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Salmon calcitonin exerts better preventive effects than celecoxib on lumbar facet joint degeneration and long-term tactile allodynia in rats

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ABSTRACT

Objective: To evaluate and compare the effects of salmon calcitonin (sCT) and celecoxib (CLX) on cartilage, subchondral bone and tactile allodynia in a rat model of lumbar facet joint (FJ) osteoarthritis (OA).
Method: Forty 3-month-old male Sprague-Dawley rats were randomly divided into four groups: 30 received surgical collagenase (type II) injections in the right L3–L6 facet joints followed by 8 weeks of treatment with normal saline, CLX or sCT, and the other 10 received sham surgery. Tactile allodynia, changes of cartilage and subchondral bone of the L4–L5 FJs, and serum biomarkers were analyzed for all rats.
Results: Both sCT and CLX ameliorated cartilage lesions, significantly increased aggrecan expression and decreased caspase-3 expression. sCT also decreased the expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4). According to the micro-computed tomography (micro-CT) analysis, sCT significantly improved microarchitecture parameters of subchondral bone and micro-CT score; and inhibited articular process hypertrophy. CLX showed better antihyperalgesic effects than sCT on days 3 and 7 postoperatively despite no statistical differences, whereas sCT possessed better analgesic effects than CLX on days 42 and 56. Besides, the sCT treatment reduced the elevated cartilage oligomeric matrix protein (COMP) concentration in rats injected with collagenase (type II).
Conclusions: Both sCT and CLX exerted preventive effects on FJ OA caused by collagenase (type II), but sCT showed more protective effects, particularly on maintaining cartilage metabolism, restraining the deterioration of the subchondral bone microarchitecture and tactile allodynia, and reducing serum COMP concentrations.

1. Introduction

Low back pain (LBP), which affects approximately two-thirds of the adult population at some period in their lifetime [1,2], is a common musculoskeletal disorder that has become one of the greatest public health problems. It is the leading specific cause of years lived with disability and results in an enormous economic burden to society globally [1,3]. Osteoarthritis (OA) of the facet joint (FJ) is considered a common cause of LBP [4], while compared to other OA phenotypes like knee or hip OA, FJ OA is less known and lack of much attention. Current treatment approaches for FJ OA include conservative therapy, intra-articular injections of steroids or anesthetics, radiofrequency denervation and surgery, but their effectiveness is limited by several problems, such as considerable adverse effects, poor long-term benefits, a heavy financial burden and other issues [6].

FJ OA is a failure of the entire joint, and cartilage and subchondral bone are affected in this pathologic process [2,5]. Cartilage degeneration includes surface irregularity and fissures, matrix loss, cluster or diminishment of chondrocytes, and even denudation of cartilage [2,7–10]. Changes in subchondral bone include subarticular bone erosions, subchondral cysts, and subchondral bone-marrow edema-like lesions [2,7,8]. The unfavorable mechanical conditions in subchondral bone could facilitate cartilage degradation [11]. Studies have indicated that increased subchondral bone resorption occurs before histopathologic changes of articular cartilage, and is in accordance with early development of cartilage degeneration [12,13]. Studies on knee OA

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have indicated that substance P (SP) nerve fibers within subchondral bone not only may be associated with the metabolism and remodeling of bone, but also may be a potential source of pain [14]. SP-immunoreactive nerve fibers have also been identified in subchondral bone of FJ OA [15], but their role is incompletely understood. Disease-modifying OA drugs (DMOADs) target cartilage and subchondral bone, and seem to be candidates for treatment of FJ OA. However, to date, few studies have focused on DMOADs for FJ OA treatment.

Calcitonin (CT) is an antiresorptive peptide hormone with specific inhibitory effects on osteoclasts. Intriguingly, CT may have a possible therapeutic benefit as a treatment for OA. CT markedly counteracts subchondral bone changes, attenuates cartilage degradation, and prevents the net loss of collagen, hyaluronan, and proteoglycan aggregates in animal models of OA [16–18]. Clinical studies demonstrate CT improves functional disability, reduces cartilage degradation, and decreases the levels of biomarkers of bone resorption and cartilage degradation in OA patients [19–21]. However, to date, no reports have examined the effects of CT on FJ OA.

Celecoxib (CLX), a nonsteroidal anti-inflammatory drug (NSAID), is recommended as an option for pharmaceutical therapy in the OA treatment guidelines of the American Academy of Orthopedic Surgeons (AAOS) [22]. CLX has been shown to retard OA progression by preventing osteophytes enlargement [23], inhibiting chondrocytes apoptosis [24] and impairing the function of inflammatory mediators [25] in animal models of knee OA. CLX also improves joint pain and stiffness and physical function in patients with knee OA [26]. However, researchers have not conclusively determined whether CLX exerts beneficial effects on FJ OA and associated pain.

Therefore, the aim of the present study was to evaluate and compare the effects of salmon calcitonin (sCT) and CLX on collagenase (type II)-induced FJ OA in rats. If sCT is more effective than CLX, it may be considered a potential treatment for FJ OA in clinical practice.

2. Methods

2.1. Animal handling

This study was approved by the Institutional Animal Care and Use Committee. Forty 3-month-old male Sprague-Dawley rats (Vital River Experimental Animal Technical Co., Ltd., Beijing, China) weighing 432 ± 15 g (mean ± SD) were randomly assigned to the Sham group (n = 10), the collagenase (type II) injection group (CI group, n = 10), the sCT treatment group (sCT group, n = 10), or the CLX treatment group (CLX group, n = 10). All rats were housed under standard laboratory conditions in a temperature-controlled room (21 ± 1 °C) with a normal 12-h light/dark cycle and allowed free access to water and a sterilized diet.

2.2. Induction of experimental FJ OA

The process used to induce experimental FJ OA replicated previously validated procedures [7–9]. Collagenase type II (1 mg) (from *Clostridium histolyticum*, enzyme activity 310 U/mg, Worthington Biochemical Corporation, Lakewood, NJ, USA) was dissolved in sterile phosphate-buffered saline (PBS) (309 μl) (pH 7.4) to a final concentration of 1 U/μl. After all rats were anaesthetized with isoflurane (2% in oxygen) under sterile operating conditions, a 3-cm midline skin incision was made on the back, and the right paraspinal muscles were retracted, exposing the right L3–L4, L4–L5, and L5–L6 FJs. Then, using a 33-gauge needle attached to a 10-μl syringe (model 1701RN; Hamilton), six microliters (6 U) of collagenase (type II) were injected (intra-articular) into each of the three right FJs of CI, sCT and CLX groups. Rats in the Sham group received the same surgical procedures as the collagenase (type II) injection rats, but a 33-gauge needle did not puncture the FJs. Finally, the fascia and the skin were closed.

2.3. Drug treatment

Drug administration was initiated on the first postoperative day. Rats in the CI group received a subcutaneous injection of normal saline, rats in the sCT group received a subcutaneous injection of sCT (Novartis AG, Switzerland) at a dose of 16 IU/kg/2 days [27], and rats in the CLX group received oral gavage of CLX (Pfizer, Inc., US) at a dosage of 50 mg/kg/day [9]. After 8 weeks of treatment, all animals were anaesthetized with isoflurane (2% in oxygen) to harvest blood and were euthanized with CO_2 to harvest the entire lumbar spine samples.

2.4. Tactile allodynia test

The test was performed before surgery (days 0) and on days 3, 7, 14, 28, 42 and 56 after surgery during the day portion of the circadian cycle (06:00–18:00 h). After allowing rats to acclimate to the apparatus with a wire mesh floor covered with a Perspex chamber until exploratory behavior and major grooming activities ceased, 50% paw withdrawal thresholds (PWT) were determined with a calibrated set of von Frey filaments (Touch-Test; North Coast Medical, Gilroy, CA, USA) ranging from 0.4 to 15 g using the method described by Chaplan et al. [28]. Beginning with 2.0 g, the filament was applied to the mid-plantar of right hind paw with sufficient force to buckle and was maintained for approximately 6–8 s. A brisk withdrawal of the paw was recorded as a positive response. If a positive response was present, the filament with the next lowest force was applied. Otherwise, the filament with the next highest force was applied when no response was observed. If continuous positive or negative responses exhausted the stimulus set, values of 15.00 g and 0.4 g were assigned, respectively [28]. The experimenter was blinded to the groups when carrying out the hyperalgesia test.

2.5. Micro-CT analysis

Fixed lumbar spine tissues were scanned with a high-resolution micro-CT (SkyScan1176 Software: Version1.1 (build 6), Bruker, Kontich, Belgium). Data were acquired at 50 KeV of energy, a 270-μA current, and 17.93-μm cubic resolution. The ROI was defined as subchondral cancellous bone within a standardized rectangular frame located immediately below the midpoint of the subchondral bone plate of the superior articular process of right L4–L5 FJs with a thickness of 0.4 mm. The following densitometry and architectural parameters were obtained: bone mineral density (BMD, mg/cm^3), bone volume/trabecular volume (BV/TV, %), trabecular separation (Tb.Sp, μm), trabecular thickness (Tb.Th, μm), trabecular number (Tb.N, 1/mm), and structure model index (SMI). In the axial position, the middle micro-CT image of the right L4–L5 FJ was used to measure the length (L), height (H) and L/H ratio of the superior articular process (Fig. 5A). Seven slices, which divided the FJ into an average of eight parts, were extracted. The FJ space of each slice was divided equally into three regions, and the average of the three regions width (w1, w2 and w3) was defined as the width of the FJ space of each slice. The average width of the FJ space of the seven slices was defined as the final width of each sample (Fig. 5A). Additionally, the severity of FJ OA was defined using a scoring system modified from grading methods published by Kalichman et al. [29] and Weishaupt et al. [5] (Table 1).

2.6. Histological examination

The lumbar spine tissues were sequentially fixed with 10% neutral buffered formalin, decalcified in 10% EDTA-2Na for 10 weeks, and then embedded in paraffin. Six-micrometer sections of the L4–L5 FJs were obtained for toluidine blue staining. The articular cartilage lesions of the superior articular process of right L4–L5 FJs were assessed using a modified Mankin grading system [7]. Two independent pathology professionals assessed the histological findings obtained from these

Table 1
Modified scoring systems for assessing the severity of FJ OA by micro-CT.

Parameter	Score
(1) Space width	
a. Normal (0.25 mm ≤ width)	0
b. Mild narrowing (0.15 mm ≤ width < 0.25 mm)	1
c. Moderate narrowing (width < 0.15 mm)	2
d. Bone to bone	3
(2) Hypertrophy	
a. Normal (5.5 ≤ W/T ratio)	0
b. Mild (4 ≤ W/T ratio < 5.5)	1
c. Moderate (2.5 ≤ W/T ratio < 4)	2
d. Severe (W/T ratio < 2.5)	3
(3) Subchondral bone	
a. Normal	0
b. Mild subarticular bone erosion	1
c. Severe subarticular bone erosion and/or subchondral cysts	2

specimens in a blinded manner. Each data point represents the mean of scores graded by these two reviewers.

2.7. Immunohistochemical assessments

The cartilage status was further investigated by examining aggrecan (AGG), collagen-II (Col-II), caspase-3, metalloproteinase-13 (MMP-13), and a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) expression, and the expression of SP in the subchondral bone was also detected. Briefly, paraffin-embedded sections were deparaffinized with xylene and rehydrated with ethanol. Subsequently, antigen retrieval was performed using 0.05% trypsin at 37 °C for 30 min, endogenous peroxidases were inactivated by incubating sections with 0.3% H_2O_2 at room temperature for 15 min, and sections were then incubated with the following antibodies overnight at 4 °C: AGG (1:500 dilution; Cat. No. GTX54920; Gene Tex Inc. USA), Col-II (1:100 dilution; II-II6B3 was deposited to the DSHB by Linsenmayer, T.F.), Caspase-3 (1:200 dilution; Cat. No. PB0183; Boster Co., Ltd., Wuhan, China), MMP-13 (1:200 dilution; Cat. No. GTX55707; Gene Tex Inc. USA), ADAMTS-4 (1:200 dilution; Cat. No. ab185722; Abcam Inc., USA), and SP (1:200 dilution; Cat. No. ab10353; Abcam Inc., USA). The following procedures were performed according to the instructions provided by the Biotinylated Anti-Guinea Pig IgG (H + L) Antibody (Cat. No. 16-17-06, SeraCare Inc. USA), PV-6000 Polink-1 and SP-9002 SPlink HRP DAB Detection System (ZSGB-BIO Corp., China), and the ZLI-9018 DAB kit (ZSGB-BIO Corp., China). Finally, sections were counterstained with hematoxylin, dehydrated, cleared with xylene and mounted.

All images were captured with a BX53 microscope system (Olympus, Tokyo, Japan). The region of interest (ROI) was defined as the middle zone of cartilage and subchondral bone from the superior articular process of right L4–L5 FJs at 100× magnification. Using Image-Pro Plus software (Media Cybernetics, Inc., US), the intensity of positive staining in the ROI was calculated and defined as the sum of integrated optical density (IOD), and the area of ROI was also calculated. The average IOD of specific proteins, reported as IOD/mm^2, was defined as the sum of IOD divided by area of ROI. The final result used for the statistical analysis was the average of values calculated by two individuals who scored the sections in a blinded manner.

2.8. Biomarker assays

Blood was collected by puncturing the abdominal aorta at the time animals were euthanized, and serum was isolated by centrifugation and stored at −80 °C. Serum concentrations of cartilage oligomeric matrix protein (COMP) and MMP-13 were determined using enzyme-linked immunosorbent assay (ELISA) kits (CSB-E13833r and CSB-E07412r; Cusabio Biotech Co., Ltd., China) according to the manufacturers' protocols. The results were read with an iMARK Microplate Absorbance Reader (Bio-Rad Laboratories Inc., US).

2.9. Data analysis and statistics

The Intraclass Correlation Coefficient (ICC) was calculated to analyze the inter-rater reliability for the histological examinations and the immunohistochemical assessments. A two-way random model ("single measures") based on "absolute agreement" was used [30]. There was a high level of agreement between the two raters (Table SI, all ICC > 0.8). All data are presented as means and 95% confidence intervals, with the exception of the von Frey threshold measurements, which are expressed as medians (25%, 75% quartiles) because these data do not follow a Gaussian distribution [28,31]. The Kolmogorov-Smirnov test was performed to confirm whether the data exhibited a Gaussian distribution. Differences in data with a Gaussian distribution between groups were determined using one-way analysis of variance (ANOVA). Fisher's least significant difference (LSD) *t*-test or Dunnett's T3 test was used for pairwise comparisons, depending on homogeneity of variance analyzed with Levene's test. Kruskal-Wallis H tests were performed to analyze the modified Mankin scores, micro-CT scores and von Frey threshold measurements at a single time interval, followed by the Dunn-Bonferroni *post hoc* test. The Friedman test was used to analyze repeated von Frey threshold measurements in the same group, followed by Dunn-Bonferroni *post hoc* test for multiple comparison. Spearman's rank correlation analyses were used to investigate correlations between the serum COMP concentration, modified Mankin scores, and micro-CT scores. Two-tailed P values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS software (SPSS 20.0, SPSS Inc.; Chicago, IL, US).

3. Results

3.1. Histological changes in articular cartilage

The Sham group displayed almost no degeneration of articular cartilage. The articular surface was smooth, and the matrix was densely stained with toluidine blue (Fig. 1A). The CI group exhibited extensive cartilage lesions, including reduced numbers of chondrocytes, surface fissures, and even denudation of the articular surface (Fig. 1B). The administration of CLX and sCT alleviated the severity of cartilage degeneration (Fig. 1C and D). The CI group presented a significantly higher modified Mankin score than the Sham group (by 800% increase, $P < 0.001$), and the sCT group had a significantly lower score than the CI group (by 50.75% decrease, $P = 0.034$). Significant differences were not observed between the CI and CLX groups ($P = 0.972$).

3.2. Immunohistochemical analysis

Significantly lower AGG expression was observed in the CI group than in the Sham, CLX and sCT groups (by 34.86%, 19.28% and 26.32% decrease; $P < 0.001$, $= 0.010$ and < 0.001, respectively), whereas no difference was observed between the sCT and CLX groups ($P = 0.270$). ADAMTS-4 was expressed at significantly higher levels in the CI group than in the Sham group (by 102.03% increase, $P < 0.001$), but ADAMTS-4 was expressed at significantly lower levels in the sCT group than in the CI and CLX groups (by 46.71% and 37.3% decrease; $P < 0.001$ and $= 0.001$, respectively). No significant differences were observed between the CI and CLX groups ($P = 0.103$). Caspase-3 was expressed at significantly higher levels in the CI group than in the Sham group (by 211.84% increase, $P < 0.001$). Lower caspase-3 expression was observed in the sCT and CLX groups than in the CI group (by 69.33% and 24.21% decrease; $P < 0.001$ and $= 0.010$, respectively), whereas the sCT group expressed lower levels of this protein than the CLX group (by 59.53% decrease; $P < 0.001$). Levels of Col-II and MMP-13 were not significantly different among the groups ($P = 0.489$

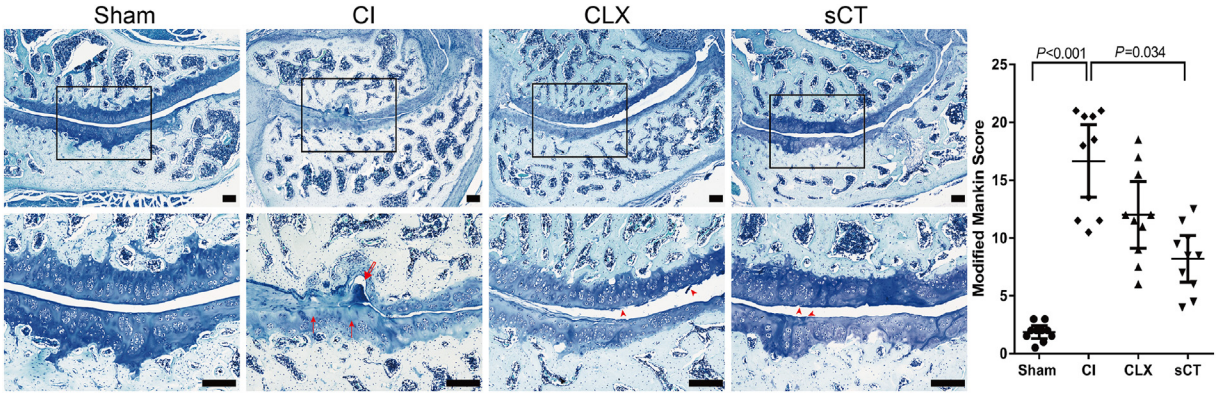

Fig. 1. Histological analysis of right L4–L5 FJs (toluidine blue staining). The Sham group displayed almost no degeneration of articular cartilage. The articular surface was smooth, and the matrix was densely stained with toluidine blue. The CI group exhibited serious cartilage lesions. Administration of CLX and sCT alleviated the severity of cartilage degeneration. Arrow indicates significantly reduced numbers of chondrocytes. Blank arrow indicates clefts that extend into subchondral bone. Arrowhead indicates surface fissures. Bars = 200 μm. Data are presented as the mean ± 95% CI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

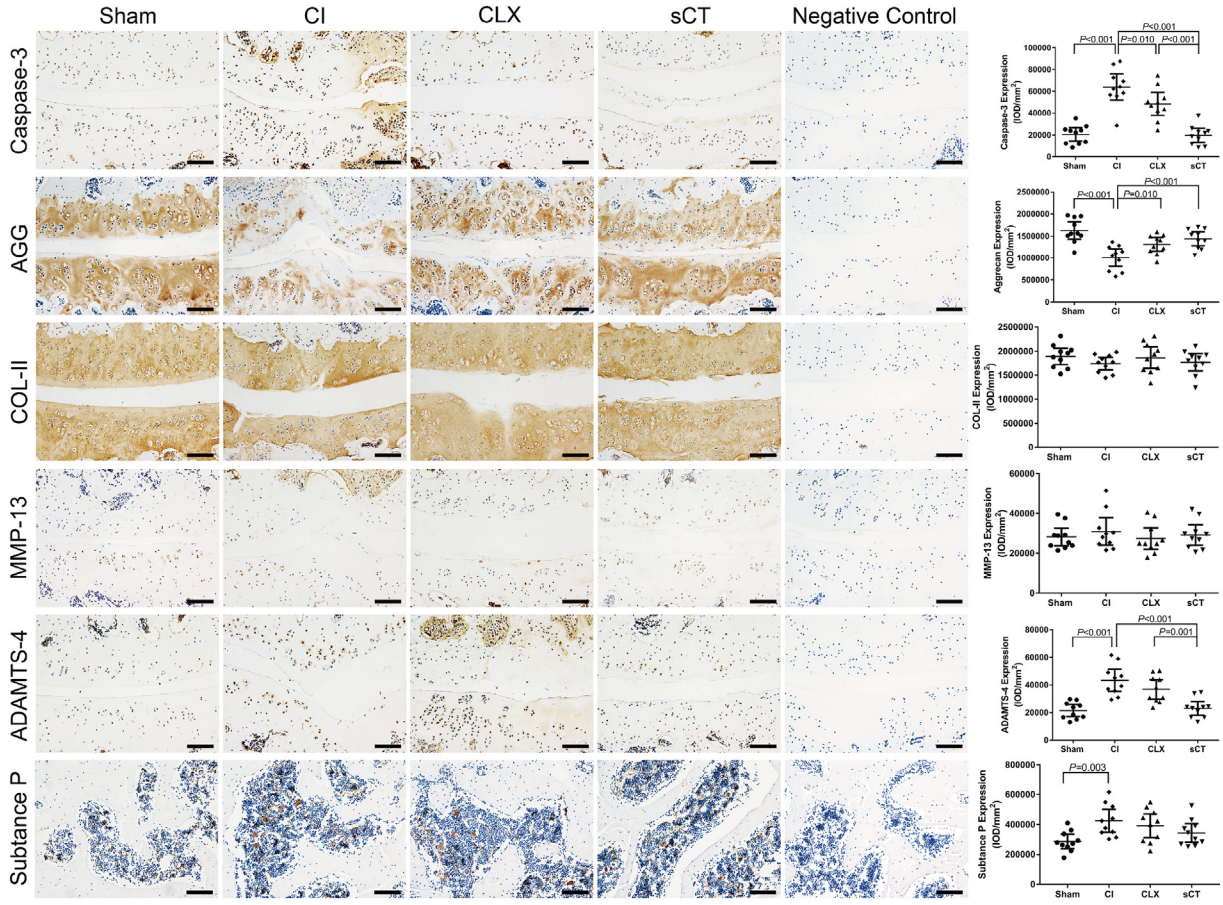

Fig. 2. Immunohistochemical staining for caspase-3, AGG, Col-II, MMP-13, ADAMTS-4 and SP in the right L4–5 FJs. Immunohistochemical staining showed caspase-3 was expressed at significantly higher levels in the CI group than in the Sham group. Lower caspase-3 expression was observed in the sCT and CLX groups than in the CI group, whereas the sCT group expressed lower levels of this protein than the CLX group. Significantly lower AGG expression was observed in the CI group than in the Sham, CLX and sCT groups. ADAMTS-4 was expressed at significantly higher levels in the CI group than in the Sham group, but ADAMTS-4 was expressed at significantly lower levels in the sCT group than in the CI and CLX groups. Levels of Col-II and MMP-13 were not significantly different among the groups. SP was expressed at significantly higher levels in the CI group than in the Sham group. Bars = 100 μm. Data are presented as the mean ± 95% CI.

and 0.753, respectively). SP was expressed at significantly higher levels in the CI group than in the Sham group (by 47.72% increase; $P = 0.003$) (Fig. 2).

3.3. Micro-CT parameters of the subchondral bone

As shown in Fig. 4, the BMD, BV/TV, and Tb.Th values were significantly decreased in the CI group compared to those in the Sham group (by 25.86%, 30.57% and 24.39% decrease, respectively; all $P < 0.001$), but the Tb.Sp and SMI were significantly increased (by

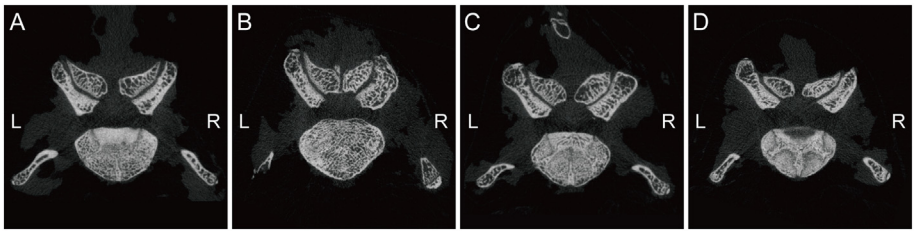

Fig. 3. 2-Dimension reconstruction of axial sections of L4–L5 FJs from each group, (A) Sham group, (B) CI group, (C) CLX group, and (D) sCT group. The right superior articular process in the Sham group developed a slender shape with a length that was significantly larger than its height, whereas the CI group showed a hypertrophic appearance, with a significantly increased height. In the CLX and sCT groups, the hypertrophic degeneration was alleviated to some degree. L and R represents left and right, respectively.

22.55% and 29.06% increase; $P = 0.002$ and 0.013, respectively). The sCT group displayed significantly higher BMD, BV/TV, and Tb.Th values than the CI group (by 15.66%, 21.85% and 14.79% increase; $P = 0.003$, 0.001, and 0.040, respectively), whereas the Tb.Sp and SMI were significantly lower (by 13.36% and 19.21% decrease; $P = 0.019$ and 0.031, respectively). The sCT group exhibited higher BMD and BV/TV values than the CLX group (by 12.23% and 17.33% increase; $P = 0.016$, and 0.004, respectively). Statistically significant differences in the Tb.N were not observed between groups ($P = 0.096$).

3.4. Morphological measurements and micro-CT score for FJs

As shown in Fig. 3, the right superior articular process in the Sham group developed a slender shape with a length that was significantly larger than its height, whereas the CI group showed a hypertrophic appearance. Specifically, the CI group displayed a 13.81% decrease in length and a 58.87% decrease in the L/H ratio (all $P < 0.001$), but a 113.38% increase in height compared to the Sham group ($P < 0.001$). Compared to the CI and CLX groups, the sCT group exhibited 60.42% and 23.63% increases in the L/H ratio ($P < 0.001$, and $P = 0.005$, respectively), but 34.14% and 15.07% decreases in height, respectively ($P = 0.001$, and $P = 0.098$, respectively). The CLX group displayed a 29.76% increase in the L/H ratio but a 22.45% decrease in height compared with the CI group ($P = 0.006$, and $P = 0.023$, respectively). No significant differences in the joint space width were observed between groups ($P = 0.081$) (Fig. 5B–E).

Significantly higher micro-CT scores were recorded for the CI group than for the Sham and sCT groups (by 723.08% and 109.8% increase; $P < 0.001$ and $= 0.002$, respectively), but statistical differences were not observed between the CLX group and either the sCT or CI group ($P = 0.685$ and 0.230, respectively) (Fig. 5F).

3.5. Tactile allodynia

As shown in Fig. 6, tactile allodynia was not observed in sham-operated rats throughout the experiments. However, rats in the other three groups developed severe tactile allodynia 3 days after surgery. In the comparison of the 50% PWT of these three groups, the CLX group presented a significantly higher value than the CI group on day 3 (by 191.3% increase, $P < 0.001$), and both the sCT and CLX groups exhibited significantly higher values than the CI group on days 7 (by 56.69% and 81.74% increase; $P = 0.041$ and 0.004, respectively) and 14 (by 78.11% and 65.68% increase; $P = 0.002$ and 0.021, respectively). The sCT group had significantly higher values than the CI group on day 28 (by 66.55% increase, $P = 0.002$), and the sCT group displayed significantly higher values than the CI and CLX groups on days 42 (by 37.19% and 42.49% increase; $P = 0.043$ and 0.025, respectively) and 56 (by 62.12% and 41.65% increase; $P < 0.001$ and $= 0.020$, respectively). In longitudinal comparisons, the CI and sCT groups both had significantly higher values on days 14, 28, 42 and 56 than those on day 3 (by 176.4%, 159.73%, 287.72% and 250.38% increase, $P = 0.023$, $= 0.028$, < 0.001 and < 0.001, respectively; by 143.91%, 114.32%, 163.54% and 181.45% increase, $P = 0.012$, $= 0.042$, < 0.001 and < 0.001, respectively), but no differences were found in comparison of values at different time point after surgery in the CLX group ($P = 0.208$).

3.6. Biomarker analysis

The serum COMP concentration was significantly higher in the CI group than in the Sham and sCT groups (by 45.29% and 34.41% increase; $P = 0.010$ and 0.031, respectively), whereas no differences were observed between the CLX and CI groups ($P = 0.282$) (Fig. 7A). The serum MMP-13 concentration was not significantly different among groups ($P = 0.055$) (Fig. 7B).

3.7. Correlation analysis

Significant positive correlations were observed between the micro-CT score and the modified Mankin score ($r = 0.807$, $P < 0.001$), between the serum COMP concentration and the modified Mankin score ($r = 0.444$, $P = 0.004$), and between the serum COMP concentration and the micro-CT score ($r = 0.370$, $P = 0.019$) (Fig. 7C–E).

4. Discussion

In the present study, we found that sCT possessed more substantial effects on maintaining cartilage metabolism, restraining the deterioration of the subchondral bone microarchitecture and tactile allodynia, and reducing serum COMP concentrations in collagenase (type II)-induced FJ OA than CLX. Notably, the doses of therapeutic agents used in the present study were converted from the clinical doses according to the guidelines issued by the FDA [32], and had been used in previous animal studies focusing on musculoskeletal disorders [9,27,33].

Since the structure of the FJ is different to that of the knee joint, FJ OA of rats is mainly induced by intra-articular injection of chemicals [7–10,34–36]. Rat models of FJ OA induced by monosodium iodoacetate [9,10] and Freund's adjuvant [34] are characterized by severe damage or even complete destruction of FJ in a short period, and these models were more suitable for pain research. Yeh et al. found that administration of a low dose of collagenase (< 10 U) was appropriate in rats, and indicated that this model is a useful tool for the evaluation of potential therapeutic modalities for FJ OA [7,8]. Therefore, we selected a collagenase (type II)-induced rat model of FJ OA in our study.

Cartilage lesions are one of pathologic changes seen in OA joints [37]. The imbalance of extracellular matrix (ECM) homeostasis in cartilage, characterized by loss of matrix components and increased levels of matrix-degrading proteases, plays an important role in the development of OA [38,39]. In our study, collagenase (type II) injections caused significant damage to FJ cartilage, consistent with previous studies [7,8]. The CLX treatment inhibited cartilage lesions to some extent, which was in agreement with the results from previous studies of knee OA [24,40].

Compared to the CLX group, the sCT group showed superior protective effects on cartilage structure and metabolism, with lower modified Mankin score, significantly decreased expression of ADAMTS-4 and caspase-3, and increased expression of AGG. Several studies have confirmed that CT exerts significant protective effects on cartilage in

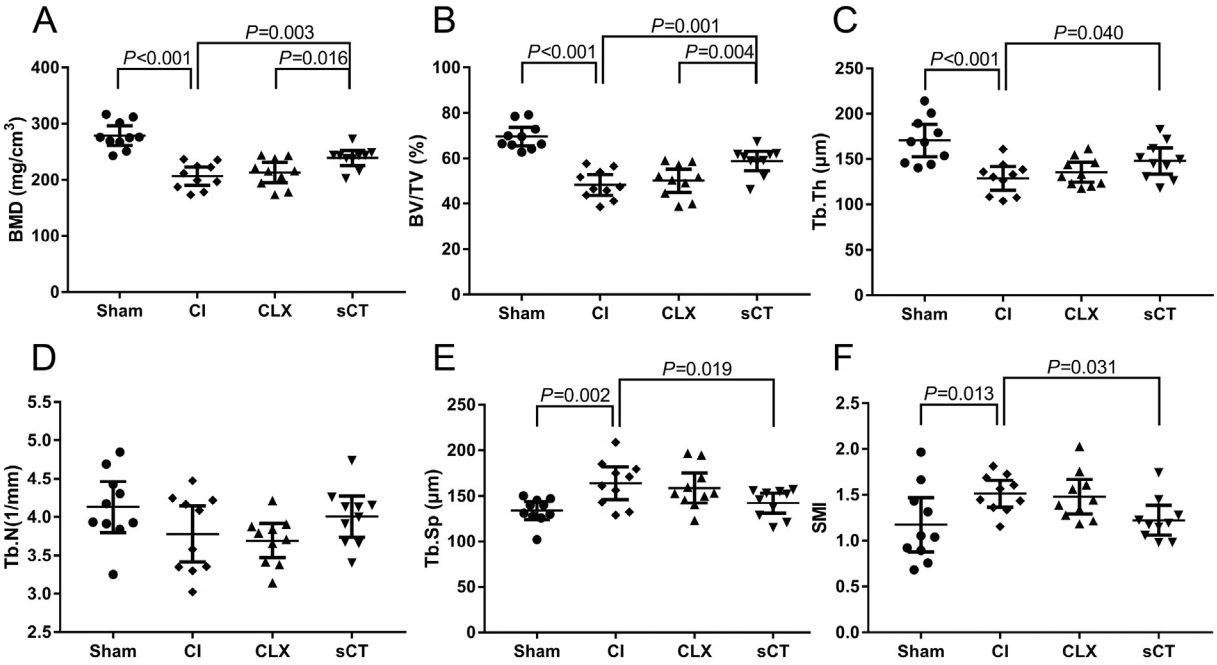

Fig. 4. Morphological parameters and micro-CT scores of the right L4–5 FJs. (A) Diagram of morphological measurements. Solid lines for w1, w2 and w3 represent the widths of each subregion of the right L4–5 FJ space. Seven slices, which divided the FJ into an average of eight parts, were extracted. The FJ space of each slice was divided equally into three regions, and the average of the three regions width (w1, w2 and w3) was defined as the width of the FJ space of each slice. The average width of the FJ space of the seven slices was defined as the final width of each sample. Solid lines for L and H represent the length and height of the superior articular process of the right L4–5 FJs, respectively. (B-E) Morphological parameters. The CI group displayed a significant decrease in length and in the L/H ratio, but a significant increase in height compared to the Sham group. Compared to the CI group, the sCT and CLX groups both exhibited a significant increase in the L/H ratio, but a significant decrease in height. The sCT group also displayed a significant increase in the L/H ratio compared with the CLX group. No significant differences in the joint space width were observed between groups. (F) Micro-CT scores. Significantly higher micro-CT scores were recorded for the CI group than for the Sham and sCT groups. Data are presented as the mean ± 95% CI.

OA [17,18,41,42]. CT not only enhances the hyaluronan and fast-sedimenting aggrecan aggregate content in cartilage[18], but also significantly up-regulates the expression of cartilage phenotype markers, along with the down-regulation of catabolic gene products [43]. CT could also inhibit chondrocytes apoptosis [43,44] and may even regenerate hyaline cartilage [41]. Regrettably, no differences in Col-II or MMP-13 expression were observed among groups. In summary, the sCT treatment, at least in part, protected chondrocytes and maintained ECM homeostasis during the development of FJ OA in rats.

Not only articular cartilage but subchondral bone is also closely

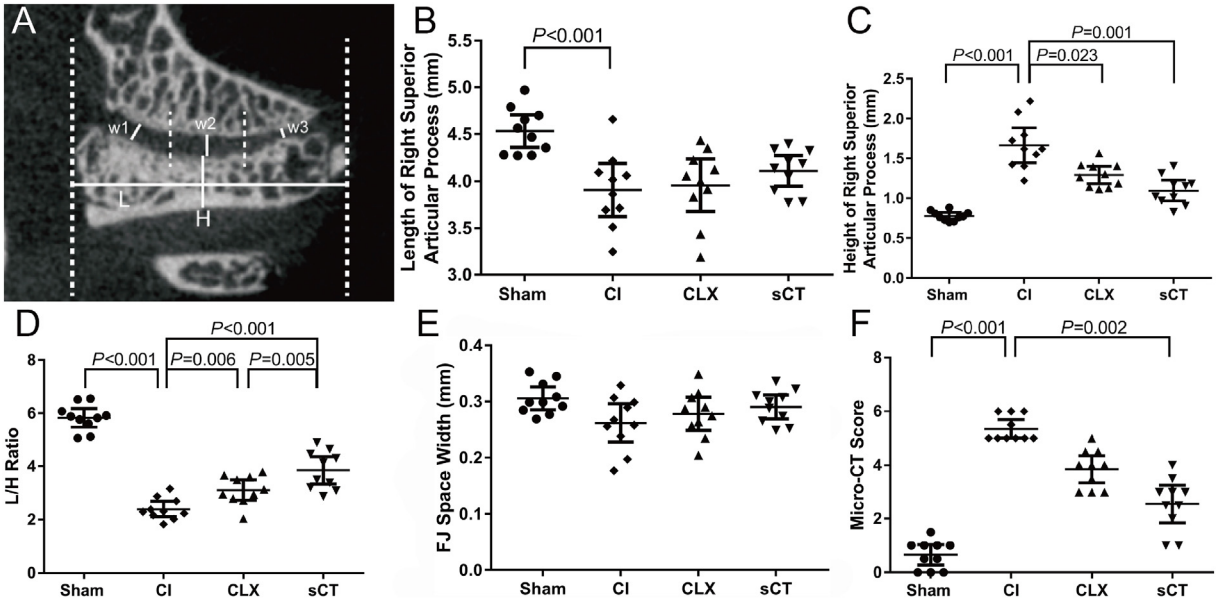

Fig. 5. Microarchitecture parameters of subchondral bone by micro-CT analysis. The BMD, BV/TV, and Tb.Th values were significantly decreased in the CI group compared to those in the Sham group, but the Tb.Sp and SMI were significantly increased. The sCT group displayed significantly higher BMD, BV/TV, and Tb.Th values than the CI group, whereas the Tb.Sp and SMI were significantly lower. The sCT group exhibited higher BMD and BV/TV values than the CLX group. Statistically significant differences in the Tb.N were not observed between groups.

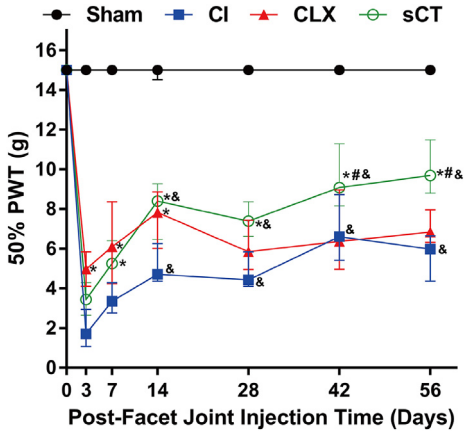

Fig. 6. Tactile allodynia assessments over a 56-day period are presented as 50% PWT in response to von Frey filaments applied to the mid-plantar right hind paw of rats in each group. The CLX group presented a significantly higher value than the CI group on day 3, and both the sCT and CLX groups exhibited significantly higher values than the CI group on days 7 and 14. The sCT group had significantly higher values than the CI group on day 28, and the sCT group displayed significantly higher values than the CI and CLX groups on days 42 and 56. In longitudinal comparisons, the CI and sCT groups both exhibited significantly higher values on days 14, 28, 42 and 56 than those on day 3. $^{*}P < 0.05$ *vs.* CI group at the same time point. $^{\#}P < 0.05$ *vs.* CLX group at the same time point. $^{\&}P < 0.05$ *vs.* values on day 3 in the same group.

associated with the development and progression of FJ OA [2]. In our study, BMD, BV/TV, and Tb.Th were significantly decreased in the CI group compared with those in the Sham group, but the Tb.Sp and SMI were significantly increased, indicating that subchondral bone was undergoing active remodeling in FJ OA progression. Notably, significantly higher SP expression in the CI group than in the Sham group implied SP nerve fibers may be involved in this active remodeling because it is believed that SP can upregulate osteoclastogenesis and facilitate bone resorption [45,46]. CLX had no impact on microarchitecture parameters, whereas sCT significantly increased BMD, BV/TV, and Tb.Th, and significantly decreased Tb.Sp and SMI. These results indicated that sCT protected subchondral trabecular structure significantly, and reduced subchondral bone resorption and trabecular thinning. These actions may be key factors in the indirect prevention of cartilage degeneration by sCT, a hypothesis that is consistent with the findings from studies on animal models of knee OA [17,42,47]. Moreover, clinical trials have demonstrated that sCT can reduce cartilage degradation and inhibit bone resorption in patients with knee OA [19,21]. Accordingly, sCT could be a candidate for treatment of FJ OA owing to its dual protective effects on cartilage and subchondral bone.

An imaging evaluation system (mainly CT and MRI) is a common and reliable approach for grading the severity of FJ OA in clinical practice [5,29]. However, to our knowledge, an imaging evaluation system specially designed for use in an animal model of FJ OA is not available. Therefore, we developed a specific scoring system to solve this problem. Hypertrophy of articular processes is a typical characteristic of FJ OA. In this model, hypertrophic changes manifested as a significantly increased height and decreased length; thus, we used the L/H ratio as a quantitative measure of hypertrophy. The CLX treatment significantly inhibited hypertrophy of articular processes but had no significant impact on the micro-CT score. sCT significantly improved hypertrophy and the micro-CT score, which may be attributed to its ability to retard the subchondral bone remodeling. In addition, a significantly positive correlation was observed between the micro-CT score and modified Mankin score, indicating the reliability of this scoring system.

The results of our study revealed significantly elevated tactile allodynia (represented by decreased 50% PWT values) and significantly higher SP expression in the CI group compared with the Sham group. Substance P nerve fibers have been observed in the subchondral bone of degenerative FJs [15], and nociceptor fibers are potentially activated by direct pressure on the subchondral bone, intramedullary hypertension or trabecular microfractures [2]. Therefore, our results indicated SP nerve fibers in the subchondral bone may be involved in the development of tactile allodynia. In the present study, a biphasic tactile allodynia profile was seen in the CI group, with initial increased levels of tactile allodynia followed by a gradual return, which was similar to

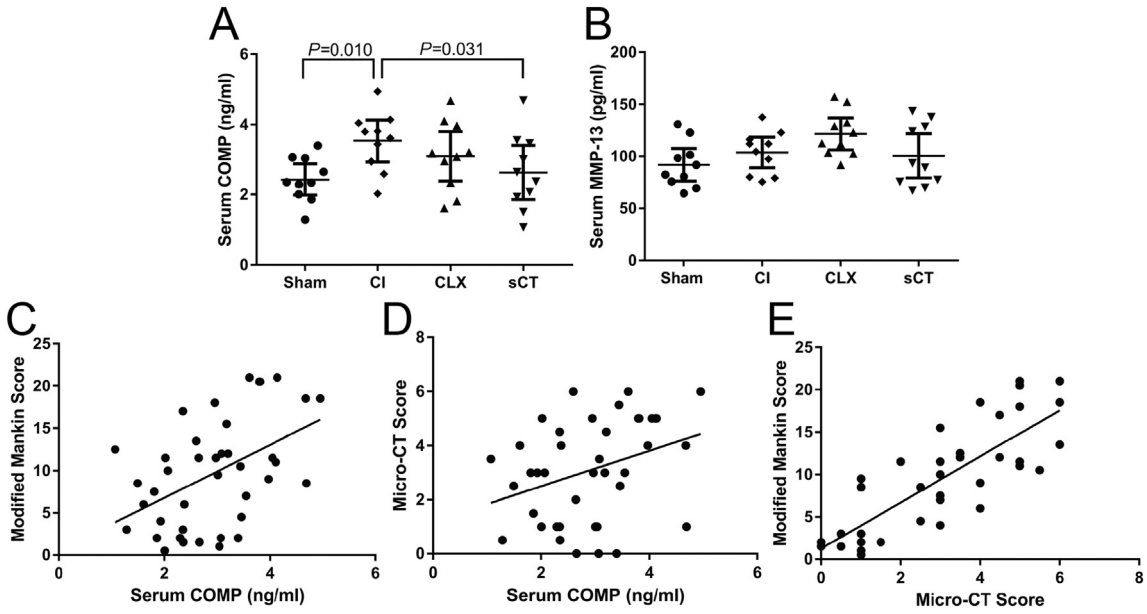

Fig. 7. Serum biomarker concentrations and Spearman's rank correlations. (A-B) Serum concentrations of COMP and MMP-13 in each group. The serum COMP concentration was significantly higher in the CI group than in the Sham and sCT groups. The serum MMP-13 concentration was not significantly different among groups. (C-E) Analysis of correlations between serum COMP concentrations, modified Mankin scores and micro-CT scores. Significant positive correlations were observed between the micro-CT score and the modified Mankin score, between the serum COMP concentration and the modified Mankin score, and between the serum COMP concentration and the micro-CT score. The regression line (solid) is shown. Data are presented as the mean ± 95% CI.

those observed in experimental studies of FJ OA [10,36,48]. However, a second phase of increased hyperalgesia following biphasic tactile allodynia was observed in one of those studies [10]. Evidence suggests that the initial increased levels of tactile allodynia may be related to inflammatory reactions in the early phase [10,48,49], and the fact that celecoxib had only early analgesic effects in our study supports this view. The second phase of increased hyperalgesia is considered to be closely related to severe degenerative changes in the joint, including exposure of subchondral bone, bone-marrow fibrosis, and marginal osteophyte formation [10,49]. Compared with FJ OA induced by monosodium iodoacetate [9,10], the slower progress and less severe degenerative changes of FJ OA induced by collagenase (type II) in our study and a previous study [7] may be why hyperalgesia in parallel with severe progressive degeneration was not found in the experimental period of our study.

CLX has been shown to be beneficial for LBP [50], but the effects of CLX on alleviation of pain from FJ OA seemed to be unsatisfactory [9,10,33]. In our study, sCT showed better antihyperalgesic effects than CLX from day 14, and particularly on days 42 and 56. The results of repeated-measures analysis revealed that sCT treatment had significantly better antihyperalgesic effects for the long-term than for the short-term, which was not observed in the CLX group. The reason why sCT exerted better antihyperalgesic effects than CLX as time progressed is difficult to obtain. We postulate that the protective effects of sCT on cartilage and subchondral bone could reduce irritation or activation of nociceptive fibers in these structures and fundamentally controlled the sources of pain. Unfortunately, no statistical differences on the SP expression were observed between the sCT and CI groups, but lower SP expression in the sCT group may imply that the antihyperalgesic effect of sCT was associated (to some extent) with its modulation on SP nerve fibers in subchondral bone, and this needs to be investigated in further studies. On the other hand, the better antihyperalgesic effects of sCT may be related to other factors. Previous studies demonstrated sCT had direct analgesic effects through raising levels of β-endorphin, interacting with specific binding sites in the central neural system and mediating serotonergic system [51,52]. Further, recent evidence suggests antihyperalgesic action of CT appears to be mediated by reduced neural serotonin (5-HT) transporter activity and increased activation of 5-HT1 receptors in the thalamus [53]. Regrettably, our study did not assess local back pain. However, since open surgery and subsequent scar formation on soft tissue may interfere with the assessment of local pain [9], an assessment of local pain may be not appropriate in this model.

Diagnostic biomarkers of knee and hip OA have been the focus of extensive research in recent years [54,55], but diagnostic biomarkers for FJ OA have largely been ignored. Serum COMP is a potential diagnostic marker for the occurrence and progression of knee or hip OA [54–56]. In our study, rats in the CI group displayed significantly increased serum COMP levels, but rats treated with sCT presented significantly decreased serum COMP levels compared to those in the CI group. Additionally, the serum COMP concentration positively correlated with the modified Mankin score and micro-CT score. These data are not only evidence that the serum COMP concentration is a promising candidate for prognosticating FJ OA but also indirect evidence that sCT exerts protective effects on cartilage.

In summary, both sCT and CLX displayed therapeutic benefits in this rat model of FJ OA. However, sCT showed more protective effects, particularly in maintaining ECM homeostasis, restraining degeneration of the subchondral bone microarchitecture and tactile allodynia, and reducing serum COMP concentrations. The serum COMP level is significantly correlated with the modified Mankin score and micro-CT score, indicating its potential utility as a diagnostic biomarker of FJ OA.

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Author contributions

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Drafting of the article: Yu Gou, Faming Tian, Liu Zhang.

Revision of the article: Yu Gou, Faming Tian, Muwei Dai, Huiping Song, Yingze Zhang, Liu Zhang.

Final approval of the version to be submitted: All authors.

Conflict of interest

The authors have no conflicts of interest pertaining to this manuscript to declare.

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