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Intra-Articular Injection of Chitin Nanofiber Attenuates Osteoarthritis: An Experimental Study in a Rat Model of Osteoarthritis

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ABSTRACT

Background This study aimed to evaluate the effect of chitin nanofibers (CNF) produced from crab shells as a medical material for the knee in an osteoarthritic rat model.

Methods The effect of intra-articular CNF injection was evaluated histologically among three groups: saline, hyaluronic acid (HA), and CNF injection groups. The Osteoarthritis Research Society International (OARSI) cartilage, subchondral bone, synovial, and meniscus scores were used for scoring.

Results At 4 weeks, the CNF group had significantly lower scores than the saline group. The Synovial score was lower in HA and CNF groups at 4 weeks than in the saline group. At 4 weeks post-treatment, the thickening of the subchondral bone plate and angiogenesis were significantly reduced in the CNF treatment group compared to those in the saline treatment group (P = 0.02).

Conclusion The anti-inflammatory effects of CNF on knee osteoarthritis were comparable to that of HA in the early stages.

Key words chitin nanofibers; osteoarthritic rat model

Intra-articular (i.a.) knee injection for osteoarthritis (OA) has been proven to be highly effective and safe,[1] and corticosteroids and sodium hyaluronate are frequently used. Unlike in Europe and the United States, sodium hyaluronate is registered as a therapeutic drug in Japan, not as an implant, and has been proven to be highly safe, with few reports of its side effects in various meta-analyses.[2] Sodium hyaluronate as a treatment for osteoarthritis comes mainly in an injectable, rather than oral form, and still holds a large share of the market today.[3] In support of this trend, a new sodium hyaluronate formulation containing diclofenac with enhanced anti-inflammatory properties has recently become commercially available.[4] In contrast, platelet-rich plasma therapy, which is positioned as regenerative medicine, has been gradually increasing in use for OA treatment in recent years. However, no alternatives to sodium hyaluronate-based agents have emerged in the past 30 years.

Chitin nanofiber (CNF) is a biomass preparation obtained by removing calcium and protein from chitin contained in crab shells and pulverizing them. It has a high dispersibility in water, is easy to process, and has excellent cost performance owing to the simplicity of the raw materials and manufacturing methods.[5] CNF is made from the 10 million tons of crab shells landed annually in the fishing ports of Tottori Prefecture, where Tottori University is located, a material that would typically be discarded. Thus, the pure cost of raw materials was zero. In recent years, the application of CNFs in medical formulations has been anticipated, and research has been conducted mainly at our university.[6] One reason is that animal experiments have shown that the expression of proinflammatory cytokines is reduced when CNF is administered to living tissues.[7] The fact that allergens below the detection limit can be removed through repeated deproteinization is another primary reason why it has attracted attention as a material that can be safely utilized in the medical field. We focused on the anti-inflammatory effects exerted by the direct exposure of CNFs to inflammatory tissues. We hypothesized that "i.a. injection of CNFs for OA suppresses local inflammation and inhibits the progression of OA." We conducted a study to prove this hypothesis by histologically examining the OA model induced by destabilization of the medial meniscus (DMM).[8]

MATERIALS AND METHODS

Experimental design

This study was conducted in accordance with the Japanese animal experiment regulations regarding animal experiments. The research protocol was approved by the Tottori University Animal Experiment Ethics Committee (17-Y-48) and conformed to the Tottori

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Abbreviations: CNF, chitin nanofibers; DMM, destabilization of the medial meniscus; HA, hyaluronic acid; i.a., Intra-articular; MML, medial meniscal ligament; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; TNF, tumor necrosis factor

University Animal Experiment Regulations. Forty-five retired bleeder Sprague-Dawley female rats (6 months old) (Charles River, Yokohama, Japan) were used. All rats were housed in plastic cages (three rats each) with ad libitum access to food and water. Lighting was controlled with a 12-h light/dark cycle, and the rearing temperature was maintained at 20–26 °C. The rats were randomly divided into three groups of 15 each for the saline, hyaluronic acid (HA), and CNF injection groups, and all rats underwent DMM surgery of the right knee.

· Saline® Group: 100 uL i.a. injection of Saline®
· HA® Group: 100 uL i.a. injection of HA®
· CNF Group: 100 uL i.a. injection of CNF

The animals underwent DMM surgery on the right knee and sham surgery on the left knee, leaving the joints intact. I.a. injections were administered in both knees weekly for 3 weeks postoperatively. Five rats in each group were euthanized 4 weeks after the first i.a. injection, another five were euthanized 8 weeks after the first i.a. injection, and the remaining five were euthanized 24 weeks after the first i.a. injection. The rats were observed daily for general health. Body weight was recorded daily preoperatively, during the first week, and once weekly until euthanasia.

A 1% concentration of HA is widely used,[9] and this concentration was used for CNF as well for comparison. As stated by Stitik[10] et al, the effect of intra-articular injections three times was evaluated; so the number of injections was set to three.

Surgical induction of OA

The DMM surgery was performed as previously described by Glasson et al.[8] Briefly, rats were sedated with sevoflurane and anesthetized with a subcutaneous injection of 2.0–2.5 mL of anesthesia mixed with midazolam, medetomidine hydrochloride, and butorphanol. Then the right knee was prepared for aseptic surgery. A longitudinal incision was made from the distal patella to the proximal tibial plateau of the right knee joint using surgical loupes. After blunt dissection of the fat pad, the medial meniscus and medial meniscal ligament (MML) were identified, and the MML was cut. This allowed the free DMM to be medially displaced. Finally, the surgical wound was closed in layers. The contralateral knee underwent a skin incision as a sham surgery. The rats were euthanized by gradually increasing the dose of CO2 to collect both knee joints.

Preparation of CNF

Chitin nanofibril gel (1%, pH 6; hereafter referred to as chitin nanofibrils) was prepared as previously described (Ifuku et al., 2009).[5] Chitin nanofibrils were prepared from dried crab shells by a simple pulverization process in an unseasoned state under acidic conditions after removing proteins and minerals (Marine Nanofiber, Tottori, Japan). The CNFs were frozen at 4 °C.

Histology

The entire bilateral knee joints were placed in 4% paraformaldehyde and demineralized in a solution of 5% formic acid and hydrochloric acid for approximately 36 h. The joints were washed with distilled water, dehydrated in a graded alcohol solution, clarified with permeating agent, and embedded in paraffin. Anterior sections (5 ± 1 μm) were taken at 30 μm intervals from the entire joint with a semiautomated microtome. For each joint, 30–35 slides were obtained. Eight slides were selected at the anteroposterior midpoint of the knee, five of which were stained with Safranin O/Fast Green and the remaining three with hematoxylin/eosin. Safranin O/Fast Green-stained slides were graded and averaged by two blinded independent investigators using their respective grading scores for the cartilage, subchondral bone, synovium, and meniscus. The cartilage score conformed to Gerwin's method [Osteoarthritis Research Society International (OARSI) cartilage score].[11] The articular surfaces of the medial and lateral femoral condyles and medial and lateral tibial condyles were divided into three equal parts, and the total score of each was used as the cartilage score. The subchondral bone score was consistent with Nagira's method.[12] Nagira's subchondral bone score was a total of 12 points, and the subchondral bone plate (Subcho.BP) (0–6 points) consisted of a combination of Subcho. BP.thickness (Subcho.BP.Th) and angiogenesis, bone volume (BV/TV) (0–3 points), and osteophytes followed by Kamekura's method (0–3 points).[13] This method scored osteophytes from 0 to 3: 0 = none; 1 = formation of cartilage-like tissue; 2 = increase in the cartilaginous matrix; and 3 = endochondral ossification. The synovial score conformed to Krenn's method.[14] The three evaluation sites were the medial recess, and the synovium of the femoral and tibial sides of the medial meniscus, and the average of each site was used as the synovial score. The meniscal score was calculated using the modified Kwok's method.[15] The total sum of the scores for all criteria (structure, cellularity, and matrix staining) in each medial and lateral menisci can range from 0 to 15. The sum of the points in the suitable coronal section of the medial and lateral middle segments was used.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 24 and R v.3.4.4. Data were reported as

beeswarm plots [mean and 95% confidence interval (CI)] or mean ± standard deviation (95% CI) at a significance level of $P < 0.05$. After checking for normal distribution (Shapiro-Wilk test) and uniformity of variance (Levene test), the data for the between-subjects factors comprising the design of the experimental model employed were analyzed. Nonparametric data were tested using the Kruskal-Wallis test. Pairwise comparisons were performed to estimate the factor effects and interactions between groups, and *p*-values were adjusted using the Bonferroni method.

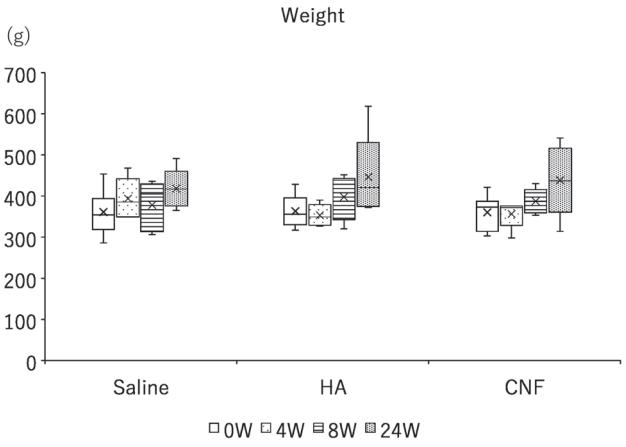

Fig. 1. Changes in weight after destabilization of the medial meniscus. A slight decrease in body weight was observed in some individuals at 4 weeks, but almost all individuals gained weight between 8 and 24 weeks. There were no obvious significant differences in the rate of change in body weight from surgery to euthanasia between the groups.

RESULTS

Clinical Observation

No experimental rats died during anesthesia induction or DMM surgery. A slight decrease in body weight was observed in almost all the animals during the first 2 weeks after surgery. All animals gained weight during the study period (Fig. 1). All animals were free of surgical wound infections, and none required additional procedures.

Histological Analysis of i.a. Tissues

Cartilage

Animals treated with saline, HA, or CNF and euthanized 4 weeks after the first i.a. injection showed different degrees of OA-like degenerative changes in response to treatment (Fig. 2). Knee joints treated with HA or CNF showed increased cartilage staining and reduced fibrillation, clones, and fibrous tissue compared to joints treated with saline. In animals euthanized 8 weeks after the first i.a. injection, joints treated with HA or CNF showed a decrease in grouped and hypertrophic chondrocytes and a decrease in fibrous tissue and fibrosis compared to the saline group. In the saline-treated knee joints, matrix cracks were observed from the surface to the midsection. In animals euthanized 24 weeks after the first i.a. injection, joints treated with HA, CNF, or saline showed decreased cartilage staining, loss of chondrocytes, and subchondral bone exposure. The OARSI cartilage score highlighted that CNF-treated knee joints presented lower scores than saline-treated joints (at 4 weeks: $P = 0.014$; at 8 weeks: $P = 0.036$)

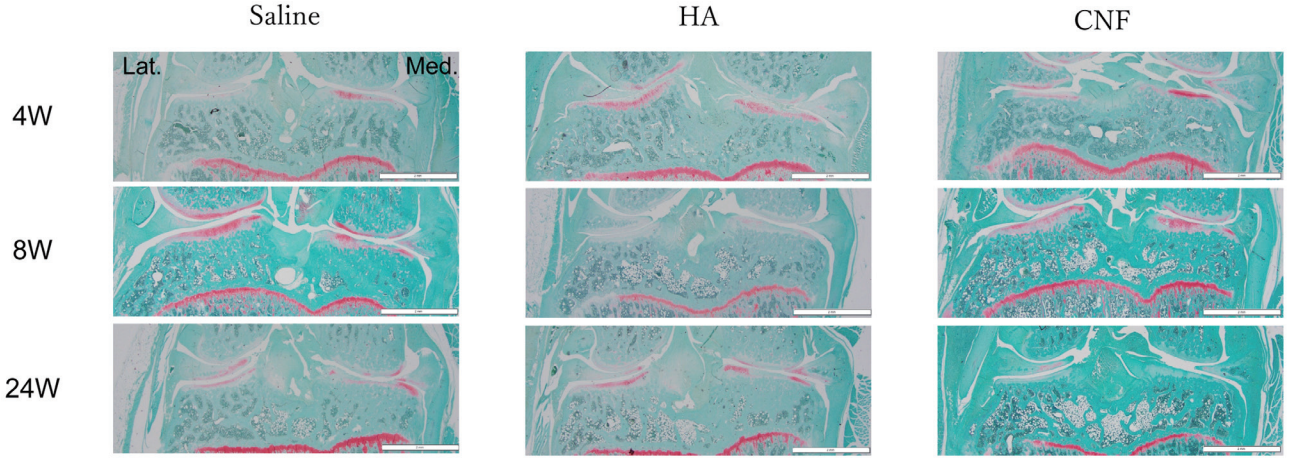

Fig. 2. Medial and lateral compartment of right knee joints after destabilization of the medial meniscus (DMM). The two groups injected with CNF or HA had less cartilage damage at 4 weeks after DMM than the saline group. Similar results were seen in the CNF group at 8 weeks. Twenty-four weeks later, osteoarthritis (OA) changes were more pronounced in all three groups. Bar = 2 mm. CNF, chitin nanofiber; HA, Hyaluronic acid.

Table 1. Results of cartilage, meniscus, synovial, subchondral bone scores at each time point after destabilization of the medial meniscus

		Time point	Saline	HA	CNF
Cartilage score (0–64)		4 w	25.2 (22–33)	18.4 (15–22)	13.8 (12–16)*
		8 w	31 (26–36)	23.8 (22–26)	21.4 (17–27)*
		24 w	41.8 (31–51)	30.8 (29–32)	38 (31–52)
Meniscus score (0–30)		4 w	21.4 (19–24)	23.2 (18–30)	23.4 (19–30)
		8 w	22 (20–28)	21.6 (18–29)	25.4 (22–30)
		24 w	28.8 (26–30)	27 (24–29)	28.2 (26–30)
Synovial score (0–9)		4 w	7.0 (6–8)	4.2 (3–5)*	3.8 (3–5)**
		8 w	4.4 (3–5)	4.6 (4–5)	4.0 (3–5)
		24 w	3.4 (3–4)	3.4 (3–4)	3.2 (2–5)
Subchondral bone score	Total (0–12)	4 w	8.4 (7–10)	8.0 (7–11)	6.4 (3–8)
		8 w	9.4 (7–11)	7.6 (6–9)	7.4 (5–10)
		24 w	9.8 (7–11)	10.6 (9–12)	11.2 (10–12)
	Plate (0–6)	4 w	4.8 (1–6)	4.2 (4–5)	3.0 (2–4)*
		8 w	5.2 (4–6)	3.8 (3–5)	3.4 (1–5)
		24 w	4.8 (4–6)	5.0 (3–6)	4.8 (2–6)
	BV/TV (0–3)	4 w	1.0 (0–2)	1.6 (0–3)	1.8 (0–3)
		8 w	2.0 (1–3)	0.8 (0–3)	2.0 (1–3)
		24 w	2.0 (0–3)	2.4 (2–3)	2.8 (2–3)
	Osteophyte (0–3)	4 w	2.4 (2–3)	2.2 (1–3)	1.8 (1–2)
		8 w	2.8 (2–3)	2.8 (2–3)	2.4 (2–3)
		24 w	3.0 (3)	2.8 (2–3)	2.8 (2–3)

The results were analyzed by Kruscal-Wallis test followed by Bonferroni post-hoc test for multiple comparisons. *$P < 0.05$, **$P < 0.01$. BV/TV, bone volume; CNF, chitin nanofiber; HA, hyaluronic acid.

(Table 1). In contrast, the knee joints treated with HA did not show lower OARSI cartilage scores than those treated with saline at 4 or 8 weeks ($P = 0.083$ and 0.062, respectively). There was no significant difference in the OARSI cartilage scores at 24 weeks in knee joints treated with HA, CNF, or saline, with an increase in the OARSI scores at 24 weeks.

Meniscus

There were no statistically significant differences in the meniscal scores between the three treatment modalities at any time point. After 4 weeks, all treatment groups showed moderate-to-remarkable changes.

Synovium

In the synovium, there was an expansion of the synovial lining cell layer and an increase in cell density after saline treatment at 4 weeks postoperatively (Fig. 3). However, synovial inflammation appeared to be reduced with HA and CNF compared to saline at 4 weeks. At 4 weeks, Synovial scores were predominantly lower for CNF- and HA-treated knee joints than for saline-treated joints (CNF: $P=0.01$; HA: $P=0.046$) (Fig. 4). However, there were no significant differences at 8 and 24 weeks (Table 1).

Subchondral bone

Subchondral bone scores increased in all treatment groups, with no significant differences at 4, 8, and 24 weeks (Table 1). However, at 4 weeks post-treatment, subchondral bone plate scores in the CNF treatment group were markedly lower than in the saline treatment group because the thickening of the subchondral bone plate and increased amount of angiogenesis were suppressed ($P = 0.02$) (Figs. 5, 6 and Table 2). BV/TV% showed a moderate-to-significant increase after 4 weeks in all treatment groups. Osteophyte formation with endochondral ossification was observed after 8 weeks in all treatment groups, with no significant between-group differences.

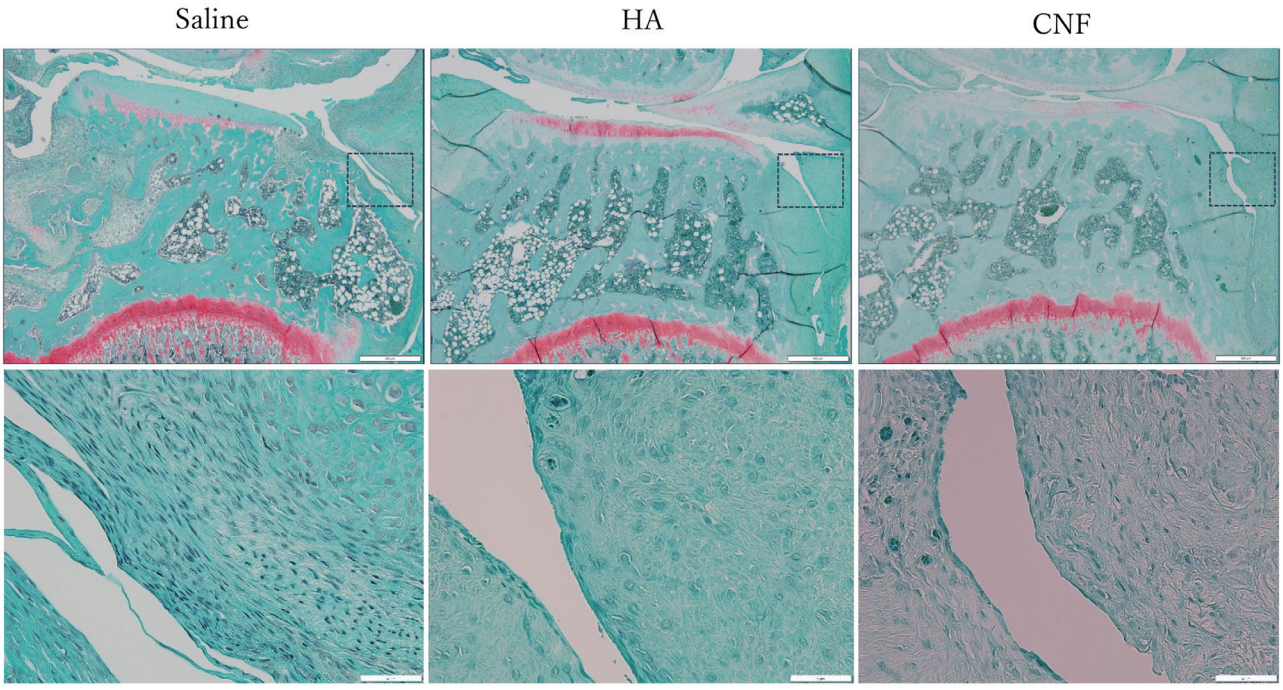

Fig. 3. Synovial membrane below the medial meniscus of the right knee at 4 weeks after destabilization of the medial meniscus (DMM). Hyaluronic acid (HA) injection and chitin nanofiber (CNF) injection resulted in less thickening of the synovial cell layer compared to saline injection. In addition, there was an increase in proliferation and cell density of synovial surface cells at 4 weeks after DMM in saline groups, whereas these changes were less pronounced in the CNF and HA groups. Bar of low magnifications = 500 μm, bar of high magnifications = 50 μm.

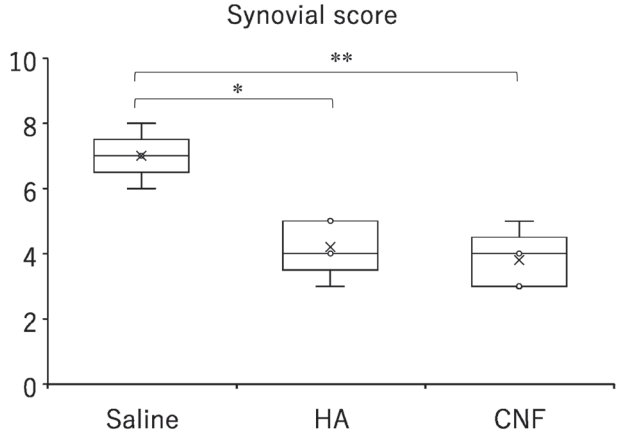

Fig. 4. Synovial score at 4 weeks after medial meniscus destabilization (DMM). The score at 4 weeks after DMM was significantly lower in the CNF group and HA group than in the saline group. *$P < 0.05$, **$P < 0.01$. CNF, chitin nanofiber; HA, Hyaluronic acid.

Osteophyte

Osteophyte thickness was increased in all groups at 4 weeks postoperatively up to 24 weeks (Table 1). There was no statistically significant difference in osteophyte score among treatment modalities. However, only in osteophytes of the knees after 4 weeks of CNF treatment showed that no specimens had reached 3 points of endochondral ossification at that time. Therefore, it is possible that osteophyte formation is suppressed compared to the other two groups.

DISCUSSION

Chitin is extracted from the outer skin tissue of crab shells as ultrafine chitin fibers. CNFs are fibrous materials that can be safely used in the medical field to remove allergens below detection limits through repeated deproteinization processes. CNF has been reported to have anti-inflammatory effects on skin keratinocytes and mucosal tissues.[16] However, no reports have examined the safety and efficacy of CNF administration in i.a. tissues. To the best of our knowledge, this is the first detailed examination of the efficacy of CNF administration in a knee OA model.

The expected therapeutic effects on knee OA include anti-inflammatory effects and protection of cartilage tissue. Recent reviews have suggested that the mechanism of OA progression may be a vicious cycle of cartilage destruction, in which overload and inflammation cause a decrease in the extracellular matrix and chondrocytes, inducing subchondral bone remodeling;

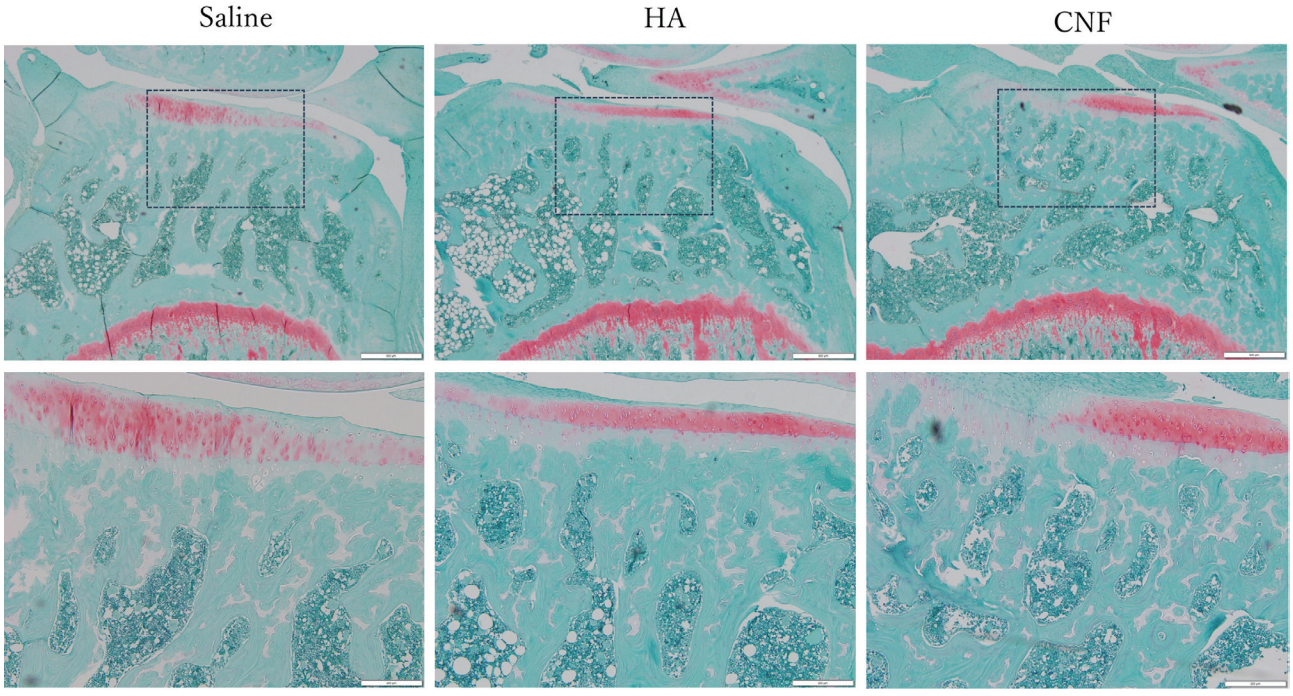

Fig. 5. Comparison of subchondral bone plate thickness of right knee at 4 weeks after medial meniscus destabilization. The CNF group showed less subchondral bone thickening and angiogenesis as well as fewer chondrocytes on the medial articular surface compared to the saline group. Bar of low magnifications = 500 μm, bar of high magnifications = 200 μm. CNF, chitin nanofiber; HA, Hyaluronic acid.

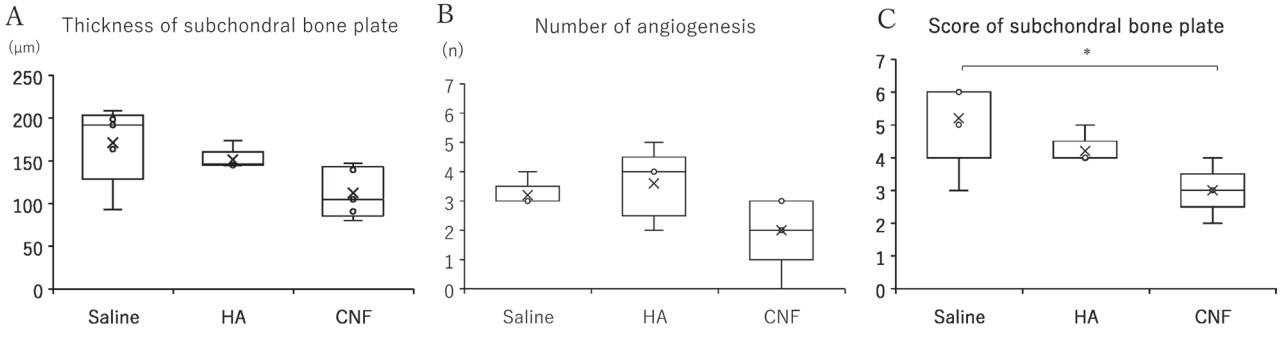

Fig. 6. Quantitative values and scores for each parameter of subchondral bone plate at 4 weeks after medial meniscus destabilization (DMM). **A**: Thickness of subchondral bone plate; **B**: number of angiogenesis in subchondral bone plate; **C**: score of subchondral bone plate. According to Nagira's system the subchondral bone plate score is evaluated by a combination of the percentage increase in subchondral bone thickness and the amount of angiogenesis. The score of subchondral bone plate at 4 weeks after DMM was significantly lower in the CNF group than in the saline group. *$P < 0.05$. CNF, chitin nanofiber; HA, Hyaluronic acid.

cytokines produced from these cells stimulate the production of inflammatory mediators in synovial fibroblasts.[17] This study's histological evaluation of synovitis showed that the CNF-treated group demonstrated the same suppression of synovitis as the HA-treated group in the short term after treatment. However, in the mid-to-long term, the therapeutic effect disappeared, and the duration was comparable to that of HA. In contrast, the subchondral bone score, which comprehensively measures subchondral bone thickening and angiogenesis, increased BV/TV%, and increased osteophyte formation was not significantly different between the treatment groups. However, subchondral bone thickening and angiogenesis were suppressed simultaneously with the suppression of synovitis.

Various mechanisms of action for high-molecular-weight HA in preventing arthritis have been reported.[18] Among them, the anti-inflammatory effect on local synovitis of OA is reported to be mediated by toll-like receptors (TLRs) and CD44 and downstream by

Table 2. Quantitative value of each parameter of subchondral bone scores except for osteophytes at each time point after destabilization of the medial meniscus

Parameters	Time point	Saline	HA	CNF
Plate thickness (μm)	4 w	171 (93–209)	151 (145–174)	113 (80–147)
	8 w	181 (130–225)	213 (168–252)	163 (97–231)
	24 w	200 (142–240)	211 (143–262)	205 (148–312)
Angiogenesis (*n*)	4 w	3.2 (3–4)	3.6 (2–5)	2.0 (0–3)
	8 w	4.4 (3–7)	3.0 (0–5)	3.2 (0–5)
	24 w	0.4 (0–2)	1.2 (0–3)	0.2 (0–1)
BV/TV (%)	4 w	63 (51–73)	74 (59–88)	75 (57–94)
	8 w	82 (68–94)	70 (53–95)	76 (65–85)
	24 w	80 (57–94)	87 (77–99)	86 (71–99)

BV/TV, bone volume; CNF, chitin nanofiber; HA, hyaluronic acid.

nuclear factor kappa B (NF-κB), interleukin (IL)-6, and tumor necrosis factor (TNF).[19, 20] However, the pathways through which CNFs exert their effects and mechanisms of action remain unclear. Since suppression of NF-κB activity has been mentioned in the analysis of anti-inflammatory mechanisms in mucosal tissues,[16] we speculated that the i.a. administration of CNF to the knee OA model in this study may have suppressed inflammatory responses through a cascade mediated by CD44, TLR, and other molecules. Conversely, direct spraying of CNF on human skin tissue was reported to widely reduce the expression of IL-1α, IL-1β, IL-6, IL-8, and TNF-α from skin cells,[21] suggesting that CNF may inhibit the production or activity of inflammatory cytokines in synovial cells.

Hepatocyte growth factor production in OA subchondral bone and transforming growth factor-β promoting matrix metalloproteinase-13 synthesis in chondrocytes have been mentioned as part of the mechanism of OA progression.[22] In the present study, it was not clear whether CNF directly inhibited OA changes in the subchondral bone. However, from CNF synchronization with the inhibition of inflammatory changes in the synovial tissue, it was inferred that CNF reduced subchondral bone changes by inhibiting the production and activation of inflammatory mediators.

The OA model used in this study showed no allergic reactions to i.a. injections in any treatment group. Chitin/chitosan must be considered the safest product because hypersensitivity reactions to crab shells can cause severe conditions.[23] CNF can be safely administered using nanofiber technology to remove allergenic substances in crab shells, which theoretically prevents antigen-antibody reactions.[24] However, like hyaluronan, CNF is a protein that is foreign to the body; therefore, a foreign-body reaction may occur. It is necessary to evaluate the biological reaction in the case of short-term administration of a small number of doses and the case of long-term administration or multiple doses.

This study demonstrated the effects of CNF in inhibiting inflammatory changes and slowing the progression of cartilage tissue destruction in an OA model. However, because immunostaining was not performed, we could not evaluate whether the effect was selective in the OA progression cascade. In addition, an effective method of CNF administration has not been established. Therefore, since CNF in this study was administered only in the early stages of knee OA at small doses, the therapeutic effects of other administration methods should be investigated. The treatment protocol for the OA model in this study may be expected to be at least as effective as in the HA treatment group. Aklog et al reported that CNF can be produced from crab shells, a prime source because it is derived from discarded previously living organisms, which can be procured at a relatively low cost, about 5,000 Japanese yen per kilogram.[25] Using CNF as a medical resource is an issue for future research, but it has the potential of being processed and used at a lower cost than hyaluronan.

There are some limitations in this study. We can't find any reports on experiments in which CNF has been injected intra-articularly in living organisms. The normal pH in knee joints is reported to be around 7.3, and it is reported to decrease under inflammatory conditions.[26] The CNF used in this study was adjusted to a pH of 6.0–7.0, which is close to neutral. In fact, the synovitis score of the chitin group at 4 weeks was lower than that of the saline group, suggesting that there might be no negative effect of intra-articular administration of chitin nanofibers. Here, we set the CNF concentration as

noted in a previous paper.[27] The optimal intra-articular administration of chitin nanofibers for OA prevention effect is a future issue.

In conclusion, few i.a. doses of CNF for early OA joints showed chondroprotective and anti-inflammatory effects without adverse events. We believe that accumulating preclinical data is urgently needed, as CNF can be purified inexpensively, which will undoubtedly contribute to reducing therapeutic resources. We believe this study contributes to developing novel disease-modifying therapies for OA.

AUTHORS' CONTRIBUTIONS

ME and KN designed the study. MO and KN performed the experiments. KN and MO analyzed the data. ME, KN, and MO wrote the paper. KN and MO collected and assembled the data. KN has statistical expertise. HN revised the manuscript critically. HN and ME approved the final manuscript. All the authors have read and approved the submitted version of the manuscript.

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The authors declare no conflict of interest.

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