RESEARCH ARTICLE

Therapeutic efficacy of intra-articular hyaluronan derivative and platelet-rich plasma in mice following axial tibial loading

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Abstract

Objective

To investigate the therapeutic potential of intra-articular hyaluronan-derivative HYADD® 4-G and/or platelet-rich plasma (PRP) in a mouse model of non-invasive joint injury.

Methods

Non-invasive axial tibial loading was used to induce joint injury in 10-week-old C57BL/6J mice (n = 86). Mice underwent a single loading of either 6 Newton (N) or 9N axial tibial compression. HYADD® 4-G was injected intra-articularly at 8 mg/mL or 15 mg/mL either before or after loading with or without PRP. Phosphate-buffered-saline was injected as control. Knee joints were harvested at 5 or 56 days post-loading and prepared for micro-computed tomography scanning and subsequently processed for histology. Immunostaining was performed for aggrecan to monitor its distribution, for CD44 to monitor chondrocyte reactive changes and for COMP (cartilage oligomeric matrix protein) as an index for cartilage matrix changes related to loading and cartilage injury. TUNEL assay was performed to identify chondrocyte apoptosis.

Results

Loading initiated cartilage proteoglycan loss and chondrocyte apoptosis within 5 days with slowly progressive post-traumatic osteoarthritis (no cartilage degeneration, but increased synovitis and ectopic calcification after 9N loading) at 56 days. Mice treated with repeated HYADD® 4-G (15 mg/mL) or HYADD® 4-G (8 mg/mL) ± PRP or PRP alone exhibited no significant improvement in the short-term (5 days) and long-term (56 days) consequences of joint loading except for a trend for improved bone changes compared to non-loaded joints.

Conclusion

While we failed to show an overall effect of intra-articular delivery of hyaluronan-derivative and/or PRP in reversing/protecting the pathological events in cartilage and synovium following joint injury, some bone alterations were relatively less severe with hyaluronan-derivative at higher concentration or in association with PRP.

Introduction

Osteoarthritis (OA) is a leading cause of disability worldwide. Post-traumatic OA (PTOA) in particular is a frequent cause of disability following trauma to the weight-bearing joints. It is estimated that 12% of the nearly 21 million Americans with symptomatic OA have a post-traumatic etiology [1]. Joint trauma can lead to a spectrum of acute lesions, including cartilage degradation, bone remodeling, ligament or meniscus tears, and synovitis, all of which are potentially associated with PTOA.

Murine models of knee injury are critical tools to study alterations associated with PTOA in the joint. Several invasive injury models such as injecting collagenase into the joint [2], using a needle to induce anterior cruciate ligament (ACL) transection in the closed knee, or using surgical techniques to transect or disrupt the ligaments or the medial meniscus of the knee [3, 4] have been widely used. However, these models do not mimic clinically relevant injury conditions due to invasive nature and non-physiologic injury methods. Therefore, the non-invasive model described in this paper and used by others [5, 6] and us in a modified form is especially attractive for studies of early and late pathological events following knee joint injury. However, the mechanisms, by which severity of injury leads to increased cartilage degeneration and accelerated synovial response, remain incompletely understood. In addition, detection of the early events in chondrocyte responses that lead to cartilage degeneration and eventually to development of PTOA has also been difficult to accomplish. With limited treatment options and no disease-modifying OA drugs, there is a critical need to develop new therapeutic approaches to repair joint injury and reduce the probability of PTOA. The wide spectrum of changes in the knee joint following tibial loading may be targets for treatment options.

Hyaluronan (hyaluronic acid, HA) is a high molecular-weight, non-sulfated, unbranched glycosaminoglycan [7] that plays a significant role in maintaining the homeostasis of the synovial joint as well as providing lubrication, shock absorption, elasticity and hydration to the joint tissues [8], and has well known anti-inflammatory [9] and anti-apoptotic effects on chondrocytes [10]. Numerous experimental and clinical studies suggest that intra-articular injection of HA and its derivatives potentially exert a protective effect on joint structure and function [11–13]. HYADD® 4-G (Fidia Farmaceutici, Abano Terme, Italy) hydrogel is a novel HA amide derivative in which an aliphatic amine is bound to HA at the carboxylic group of the glucuronic acid in only 2% of the carboxylic groups, therefore 98% remains unmodified HA. This small chemical modification on the molecule increases the viscoelastic properties of HA and thereby increasing beneficial effects in human OA as well as in surgical models of PTOA in large animals [12, 14]. Increasing the viscoelastic properties of HA provides superior lubricant properties and thus reduces the coefficient of friction as well as possibly limits articular cartilage damage during joint activity [15].

Platelet-rich plasma (PRP) is another option for the intra-articular treatment of OA, considering the high content of growth factors in the PRP preparations. The growth factors, stored in the granules of the platelets are released after platelet activation, which can occur before

injection by adding thrombin or $CaCl_2$. Most of these factors are implicated in the regulation of articular chondrocyte anabolic activity. In particular, platelet-derived growth factor (PDGF) stimulates chondrocyte proliferation and proteoglycan synthesis (reviewed in [16]) and transforming growth factor beta 1 (TGF-β1) affects chondrocyte proliferation [17] and stimulates chondrogenesis of stem cells.

A systematic review of the efficacy of PRP has concluded that multiple sequential intra-articular PRP injections might have beneficial effects in the treatment of mild to moderate knee OA in adult patients [18] in comparison to HA or saline. However, in animal models while the HA efficacy on experimental OA is widely reported [19, 20] data on the PRP effect are scarce [21].

To date, there is no study that has tested the potential (therapeutic) effects of HA or PRP in the injured mouse knee joint after mechanical loading. Here we have analyzed joint tissues to determine whether treatment with HA or growth factor-rich preparations administered at the time of injury or later could reduce the immediate effects of loading and/or the long-term effects of joint injury.

Materials and methods

Study design

Study procedures were approved by Washington University Institutional Animal Care and Use Committee. Mice (C57BL/6J) were procured from the Jackson Laboratory (Bar Harbor, ME) and housed under standard sanitary and husbandry conditions as described previously [22]. Briefly, mice were housed in a designated mouse facility operating at controlled temperature (21˚C-22˚C) and controlled lighting (12 h light, 12 h dark) conditions with high standards of sanitation. On an average 4–5 mice were kept in individually ventilated cages. Mice were provided standard rodent chow (Purina 5053, Purina Mills St. Louis, MO) and water *ad libitum*. In total, we evaluated 150 knees from 86 mice (5–14 knees per group). Some of the knees were excluded from the analysis due to the lack of an ACL rupture (in 9N loading group, in 6N loading group no ACL tear occurred) or due to lack of extraction of reliable data–for example poor tissue preparation, problems with embedding, poor orientation of sections and improper scanning. The number of mice in each group, time points and treatment options are depicted in Table 1, and an overview of the experimental design is shown in Fig 1.

HYADD® 4-G (HA-derivative)

HA-derived HYADD® 4-G hydrogel is a non-cross-linked HA (500–730 kDa) of fermentative origin. Fidia Farmaceutici (Abano Terme, Italy) provided HA-derived HYADD® 4-G hydrogel in sealed syringes with two different concentrations: 8 mg/mL and 15 mg/mL. For the sake of simplicity, we used the term HA-derivative for this product. HYADD® 4-G (8 mg/mL) is commercially available with the brand name HYMOVIS® (http://hymovis.com).

Preparation of PRP

We prepared PRP from mouse blood for therapeutic use alone or combined with the HA-derivative. PRP was prepared as described previously [23]. Briefly, intra-cardiac whole blood sample was drawn from 8-week old C57BL/6J mice (n = 10) using sterile syringe pre-loaded with heparin. First, the blood was centrifuged at 200g for 20 min to separate the platelet-poor plasma from the red and white blood cells. In second step, the plasma was centrifuged at 2000g for 10 min. Platelets were re-suspended in supernatant to obtain PRP (about 10% volume of the pooled whole blood). The concentrations of the growth factors were assessed by enzyme-

Table 1. Loading regimens, treatment options, time points and sample size.

Force	Description of treatment	Volume injected[#]	Time point (day)	Mouse (*n*)	Knee (*n*)
	Right knees loaded and injected only				
6N	HYADD-4G 8 mg/mL pre-loading	15 µL	5	5	5
6N	HYADD-4G 8 mg/mL post-loading	15 µL	5	6	6
6N	PBS, control	15 µL	5	6	6[a]
6N	HYADD-4G 15 mg/mL post-loading	15 µL	5	5	5
6N	PBS control	15 µL	56	6	6[b]
6N	HYADD-4G 8 mg/mL post-loading	15 µL	56	6	6
	Both knees loaded and injected				
9N	HYADD-4G 15 mg/mL (right knees) and PBS (left knees) post-loading	15 µL	5	10	6 (Right)[c], 7 (left)[d]
	Both knee loaded and injected				
9N	HYADD-4G 8 mg/mL, 3 injections post-loading	15 µL	56	10	14[e]
9N	HYADD-4G 15 mg/mL, 3 injections post-loading	15 µL	56	8	15*
9N	PBS control, 3 injections post-loading	15 µL	56	8	14*
9N	PRP, 3 injections post-loading	15 µL	56	8	13*
9N	PRP + HYADD-4G 8 mg/mL, 3 injections post-loading	5 µL + 10 µL mixture	56	8	13*

a: 6 left knees from this group were used for non-loaded control at day 5

b: 6 left knees from this group were used for non-loaded control at day 56

c: 3 knees were excluded due to lack of micro-CT data, 1 knee without ACL rupture was also excluded.

d: 1 knee was excluded due to lack of micro-CT data, 2 knees without ACL rupture were also excluded.

e: 2 knees were excluded due to lack of micro-CT data, 4 knees without ACL rupture were also excluded.

*: all the knees without ACL rupture were excluded
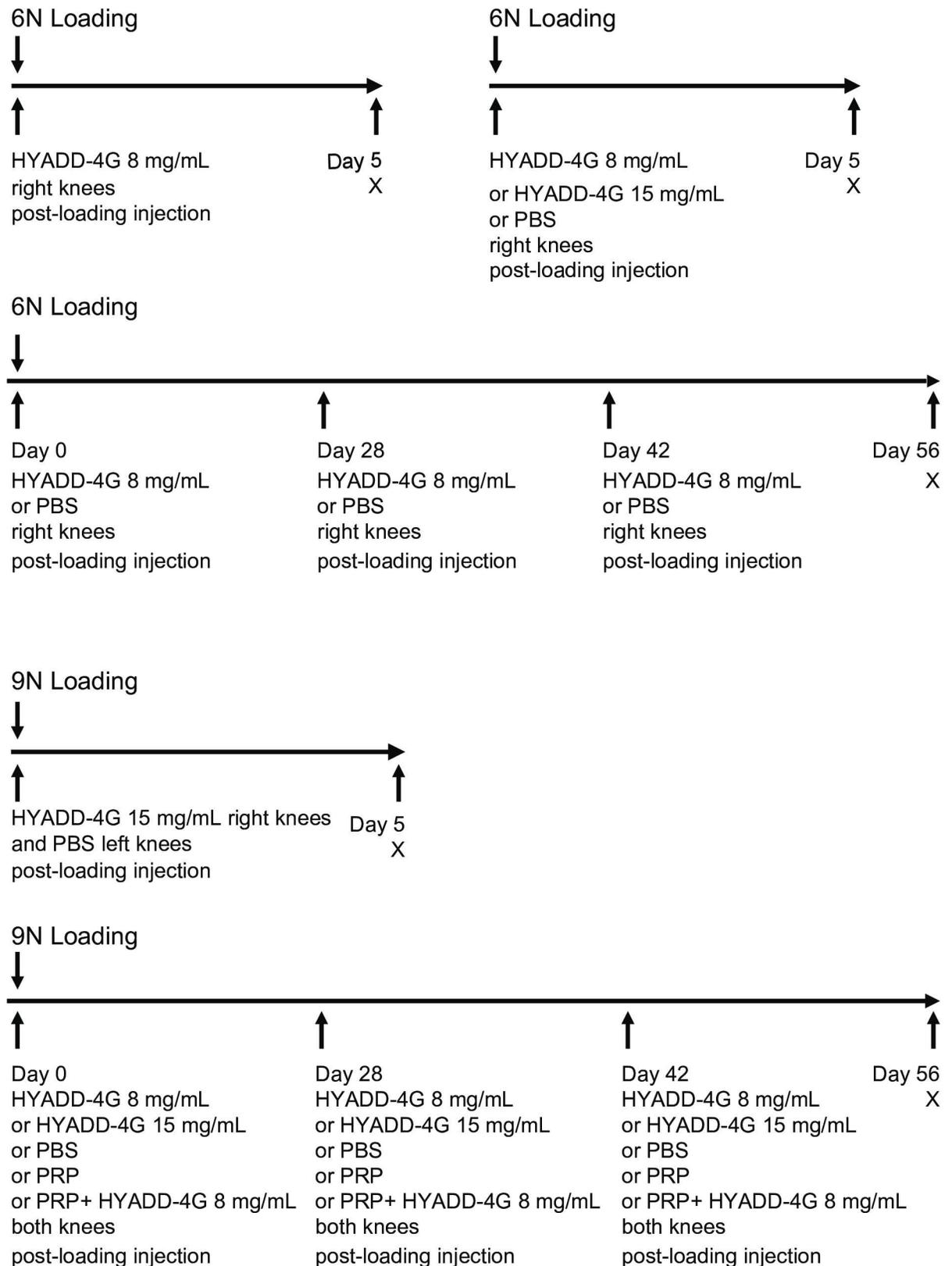
#: volume that was injected into each knee

linked immunosorbent assay using 3 replicates. The PRP preparation had higher concentrations (mean and 95% confidence interval, CI) of growth factors namely TGF-β1 (46.87 ng/ml, 95% CI, 39.97–53.77) and PDGF AB (2.68 ng/ml, 95% CI, 2.67–2.69) compared to platelet-poor plasma, which had significantly lower concentrations (4.39 ng/ml, 95% CI, 4.24–4.54 and 0.04 ng/ml, 95% CI, 0.01–0.07 respectively).

Axial tibial loading and intra-articular injection

Mouse knees underwent 6N or 9N of compressive loading as detailed previously [24, 25]. Briefly, under anesthesia (2.5% isoflurane in 4 L/min oxygen; Highland Medical Equipment), the right tibia was held vertically in an upside-down position between the two custom-made cups of a materials testing machine (Instron ElectroPulse E1000). Axial tibial load was applied through the ankle. Cyclic loads consisting of 0.34 sec of rise and fall time and a baseline hold time of 10 sec between each cycle for 60 cycles. Peak loading force (6N or 9N) with a 0.5N pre-load force (to maintain the tibia in position) was applied. Some contralateral non-loaded knees served as control.

While mice were under anesthesia, they were injected with the indicated treatment(s) (Table 1). Intra-articular injection was performed as reported by Diekman et al., [26]. Briefly, the mouse was positioned for lateral entry of needle with the left hind limb extended to facilitate injection of 15 µL into the joint space through the patellar tendon. This delivery technique does not result in initiation of OA [27]. As stated above, the volume of each treatment injected was 15 µL, however, when we combined the treatments, we mixed 5 µL of PRP + 10 µL of HYADD-4G 8 mg/mL.

6N Loading

HYADD-4G 8 mg/mL
right knees
post-loading injection

Day 5
X

6N Loading

HYADD-4G 8 mg/mL
or HYADD-4G 15 mg/mL
or PBS
right knees
post-loading injection

Day 5
X

6N Loading

Day 0
HYADD-4G 8 mg/mL
or PBS
right knees
post-loading injection

Day 28
HYADD-4G 8 mg/mL
or PBS
right knees
post-loading injection

Day 42
HYADD-4G 8 mg/mL
or PBS
right knees
post-loading injection

Day 56
X

9N Loading

HYADD-4G 15 mg/mL right knees
and PBS left knees
post-loading injection

Day 5
X

9N Loading

Day 0
HYADD-4G 8 mg/mL
or HYADD-4G 15 mg/mL
or PBS
or PRP
or PRP+ HYADD-4G 8 mg/mL
both knees
post-loading injection

Day 28
HYADD-4G 8 mg/mL
or HYADD-4G 15 mg/mL
or PBS
or PRP
or PRP+ HYADD-4G 8 mg/mL
both knees
post-loading injection

Day 42
HYADD-4G 8 mg/mL
or HYADD-4G 15 mg/mL
or PBS
or PRP
or PRP+ HYADD-4G 8 mg/mL
both knees
post-loading injection

Day 56
X

Fig 1. Study design. An overview of treatment(s) before and/or after loading, time points of repeated injections and analysis of 6N and 9N loading is shown. X = sacrifice, PBS = phosphate buffered saline, PRP = platelet-rich plasma.

Knee joint preparation

Mice were sacrificed using CO_2 chamber at indicated time points (Fig 1). Knee joints were dissected and fixed in 10% neutral buffered formalin. Joints were then washed with distilled water and incubated in 70% ethanol until scanning for micro-CT or decalcification. Based on our and others studies [5, 25], all of the following parameters were studied in femur.

Micro-CT analysis

Prior to decalcification, knees were scanned using vivaCT-40 micro-CT scanner (Scanco-Medical) for analysis of 3-dimensional bone structure for trabecular bone volume fraction (BV/TV) [22, 28]. Subchondral bone plate thickness was measured via custom-written MATLAB-2015b (Mathworks) program [29].

Histology and scoring of proteoglycan loss

Knee joints were decalcified using Immunocal (StatLab, McKinney, TX) and paraffin embedded. Five-micron thick sections were cut in sagittal plane throughout the joint with every third section stained with Safranin-O. As there was no appreciable structural loss of non-calcified cartilage, we scored articular cartilage for proteoglycan depletion (loss) using a supplementary OARSI (Osteoarthritis Research Society International) scoring system (called proteoglycan scoring system) as described by Glasson and co-workers [30]. Synovial pathology was measured as before [25, 31] using three selected sections from each knee.

Detection of chondrocyte apoptosis

Chondrocyte apoptosis was detected by in situ terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay (In situ Cell Death Detection Kit, Roche, Indianapolis, IN). In order to compare the number of apoptotic cells, we examined the numbers of TUNEL-positive cells in non-calcified cartilage as it is commonly thought that apoptosis naturally occurs in calcified cartilage [32]. Images acquired from three TUNEL-stained slides were evaluated by two blinded observers as described previously [33].

Immunostaining of extracellular matrix proteins and CD44

To assess the expression pattern of extracellular matrix proteins such as aggrecan, and cartilage oligomeric matrix protein (COMP), and CD44 (a cell surface antigen for hyaluronic acid binding), we used antibody-specific immunostaining as described elsewhere [24, 25]. Selected slides were set for baking at 60°C for 4 h followed by deparaffinization in xylene twice for 5 min. Slides were then rehydrated in descending concentrations of ethanol with final washes with distilled water and phosphate buffered saline (PBS). For antigen retrieval, the slides were digested with 10 μg/mL proteinase K at 37°C for 20 min in a customized humidifying chamber. After washing with PBS, slides were blocked in 10% normal goat serum in PBS at room temperature for 1 h. After draining off the blocking buffer, slides were incubated with following primary antibodies (procured from Abcam, Cambridge, MA unless indicated otherwise) for respective proteins at 1:100 dilutions using 1.5% goat serum in PBS: rabbit anti COMP (Kamiya Biomedical Co., Seattle, WA, PC-140), rabbit anti aggrecan for G2 domain (generous gift from Dr. Amanda Fosang, Melbourne, Australia), rabbit anti CD44. Slides were incubated at 4°C overnight followed by 3 washes with PBS. The secondary antibodies were used (procured from Abcam, Cambridge, MA) at 1:200 dilutions using 2.5% goat serum in PBS. Slides were incubated at room temperature for 60 min. Finally, the slides were rinsed with PBS and distilled water before being sealed with VECTASHIELD antifade mounting medium with

DAPI (Vector Laboratories, Burlingame, CA). Sections were imaged using Eclipse E800 microscope (Nikon, Tokyo, Japan) with QImaging Retiga 2000R Fast 1394 camera and Meta-Morph software v7.7 (Molecular Devices, Sunnyvale, CA). All antibodies used in this study have been reported by Duan et al. [25].

Statistical analysis

Data collection and analysis were performed in a blinded fashion. As noted above (Table 1), we used a sample size of 5–14 per group. Post-hoc power analysis showed that this sample size is sufficient to detect an effect size ranging between 1.1 to 2.0 at a power of 80% and $\alpha = 0.05$ for any given parameter. Therefore, our interpretation of results and conclusion drawn are based on this level of effect size. We used one-way analysis of variance with Tukey's honestly significant difference (HSD) post-hoc test to compare each group to all other groups in a particular parameter using GraphPad Prism v.5.04 (GraphPad Software Inc., La Jolla, CA). Data are expressed as mean ± 95% CI. Differences were considered statistically significant at $P < 0.05$.

Results

### Effect of treatments on cartilage injury and proteoglycan loss

The cartilage injury site was located at the posterior aspect of the lateral femoral condyle and was characterized by the loss of Safranin-O staining without disrupting nuclear structure. We observed complete loss of Safranin-O staining indicative of proteoglycan depletion in the non-calcified cartilage extending up to 50% of the articular surface (Fig 2A and 2B). The cartilage injury was identical in 6N and 9N loading groups. We found that treatment with HA-derivative or PRP alone or in combination did not confer any protective response on cartilage proteoglycan loss (Fig 2A and 2B). We also failed to appreciate any significant differences in proteoglycan loss (at 56-days) between any treatment modalities used or between 6N and 9N loading groups (Fig 2C).

Effect of treatments on COMP and apoptosis

Non-immune controls are shown in the S1A and S1B Fig. Immunostaining of COMP (Fig 3A–3C, red) showed its presence around the chondrocytes in the intact area and in the lacuna left by the apoptotic chondrocytes in the injured area at the early time point (day 5) post-injury. This distribution pattern of COMP is parallel with our previous findings [24, 25]. COMP staining became weaker over time and was hardly seen in the injured area at day 56 post-loading. We also observed that cells at the site of injury underwent apoptosis as TUNEL assay detected fragments of damaged DNA (Fig 3A–3C, green). There was no apoptosis in the non-calcified layer of the cartilage in the non-loaded knees and in the non-injured cartilage of the loaded knees. Apoptotic chondrocytes were randomly seen in the calcified layer of the cartilage of both loaded and non-loaded knees across all the time points. There were more apoptotic chondrocytes detected at day 5 in the non-calcified layer of the injured cartilage, which were largely cleared by day 14 (not shown) and absent at day 56. HA-derivative or PRP alone or in combination did not protect or reverse chondrocyte apoptosis.

Effect of treatments on aggrecan and CD44

As reported by us [24, 25] and as our current data suggest, aggrecan, similar to COMP, was expressed around the chondrocytes in the intact cartilage area in loaded and non-loaded knees. After injury, increased aggrecan was observed in the lacunae created by apoptotic
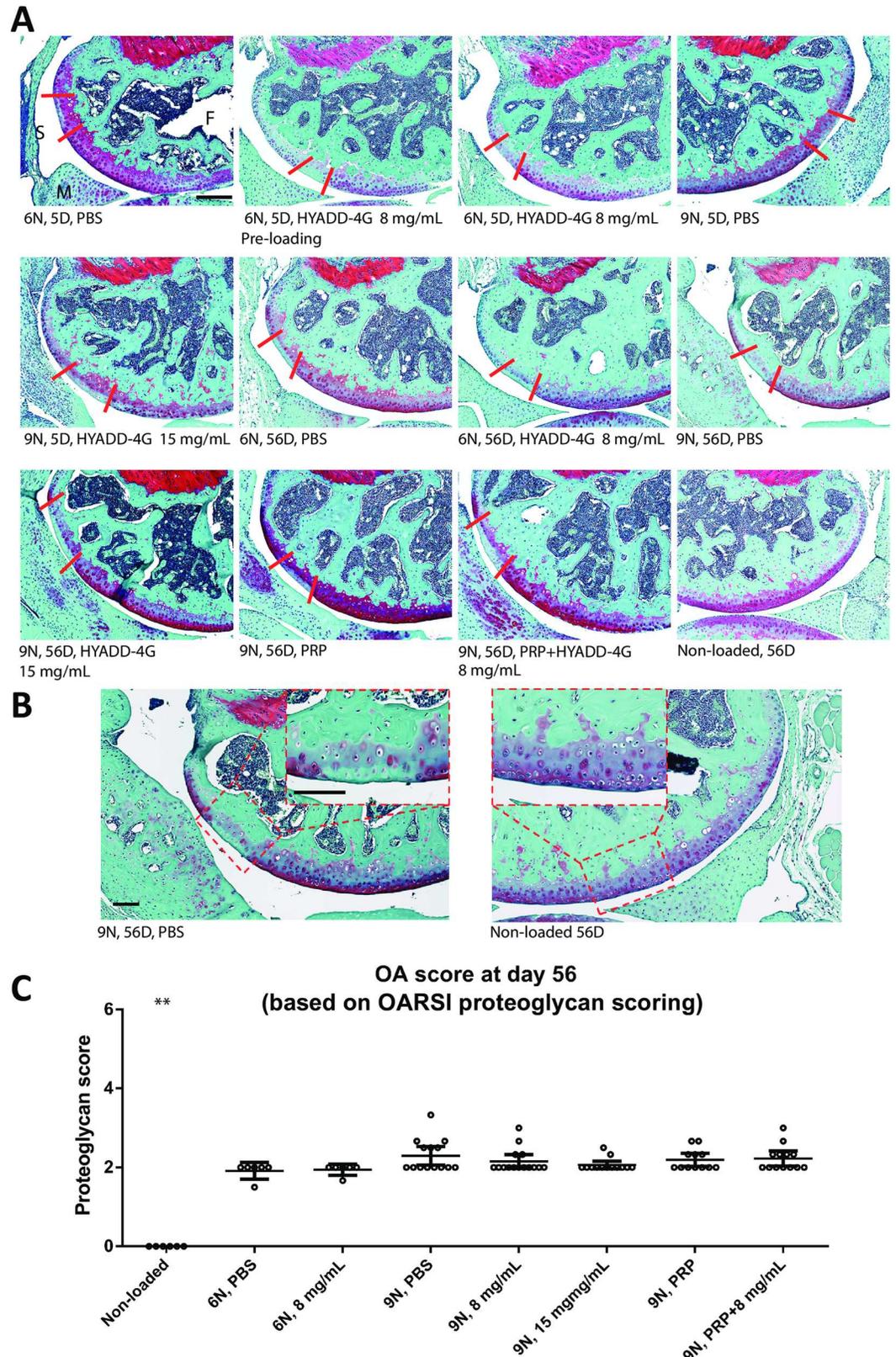
Fig 2. Cartilage injury. Representative images of cartilage injury of the lateral femoral condyle in each group showing loss of Safranin-O staining and absence of normal nuclear staining are provided for higher (A) and lower

(B) magnification. There was no therapeutic effect of HYADD® 4-G or PRP on cartilage injury and results were not different from PBS treatment. The area between two red lines in each image shows the injury site of each knee. OA scored based on proteoglycan loss on day 56 after loading showed that there was no significant effect of any treatment modality on proteoglycan replacement (C). Bar = 50 μm, ** indicates P < 0.01 compared with other groups. Error bars represent 95% confidence interval. PBS = phosphate buffered saline, PRP = platelet-rich plasma, F = femur, M = meniscus, S = synovium.

chondrocytes in the injured cartilage (Fig 4A–4C and S2A Fig, red). Furthermore, CD44, which is a cell surface receptor for HA, demonstrated a unique expression pattern (Fig 4A–4C and S1A Fig, green). Its fluorescent intensity increased in the area closely surrounding the injured cartilage at day 5 in all treatment groups (Fig 4A–4C arrows). However, this expression pattern of CD44 disappeared at day 56 (Fig 4B). CD44 chondrocytes were mostly seen in the hypertrophic cells in the calcified cartilage in all groups. As stated above for COMP and apoptosis, none of the treatments modified the expression pattern of aggrecan and CD44.

## Effect of treatments on synovitis and ectopic calcification

We observed that non-loaded knees had normal synovium as before [25]. Both 6N and 9N loading resulted in some degree of synovitis (enlargement of synovial lining and increased density of cells in synovial stroma) at day 5 persisting to day 56 post-loading (Fig 5A). Quantification of synovitis score at day 5 and 56 post-injury indicated that synovitis was affected by time and loading force. At day 5 post-injury, 6N loaded + PBS treated group had a significant lower synovitis compared to 9N loaded + PBS treated group (Fig 5B, c: P < 0.01). However, the long term (56 day) results showed an increase in the severity of synovitis in 9N loaded + PBS treated group (Fig 5B, b: P < 0.01) compared to 6N loaded + PBS treated group (Fig 5B, a: P < 0.01). At each time point, the HA-derivative treated or PRP treated groups revealed no significant differences in synovitis compared to PBS groups, indicating that none of the treatment modality resolved synovitis (Fig 5B). We also observed the formation of calcified synovial nodules in the joint that were assessed by micro-CT (Fig 5C). These nodules were more pronounced in the 9N loading group compared to 6N loading group and non-loaded knees but we failed to observe any significant effect of HA-derivative and/or PRP treatments in resolving ectopic calcification (Fig 5D), even if the number of ectopic nodules decreased by 1/3 in comparison to PBS treated joints (in 9N group).

Effect of treatment on bone parameters

We observed some differences in bone volume fraction (BV/TV) between non-loaded and loaded knees, but the only significant difference was noted between non-loaded knees and 9N loaded knees at 56 days (Fig 6A and 6B). No appreciable differences were seen in subchondral bone plate thickness between loaded and non-loaded knees (Fig 6C). The subtle difference in BV/TV in loaded and non-loaded knees were mitigated in HA-derivative at 8 mg/mL + PRP group and in HA-derivative at 15 mg/mL group without reaching statistical significance. Furthermore, no significant differences in these groups were seen in comparison to non-loaded joints.

Discussion

In this study, we used known short-term parameters of the compression model to determine whether there were effects of HA-derivative and/or PRP. In addition, we extended the time after injury to 8 weeks (56 days) to determine long-term consequences of the single injury and whether there were long-term effects of the treatments.
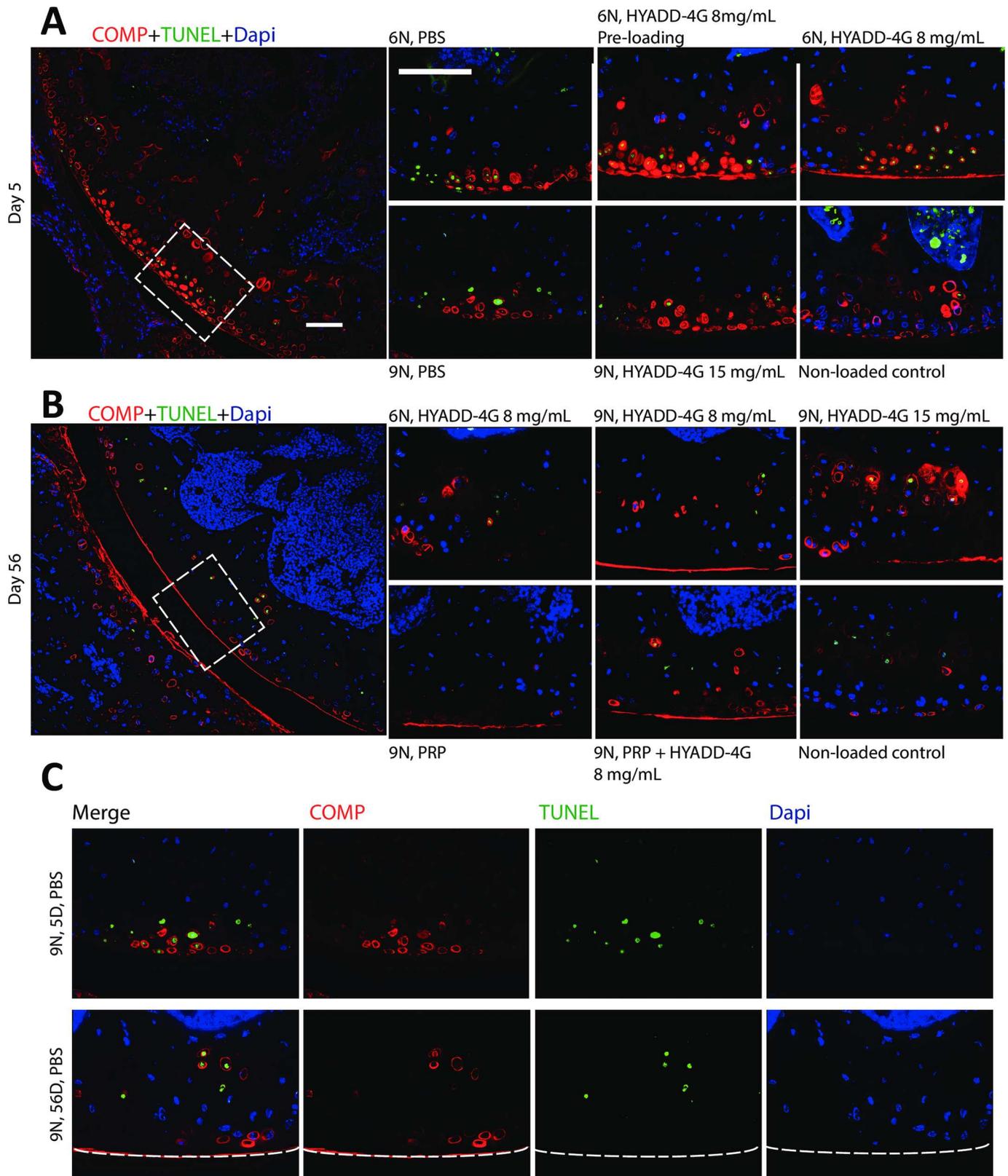
Fig 3. Chondrocyte apoptosis and COMP staining. Representative images of chondrocyte apoptosis (green), COMP (red) and DAPI (blue) immunostaining are shown. At day 5 (A), TUNEL assay identified apoptotic chondrocytes (green) in the injured cartilage area in all loaded groups.

COMP (red) was found around the chondrocytes in the intact area and in the lacuna left by the apoptotic chondrocytes in the injured area. At day 56 (B), TUNEL positive chondrocytes in the non-calcified layer of injured cartilage disappeared. No new developed apoptosis was observed. COMP (red) was hardly seen in the injured region and its expression remained the same as day 5 in the adjacent healthy cartilage surface. None of the treatments conferred any protective response on chondrocyte apoptosis or the expression pattern of COMP at day 5 (A) and 56 (B) after loading. White boxes indicate the injured area where enlarged images were taken. Representative images at days 5 and 56 for 9N loading with split channels to show the changes of chondrocyte apoptosis (TUNEL positive cells) and distribution of COMP (red) are also presented (C). Bar = 100 μm, dotted white lines indicate the joint surface. COMP = cartilage oligomeric matrix protein, PBS = phosphate buffered saline, PRP = platelet-rich plasma, TUNEL = terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling.

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This study confirmed our previous findings in the compression model, which was largely characterized by the focal loss of proteoglycan at the site of cartilage injury on the posterior aspect of femoral condyle, chondrocyte apoptosis in this area, changes in the pattern of aggrecan expression, synovitis and ectopic calcification at early time points [24, 25]. We did not find pronounced progression in cartilage loss, cartilage fibrillation, and PTOA with 9N loading even over 56-day time period. This observation is in definite contrast with the DMM (destabilization of the medial meniscus) model of OA in which the medial meniscal tibial ligament is disrupted through a surgical procedure. In loading model, while chondrocytes in the injured area have a high degree of cell death, the cartilage remains intact (other than complete loss of Safranin-O staining in the non-calcified cartilage extending to <50% of the articular surface) even 8 weeks post-injury.

Several new studies have shown that acute chondrocyte death caused by in vitro impact injuries results from apoptosis [24, 34]. Lisignoli et al. [10] and Zhou et al. [35] have shown that HA can inhibit chondrocyte apoptosis via CD44 and CD54 activation. In our study, regardless of the injection time (e.g. pre-loading or post-loading), dosage of HA-derivative (e.g. 8 mg/mL or 15 mg/mL) ± PRP and loading force (e.g. 6N or 9N) did not reveal any obvious immediate preventive effect towards chondrocyte apoptosis emerging from tibial compression. This discrepancy could be attributed to the nature of the factors (experimental conditions) that triggered the apoptotic process. In our study, in vivo mechanical loading triggered apoptosis, while Fas ligand or IL-1β stimulated chondrocyte apoptosis in the aforementioned in vitro studies.

Our findings differ with other studies that have reported a beneficial effect of repeated injections of both HA and PRP in the rabbit joints after impact to patella and femoral condyle [36, 37]. In these studies, cell apoptosis was found to be significantly reduced after repeated (total 8 injections) intra-articular injections of 1 mg/kg HA at 30 days after knee impact, or at 10 days after a single post-loading injections of PRP. No other parameters were evaluated in these studies to detect the whole joint damage, and the differences in observations compared to our study could be related to the magnitude and frequency of compression applied during the impact loading, severity of joint damage or differences in animal model. In another study [38], presence of HA and chondroitin sulphate reduced the number of apoptosed chondrocytes in equine cartilage explants after single-impact loading. However, no changes in type II collagen or proteoglycan metabolism were noticed.

HA interacts with its surface receptors, CD44, on chondrocytes [10]. CD44-HA interactions link chondrocytes to their matrix and these interactions modulate cartilage metabolism [39]. Inhibition of CD44 expression causes substantial loss of aggrecan and promotes matrix remodeling [40]. Our previous study has shown a change in the pattern of CD44 expression in the injured cartilage [25] at early time-point after loading, with an increased expression a few days after impact. The data of PBS group in the current study are consistent with our previous findings [25] and no difference was evident between PBS, HA-derivative and PRP treated
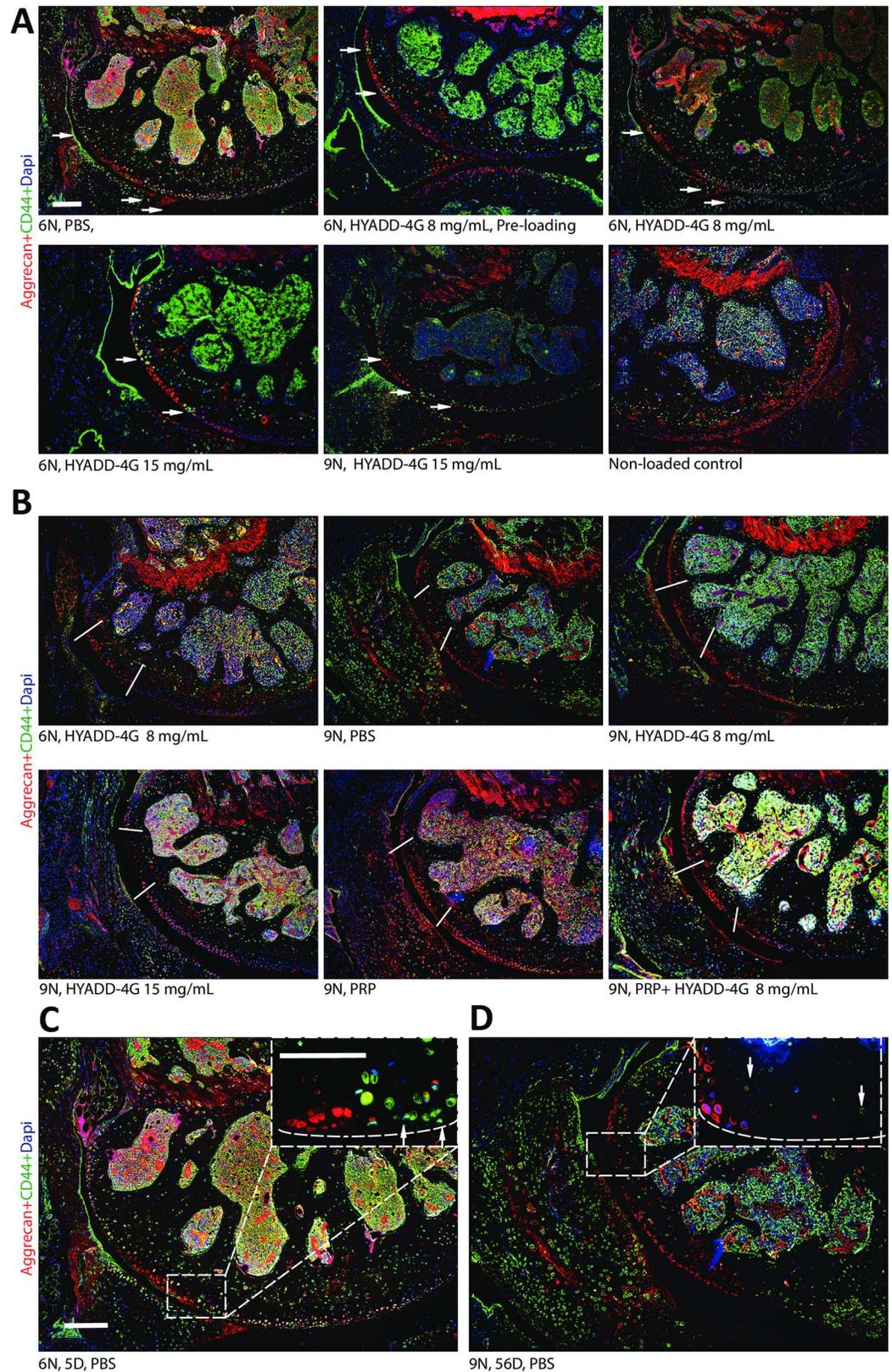
Fig 4. CD44 and aggrecan staining. At day 5, CD44 (green) was mainly seen in the calcified zone of articular cartilage in control knees with only a few CD44-positive signals around hypertrophic chondrocytes (A). CD44

signals were not found at the site of cartilage injury, albeit higher signals were discovered in the chondrocytes immediately around the injured zone (white arrows). There was no difference in the pattern of CD44 expression between different treatment groups. Aggrecan staining (red) was found around the chondrocytes in the intact area and in the lacuna left by the apoptotic chondrocytes in the injured area. Bar = 100 μm. At day 56, CD44 in the loaded knees, same as non-loaded control knees, was mainly seen in the calcified zone of articular cartilage with only a few CD44-positive signals around hypertrophic chondrocytes. No substantial CD44 signals were found in the chondrocytes immediately around the injured zone as day 5. There was no difference in the pattern of CD44 expression between different treatment groups. Aggrecan staining (red) was much weaker in the injured region but remain the same in the intact area of the loaded cartilage. White bars indicate the injured areas (B). Representative images of day 5 (C) and day 56 (D) of injured knees. White dotted boxes indicate the enlarged area. The white arrows in C indicate the higher CD44 signals in the chondrocytes immediately around the injured zone at day 5 while the white arrows in D show the expression of CD44 in the hypertrophic chondrocytes in both loaded and non-loaded knees at day 56. Also, low intensity of aggrecan signal in the injured region at day 56 was noticed. Dotted lines indicate the joint surface. PBS = phosphate buffered saline, PRP = platelet-rich plasma.

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animals for CD44 expression on chondrocytes. Together with our observation, it suggests that the early events after impact loading do not seem to be modified by exogenous HA.

The role of ectopic ossification and osteophytes in OA development is not completely clear, although it is thought that they mechanically stabilize an osteoarthritic joint, thereby preventing structural progression [41]. Felson and co-workers [42] have shown that osteophytes are predictors for progression of OA, due to their strong association with joint malalignment. In a number of clinical investigations, it has been reported that osteophytes are strongly associated with OA joint pain and its progression [43]. In our study, histological and micro-CT evaluations revealed a reduction, though not statistically significant, in ectopic calcification/osteophyte formation at the 8-week time point after HA-derivative and PRP injections. Consequently, HA-derivative may relieve OA pain in part by reducing ectopic calcification and osteophyte formation. Additional preclinical and clinical studies will be required to test this hypothesis.

Christiansen and colleagues [5] have reported changes in knee joint following a one-time 12N loading as they observed a rapid loss of trabecular bone in injured knees at day 7 post-loading, which persisted until day 56. We also observed a loss of trabecular bone only at 56-day time point in the mice that underwent 9N loading. Since we used a lower loading force, our short-term (day 5) data did not show any significant changes in bone volume fraction and subchondral bone plate thickness. The effect of HA on bone formation/remodeling remains controversial. Sasaki and Watanabe [44] showed that high molecular weight HA was osteoinductive, while Cho et al. [45] demonstrated that HA failed to promote bone formation in distraction osteogenesis. Interestingly, we did not find any significant effect of HA-derivative alone or in combination with PRP on trabecular bone volume fraction. This lack of effect can be attributed to an increase weight bearing in the joints of the HA-derivative treated groups.

Numerous PRP studies have been performed in laboratory and clinical research in last two decades. Of these, some studies have reported that PRP increases chondrocyte viability and proliferation [46], and enhances the deposition of extracellular matrix proteins [47]. Other studies have reported PRP decreased type II collagen synthesis and stimulated the expression of catabolic (inflammatory) molecules [48]. One potential reason for these disparities could be attributed to the variations in the composition of PRP, including different protocols employed for the preparation and activation of PRP.

Although PRP has been used for over 40 years for treatment of acute and chronic injuries of bone and cartilage [49], only a few studies have shown its therapeutic effect in injury compared to HA [50, 51], thus leaving the therapeutic efficacy of PRP in OA treatment controversial. The combination of HA+PRP has been proposed as a therapy by Chen et al. [52] for tissue
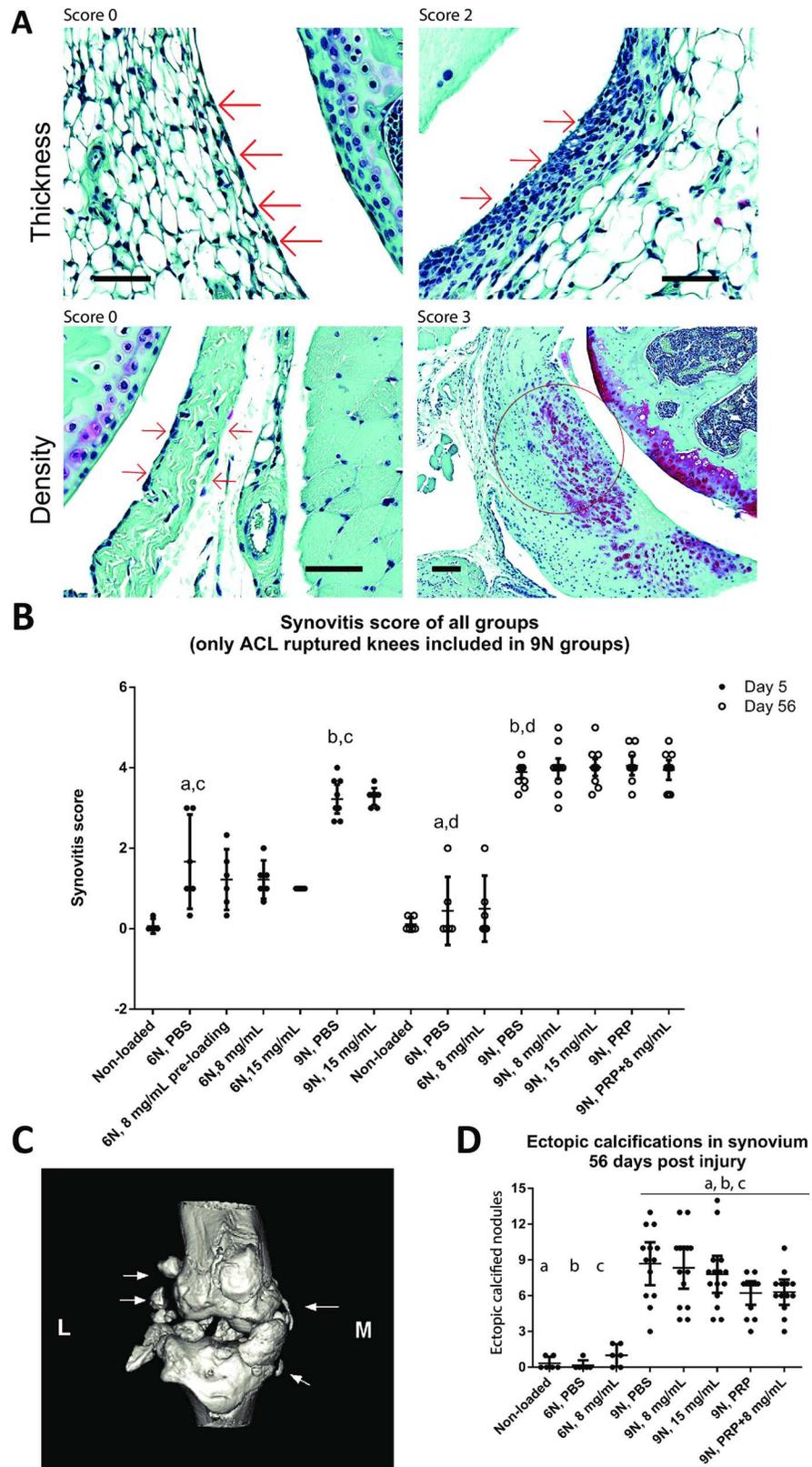
Fig 5. Synovitis and ectopic ossification. Enlargement of the synovial lining cell layer and density of the cells in the synovial stroma (red arrows) were individually assessed. The red circle shows a representative

ectopic ossification nodule (A). Quantification of synovitis score in each group indicated that synovitis progressed with time but no significant differences were found in the same loading force groups at the same time point. a, b, c, and d: P < 0.01 (B). A representative micro-CT image showing synovial ectopic calcification is shown in which white arrows show the nodules around the injured knee (C). Quantification of ectopic nodules in each group showed higher number of calcified nodules in the 9N group compared to non-loaded knees and knees loaded with 6N force. There were no significant differences observed within the same loading groups. a, b and c P < 0.01 (D). L = lateral side, M = medial side, bars = 100 μm. Error bars represent 95% confidence interval. PBS = phosphate buffered saline, PRP = platelet-rich plasma.

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regeneration in OA. The therapeutic efficacy of the combined products was demonstrated in 3-dimensional arthritic neo-cartilage and in an OA model of ACL tear. The cartilaginous extracellular matrix was retrieved from inflammation-induced degradation by HA+PRP. The intra-articular injection of HA+PRP could strongly rescue the meniscus tear and cartilage breakdown and promote cartilage regeneration and inhibit inflammation. In our study, the combination of HA-derivative and PRP may indicate that the association can have synergistic effect only on the trabecular bone loss at longer term after knee injury.

This study has some limitations. There are no studies to directly compare the effect of PRP or HA with our study, so we used the literature from other study designs such as the in vitro effects of HA in IL-1β-treated human chondrocytes, in vitro equine impaction-induced apoptosis and in vivo loading induced apoptosis in rabbit joints after impact to patella and femoral condyle. It is likely that HA has effect on other functional parameters that are beyond the scope of present study. Therefore, our findings do not represent direct comparison with the other studies. Our study also lacks appropriate positive control, as there are currently no disease modifying OA drugs or the use of some treatments has been evaluated for functional outcomes such pain and gait pattern, which we did not undertake in our study. Furthermore, the analyses were performed on the femur side as this model results in changes that occur on the femur. It would have been ideal if our study could provide more specific information on focal subchondral or trabecular bone analyses given the focal nature of the joint injury, however, this was not possible, as we could not detect any focal changes in the bone on micro-CT. Lastly, the outcomes of our study were centered on joint structure, whereas clinical findings on the effectiveness of HA and PRP are often focused on patient-reported outcome measures (symptoms) [53] or biomarker assessment [54]. To quantify these parameters in rodent studies, other outcome measures will be used such as gait analysis, pain assessment and serum and/or synovial biomarkers. On the flip side, patient reported outcome measures ascertain whether patients feel better with HA or PRP treatment, whereas in animal studies we can measure actual structural changes.

Conclusions

In this study, contrary to our hypothesis, there was no protective effect of HA-derivative (HYADD® 4-G) at two different concentrations (8 mg/mL and 15 mg/mL) at two different loadings (6N and 9N) and at two different time points (5 days and 56 days) on these parameters: cartilage proteoglycan loss, chondrocyte apoptosis, expression pattern of aggrecan, COMP, and CD44, synovitis, ectopic calcification, subchondral bone plate thickness. Bone volume trabecular fraction was found to be significantly different between loaded (9N group) and non-loaded joints. The repeated injections of HA-derivative at 15 mg/mL and HA-derivative at 8 mg/mL+PRP, could reduce but not significantly improve the bone changes. Additionally, the injections of only PRP conferred no therapeutic efficacy, and the results were not significantly different from PBS. Other potential treatment options need to be investigated to reduce the deleterious immediate and long-term effects of traumatic injury to the knee joint.
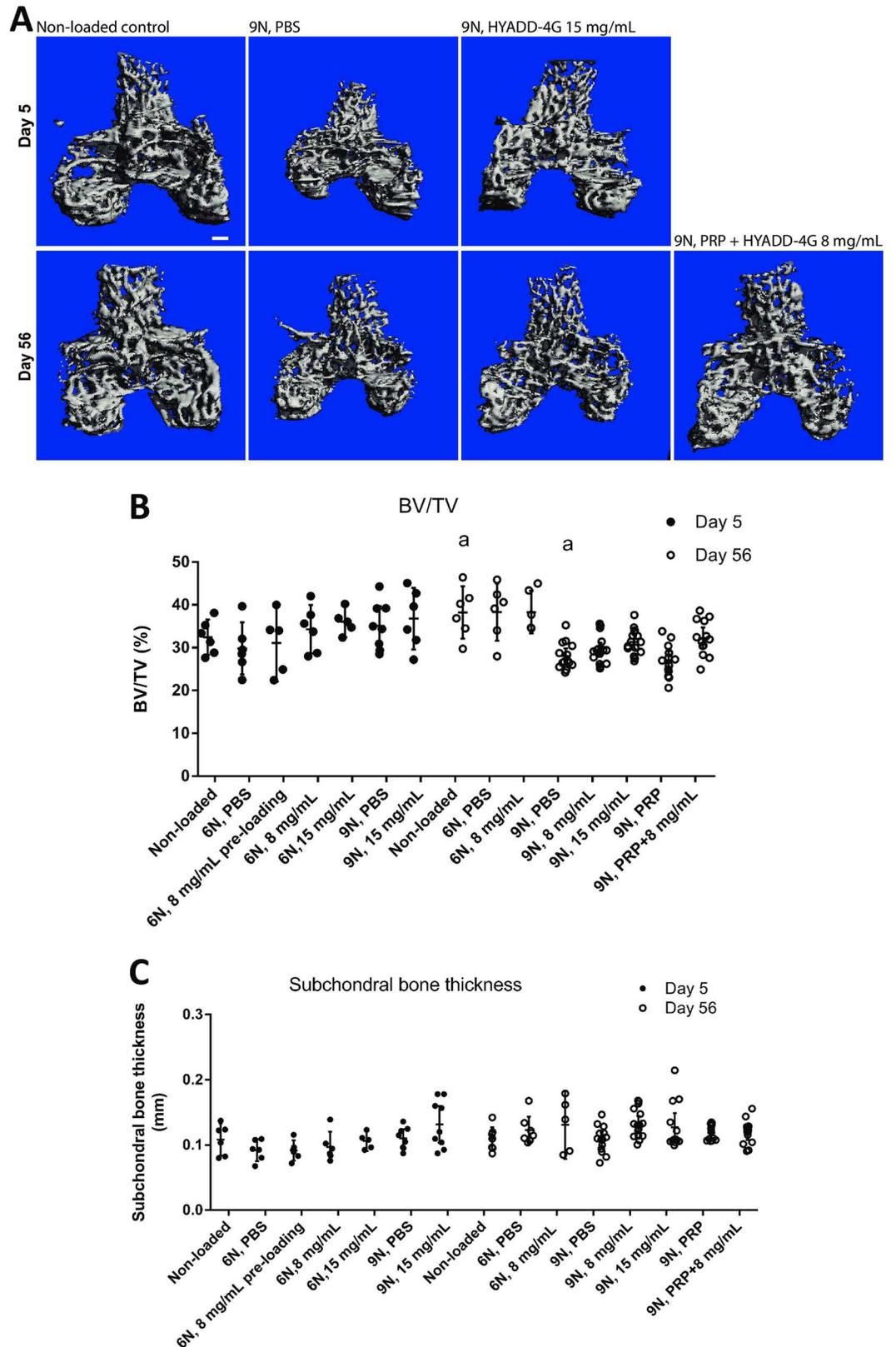
Fig 6. Micro-CT analysis. Micro-CT results showed no preventive effect of treatments on BV/TV at day 56 following loading. Trabecular bone was analyzed at the femoral epiphysis at day 5 (top row) and day 56 (bottom

row). All images are presented as superior views (A). Quantification of femoral epiphysis trabecular bone volume (BV/TV) showed that there was no significant difference between treatment groups and the PBS at this time point (B). a: P = 0.02. Interestingly, at day 56 (9N group) receiving Hyadd-4G at 15 mg/mL and PRP + HYADD® 4-G at 8 mg/mL group showed no statistical differences at the 56 days, non-loaded group. Quantification of subchondral bone thickness, showing no preventive effect of treatments on bone loss at 56 days after loading is presented (C). Error bars represent 95% confidence interval. PBS = phosphate buffered saline, PRP = platelet-rich plasma.

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Supporting information

S1 Fig. Non-immune controls for TUNEL assay and CD44 staining. A. Non-immune control for TUNEL assay. **B**. Non-immune control for CD44 staining. The area indicated by white dotted line boxes is shown at higher magnification on the right. The dotted lines indicate the edges of the cartilages. Bars = 100 μm.
(TIF)

S2 Fig. Split channels of CD44 staining. A. Images with split channels from Fig 4C. **B**. Images with split channels from Fig 4D. Red = aggrecan, green = CD44, blue = DAPI. Bars = 100 μm.
(TIF)

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Visualization: XD LJS NC NH MJS AS MFR.

Writing – original draft: XD LJS NC NH MJS AS MFR.

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