (95% confidence interval). Scale bar = 100 μm.

Figure 6 shows the distribution of phospho-ERK-positive cells in cartilage from the control [Fig. 6(A)], OA [Fig. 6(B)], OA + glucosamine [Fig. 6(C)], and glucosamine alone groups [Fig. 6(D)]. When compared with the control group, the number of positive phospho-ERK chondrocytes was upregulated in the OA + glucosamine and glucosamine alone groups ($P < 0.001$). A slightly decreased phospho-ERK expression was seen in the OA specimen at 18 weeks after ACLT [Fig. 6(F), $P = 0.438$]. In all four individual treatment groups, no staining was observed in the phospho-p38, phospho-JNK, or phospho-ERK negative controls.
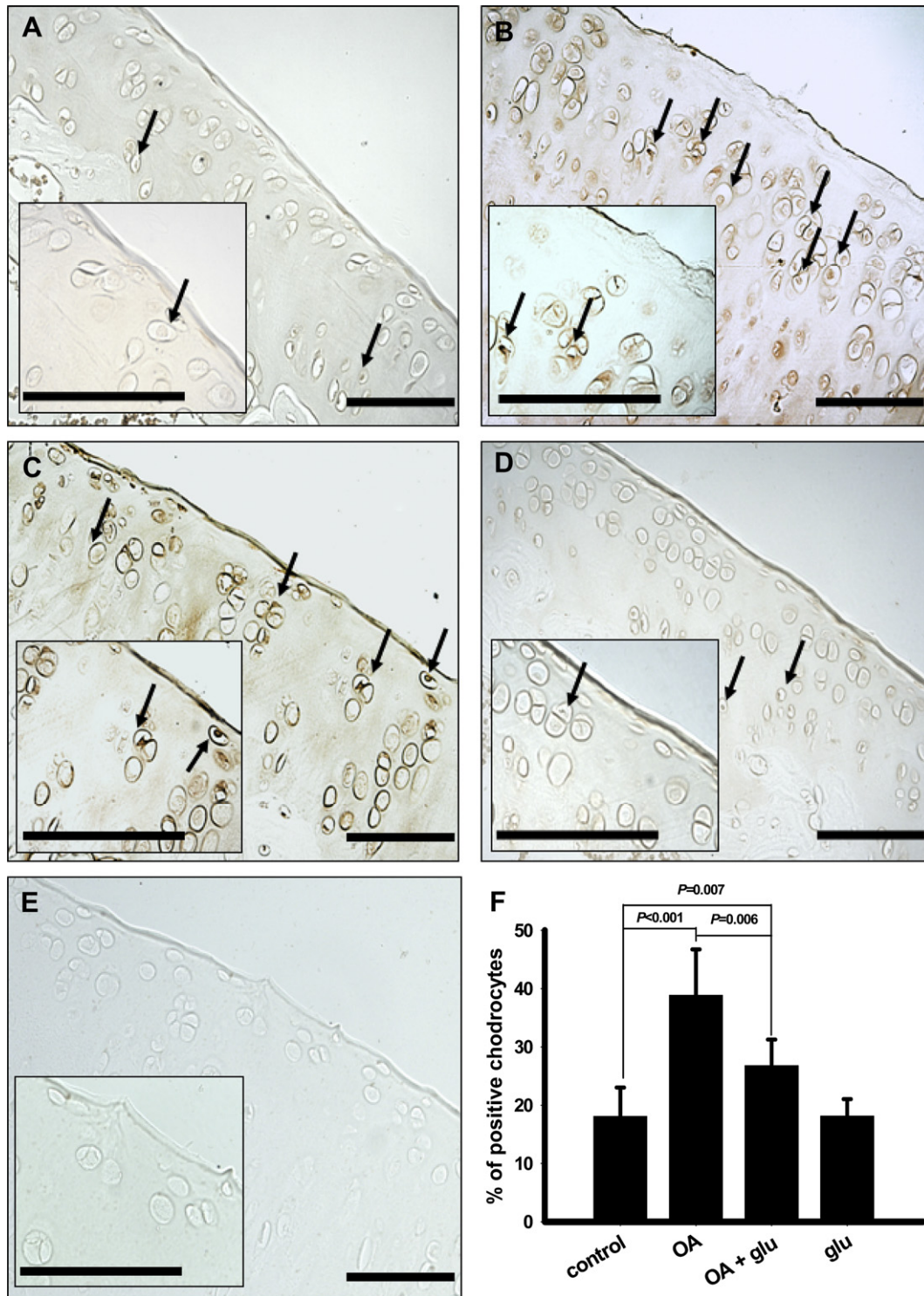
Fig. 5. Distribution of phospho-JNK protein immunoreactivity in the cartilage of the control, OA, OA + glucosamine, and glucosamine alone groups. Positive immunoreactivity of the JNK protein is indicated by the red-brown color (arrows). Distribution of anti-phospho-JNK immunoreactivity in the cartilage of the (A) control, (B) OA, (C) OA + glucosamine, and (D) glucosamine alone groups and (E) the negative control from OA. All were stained with antibodies against the phospho-JNK protein. Scale bar = 100 μm. (F) Quantitative analysis showed that glucosamine sulfate significantly reduced the ACLT-induced increase in the number of phospho-JNK-positive chondrocytes in cartilage of the OA knee. The data were presented as mean (95% confidence interval). Scale bar = 100 μm.

Discussion

To our knowledge, this is the first study to demonstrate that oral glucosamine sulfate can attenuate the development of OA and associated nociceptive behaviors (such as mechanical allodynia and weight-bearing distribution) in an experimental rat OA model.

More interestingly, glucosamine sulfate inhibited p38 and JNK but enhanced ERK MAPK expression in the articular cartilage chondrocytes in the experimental OA model.

The destabilizing effect secondary to the ACLT has been used as a model for the study of joint alterations that closely resemble those of human OA[28]. This include superficial fibrillation,
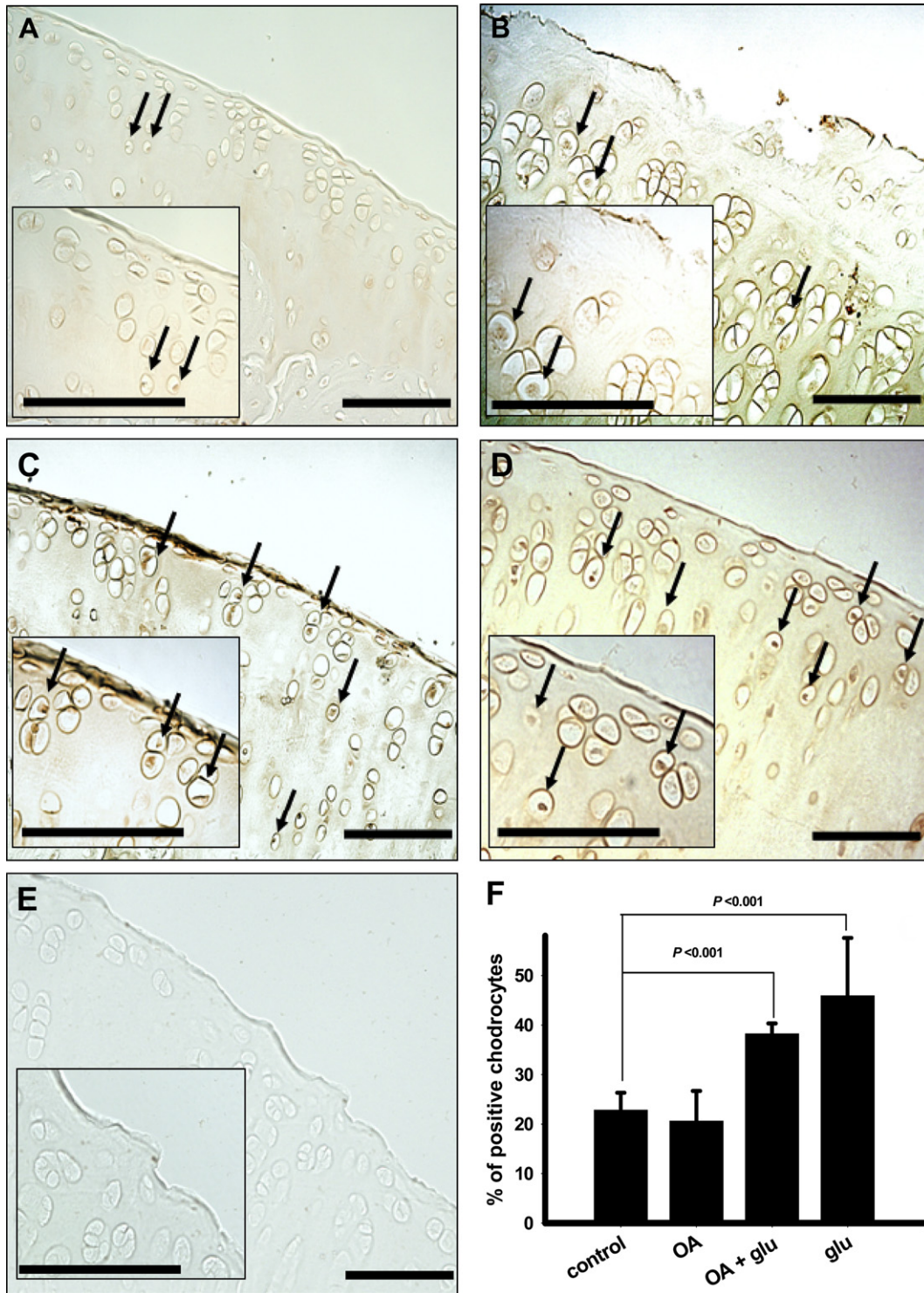
Fig. 6. Distribution of phospho-ERK protein immunoreactivity in the cartilage of the control, OA, OA + glucosamine, and glucosamine alone groups. Positive immunoreactivity of the phospho-ERK protein is indicated by the red-brown color (arrows). Distribution of anti-phospho-ERK immunoreactivity in the cartilage of the (A) control, (B) OA, (C) OA + glucosamine, and (D) glucosamine alone groups and (E) the negative control from OA + glucosamine. All were stained with antibodies against the phospho-ERK protein. Scale bar = 100 μm. (F) Quantitative analysis showed that glucosamine sulfate significantly enhanced the number of phospho-ERK-positive chondrocytes in cartilage of the ACLT-induced OA knee. The data were presented as mean (95% confidence interval). Scale bar = 100 μm.

disorganization of the collagen and proteoglycans network, joint capsule thickening, and osteophyte formation[28]. Glucosamine protects against subchondral bone change during the development of early phase of ACLT-induced OA suggest a possible mechanism of glucosamine to partially protect cartilage from

degeneration[29]. In our present study, glucosamine sulfate attenuated the development of OA compared to placebo treatment (Tables I and II; Fig. 3). This is in accordance with a previous animal study, that glucosamine is able to reduce cartilage degradation following ACLT in rabbits[30].

Patients with arthritis of the knee joints usually experience a pain sensation when the inflamed knee joints bear weight or are mechanically stimulated by being moved or pressed[31]. Lee *et al.* showed that intra-articular injection of magnesium sulfate could attenuate the nociceptive behavior such as mechanical allodynia and thermal hyperalgesia in a collagenase induced OA rat model[26]. In the present study, ACLT-induced nociceptive behavior was characterized by a decrease in mechanical allodynia threshold and decrease weight-bearing distribution in the injured hind paw (Fig. 1). In the OA + glucosamine group, significant improved mechanical allodynia at 15 and 18 weeks, and hind paw weight-bearing distribution were noted at 12, 15, and 18 weeks after ACLT, respectively, when compared with OA group (Fig. 1). This is partial in accordance with a previous study that used a quantitative joint pain associated to weight bearing in the ACLT model in rats[32]. We believe that pain on weight bearing, as assessed in the present study, represents a major component of the multifactorial mechanism of joint pain in OA.

The ability of glucosamine-containing nutraceuticals to reduce proteoglycan loss, impede cartilage degeneration, delay joint-space narrowing, and improve pain has been extensively reported[5,33]. Glucosamine treatment upregulates transforming growth factor (TGF)-β1 mRNA levels in chondrocytes, and it can preserve cartilage and promote its repair upon damage[34]. Glucosamine has been shown to suppress prostaglandin E_2 (PGE_2) production in chondrocytes and synoviocytes from OA joints[35]. It also blocked IL-1-induced expression of matrix-specific proteases such as matrix metalloproteinase (MMP)-3, MMP-9, MMP-10, MMP-12, and aggrecanase[36]. Glucosamine inhibits aggrecanase activity *via* suppression of glycosylphosphatidylinositol-linked proteins[37]. The transcription factors nuclear factor (NF)-κB and activation protein (AP)-1 have been implicated in OA and are downregulated by glucosamine[38,39]. In OA, episodic synovial inflammation at the clinical stage is a well-documented phenomenon and is believed to be involved in disease progression in OA[1,40]. Inflammatory changes associated with the ACLT model include joint effusion and synovial membrane hyperplasia, which represent important mechanisms of joint pain in patients with OA[41]. In the present study, moderate synovitis was noted in ACLT knees, and glucosamine administration could decrease the severity of synovitis (Table II). Joint width could be measured in patients to determine the extent of tissue swelling as an index of inflammation[19]. Oral administration of glucosamine inhibited swelling in both hind paws of mice in which Freund's complete adjuvant arthritis was induced[42]. In the present study, the OA + glucosamine group showed a smaller increase in knee joint width as compared to the OA group (Fig. 2). Oral glucosamine may therefore decrease inflammation in the ACLT knee. Taken together, the above studies support a role for glucosamine in the protection of the cartilage matrix and chondrocyte metabolism, suggesting a possible mechanism by which glucosamine may help to alleviate clinical signs and retard progression of OA.

MAPKs are activated in joint diseases[10,15], and its inhibitors would attenuate arthritis-induced NF-κB activation and upregulation of proinflammatory inducible nitric oxide synthase (iNOS) and COX-2 in rats[43,44]. p38 and JNK are phosphorylated in response to proinflammatory cytokines such as TNF-α and IL-1β[45], resulting in activation of transcription factors, including AP-1 complex[46], which are involved in expression of MMPs and aggrecanase[47,48] Inhibitors of JNK, p38, and ERK pathways downregulate IL-1-induced COX-2 expression and PGE_2 production in human chondrocytes[49]. Brown *et al.* reported that p38 inhibitor acts as a potential therapeutic for the treatment of joint degeneration and pain associated with OA[15]. The ERK pathway is critical in transducing the IL-1β and TNF-α signal to induce expression of MMPs responsible for cartilage destruction[50]. Glucosamine inhibited IL-1β-stimulated MMP-1,

MMP-3, and MMP-13 production in human chondrocytes by inhibition of JNK and p38 phosphorylation[39]. Glucosamine is capable of inhibiting iNOS and COX-2 expression in lipopolysaccharide (LPS)-induced RAW264.7 cells *via* attenuation of NF-κB signaling by p38 and JNK, but not by ERK[13]. In the present study, p38 and JNK expression was increased in the OA group, but ERK expression showed no significant difference between the OA and control groups (Figs. 4–6). Treatment with glucosamine could decrease the expression of p38 and JNK in OA cartilage chondrocytes (Figs. 4 and 5). More interestingly, treatment with glucosamine sulfate increased ERK expression in the OA + glucosamine group and glucosamine alone groups of rats (Fig. 6), and this is the first finding that treatment with glucosamine sulfate could increase ERK expression in the chondrocytes of OA and naïve rats chondrocytes. Our observation was partially in line with previous report that in a dog model of ACLT-induced OA, p38, JNK, and ERK were all activated to a greater degree in OA compared with normal tissue[14]. Another report documented that phosphorylation levels of p38 and JNK were higher in OA cartilage than in normal tissue, whereas ERK was found in both[10,11]. The reasons for these discrepancies with our results regarding ERK in OA are presently unclear but are most likely due to cell-type differences or to differences in experimental conditions. We suppose that p38, JNK, and ERK might, at least partially, play a role in OA development and nociception in rat OA. These findings support a theory that attenuation of MAPK signaling can lead to modulation of nociceptive transmission in arthritis. However, the exact mechanism of MAPK activity on pain reduction in OA needs further study.

In conclusion, oral glucosamine sulfate can attenuate the development of OA and associated nociceptive behavior such as mechanical allodynia and weight-bearing distribution, in an experimental rat OA model. More interestingly, glucosamine sulfate inhibited p38 and JNK but enhanced the expression of ERK in the articular cartilage in the experimental OA model. These findings may have the way for further investigations of glucosamine sulfate as a potentially therapeutic target for the treatment of the inflammatory component in OA.

Author contributions

Dr Jean had full access to all of the study data and assumes responsibility for its integrity and the accuracy of the data analysis.
Study design: Wen ZH, Chang YC, Lee CH, and Chen WF.
Data acquisition: Lee CH, Wen ZH, Chang YC, Neoh CA, and Huang SY.
Data analysis and interpretation: Tang CC, Wen ZH, Hsieh SP, Ng HF, and Chang YC.
Manuscript preparation: Hsieh CS, Chen WF, Tang CC, and Wen ZH.
Statistical analysis: Tang CC, Wen ZH, and Chang YC.

Conflict of interest
The authors report no conflict of interest with this study.

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