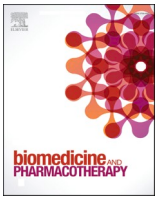
Biomedicine & Pharmacotherapy 189 (2025) 118239

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



The effect of a low transition temperature mixture for enhanced bioavailability of celecoxib in combination with hyaluronic acid in a rat model with post-traumatic knee osteoarthritis

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ARTICLE INFO

Keywords:
Osteoarthritis
Low Transition Temperature Mixtures/Deep Eutectic Systems
Hyaluronic acid
Celecoxib
Non-steroidal anti-inflammatory drugs
Chondroprotective

ABSTRACT

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of disability worldwide. Current therapies include pain relief with oral uptake of non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections of hyaluronic acid (HA), to restore the lubricant and protective properties of the joint (viscosupplementation). The administration of both therapies is limited, especially for NSAIDs, given the systemic side-effects. This work intended to explore the potential of a novel injectable gel for osteoarthritis treatment consisting of hyaluronic acid (HA) combined with celecoxib (CEX) incorporated in a glycerol:sorbitol (GS)-based Low Transition Temperature Mixture (LTTM) for enhanced bioavailability. The efficacy of HA+GS+CEX versus HA, HA+CEX and saline control was tested in a post-traumatic osteoarthritis rat model (female Sprague-Dawley rats, n = 6 per group), induced by unilateral anterior cruciate ligament transection and partial medial meniscectomy (ACLt+pMMx). Outcome measures included knee edema, pain-associated behaviour, systemic CEX levels, bone changes as detected by micro-computed tomography, joint degeneration by Mankin score and synovitis by Krenn score. No changes were observed in knee edema between treatments. All HA-containing formulations effectively reduced synovitis, and alleviated pain-associated behaviour. HA+GS+CEX injection limited the peak CEX systemic exposure, prevented the loss of integrity of the subchondral bone plate, and inhibited cartilage degeneration. Overall, although all HA-based formulations reduced pain and inflammation, only the combination of HA+GS+CEX inhibited joint degeneration, suggesting an added therapeutic benefit over HA or HA+CEX alone.

1. Introduction

Osteoarthritis (OA) is a musculoskeletal inflammatory disease that affects about 7 % of the global population [1]. It is associated with cartilage degeneration, bone remodelling, inflammation, pain and, in advanced stages, loss of joint mobility [2].

Intra-articular (IA) injections of corticosteroids or hyaluronic acid (HA) are conditionally recommended in individuals with knee OA [3]. HA injectables, known as viscosupplementation, aim to restore the physiologic properties of synovial fluids, while corticosteroids are administered for anti-inflammatory action. Both treatments reduce pain and improve joint function, but their efficacy is short-lived and heterogeneous as it depends on several factors such as age and OA stage [3,4]. Corticosteroids typically provide an early stronger but shorter lasting

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https://doi.org/10.1016/j.biopha.2025.118239
Received 24 March 2025; Received in revised form 29 May 2025; Accepted 10 June 2025
Available online 16 June 2025

effect compared to viscosupplements [4,5]. The co-administration of HA and corticosteroids was also suggested to have some improved therapeutic outcomes, including enhanced pain relief and function, compared to HA alone [6,7]. However, IA corticosteroid injections have a limited number of applications due to the occurrence of several adverse effects such as tendon rupture, joint deterioration, hyperglycaemia, osteoporosis, avascular necrosis, and even accelerated OA progression [8,9].

An alternative to IA injection of corticosteroids could be the use of non-steroidal anti-inflammatory drugs (NSAIDs), conventionally used for OA pain management through oral or topic administration. Among NSAIDs, celecoxib (CEX) is a selective cyclooxygenase-2 (COX-2) inhibitor, prescribed for OA pain relief. However, the low bioavailability of NSAIDs limits the amount reaching the joints and the high dosages required for efficacy often lead to systemic side effects [3,10,11]. Administering CEX via local intra-articular injection could enhance therapeutic efficiency using lower doses, and minimize systemic side effects [12]. In addition to pain relief, CEX has been reported to have chondroprotective action [13,14], inhibition of inflammation, [13–16] and prevention of cyst and osteophyte formation, when administered intra-articularly in controlled release formulations [16,17]. However, this approach does not provide for restoration of natural lubrication, which may yield suboptimal pain management and particulate drug carriers may incite an innate immune response. Combined IA injection of CEX and HA may lead to cartilage protection [18,19], inflammation reduction [18] and pain control [19], in comparison to monotherapies. However, the available preclinical data on the topic remain insufficient and inconsistent due to variations with several factors, such as dosage, delivery approach (e.g.: dispersed drug vs particle-based drug delivery systems) and animal models used [14–17,20]. One of the likely reasons for this inconsistency is CEX's poor solubility in physiological environments, affecting its bioavailability and efficacy [21].

To address these challenges, Low Transition Temperature Mixtures (LTTMs) have emerged as a highly versatile new class of solvents. LTTMs consists of mixtures of two or more components (e.g.: sugars, alcohols, aminoacids, organic acids, etc), that form a liquid system through interactions at certain molar ratios and temperatures. It comprises but is not restricted to Deep Eutectic Systems (DES), since it is not restrained to a distinctive melting temperature, as characteristic of DES [22,23]. They can be engineered to be non-toxic, biodegradable, biocompatible, and tuned to the desired applications, such as drug delivery [22–25]. As a new, green solvent platform, their potential to increase drug solubility and bioavailability is of high interest to improve the efficiency of existing drugs [26]. Recently, a glycerol:sorbitol (GS)-based LTTM was identified with superior CEX solubility, compatibility with saline media, and suitability for IA injection due to its physiological pH (around 7.4) and the safety of its constituents [27]. Notably, glycerol and sorbitol are both approved by the USA Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for use in human clinical products [28,29]. Additionally, the GS LTTM exhibit inherent lubricant properties [30]. Based on these features, the GS-based formulation was previously developed to further incorporate natural joint biomaterials such as HA. A final injectable gel formulation combining GS, CEX and HA showed proper biocompatibility *in vitro* and rheologic properties within the range of commercial viscosupplements and healthy synovial fluids [31]. Following these achievements, the goal of this study was to evaluate the possible added value of the HA+GS+CEX formulation compared to HA+CEX or HA alone in an OA rat model of anterior cruciate ligament transection combined with partial medial meniscectomy (ACLt+pMMx). This well-established preclinical model replicates key aspects of human OA, including cartilage degeneration and subchondral bone remodeling [32].

2. Methods

2.1. Preparation of injectables

Four injectables were prepared: PBS (phosphate buffer saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, 25 °C) for the control group, HA solution in PBS (HA), a solution of HA and celecoxib in PBS (HA+CEX) and a solution of HA and celecoxib in GS:PBS 2:1 (w/w) (HA+GS+CEX). Celecoxib was added at 1.82 mg/mL and HA at 1 % (w/w). All the reagents used were pharmaceutical grade. Glycerol:sorbitol 2:1 (GS, molar ratio) was prepared by heating and stirring until a translucid liquid was obtained, and the GS:PBS 2:1 ratio selected for enhanced CEX solubility, as reported elsewhere [27].

2.2. Animal procedures

The *in vivo* experiment procedures (see timeline in Fig. 1) were performed in agreement with the Dutch Law of Animal Experimentation and approved by the Animal Ethics Committee in Utrecht, the Netherlands (project number AVD11500202114837), and are reported in compliance with the ARRIVE guidelines [33] and with the recommendations made by the Biomedicine & Pharmacotherapy journal.

Twenty-four female, 12-weeks old Sprague-Dawley rats (Crl:CD(SD) strain code 001, Charles River, Germany), were housed in groups under standard laboratory conditions (open cage), with water and food [regular chow diet ("rat and mouse breeder and grower 801730"; 22 kcal% protein, 9 kcal% from fat, 69 kcal% from carbohydrates of which 8.4 % sucrose, SDS Diets, UK)] *ad libitum*. The animals were acclimatized prior to the study for a period of 7 days, monitored and weighed weekly through all study.

Knee osteoarthritis was induced unilaterally by the combination of anterior cruciate ligament transection (ACLt) and partial medial meniscectomy (pMMx) in the right knee (ipsilateral), under isoflurane inhaled anaesthesia (4 % for induction, 1.5–3 % for maintenance). Regarding analgesia, buprenorphine (0.03 mg/kg) was subcutaneously administered at least 30 min prior surgery and 6 h after the first administration, and carprofen (4 mg/kg) was subcutaneously administered at least 30 min prior surgery, and at day −27 and −26 (day 1 and 2 after OA surgery induction).

Four weeks after OA induction (day 0), the animals were randomized in 4 groups of 6 rats each; block randomization by cage, sample size calculation from previously reported data [34], using G-Power v3.1.9.7 [35,36]. The primary outcome variable used for the G-Power calculation was synovitis (Krenn Score) – detailed information on these can be found in supplementary data). Each rat received a 25 μL unilateral intra-articular injection (30 G needle), through the patellar tendon, in the OA-induced knees, according to the respective groups: HA, HA+CEX, HA+GS+CEX, and control (PBS). Syringes were prepared and coded by a third party not involved in the procedures.

Euthanasia was performed at day 56 by aortic excision under deep anaesthesia. One rat of PBS and HA+CEX groups were sacrificed at days 1 and 48, respectively, as they reached the humane endpoint due external factors not related to the OA induction. All data collected prior to sacrifice was used since no influence in the parameters analysed was observed, except for the weight bearing analysis, for which the sacrificed rat from the HA+CEX group was excluded, due to a tumour that could have affected the weigh distribution. Upon sacrifice, knees were harvested for further analysis. All assays and data analysis were performed in random order by blinded observers.

2.3. Blood celecoxib concentration

Systemic celecoxib was analysed by ELISA (ref. 180719, Neogen, USA) for the two groups receiving injections with the drug (HA+CEX and HA+GS+CEX). For that, blood samples were drawn via tail end at 2,

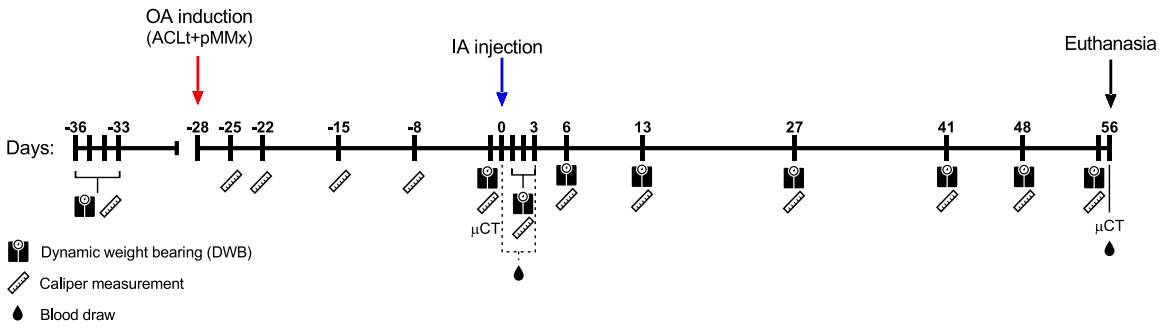

Fig. 1. Experimental timeline. Knee OA induced unilaterally in rats at day −28 and submitted to intra-articular injection at day 0. Pain-behaviour, knee edema, and systemic celecoxib were assessed at the days indicated by the respective symbols: dynamic weight bearing (DWB, scale icon), caliper measurements (ruler icon), blood draw (drop icon). Baseline for DWB and caliper measurements consisted of the average data from 3 day-measurements before OA induction. Micro-computed tomography scans (µCT) were performed at the day prior injection (day −1) and after euthanasia (day 56) to assess bone changes. The animals were euthanized at day 56 (endpoint) and the knee joints were harvested for posterior histological analyses.

4, 6, 8, 24, 48 and 72 h after IA injection. Blood was collected in lithium heparin plasma tubes (BD Microtainer, USA), which were placed on ice and then centrifuged (15 min, 2000 g) within 30 min after collection. Plasma aliquots were stored at − 80 °C until analysis.

2.4. Caliper measurements

To assess knee swelling, a digital caliper (Kunzer 7EMS01, Germany) was used to measure rat knees' diameter at predetermined time points during the study (Fig. 1). Changes in the knees' diameter were determined by the mean value from three consecutive manual measures for each timepoint, upon subtracting the baseline measurements. Baseline was considered as the average of three days (day –35 to –33) measured before OA induction. Data are presented as mean ± SD.

2.5. Dynamic weight bearing (DWB)

Pain-associated behaviour was assessed through DWB analysis. Rats were individually placed in a 22 × 22 × 30 cm plexiglass chamber with floor sensors containing pressure transducers (44 ×44 captors, 10.89 mm per captor), and allowed to move freely during all procedure. Five min videos were recorded using a high-resolution camera (640 ×480 pixel), coupled to DWB software (Bioseb, module v1.4.2.98, France) that measured, in grams, the average weight exerted by each limb. The weight-bearing limbs were validated by an observer blinded for treatment group, for at least 1.5 min video frames, through association between rat position and sensor activation. The average of 3 day-measurements (day –36, −34, –33) before OA induction were considered as baseline. Data were expressed as the weight on the contralateral limb as a percentage of total weight distributed by both hind limbs (mean ± SD).

2.6. Micro-computed tomography

Micro-computed tomography scans (µCT) were acquired in a Quantum FX CT scanner (PerkinElmer, Massachusetts), one day prior the intra-articular injection (day −1) and post-mortem (endpoint). The *in vivo* measurements were carried out under isoflurane anaesthesia (3.5–4 % induction, 1.5–2.5 % maintenance). Both hind limbs were positioned in extension and scanned for 3 min at an isotropic voxel size of 42 µm, 90 kV voltage, 180 µA current and a field of view of 21 mm. 3D reconstructed images were obtained, and 2D images were reconstructed using software Analyze 11.0 (PerkinElmer; RRID:SCR_009120). 512 serial slides (2D) were generated, and then 70 serial slides analysed using ImageJ software (ImageJ, 1.50i, NIH, USA). Images were converted to 8-bit, brightness/contrast parameter set from 100 to 255 and, an autolocal threshold algorithm (v1.16.12, Bernsen method, 5 radius) was applied. Semi-manual segmentation for the tibial subchondral bone plate and trabecular bone was done as previously reported [37], and segmented (polygon selection) regions of interest (ROI) registered. ROI were generated each 5 slides, starting from the union of the medial and lateral side of the tibial epiphysis (back to front joint direction). Interpolation was applied and corrected when necessary. Changes in the tibial subchondral bone plate (SBP) and trabecular bone (TB) were assessed by comparing data of the endpoint (day 56) to the one obtained pre-injection (day −1, n = 24). Post hoc comparison of group effects was performed upon data normalization by dividing the endpoint by the pre-injection data. Results were expressed as mean ± SD.

2.7. Histology

Knees were harvested and fixed in 4 % neutral buffered formaldehyde (NBF) for 5 days. Thereafter, joints were decalcified in 0.5 M EDTA solution for a total of 7 weeks, with a refixation step overnight in 4 % NBF every week. The decalcified knees were then dehydrated in a graded series of alcohols using a tissue processor (Leica biosystems, Netherlands), cleared in xylene, and further infiltrated and embedded in paraffin wax. 5 µm transversal sections were obtained using a Leica micrometre (RM2245, China) and stained (Leica autostainer XL, Germany) with Safranin-O, Fast Green and haematoxylin (Saf-O), as well as with Hematoxylin and Eosin (HE). HE-stained sections were used to assess synovitis, by the Krenn score, as previously described [38]. Briefly, the parameters of synovial lining cell layer enlargement, resident cells density and inflammatory infiltrate were scored from 0 to 3, reaching a total of 0–9, where 0–1 means no synovitis; 2–4: low-grade synovitis and 5–9: high-grade synovitis. In turn, joint degeneration was evaluated in Saf-O stained slides through the Mankin score, as stated elsewhere [39]. In short, 4 categories were scored as follows: cartilage structure (0−6), cellularity (0−3), Saf-O staining (0−4), tidemark integrity (0−1). Their sum provides a total score ranging from 0 (healthy) to 14 (severe joint degeneration). Both score systems were randomly and individually attributed by two blind evaluators. Averaged scores were used for data representation and plotted as mean ± SD.

2.8. Statistics

Statistical analysis was performed with GraphPad Prism 8.0.1. Normality was assessed by Shapiro-Wilk tests and Q-Q plots. Knee diameter measurements were assessed by mixed model analysis for group comparison. Dynamic weight bearing was analysed by 2-way-ANOVA for both group and timepoints. Systemic CEX levels were analysed by 2-way-ANOVA with factors treatment and timepoints, followed by post hoc comparison between treatments at each timepoint using the uncorrected Fisher's LSD test. µCT analysis was performed using Brown-Forsythe and Welch's ANOVA tests for normal data while non-normal data was assessed by Kruskal-Wallis tests. The histological scoring was

analysed by 2-way-ANOVA to account for both the effect of treatment and inter-observer variability. P-values ≤ 0.05 were considered significant. Mean differences (MD) within the 95 % confidence interval (CI) were provided as MD [lower CI to upper CI].

3. Results

3.1. Systemic CEX peak exposure reduced by HA+GS+CEX vs HA+CEX

The systemic CEX levels were taken as an indirect measure of intra-articular concentration, given the low amount of synovial fluid in rats, which did not allow to locally monitor CEX concentrations.

CEX systemic levels (Fig. 2) in both groups increased in the first 6 h and decreased thereafter. Residual CEX was detected at 72 h for both groups. HA+GS+CEX resulted in a lower systemic CEX level than HA+CEX (MD [lower CI to upper CI]: 14.5 [7.5−21.5], p = 0.033). Post-hoc comparisons (Table S1) depicted significant differences between treatments at 2 h (MD [lower CI to upper CI]: 20.2 [1.9–38.5], p = 0.031), 4 h (38.6 [20.3–56.9], p < 0.0001), 6 h (21.4 [3.1–39.7], p = 0.022) and 8 h (19.2 [0.9–37.5], p = 0.04).

3.2. Alleviation of OA-induced pain by HA-based treatments without noticeable influence in knee edema

Knee edema and pain associated behaviour were evaluated by knee diameter measures and dynamic weight bearing (DWB) analysis, respectively. No significant OA-induced knee edema and no significant differences between groups after treatment were identified throughout the study (Fig. 3, Table S2).

Weight bearing, as measured by differences in loading between the non-affected hindlimb (contralateral) and OA hindlimb (ipsilateral) was used as indicator of pain associated behaviour (Fig. 4, detailed statistics in Tables S3 and S4). Before OA induction, there was an equivalent weight load distribution on both hindlimbs (50.6 ± 5.1 %). One day prior to injection (3-weeks after OA induction), there was a significant increase of the weight load on the contralateral hind paw (60.7 ± 6.1 %) in comparison to baseline (MD [lower CI to upper CI]: 10.7 % [8.2–13.1], p < 0.0001), which indicates unloading of the ipsilateral hind paw as a response to OA-induced pain. Overall, all treatments depicted normalisation of loading to baseline over time (symmetric loading), from day 6 for HA, followed by HA+GS+CEX from day 13, HA+CEX from day 27, and at day 55 for PBS (Fig. 4, Table S3). Additionally, as opposed to PBS, all treatments decreased loading of the contralateral paw compared to pre-injection (day −1), which was significant from day 3 to day 55 upon HA or HA+CEX injection; HA+GS+CEX also showed successive reduction from day 3, significant from day 6 to day 55 (Table S3).

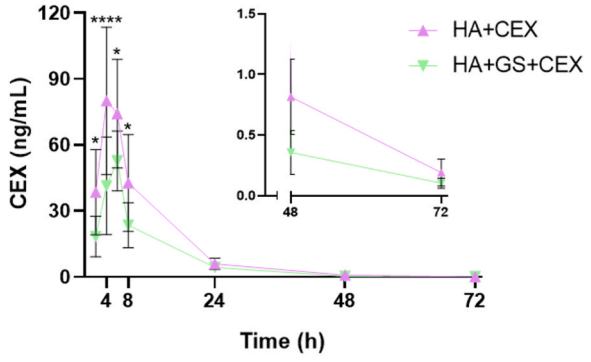

Fig. 2. Celecoxib quantified in plasma 2–72 h upon intra-articular injection. Overall, HA+CEX (n = 6; 24 h: n = 5) and HA+GS+CEX (n = 6) presented similar release profiles overtime, however systemic peak exposure was higher for HA+CEX in comparison to HA+GS+CEX. ****p < 0.0001; *p ≤ 0.05.

In terms of group-related differences (Table S4), contralateral load reduction for HA in comparison to control (PBS) was significant for day 1 (MD [lower CI to upper CI]: 6 % [0.8–11.1], p = 0.023), day 3 (7.8 % [2.6–12.9], p = 0.003), day 6 (8.9 % [3.7–14.1], p < 0.001), and day 13 (6.1 % [0.9–11.3], p = 0.021). HA+CEX also reduced the contralateral load bearing in comparison to control, significantly for day 3 (7.5 % [2.2–12.9], p = 0.006), day 6 (6 % [0.7–11.4], p = 0.028), and day 27 (5.5 % [0.1–10.9], p = 0.046). HA+GS+CEX significantly reduced the contralateral load in comparison to control from day 3 (5.7 % [0.6–10.9], p = 0.030), day 6 (5.7 % [0.6–10.9], p = 0.030), day 13 (7.5 % [2.4–12.7], p = 0.005) and day 55 (5.2 % [0.1–10.4], p = 0.045).

3.3. Subchondral bone plate integrity preserved by HA+GS+CEX treatment

The integrity of the subchondral bone plate (SBP) can be represented as bone volume fraction (BV/TV) and is expected to depict a value of 1 in healthy subjects, as the volume of mineralised bone should match the total volume. OA progression in terms of loss of SBP integrity, was more evident for the tibia medial compartment (data for lateral compartment in Tables S6 and S8), and was not prevented by the injection of PBS, HA and HA+CEX, as demonstrated by a significant reduction in SBP at day 56 compared to pre-injection (day −1). However, no significant reduction in SBP was found in the HA+GS+CEX group (Fig. 5A, Table S5). Post hoc group comparison for normalised data (endpoint/baseline) further supported that HA+GS+CEX preserved SBP integrity when compared to the PBS control group (Fig. 5B-D, Table S5, mean rank difference: −8.8, p = 0.0252).

Regarding the SBP medial thickness, no significant differences between treatments were noted nor in comparison to OA pre-injection for the affected limb (Table S7).

3.4. HA+GS+CEX treatment potentially prevents bone changes

Since in rats the growth plate does not close with adulthood, growth-related changes are expected as chondrocytes undergo hypertrophy and calcification along animals growing [40]. On the ipsilateral medial compartment, there was an increase of the trabecular bone volume fraction (BV/TV) compared to the pre-injection, significant for the PBS group, and tendentially lower for the HA+GS+CEX group (Fig. 6A, Table S9). When data was normalized, a trend towards a reduction of the trabecular bone volume fraction in the medial compartment for the HA+GS+CEX group was observed (although not significant) (Fig. 6C, Table S9). For the contralateral hindlimb, there was a significant increase of the trabecular bone volume fraction for the PBS, HA, and HA+CEX when compared to the pre-injection data, while no differences were detected between HA+GS+CEX and pre-injection (Fig. 6B, Table S9). No changes were detected between groups in the contralateral hindlimb after data normalization (Fig. 6D, Table S9). Additionally, in comparison to pre-injection, all injections tended to increase the medial ipsilateral thickness, which was significant for HA and HA+CEX (Fig. 6E, Table S11). For the contralateral limb, the medial thickness was increased in the animals receiving PBS, HA, and HA+CEX when compared to the pre-injection values, and no significant changes were detected for HA+GS+CEX injection (Fig. 6F, Table S11). When data were normalized by the pre-injection values, no significant differences were detected between groups (Fig. 6G-H, Table S11). Results for the lateral compartment can be found in Table S10 and S12.

3.5. HA-based treatments inhibited synovial inflammation whereas joint degeneration was only inhibited by HA+GS+CEX

Synovial inflammation as assessed by the Krenn score, was significantly lower for all the HA-based treatments in comparison to the control (PBS) group (Fig. 7A, Table S13).

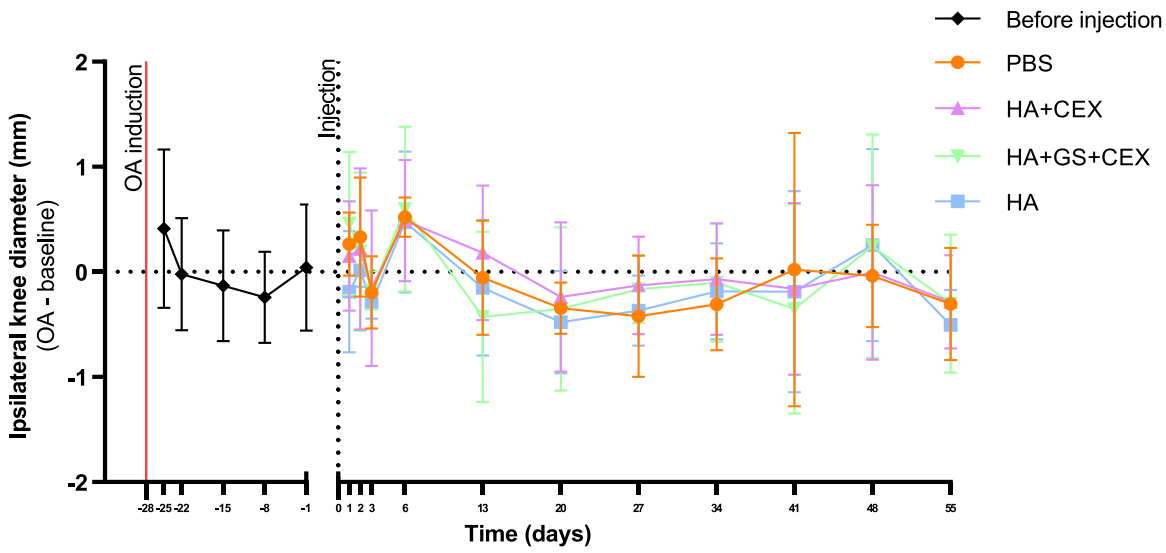

Fig. 3. Ipsilateral knee diameter changes upon subtracting baseline (healthy, prior OA induction) measurements. Following OA induction (days −25 to −1) and after IA injection (days 1–55), no relevant knee edema or significant differences between groups were detected (PBS: n = 6 day 1, n = 5 from day 2; HA: n = 6; HA+CEX: n = 6, n = 5 at day 55; HA+GS+CEX: n = 6).

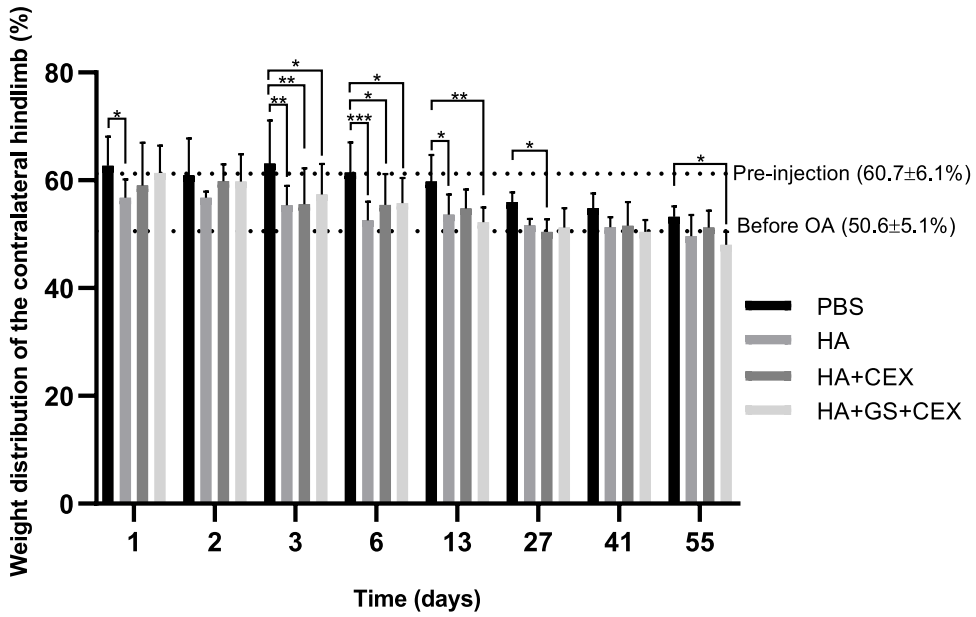

Fig. 4. OA-induced pain behaviour. Dynamic weight bearing analysis of the weight on the contralateral hindlimb as a percentage of total weight distributed on both hindlimbs. HA-based treatments reduced pain-associated load on the contralateral paw (PBS: n = 5, HA+CEX: n = 5, HA: n = 6, HA+GS+CEX: n = 6). $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

As expected for this rat model, joint degeneration was more severe on the medial tibia plateau [41], for which the histologic results are depicted in Fig. 7B-D, Table S14 (data for lateral tibia plateau is presented in Table S15). Only HA+GS+CEX inhibited joint degeneration (Fig. 7B) as compared to the control group, (MD [lower CI to upper CI]: 3.7 [1.1–6.3], p = 0.007), HA (2.3 [-0.2–4.8], p = 0.066), and HA+CEX (5 [2.4–7.6], p < 0.001), especially in terms of cartilage structure (Fig. 7C,D). In addition, the total Mankin score was lower in HA-treated knees in comparison to HA+CEX injection (-2.7 [-5.3–0], p = 0.047).

4. Discussion

Hyaluronic acid (HA) and celecoxib (CEX) have been reported to be efficacious in OA treatment, especially in alleviating pain [4,42]. However, no intra-articular (IA) injectables with CEX, let alone HA+CEX, are yet clinically available, likely attributed to CEX's poor solubility and consequent bioavailability limitations. A formulation was priorly designed to overcome such limitations by including a new biocompatible LTTM solvent (glycerol:sorbitol, GS). LTTMs have shown promising potential as bioavailability enhancers of poor soluble and/or permeable drugs [26]. By exploring such principle, the LTTM glycerol: sorbitol made possible to develop an injectable gel combining HA and CEX with enhanced solubility, representing a breakthrough in the design of a 2-in-1 local injection for OA therapeutics. In addition, the LTTM solvent has a lubricant role, adding to HA as natural joint constituent restoring synovial fluid properties. The development of this combined formulation allowed not only to enhance CEX solubility, as well as to modulate its delivery [27,31]. Here, the effects of such IA injectable

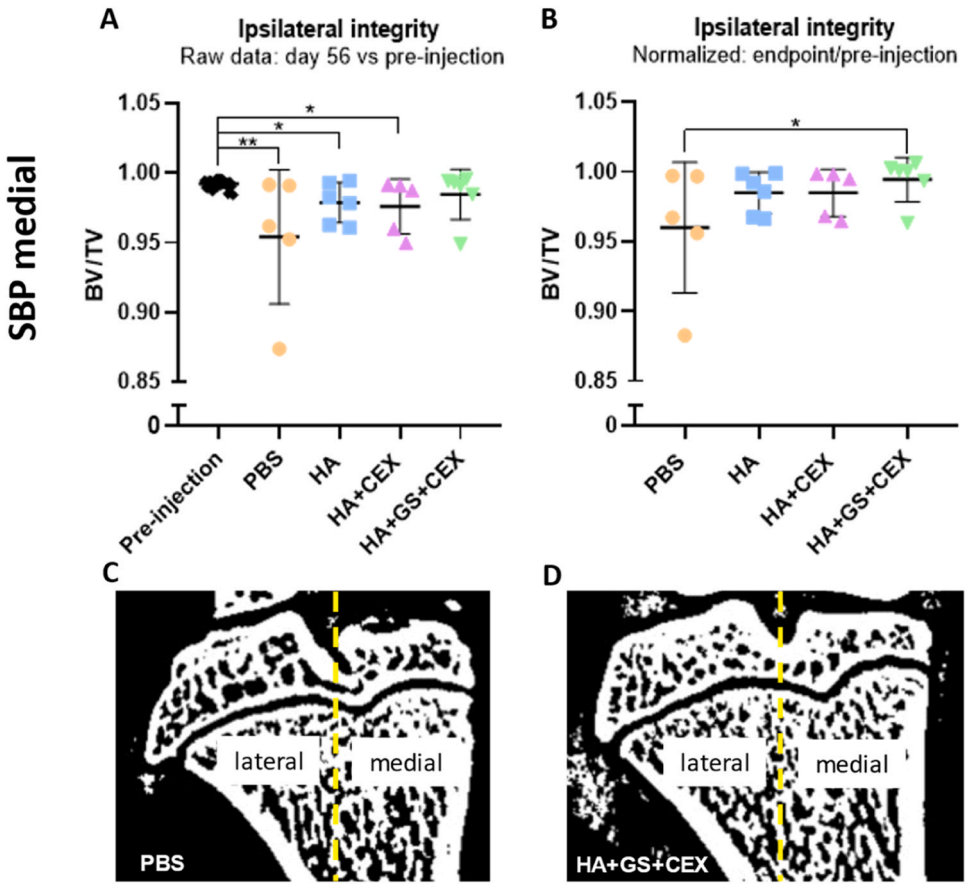

Fig. 5. Integrity of the subchondral bone plate (SBP). OA progression assessed by micro-computed tomography (μCT) analysis (PBS: n = 5; HA: n = 6; HA+CEX: n = 5; HA+GS+CEX: n = 6). (A) Ipsilateral integrity as represented by the bone volume fraction (BV/TV) of the tibia plateau was significantly reduced for PBS ($p = 0.006$), HA ($p = 0.031$), and HA+CEX ($p = 0.02$) compared to pre-injection. (B) Ipsilateral integrity normalised to pre-injection values was significantly lower after PBS injection compared to HA+GS+CEX ($p = 0.025$). (C) μCT frame of region of interest for an ipsilateral knee injected with PBS (no treatment), compared to (D) psilateral μCT of knee injected with HA+GS+CEX. $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

composed of HA and CEX with enhanced CEX solubility (HA+GS+CEX) were investigated against HA+CEX and HA alone. Although knee edema after OA induction was not evident, pain-related behaviour was detected by weight load redistribution from the affected to the non-affected limb. The HA formulation exhibited the most rapid analgesic response, followed by HA+GS+CEX and HA+CEX. However, animals receiving PBS control showed reversion to symmetrical loading by the end of the study, suggesting spontaneous resolution of pain-like behaviour. This phenomenon aligns with previous findings in murine models, where loading of the ipsilateral and contralateral hindlimbs equilibrated over time [43,44]. Such spontaneous reversion to symmetrical loading may limit the sensitivity of DWB analysis to assess OA-related pain and treatment efficacy, at later time points. Thus, this model might not be optimal to evaluate pain over extended observational periods post-OA induction.

When using the GS formulation, the CEX systemic peak exposure was reduced compared to the HA+CEX injectable without it, most likely due to extended retention in the joints, and therefore slower loss to the circulation. However, the detection limits of CEX measurement in serum precluded proof for this hypothesis. Additionally, since the rat model does not allow for direct synovial fluid measurements and synovial fluid aspiration would also remove (parts of) the controlled release formulation, this cannot be stated with certainty. Nonetheless, the hypothesis of extended joint retention is consistent with longer therapeutic action, which may explain, at least partially, the higher therapeutic potential of HA+GS+CEX in comparison to HA+CEX in terms of the ability to inhibit bone changes and cartilage degeneration. This can be further addressed in more complex design studies, to strongly infer causality. The OA-induced decreased integrity of the medial subchondral bone plate could not be inhibited by HA or HA+CEX, as opposed to HA+GS+CEX, suggesting a protective effect of this particular formulation. Trabecular bone changes were also potentially inhibited by HA+GS+CEX, but not the other treatments. As some of these effects were also observed in the contralateral (non-OA) joint, it is unclear what mechanism would cause such changes, but we postulate that it might be related to CEX inhibiting bone growth. CEX has been previously associated to inhibition of bone changes related to decreased chondrocyte hypertrophic differentiation [16,45]. Chondrocyte hypertrophy, a hallmark of osteoarthritis, is involved in ossification and precedes cartilage calcification [46], which is associated with histological degeneration of the knee joint [47,48]. In the present study, histological degeneration was efficiently inhibited by HA+GS+CEX in comparison to the other treatments. This correlation between the inhibition of cartilage degeneration and bone changes is consistent with inhibited endochondral bone formation and thus, chondroprotective action attributed to HA+GS+CEX.

Synovitis was effectively reduced by all HA-based treatments, which may be related to the intrinsic anti-inflammatory action of HA [49], partially masking the effect of the dose of CEX used. Still, the anti-inflammatory action of intra-articular delivered CEX was previously demonstrated from lower to higher doses [13–17,20] compared to the one herein used (~45 μg CEX injected). For instance, in a similar OA rat model, early assessments after sustained IA delivery of 15 μg CEX from polyester-amide (PEA) microspheres confirmed the anti-inflammatory action of CEX by decreased levels of prostaglandin E2 (PGE_2), an inflammatory mediator [20]. Subsequent studies using PEA

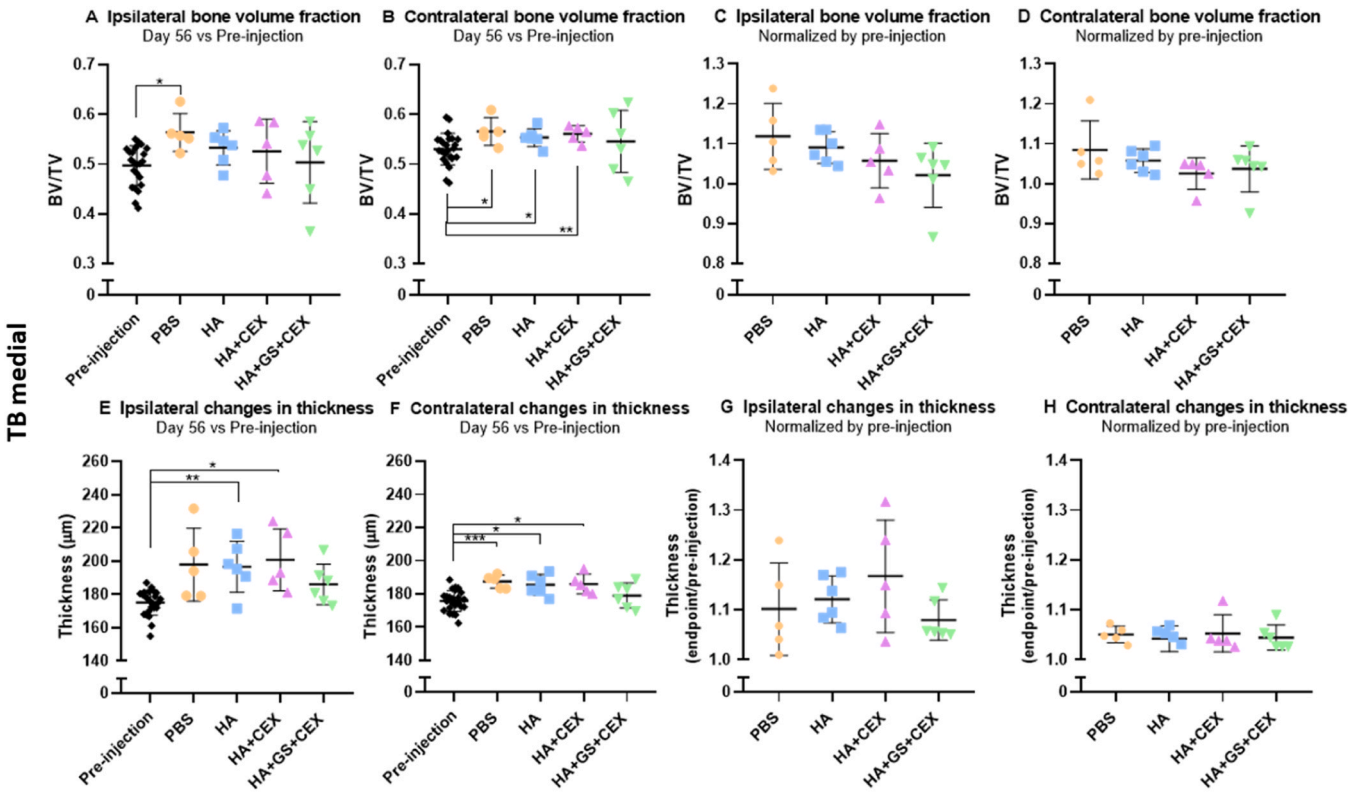

Fig. 6. Trabecular bone (TB) volume fraction and thickness of the medial tibia plateau. µCT analysis (PBS: n = 5; HA: n = 6; HA+CEX: n = 5; HA+GS+CEX: n = 6). (A) Ipsilateral bone volume fraction (BV/TV) reduced for PBS ($p = 0.013$) and HA ($p = 0.059$) compared to OA pre-injection. (B) Contralateral BV/TV significantly increased 2 months after injection with PBS ($p = 0.041$), HA ($p = 0.031$), and HA+CEX ($p = 0.008$), but not HA+GS+CEX. (C) Trend towards ipsilateral trabecular BV/TV reduction for HA+GS+CEX compared to PBS ($p = 0.083$) and HA ($p = 0.1$). (D) The injectables did not significantly change the contralateral trabecular BV/TV. (E) Ipsilateral cortical trabecular bone thickness increased for all groups injected in comparison to OA pre-injection (PBS: $p = 0.078$; HA: $p = 0.017$; HA+CEX: $p = 0.035$; HA+GS+CEX: $p = 0.078$). (F) Contralateral thickness significantly increased 2 months after injection of PBS ($p < 0.001$), HA ($p = 0.01$), or HA+CEX ($p = 0.013$), but not HA+GS+CEX, in comparison to pre-injection. (G) No significant differences between groups for the trabecular bone thickness after data was normalized by the pre-injection values for both ipsilateral and (H) contralateral limbs. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

as CEX-delivery system in the same OA rat model reported decreased local joint inflammation upon CEX injections of 30–390 µg. Those doses were also effective in reducing subchondral bone sclerosis, and other changes such as the formation of cysts, osteophytes and calcified loose bodies. Dose-dependent effects were also detected [16], but none of the CEX dosages delivered in those studies [16,20] could inhibit cartilage degeneration. Moreover, no pain readout parameters were included. In contrast, the formulation of HA+GS+CEX herein evaluated inhibited cartilage degeneration with CEX doses ranging from 0.1 to 3-times of those previously studied.

Two other studies reported a chondroprotective effect upon CEX intra-articular injection. In a rabbit OA model induced by anterior and posterior cruciate ligaments transection and medial meniscus excision, a total of 5 weekly CEX injections were administered, corresponding to a 3-fold higher CEX dose than the used in the current study. Delayed cartilage degeneration was demonstrated for both CEX or HA treatments in comparison to control (saline). Their study emphasizes that a continued CEX joint exposure may confer a chondroprotective effect [14]. A chondroprotective effect was also reported 12-weeks upon a CEX IA-injection in a male Lewis OA rat model, surgically induced by ACLT/pMMx. CEX injections were administered in both the affected and non-affected knees, with each knee receiving twice the amount herein injected [13]. Both aforementioned studies confirm the chondroprotective potential of CEX intra-articular injections, but their findings are not directly comparable to ours, since different animal strains, CEX concentrations, and study duration were used, and thus different OA phenotypes and responses to treatment. For instance, Sprague-Dawley rats were reported to be more prone to bone changes than Wistar rats in a OA model induced by unilateral groove surgery under a high-fat high-sucrose diet [50]. Thus, Sprague-Dawley rats are potentially more sensitive to study bone-related effects. Moreover, even though CEX-induced inhibition of SBP changes, such as sclerosis, have been previously reported, to the best of our knowledge, this is the first study reporting an intra-articular CEX formulation able to inhibit subchondral bone deterioration, at a single dose, 180 to 5000 times lower than the oral doses required to inhibit bone loss in arthritis rat models [51–55]. Whereas subchondral bone defects occur upon deep cartilage deterioration (osteochondral defects), cartilage lesions can occur without disrupting the structural integrity of the subchondral bone (chondral defects) [56]. As such, the findings support that HA+GS+CEX seems to have not only provided chondroprotective action, but it also prevented bone lesions, emphasising as major novelty the added value of GS in potentiating those effects, thus retarding OA-progression. Since both celecoxib and HA are known to inhibit inflammation by inhibiting PGE_2 and proinflammatory cytokine production, respectively [49,57,58], the beneficial action attributed by GS when combined to HA+CEX, may be partially associated to at least 2 routes: enhanced CEX bioavailability conferred by GS, and the intrinsic GS lubricant properties [30]. However, the relative extent of these effects, for instance in terms of causality, mechanisms, and durability is not yet understood. Pharmacokinetics and pharmacodynamics studies will help to clarify such influence, when conducted in further preclinical studies, as required for regulatory safety validation before moving to clinical trials.

In conclusion, this study showed that the intra-articular administration of HA+GS+CEX effectively reduced pain and inflammation. Moreover, this formulation had additional therapeutic activity, i.e.

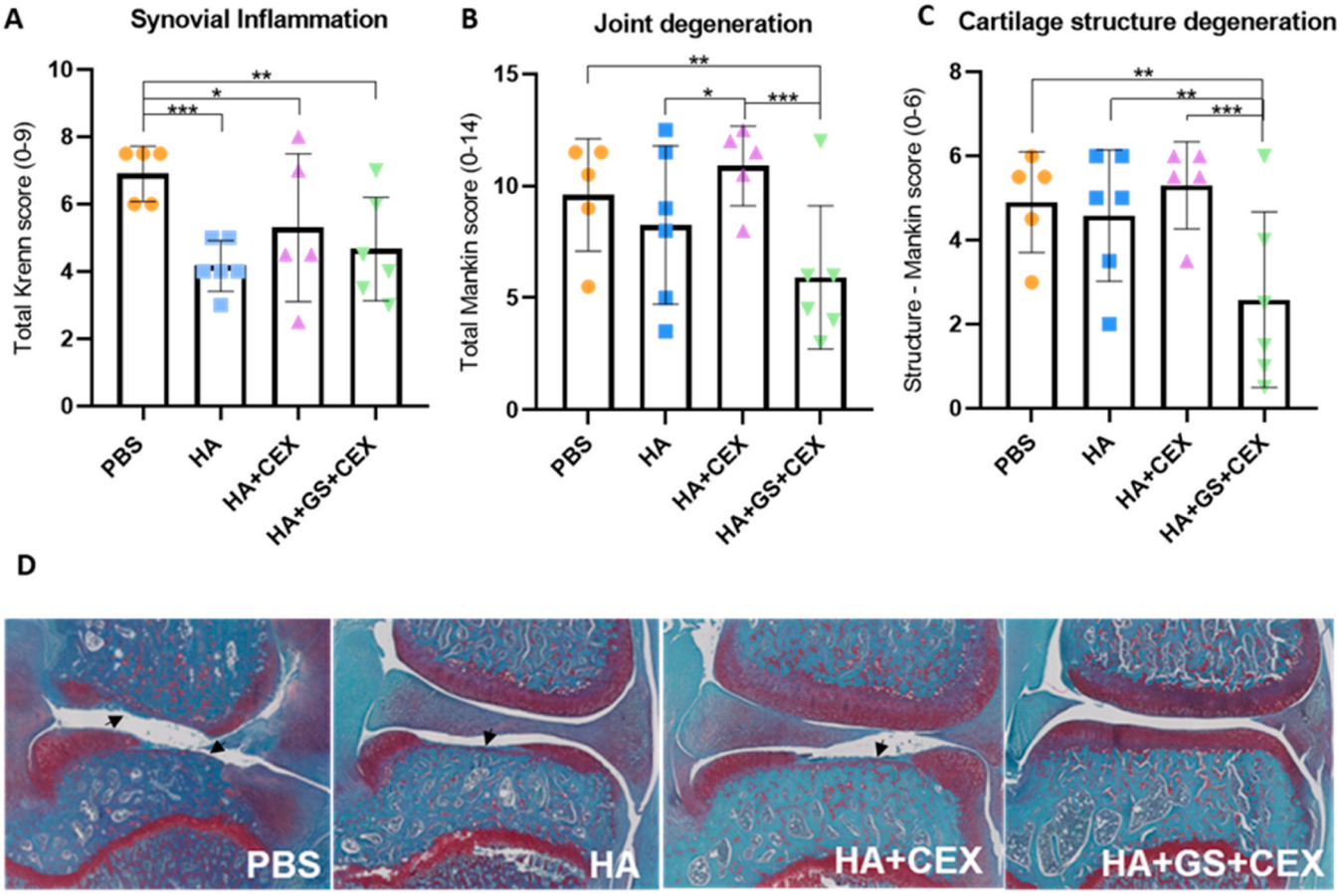

Fig. 7. Histological effects of treatment on synovitis and joint degeneration (PBS: n = 5; HA: n = 6; HA+CEX: n = 5; HA+GS+CEX: n = 6): (A) Synovial inflammation significantly reduced for all treatments (HA: $p < 0.001$; HA+CEX: $p = 0.046$; HA+GS+CEX: $p = 0.005$) in comparison to control (PBS). (B) Joint degeneration reduced by HA+GS+CEX compared to PBS: $p = 0.007$; HA: $p = 0.066$ and HA+CEX: $p < 0.001$; HA significantly reduced joint degeneration in respect to HA+CEX: $p = 0.047$. (C) Degeneration of joint structure significantly diminished for HA+GS+CEX in comparison to PBS: $p = 0.002$; HA: $p = 0.005$ and HA+CEX: $p < 0.001$. (D) Representative Saf-O-stained medial joint sections. Black arrows indicate cartilage degeneration. $^{***}p \leq 0.001$; $^{**}p \leq 0.01$; $^{*}p \leq 0.05$.

inhibition of cartilage and bone degeneration, not conferred by HA alone or HA+CEX without GS. These exploratory findings suggest that HA+GS+CEX may be a superior alternative to common HA viscosupplements. Follow-up research is necessary to understand the role of GS, further validate HA+GS+CEX in terms of long-term effects, safety, different dose regimens, sex differences, and to explore how these findings translate to relevant large animal models and humans.

CRediT authorship contribution statement

Niels Eijkelkamp: Writing – review & editing, Resources. **Alexandre Paiva:** Writing – review & editing, Supervision, Conceptualization. **Jie Du:** Writing – review & editing, Investigation. **Zhiming Wu:** Writing – review & editing, Investigation. **Jaqueline Lourdes Rios:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Yeter Dilek:** Writing – review & editing, Methodology, Investigation, Data curation. **Ana Rita C. Duarte:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Laura B. Creemers:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Ana Roda:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kelly Warmink:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Remei Escudero:** Writing – review & editing, Investigation, Data curation. **Katrin A. Muenzebrock:** Writing – review & editing, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ana Roda reports equipment, drugs, or supplies was provided by Bloomage Biotechnology Corp., Ltd. Ana Rita C. Duarte reports a relationship with Des Solutio Lda that includes: equity or stocks. Ana Roda, Ana Rita C. Duarte, Alexandre Paiva, Jaqueline L. Rios, Laura Creemers has patent Injectable composition, methods and uses thereof pending to Assignee. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Saskia van Essen for helping with the histological staining. The authors thank Bloomage Biotechnology Corp., Ltd. for kindly providing the hyaluronic acid. This project was supported by portuguese funds from FCT/MCTES [doctoral grant SFRH/BD/144424/2019]; by the European Union (ERC Consolidator Grant) from Horizon 2020 [ERC-2016-CoG 725034 Des.Solve]; and supported by the Associate Laboratory for Green Chemistry-LAQV, financed by portuguese funds [FCT/MCTES (UIDB/50006/2020)]. We also thank the funding agencies: Dutch Arthritis Association, LLP12; Turkish Government; Marie Sklodowska-Curie RESCUE CoFund GA801540 and CARTHAGO GA955335; China Scholarship Council. The funding bodies had no role in study design, data collection, data analysis, data interpretation and

writing the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2025.118239.

Data availability

Data will be made available on request.

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