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学 位 論 文

Study on the local administration of the nerve growth factor antibody on knee joints' pain in a rat osteoarthritis model

(ラット変形性関節症モデルの膝関節痛に対する神経成長因子抗体局所投与に関する研究)

2020 年 9 月

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Tian Y., Onodera T., Terkawi M. A., Iwasaki K., Hishimura R., Miyazaki T., Liang D.W., Iwasaki N.

Local administration of low-dose nerve growth factor antibody reduced pain in rat OA model [Osteoarthritis and Cartilage] (Under Review)

List of Presentations

1 Tian Y., Onodera T., Terkawi M. A., Iwasaki K., Hishimura R., Miyazaki T., Liang D.W., Iwasaki N. Effects of nerve growth factor antibody intra-articular injection in an osteoarthritic rat model

136th Hokkaido Society of Orthopaedics and Traumatology. January 26, 2019. Sapporo, Japan.

2 Tian Y., Onodera T., Terkawi M. A., Iwasaki K., Hishimura R., Miyazaki T., Liang D.W., Iwasaki N. Local administration of low-dose nerve growth factor antibody reduced pain in rat osteoarthritis model. 15th International Cartilage Regeneration & Joint Preservation Society World Congress. October 6. Vancouver, Canada.

3 Tian Y., Onodera T., Terkawi M. A., Iwasaki K., Hishimura R., Miyazaki T., Liang D.W., Iwasaki N. Local administration of low-dose nerve growth factor antibody reduced pain in rat OA model. The 34th Annual Research Meeting of the Japanese Orthopaedic Association. October 17. Yokohama, Japan.

Summary

Background and Purpose:

Osteoarthritis (OA) is the most common arthritis which contributes to an economic burden on patients and society. Pain is an important symptom of all clinical manifestations of OA, causing poor quality of life, social labor loss, and long-term hospitalization. Current pharmacological treatment for osteoarthritis pain are partly effective and accompanied by serious adverse effects. Recently, a humanized IgG2 monoclonal nerve growth factor (NGF) antibody, which has been used as an analgesic agent for OA, has gained a great achievement in relieving osteoarthritic pain of patients in clinical trial. Single intravenous injections of NGF antibody can effectively relieve joint pain in patients with OA of knee for weeks. However, the clinical experiment of tanezumab (commercial anti-NGF antibody) has been on hold by Food and Drug Administration, because of the occurrence of adverse effect that most patients presented progressively worsening OA resulting in requirement of total joint replacement. Also, the high frequency of adverse effects occurred after systemic administration of tanezumab such as paresthesia, arthralgia, pain in extremity, and headache also remains safety concern for patients.

Even so, because of the high effectiveness of anti-NGF treatment for reliving OA pain, researchers keep focusing on developing the anti-NGF treatment. Since OA only affects limited number of joints, intra-articular injection therapy appears to be more attractive alternative for patients. Local injection can largely decrease the risk of systemic exposure and the incidence of adverse effects and release an economic burden on patients. In fact, local injection can minimize adverse effects generated by given in lower concentrations. Therefore, we hypothesized that intra-articular injection of anti-NGF antibody with a lower dose may reduce osteoarthritic pain and avoid the systemic adverse effects. The purpose of our study is to evaluate the efficacy of intra-articular injection of nerve growth factor antibodies using a rat osteoarthritic model.

Materials and Methods:

OA-like pain model was established by single injection of 0.5 mg mono-iodoacetate (MIA) into right knee joints of 8-week-old male sprague dawley rats. Saline and different dose of anti-NGF antibody were injected into right knee once a week from the end of week 2 after injection of MIA. Evaluations included behavior tests, macroscopic scoring and histological evaluations. Behavior tests including weight-bearing test and von Frey filament test, were performed before and after MIA injection and continue for 6 weeks. Rats were sacrificed at the end of week 6

after MIA injection and the right knee joints were collected. Macroscopic score was performed right after collection and evaluated by Likert scale. The degeneration of cartilage was microscopically examined and scored by Mankin scoring. Fluorescent staining was performed to confirm the expression of NGF.

Results:

Florescent staining indicated that MIA injection can increase the concentration of NGF in the synovium tissue surrounding affected joints. Injection of anti-NGF antibody neutralized the NGF in tissue and therefore reduced the signal of NGF staining. Results of behavior tests showed that MIA injection can cause impaired weight bearing ($P<0.005$) and allodynia ($P<0.01$) as signs of pain from 1st week. 100 μg anti-NGF antibody treatment relieved the pain of rat OA model as evidenced by weight bearing performance but not allodynia.

Results of macroscopic evaluations showed that MIA injection can induced damage of cartilage and the injection of anti-NGF antibody has no obvious adverse effects on joint. Consistent with these results, histological evaluations of hematoxylin and eosin and safranin O showed that injection of anti-NGF antibody has no negative effects on cartilage pathological progression.

Results showed that MIA injection impaired weight bearing and allodynia as signs of pain. Of note, anti-NGF antibody treatment relieved the pain of rat osteoarthritis model as evidenced by improved weight bearing performance but not allodynia. In contrast, no significant differences were observed in macroscopic and histological scores of knees in anti-NGF antibody-treated rats and controls.

Discussion:

Anti-NGF antibody has recently been used as an analgesic drug for chronic pain, but the adverse effects of the progressively worsening cartilage degeneration are particularly considered to be problematic for OA. According to the evaluation of safety data of systemic treatment of anti-NGF antibody, the issues associated with safety were detected in both clinical and nonclinical. In this study, we found that low dose of 0.1 mg nerve growth factor antibody could alleviate the MIA-induced pain without deterioration of OA, suggesting that intra-articular administration of anti-NGF antibody may be an effective treatment for OA, especially in mono- or oligo-articular OA.

Moreover, intra-articular injection can reduce the effective dose of anti-NGF antibody (0.1 mg / knee) compared to the dose used for systemic injection. Systemic treatment of anti-NGF antibody for chronic pain using animal osteoarthritic model usually requires 10 mg/kg,

approaching to 5mg/ injection. Intra-articular injection has been widely used as cost-effective treatment for OA comparing with systemic treatment. This direct delivery of drugs requires low effective dose which can decrease the risk of side effects and damage to other unimpaired tissue. Therefore, we can assume that local treatment of anti-NGF antibody may be an appropriate strategy to reduce the dosage and thereby reduce the incidence of side effects and the exposure to the whole body. This is the first study to elaborate this hypothesis.

Systemic injection is known to be able to improve both weight-bearing asymmetry and mechanical allodynia, the intra-articular injection of anti-NGF antibody only showed effect on weight bearing while didn't present any effect on improving allodynia of rat hind paw which induced by MIA injection. Mechanical allodynia is associated with the involvement of alterations in mechano-transduction and sensory neurons in the central nervous system. Our current results suggest that intra-articular injection of anti-NGF antibody may provide insufficient analgesic effect on allodynia-related pathologies such as complex regional pain syndrome. Whether local injection of anti-NGF antibody can effectively reverse the central nervous system changes established during the NGF rising phase needs further exploration and experiments.

An intra-articular injection treatment may have some disadvantages such as infection, rejection reaction, damage of tissue. Also, the progression of OA pathological such as cartilage degeneration still needs to be treated. More experiments is required to clarify The mechanism of anti-NGF antibody does not suppress allodynia, but does not exacerbate OA.

Conclusions:

The conclusion of this research is that intra-articular injection largely reduced effective dose of anti-NGF antibody as compare to systemic injection without accelerating the OA progression. This might be alternative approach to overcome the drawback of systemic injection for treatment of OA patients. Further experiments are needed to improve intra-articular injection treatment of anti-NGF antibody and elucidate the mechanism of allodynia in OA.

List of Abbreviations

ACLT	anterior cruciate ligament transection
CNS	central nervous system
CRPS	complex regional pain syndrome
H&E	Hematoxylin and Eosin
MIA	mono-iodoacetate
NGF	nerve growth factor
OA	osteoarthritis
SD	Sprague Dawley

Introduction

OA is the most common type of arthritis that affects more than 300 million people globally and contributes to an economic burden on both patients and the society (Kloppenburg, M. and Berenbaum, F.,2020, p. 242; Chua, J.R., et al, 2019, p. 1276). According to the recent Osteoarthritis Research Society International white paper, OA has been considered a serious disease because of the lack of efficient treatments for OA (March, L., 2016, p. 1). Clinically, the symptoms of OA includes pain, joint stiffness, and disability. Pain is particularly important in all clinical problems as it is not only the cause of hospital visits for treatment, but also the main cause of poor quality of life and social labor loss (Perrot, S., 2015, p. 90). Current pharmacological treatment for OA pain using traditional analgesics, such as non-steroidal anti-inflammatory drugs and acetaminophen, is partly effective and accompanied by serious side effects such as disruption of the gastrointestinal mucosa and ulceration, cardiovascular toxicity and suppression of platelet aggregation (Krebs, E.E., et al, 2018, p. 872; da Costa, B.R., et al, 2017, p.e21). Therefore, more effective treatments to relive OA pain are required.

Recently, a humanized immunoglobulin G2 monoclonal NGF antibody, which has been used as an analgesic agent for OA, has gained a significant relevance in relieving OA-associated pain in a clinical trial (Lane, N.E., et al, 2010, p. 1521). Single intravenous injections can effectively relieve chronic pain in patients with OA of the knee joint and be effective for several weeks. However, the clinical phase III experiment of tanezumab (commercial name of anti-NGF antibody) has been on hold by the Food and Drug Administration in 2010 because of its adverse effect, with most of the patients presenting with progressively worsening OA and subsequently requiring total joint replacement (Lane, N.E., et al, 2010, p. 2248). Moreover, other adverse effects, such as paresthesia, arthralgia, pain in the extremity, and headache, were also observed after the systemic administration of tanezumab, and these effects remain a safety concern for patients (Kivitz, A.J., et al, 2013, p. 1009; Katz, N., 2011, p. 2248).

Even so, because of the high effectiveness of anti-NGF antibody treatment for relieving

chronic pain such as OA pain, researchers keep focusing on developing the anti-NGF antibody treatment. As OA only affects a limited number of joints, intra-articular injection therapy appears to be a more attractive alternative treatment for patients compared with other alternative treatments (Jones, I.A., et al, 2019, p. 77). Local injection can largely decrease the risk of systemic exposure and the incidence of adverse effects. Moreover, local injection can reduce the effective dosage, possibly preventing the aggravation of adverse effects and reducing the economic burden on patients (Evans, C.H.,2014, p. 11; Rosen, J., et al, 2016, p. 998). Therefore, the local administration of anti-NGF antibody, such as its intra-articular injection into the OA joints, might be a preferable way to maintain its effectiveness for the treatment of chronic pain and to reduce the incidence of adverse effects.

However, the hypothesis regarding the local treatment of anti-NGF antibody has not been verified yet. Hence, this study aimed to investigate whether the low-dose intra-articular injection of anti-NGF antibody can both reduce OA pain and avoid the adverse systemic effects using a rat OA model.

Methods

Ethics statement

All procedures for animal experiments were approved by the Institute of Animal Care and Use Committee of the Hokkaido University Graduate School of Medicine (no. 17-0136).

For establishing the OA pain model

The ACLT model is most used model for an OA induced animal model because that the results are highly reproducible and the most consistent OA pathogenesis (Pickarski, M., et al, 2011, p. 197). However, the ACLT surgery will ruin the integrity of joints and affect the results of intra-articular injection. MIA-induced model has been widely used as an OA pain model and the pathological process of arthritis is dose- and time-dependent. Because of this, we can control the damage of cartilage into an appropriate progression to observe the effects of NGF antibody

on the osteoarthritic progression (Guingamp, C., et al, 1997, p.1670). For observing the analgesic effect of NGF antibody and pathological progression changes, the appropriate dose of MIA and the injection time point should be decided. M Udo et al suggested that the injection of MIA into SD rat can induced mild cartilage degeneration and less bone destruction comparing with other commonly used species of experimental rat (Udo, M., et al, 2016, p.1284). Mild damage of joints is appropriate for observing the changes of cartilage damage and keeping high reactivity to the analgesic. The usual dose of MIA is 1mg/25μl, but this dose can induce serious cartilage degeneration in 2 weeks (Mapp, P.I., et al, 2013, p.1336). Whether lower dose, 0.5 mg/25μl can induce SD rats' pain behavior and observable arthritis progression still needs experiments to be decided. Therefore, the pre-experiments were performed for choosing the dose of MIA and time points for injection of anti-NGF antibody.

Eight-week-old male SD rats (CLEA Tokyo, Japan) were group housed on a 12 h light/dark cycle, had free access to food and water. Rats were anaesthetized and 0.5 mg and 1.0 mg of MIA (dissolved in 25 μl saline solution, Nacalai tesque, Kyoto, Japan) were injected one time into rat right knee joint capsule through the infrapatellar ligament (Figure 1).

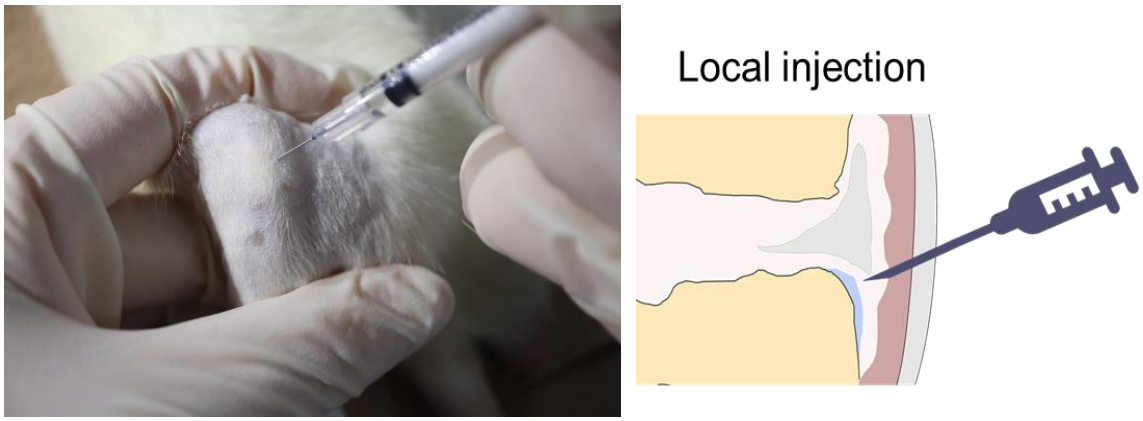

Figure 1. intra-articular injection through the infrapatellar ligament

The rats were sacrificed at the end of 1st week, 2nd week and 6th week (n=3) and the right knee joints were collated and evaluated by macroscopic score and histological score. Behavior

test were performed 2 times a week (Figure 2).

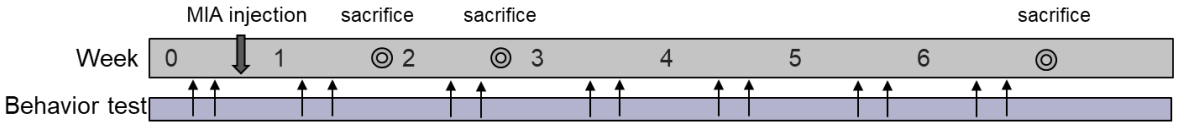

Figure 2. Schedule of MIA injection, pain behavior test and sacrifice. 0.5 mg and 1.0 mg of MIA were injected one time into rat right knee joint. Rats were sacrificed at the end of 1st week, 2nd week and 6th week (n=3). Behavior tests were performed 2 times a week.

Behavior tests

For defining pain behaviors in an in vivo model, the weight bearing of two limbs changes were utilized as an index of joint discomfort in the osteoarthritic knee (Bove, S.E., et al, 2003, p. 821). Weight bearing changes in hind paw represent the weight distribution between the right (osteoarthritic) and left (contralateral control) limbs as a direct index of joint pain in the osteoarthritic knee (Iwasaki, K., et al, 2016, p. 2026). A static weight-bearing test device (Bioseb, Chaville, France) was used to determine the hind paw weight distribution. Rats were placed in an angled chamber so that each hind paw rested on a separate force testing plate (Figure 3). The force exerted by each hind limb (measured in grams) was averaged over a 5-s period. Each data point was the mean of the three-time test of 5-s readings. Results were presented as the distribution in weight bearing between the left (control) limb and right (osteoarthritic) limb as calculated by the following equation: weight of treated leg/weight of both legs ×100%.

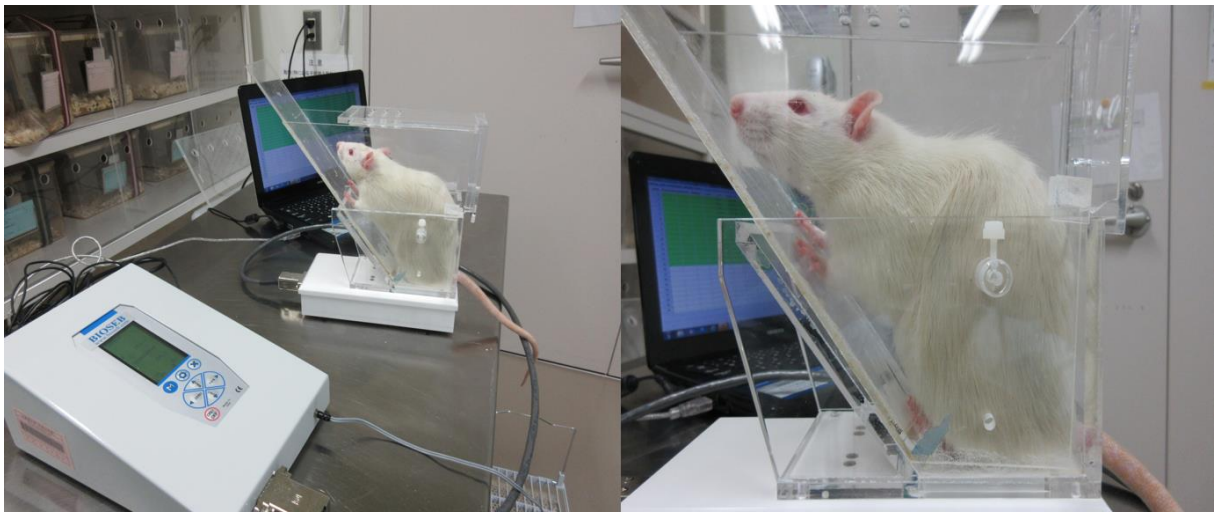

Figure 3. Incapacitance tester for determination of hind paw weight distribution.

Von Frey filament (SHINFACTORY, Fukuoka, Japan) was used to measure allodynia which induced by mechanical stimulate (Pitcher, G.M., 1999, p.185). Unanesthetized animals were placed in a chamber with a mesh bottom, which allowed access to the plantar surface of each hind paw (Figure 4). The animals were allowed to acclimatize in the chamber for 10 mins before testing. The mechanical threshold of the ipsilateral hind paw was assessed using the modified up–down method (Chaplan, S.R., et al, 1994). A von Frey hair was applied perpendicularly to the plantar surface of the ipsilateral hind paw until the hair flexed and subsequently held in place for 3 s. The von Frey hair (range, 0.4-60.0 g) was applied in ascending order to observe the withdrawal reaction of rats. If a rapid withdrawal reaction was observed, the von frey hair was subsequently applied in descending order until the withdrawal reaction was no longer observed. This method was repeated three times with an interval of 10 min. Allodynia was confirmed by the decrease of mechanical threshold.

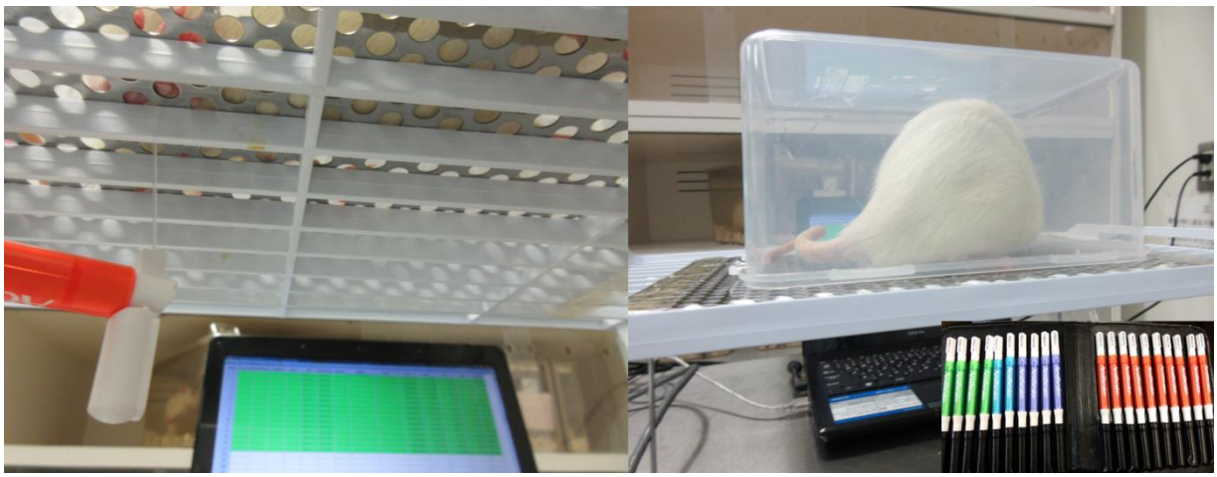

Figure 4. Von Frey filament for measuring allodynia which induced by mechanical stimulate

These two kinds of behavior tests are most used in many researches of pain behavior evaluations in animal osteoarthritic models (Pitcher, T., et al, 2016, p.185; Combe, R., et al, 2004, p. 236).

MIA-induced rat OA pain model

Eight-week-old male Sprague–Dawley rats (CLEA Tokyo, Japan) were grouped and housed on a 12-h light/dark cycle and had free access to food and water. Furthermore, 0.5-mg mono-iodoacetate (MIA) (dissolved in 25-µl saline solution, Nacalai Tesque, Kyoto, Japan) was injected one time into the rats' right knee joint capsules through the infrapatellar ligament to induce OA-like pain in the rats' knee joints (Mapp, P.I., et al, 2013, p.1336). Same volume of saline was injected into rats' right knee joints as sham treatment. To confirm the effect of MIA, behavioral tests including of the weight-bearing test and von Frey filament test were performed before and after MIA injection and were continuously performed for 6 weeks. Rats were randomly divided into 6 groups including 4 MIA injection groups and 2 sham groups (saline injection). Each group contained a minimum sample size of 6 rats.

Treatment of anti-NGF antibody

To observe the effect of the local administration of anti-NGF antibody on OA pain, saline

and different doses (1 μg, 10 μg, and 100 μg dissolved in 25- μl saline solution) of anti-NGF antibody (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) were injected into the rats' right knee joint capsules through the infrapatellar ligament at week 2 after MIA injection (MIA groups). In order to observe the effect of the local administration of anti-NGF antibody on rats' knee joints tissues, saline and 100 μg anti-NGF antibody were intra-articularly injected into the right knee joints of rats received saline injection (sham groups). The injection was performed one time a week for four times until the end of 6th week.

Macroscopic and Histological evaluations

Rats were sacrificed at the end of 6th week after MIA injection. Right knee joints were collected and fixed in 10% formalin. Macroscopic score was performed right after collection and evaluated by Likert scale (table 1, Guingamp, C., et al, 1997, p.1670).

Table 1 – Likert scale (Guingamp macroscopic lesions)

Grade
0 = normal appearance
1 = slight yellowish discoloration of the chondral surface
2 = little cartilage erosion in load-bearing areas
3 = large erosions extending down to the subchondral bone
4 = large erosions with large areas of subchondral bone exposure.

For conducting the knee joint histological examination, knee joints were decalcified in decalcifying solution B (Wako, Osaka, Japan) for 3 d before embedded in paraffin. Sections of 5 μm size were prepared and subjected to staining with hematoxylin eosin and safranin O. The degenerations of cartilage were microscopically examined using an all-in-one microscope (Keyence, Osaka, Japan) and scored by modified Mankin scoring system (table 2, Mankin, H.J., et al, 1971, p.523).

Table 2 – modified Mankin scoring system

Cartilage structure

Normal	0
Surface irregularities	1
Pannus and surface irregularities	2
Clefts to transitional zone	3
Clefts to radial zone	4
Clefts to calcifiied zone	5
Complete disorganization	6
Cartilage cells	
Normal	0
Pyknosis, lipid degeneration hypercellularity	1
Clusters	2
Hypocellularity	3
Safranin-O	
Normal	0
Slight reduction	1
Moderate reduction	2
Severe reduction	3
No staining	4
Tidemark integrity	
Intact	0
Destroyed	1

Immunofluorescence staining

For 1 h, 5-μm sections of knee joints were prepared and blocked with horse serum. Subsequently, the sections were incubated overnight at 4°C with a primary NGF antibody (1:500, Mochida Pharmaceutical Co., Ltd.). The primary antibodies were detected by treatment with goat anti-mouse Alexa Fluor Plus 594 (Invitrogen, Waltham, MA USA) for 60 min at 37°C. The nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (Invitrogen). Finally, the sections were mounted, covered by cover slides, and examined using a fluorescence microscope (Keyence).

Statistical analysis

All data are presented as means ± standard errors of the means. For behavior evaluations, Two-way analysis of variance followed by Tukey's test was used for comparison of each group.

For macroscopic and histological evaluations, one-way analysis of variance followed by Tukey's multiple-comparison procedure was used to compare differences among groups (GraphPad Software, La Jolla, CA, USA). Results presented as means ± standard errors of the means were considered statistically significant when $p < 0.05$.

Results

Results of pre-experiment

The behavior evaluations showed that both 0.5 mg and 1.0 mg MIA injection can induce pain behavior represented by weight bearing asymmetry and mechanical threshold reduction from 1st week (Figure 5). The results showed that 0.5 mg MIA is enough to induce pain behaviors in rats.

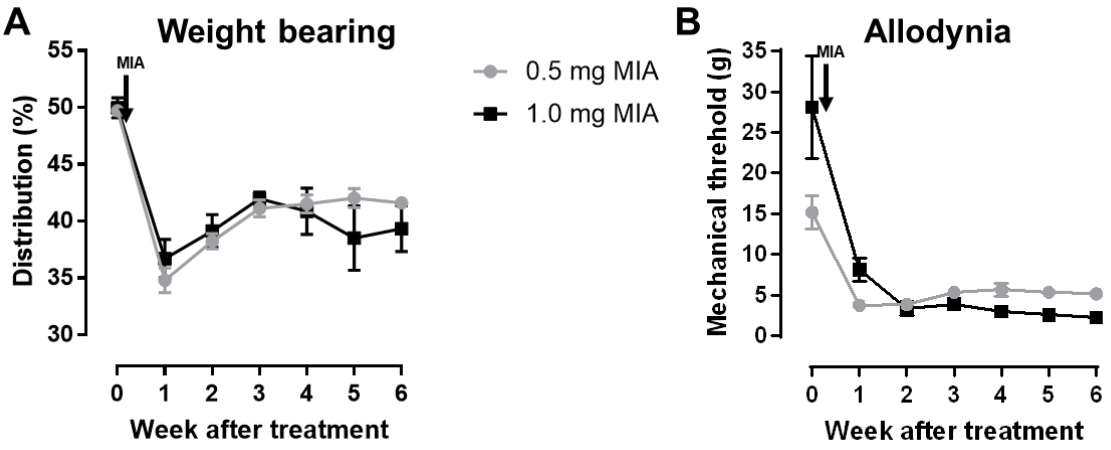

Figure 5. Behavior evaluations of rats received 0.5 mg and 1.0 mg MIA injection. (A) MIA injection can induce weight-bearing asymmetry. The results represent as means ± standard errors of the means for the 3 rats. (B) MIA injection had significantly low rat's hind paw withdrawal mechanical thresholds. The results represent as means ± standard errors of the means for the 3 rats.

The results of macroscopic scoring showed that both 0.5 mg and 1.0 mg MIA injection can induce cartilage degeneration (Figure 6A). However, 1.0 mg injection caused severe OA

typified by rapid deterioration that extended to subchondral bone tissue. While in 0.5 mg group, only mild and slow developing but observable cartilage degeneration was presented. At the end of 6th week, significant worse (p=0.0009) articular cartilage damage can be observed in 1 mg group. (Figure 6B).

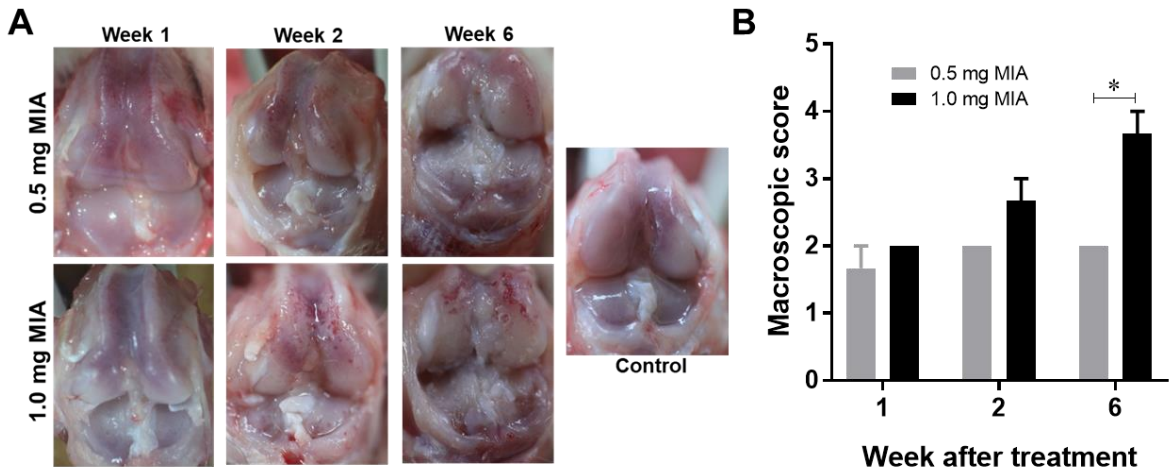

Figure 6. Macroscopic evaluation of knee joints from rats received 0.5 mg and 1.0 mg MIA injection. (A) The macroscopic figures showed that injection of 0.5 mg MIA can induce mild cartilage degradation while in 1.0 mg MIA injection group, severe damage of cartilage can be observed at week 2. (B) Macroscopic score using Likert scale showed that injection of NGF antibody reveal that 1.0 mg MIA injection induced rapidly progressing cartilage degeneration. 0.5 mg MIA injection induced slowly progressing cartilage degeneration. The results represent as means ± standard errors of the means for the 3 rats. * indicates a significant difference, as determined by one-way analysis of variance, followed by the Tukey's multiple-comparison procedure ($p \leq 0.05$).

Consistent with these results, histological results showed that cartilage degeneration started from 1st week in 1.0 mg MIA injected rats and by the end of 6th week, damage was extended to the subchondral bone resulting in bone destruction (Figure 7A, 7B). However, in 0.5 mg injected group, reduction of safranin O as a sign of cartilage degeneration started from

2nd week. Also, the score evaluated by Mankin scoring system indicated significant differences between 0.5 mg and 1.0 mg MIA injection group at the end of 2nd week ($p < 0.0001$) and 6th week ($p = 0.0004$) ($n = 3$) (Figure 7C).

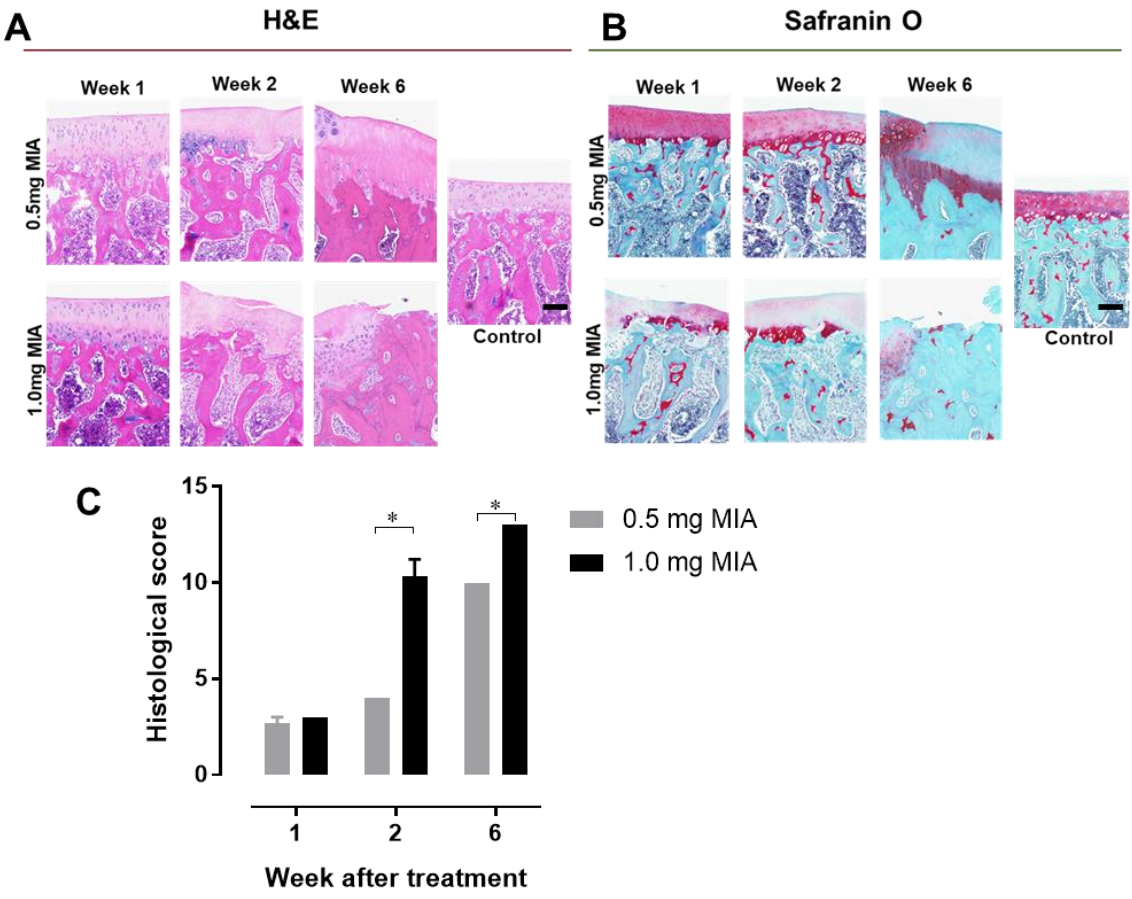

Figure 7. Histological evaluation of knee joints from rats received 0.5 mg and 1.0 mg MIA injection. (A) H&E staining showed that cartilage degeneration started from week 1 and bone destruction can be seen in 1.0 mg MIA group at 6th week. Scale bar is 100 µm. (B) Severe reduction of safranin O staining can be observed in 1.0 mg MIA injection at 1st week. In 0.5 mg group, reduction started from 2nd week. Scale bar is 100 µm. (C) The results of Mankin score showed significant difference between 0.5 mg and 1.0 mg MIA groups at 2nd week and 6th week. The results represent as means ± standard errors of the means for the 3 rats. * indicates a significant difference, as determined by one-way analysis of variance, followed by the Tukey's multiple-comparison procedure ($p \leq 0.05$).

According to the above results, the appropriate MIA-induced OA model should be 0.5 mg MIA injection and the injection of NGF antibody should start after observable cartilage degeneration which started at 2nd week.

Anti-NGF antibody can relieve the pain behavior of weight bearing but not allodynia

To observe the effect of analgesic local treatment on OA, a MIA-induced OA murine model was used for further experiments. To confirm the effect of local treatment of anti-NGF antibody, different doses (1 μg, 10 μg and 100 μg) of anti-NGF antibody were intra-articularly injected into the rats' right knees once a week from the end of 2nd week. Behavior tests were performed two times a week and knee joints were collected after the end of 6th week for macroscopic and histological evaluations. Immunofluorescence staining for NGF indicated that MIA injection can increase the concentration of NGF in the synovial tissue surrounding the affected joints (Figure 8). The injection of anti-NGF antibody neutralized the NGF in the tissue and therefore reduced the signal of NGF staining (Figure 8).

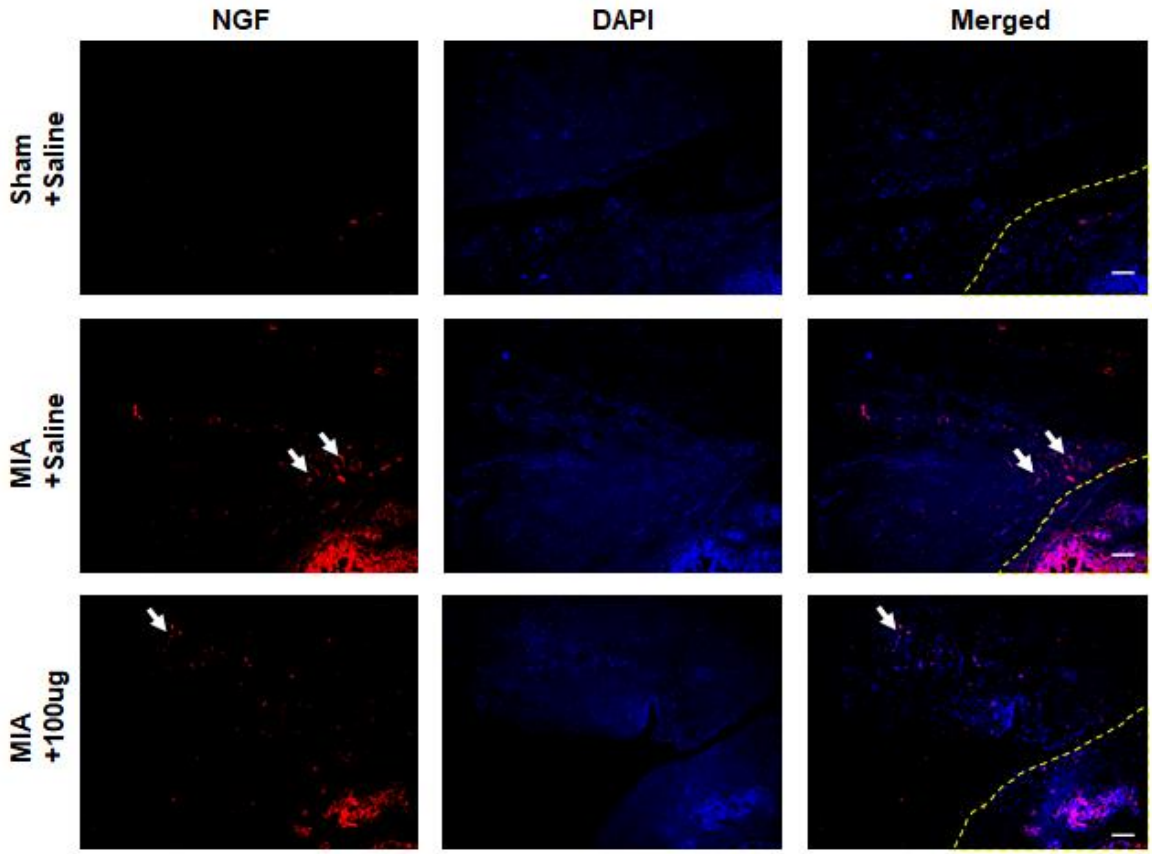

Figure 8. Fluorescent staining of knee joints indicates NGF concentration changes before and after treatment. MIA injection induced NGF increase which appears as high NGF positive area. After injection of 100 μg NGF, there is a decrease of NGF positive reaction. Arrows indicates the positive reaction for NGF. The yellow dotted line indicates area of articular cartilage and subchondral bone tissue. Scale bar is 100 μm.

The results of the behavioral tests showed that MIA injection can cause impaired weight bearing ($p < 0.005$) and decreasing mechanical threshold regarded as allodynia ($p < 0.01$) as signs of pain from the 1st week, and rats receiving saline injection did not present any pain behavioral changes. After the intra-articular injection of anti-NGF antibody, 100 μg anti-NGF antibody treatment effectively relieved the pain of the OA model rats, as evidenced by improved weight-bearing performance (Figure 9A, saline, 1 μg, 10 μg vs 100 μg from week 3, $p < 0.001$), whereas 1 μg and 10 μg showed no effect on relieving pain. However, allodynia, which was

also induced by MIA injection, did not improve with anti-NGF antibody injection of all doses (Figure 9B). These results showed that only 100 µg anti-NGF antibody can relieve the OA pain induced by MIA injection, as confirmed by the improvement of weight-bearing asymmetry but not allodynia.

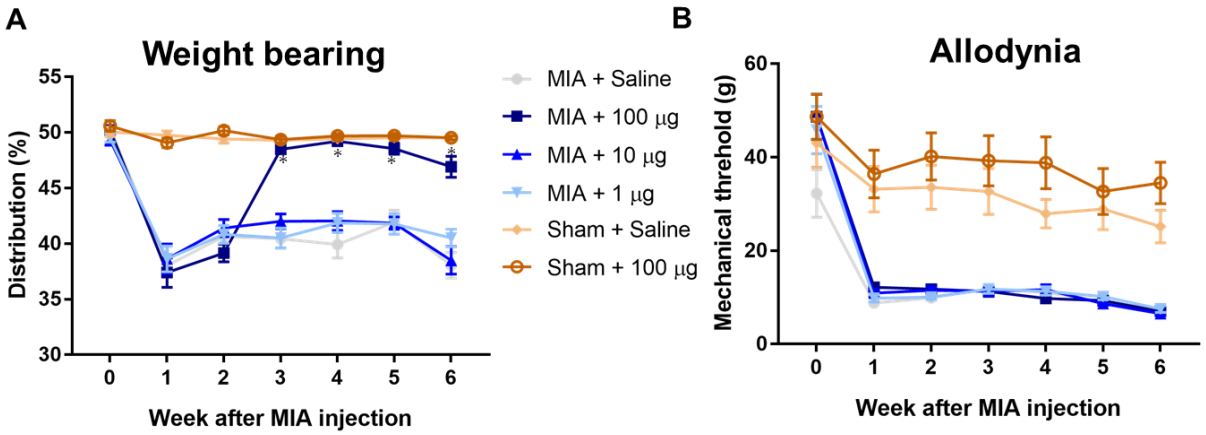

Figure 9. Anti-NGF antibody treatment relieved the pain of rat OA model as evidenced by improved weight bearing performance but not allodynia. (A) MIA injection can induce weight-bearing asymmetry, whereas saline injection did not show any significant changes in the rat's weight-bearing asymmetry. Moreover, 100 µg anti-NGF antibody treatment reduced pain in the rat OA model as evidenced by improved weight-bearing performance ($p < 0.0001$). Furthermore, 1 µg and 10 µg antibody treatment could not improve the weight-bearing asymmetry after MIA injection. The results represent as means ± standard errors of the means for the 6 rats. * indicates a significant difference, as determined by two-way analysis of variance, followed by the Tukey's multiple-comparison procedure ($p \leq 0.05$). (B) MIA injection had significantly lower rat's hind paw withdrawal mechanical thresholds than saline injection. No significant improvement of allodynia was observed after the injection of NGF antibody of all doses. The results represent as means ± standard errors of the means for the 6 rats.

These results showed that only 100 µg anti-NGF antibody can relive the osteoarthritic pain induced by MIA injection confirmed by improvement of weight bearing asymmetry but not

allodynia.

Anti-NGF antibody injection has no negative effect on cartilage

For observing the effect of anti-NGF antibody on the joint pathological progress, macroscopic and histological were evaluated. Results of Macroscopic evaluations showed that MIA injection can induced damage of cartilage imitating osteoarthritis characterized by cartilage erosions (Figure 10A). The injection of anti-NGF antibody of all dose and saline has no obvious adverse effects on joint cartilage (Figure 10B).

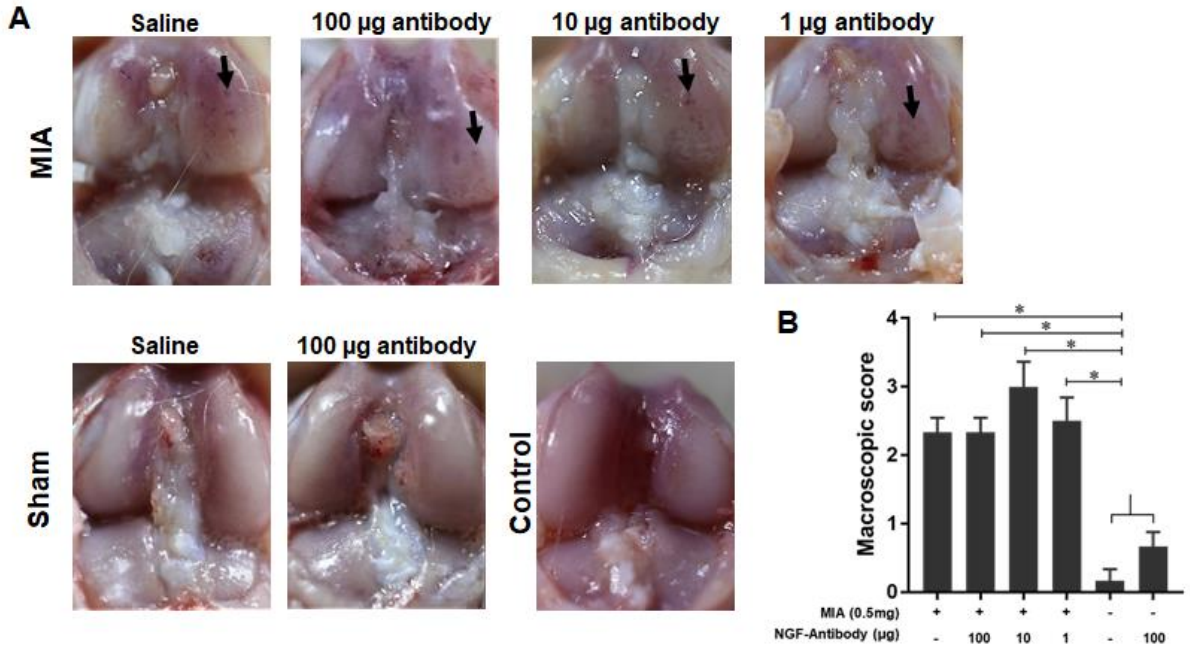

Figure 10. Macroscopic evaluation of rat affected knee joints indicates no differences among MIA injection groups. (A) The macroscopic figures showed that MIA injection can induce cartilage degradation, whereas saline injection has no effect. Arrows indicate cartilage erosions. (B) Macroscopic score using the Likert scale showed that nerve growth factor antibody injection revealed no evident difference between each MIA group. The results represent as means ± standard errors of the means for the 6 rats. * indicates a significant difference, as determined by one-way analysis of variance, followed by the Tukey's multiple-comparison procedure ($p \leq 0.05$).

Consistent with these results, histological evaluations of H&E staining (Figure 11A) and safranin O staining (Figure 11B) showed that injection of anti-NGF antibody has no negative effects on cartilage pathology (Figure 11C).

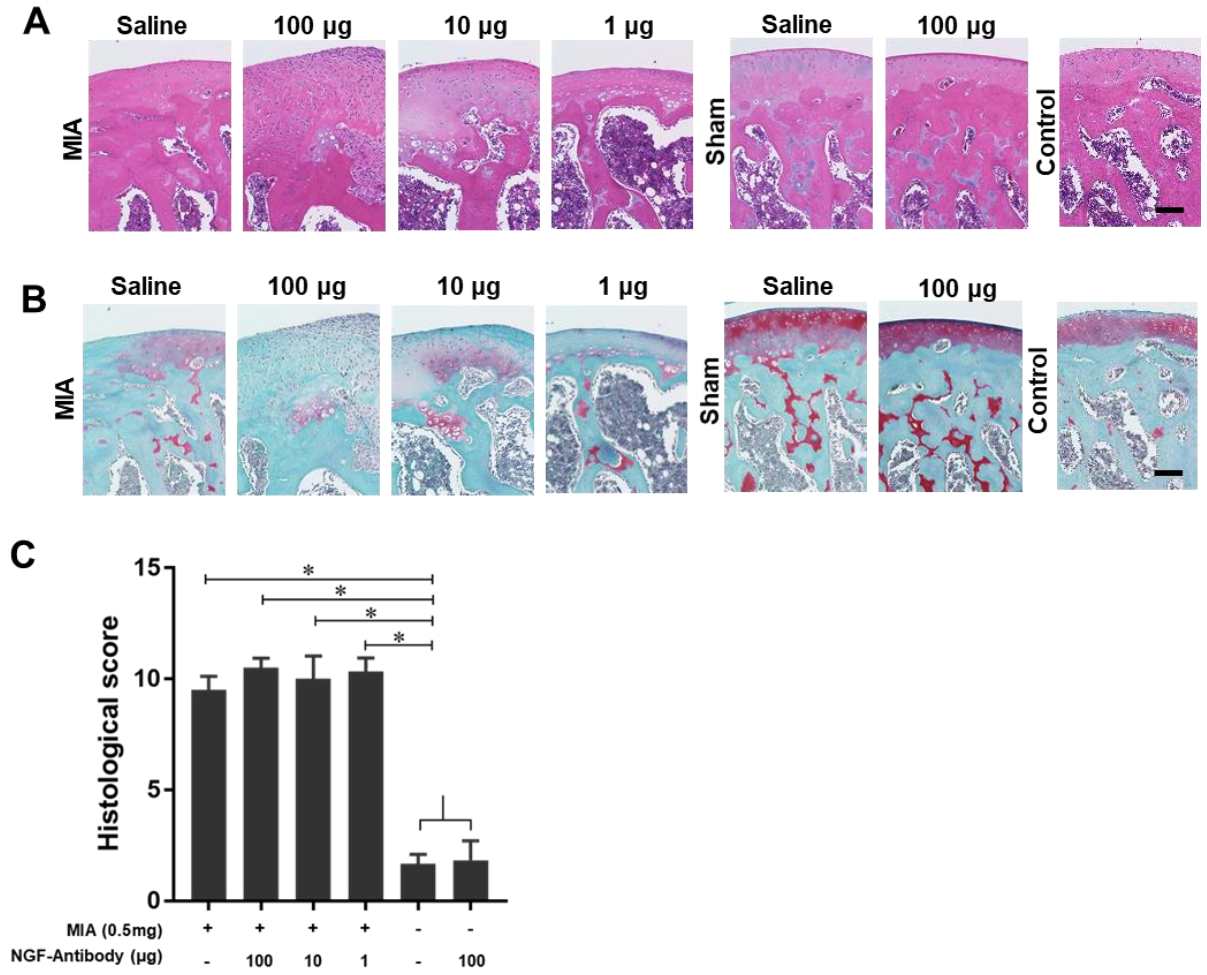

Figure 11. Histological evaluation of rat affected knee joints agreed with macroscopic evaluation. (A) Hematoxylin–eosin staining showed that the inflammation formed around the cartilages and chondrocytes was disorganized and no longer observed after MIA injection. Scale bar is 100 µm. (B) Results of safranin O staining showed that MIA injection can induce cartilage degradation characterized by cartilage irregularities and staining reduction. Scale bar is 100 µm. (C) The results of the Mankin score showed no significant difference among the MIA groups or between the sham groups, revealing that the treatment of anti-NGF antibody did not exacerbate the pathological progression of OA joints. The results represent as means ± standard errors of the means for the 6 rats. * indicates a significant difference, as determined by one-way

analysis of variance, followed by the Tukey's multiple-comparison procedure ($p \leq 0.05$).

These results showed that injection of anti-NGF antibody didn't accelerate the progression of OA.

Discussion

The results of this study suggested that the local administration of anti-NGF antibody at low dose was effective for pain relief in a MIA-induced OA pain without accelerating the progression of cartilage degeneration in the affected joint. The results of fluorescent staining revealed that the increased anti-NGF levels in the synovial tissue triggered by MIA was effectively neutralized by the local anti-NGF antibody injection. The intra-articular injection of a low dose (100 μg) of anti-NGF antibody that neutralizes NGF results in pain relief, as evidenced by the improvement of weight-bearing asymmetry caused by MIA injection. Macroscopic evaluation showed that the injection of anti-NGF antibody rarely accelerated the progression of MIA-induced OA. Similarly, histological evaluation also revealed that the local administration of anti-NGF antibody had no adverse effects.

Anti-NGF antibody has recently been used as an analgesic drug for chronic pain, but adverse effects, including progressively worsening cartilage degeneration, are considered particularly problematic in OA (Katz, N. et al, 2011, p.2248; Miller, R.E., et al, 2018, p.199). M.C. Hochberg revealed that the syndrome of rapid progression of OA is observed after the systemic treatment of anti-NGF antibodies and concluded that it is necessary to use the low effective doses to control the risk of adverse effects (Rosen, J., et al, 2016, p. 998). Moreover, Patrice Bélanger et al. reviewed the evaluation of safety data of systemic treatment of anti-NGF antibody and found out that issues associated with safety were detected in both clinical and nonclinical cases (Bélanger, P., et al, 2018, P. 1). In this study, we found that low dose of 0.1-mg anti-NGF antibody could alleviate the MIA-induced pain without deterioration of OA,

suggesting that intra-articular administration of low effective dose of anti-NGF antibody may be an effective treatment for OA, specifically in mono- or oligo-articular OA.

Moreover, the effective dose of anti-NGF antibody (0.1 mg/knee) is lower in intra-articular injection than that in systemic injection. Several studies have focused on the efficacy and safety of anti-NGF antibody for chronic pain using an animal OA model through systemic treatment. For systemic injection, it usually requires 10 mg/kg, approaching to 5 mg/injection (Xu, L., et al, 2016, P. 1587; Halvorson, K.G., et al, 2005, P. 9426). In fact, intra-articular injection has been considered a more cost-effective treatment for OA compared with systemic injection (Katz, N., et al, 2011, p. 2248; Belzile, E.L., et al, 2017, n. pag.). Direct delivery of drugs requires low effective dose, which can decrease the risk of side effects and damages to other unimpaired tissues. Therefore, we can hypothesize that the local treatment of NGF antibody may be an appropriate strategy to reduce the dosage and thereby the incidence of side effects and exposure of the whole body to the antibody. This is the first study to elaborate the hypothesis regarding the local treatment of anti-NGF antibody.

However, systemic injection is known to be able to improve both weight-bearing asymmetry and mechanical allodynia (Xu, L., et al, 2016, p. 1587). The intra-articular injection of NGF antibody only showed an effect on weight bearing. However, it did not present any effect on improving the allodynia of the rat's hind paw. Mechanical allodynia is a painful sensation stimulated by light touch. Although the mechanism of allodynia is incompletely understood, the involvement of alterations in mechano-transduction and sensory neurons in the CNS has been described by several studies (Hochberg, M.C., 2015, p. s18; Lolignier, S., et al, 2015, p. 133; Orita, S., et al, 2011, p. 134). The increasing expression of NGF under OA condition, contributes to the change in receptor sensitivity, or the changes in the dorsal root ganglion may result in central sensitization (Woolf, C.J.,2011, p. s2; Woolf, C.J., et al 1994, p.327). Our current results suggest that the intra-articular injection of anti-NGF antibody may provide insufficient analgesic effect on allodynia-related pathologies such as CRPS. Whether

the local injection of anti-NGF antibody can effectively reverse the CNS changes established during the NGF rising phase needs further exploration and experiments.

On the contrary, intra-articular injection treatment requires superb operation skills and experience to avoid the risks of infection, rejection reaction, and tissue damage (Lane, N.E., et al, 2010, p. 1521; Cheng, J. and Abdi, S., 2007, p.141; McAlindon, T.E., et al, 2017, p. 1967). Furthermore, although OA pain has been relieved by anti-NGF antibody injection, complications of OA, such as cartilage degeneration, still require treatment. The mechanism by which topical administration of anti-NGF antibody does not suppress allodynia, but does not exacerbate OA, is still unknown. Further study is required to clarify the association between these two phenomena.

Conclusion

1. Local administration can largely reduce the effective dose of anti-NGF antibody for reliving OA pain
2. Local administration of low dose anti-NGF antibody can relive OA pain evidenced by weight bearing performance but not allodynia
3. Local administration of low dose anti-NGF antibody did not interrupt the pathological progression of OA
4. Further experiments are needed to explore the mechanism by which topical administration of anti-NGF antibody does not suppress allodynia

In conclusion, based on the results of this study, the effective dose of anti-NGF antibody in intra-articular administration was lower than that of systemic injection in previous reports without accelerating OA progression. Moreover, intra-articular injection might be an alternative approach to systemic injection for the treatment of patients with OA. Further experiments are required to improve the intra-articular injection treatment of anti-NGF antibody and elucidate the mechanism of allodynia in OA.

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Conflict of interest

The author has received NGF antibody using for research from MOCHIDA PHARMACEUTICAL CO., LTD. Tokyo Japan.

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