Research Article

Title: Subchondral bone fragility with meniscal tear accelerates and parathyroid hormone decelerates articular cartilage degeneration in rat osteoarthritis model†

Running title: Bone fragility increases cartilage degeneration

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Abstract: The aims of this study were to investigate the influence of subchondral bone fragility (SBF) on the progression of the knee osteoarthritis by using a novel rat model, and to examine the preventive effect of parathyroid hormone (PTH) on cartilage degeneration. First, 40 rats were assigned to the following four groups: Sham, SBF, Medial meniscal tear (MMT), and MMT+SBF groups. In SBF and MMT+SBF groups, we induced SBF by microdrilling the subchondral bone. Second, 10 additional rats were randomly assigned to the following two groups: MMT+SBF+saline and MMT+SBF+PTH groups. Osteoarthritic changes in the articular cartilage and subchondral bone were evaluated using safranin-O/fast green staining, matrix metalloproteinase-13 (MMP-13) and type X collagen immunohistochemistry, toluidine blue staining and micro-CT scanning. The combination of SBF and meniscal tear increased the number of mast cells in the subchondral bone, and led to the abnormal subchondral bone microarchitecture, such as abnormally decreased trabecular number and increased trabecular thickness, compared with meniscal tear alone. Moreover, SBF with meniscal tear enhanced articular cartilage degeneration and increased the expression of MMP-13 and type X collagen, compared with meniscal tear alone. The administration of PTH decreased the number of mast cells in the subchondral bone and improved the microstructural parameters of the subchondral bone, and delayed the progression of articular cartilage degeneration. These results suggest that SBF is one of the factors underlying the osteoarthritis development, especially in knees with traumatic osteoarthritis, and that the administration of PTH is a potential therapeutic treatment for preventing OA progression. This article is protected by copyright. All rights reserved

Key words: osteoarthritis; subchondral bone; articular cartilage; parathyroid hormone; cartilage degeneration
Introduction

Osteoarthritis (OA) is a common chronic joint disorder. It is characterized by articular cartilage degeneration and subchondral alterations\(^1\). In several epidemiological studies, age, gender, obesity, genetics, bone metabolism, occupation, and trauma were recognized as risk factors for OA\(^2\). Despite the prevalence of OA in the elderly, its pathophysiology remains unclear. Furthermore, the therapeutic approaches available currently for the early stages of OA are often limited to the use of analgesic medications. Therefore, it is important to clarify the mechanism of OA initiation and to develop disease-modifying drugs for OA.

Although changes in subchondral bone (SB) have traditionally been considered to follow cartilage destruction, the question of whether SB microarchitecture alterations merely represent adaptation to an altered biochemical and mechanical environment, or are a driver of OA progression remains controversial\(^1\). At the cellular level, articular chondrocytes exhibit increased expression of matrix metalloproteinases in the state of co-culture with OA SB osteoblasts\(^3\), and in humans, MRI studies revealed that bone marrow lesions in SB are predictive of cartilage loss in the same subregion\(^4\).

In animal studies, osteoporosis models induced by ovariectomy have been used, in order to investigate the influence of subchondral bone fragility (SBF) on the
development of cartilage degeneration\textsuperscript{5, 6}. In these models, it was suggested that microstructure impairment in SB enhanced cartilage degeneration. However, ovariectomy induces estrogen deficiency, and has direct effects not only on SB, but also on articular cartilage and other tissues\textsuperscript{7}. Previous studies showed that estrogen receptors were expressed by articular chondrocytes, and that estrogen increased glycosaminoglycan synthesis and decreased cyclooxygenase-2 mRNA expression in articular chondrocytes\textsuperscript{8, 9}. So, we made a new SBF model in which we made changes only in the subchondral trabecular bone without changing any other environments, in order to determine whether SBF directly increases articular cartilage degeneration.

Recently, expectations over the therapeutic potential of bone-modifying agents such as bisphosphonates, strontium ranelate and parathyroid hormone [1-34] (PTH) in the treatment of OA have risen\textsuperscript{10-14}. PTH is a bone-forming drug used to treat patients with osteoporosis\textsuperscript{15}. Intermittent administration of PTH increases the number and activity of osteoblasts and decreases their apoptosis\textsuperscript{16}, thereby improving the microstructure of trabecular bone. In the naturally occurring guinea pig OA model, the administration of PTH retarded microarchitectural changes in trabecular SB from a rod-like to a plate-like structure and increased type-II collagen expression in the cartilage\textsuperscript{10}. In a rabbit OA model of low bone mass surgically-induced by ovariectomy, the administration of PTH...
improved the microstructural parameters at the SB and prevented the progression of cartilage damage\textsuperscript{11}. Moreover, PTH was reported to have direct effect on chondrocytes via PTH receptor in vitro\textsuperscript{17}. PTH treatment could decelerate articular cartilage degeneration in the surgery-induced murine OA model and its chondroprotective effect was also associated with PTH receptor upregulation in chondrocytes\textsuperscript{18}. However, whether a bone-forming agent, PTH, can prevent the progression of OA remain largely unknown.

The aims of this study were, using a novel rat model, to determine (1) whether SBF alone produces articular cartilage degeneration, (2) whether SBF in combination with meniscal tear enhances the development of articular cartilage degeneration, and (3) whether the administration of PTH prevents the development of articular cartilage degeneration related to SBF.

Materials and methods

Surgical procedure

To investigate the effect of SBF on OA initiation and progression, 40 male 12-week-old Sprague Dawley rats were randomly assigned to four groups as follows: Sham, SBF, Medial meniscal tear (MMT), and MMT+SBF groups (n = 10/group). The rats were
anesthetized with isoflurane and their right hind limbs were shaved. In the SBF group, the right knee joint was exposed after a medial skin and capsular incision was performed. Subsequently, partial trabecular SB defects in the proximal medial tibia were created using a microdrill (diameter, 0.5 mm; depth, 2 mm) inserted through three points in seven directions (Fig. 1). After irrigation with saline, the medial capsule was sutured with 5-0 polyglycolic acid absorbable sutures and the skin was sutured with 5-0 nylon sutures. In the Sham group, the right knee joint was exposed after a medial skin and capsular incision was performed. Subsequently, only superficial cortical bone defects were created at the same three points, without trabecular SB defects. Microdrilling was stopped as soon as bleeding from the cortical bone defects was observed. After irrigation, the medial capsule and skin were sutured. In the MMT+SBF group, MMT surgery was performed as described previously\(^1\) and trabecular SB defects were created. Briefly, we transected the medial collateral ligament of the right knee, reflected the medial meniscus toward the femur, and cut through it at its narrowest point. Subsequently, we created trabecular SB defects in the same way as that described for the SBF group. In the MMT group, MMT surgery was performed, and only superficial cortical bone defects were created at the same three points, without trabecular SB defects. Five rats from each group were sacrificed at 3 and 6 weeks after
the surgery. The experimental design used in this study was approved by the animal research committee of Kyoto University (Med kyo17221).

**Pharmaceutical treatment**

To investigate the therapeutic effect of a bone-forming agent: PTH on OA progression, we used 10 male 12-week-old rats. All 10 rats underwent MMT surgery, and trabecular SB defects were created in the same way as that described for the MMT+SBF group.

Subsequently, rats were randomly assigned to two groups as follows: MMT+SBF+saline group and MMT+SBF+PTH group (n = 5/group). In the MMT+SBF+PTH group, rats received a subcutaneous injection of PTH (teriparatide) (30 µg/kg/day, 3 days/week; Asahi Kasei Pharma Corporation, Tokyo, Japan). In the MMT+SBF+saline group, rats received a subcutaneous injection of saline solution.

These agents were administered for 6 weeks after the surgery. All rats in MMT+SBF+saline and MMT+SBF+PTH groups were sacrificed at 6 weeks after the surgery.
Bone morphological analysis

The right knee joints were fixed in 4% paraformaldehyde overnight, and scanned using a micro-CT system (SMX-100CT, Shimadzu, Kyoto, Japan). Morphological alterations in the SB were evaluated using micro-CT. Scans were performed using the following parameters: voltage, 43 kV; current, 43 μA; and voxel size, 21 μm$^2$. All data were exported to VG Studio Max 2.0 (Visual Science, Tokyo, Japan) for three-dimensional reconstruction. Alterations in the SB were then observed qualitatively in sagittal and frontal sections. To further analyze the proximal tibial SB, a subset (2.0 mm ventro-dorsal length) of the weight-bearing lesion at the medial tibial plateau was taken as the region of interest$^{22}$. The following morphometric parameters were calculated semi-automatically using VG Studio Max 2.0: bone volume fraction (BV/TV, calculated as bone volume/total volume), trabecular thickness (Tb.Th, calculated as $2/($bone surface/bone volume$)$), trabecular number (Tb.N, calculated as (BV/TV)/Tb.Th), and trabecular separation (Tb.Sp, calculated as $(1/Tb.N)-Tb.Th$).

Histological analysis

After the evaluation by micro-CT scanning, the fixed right knee joints were decalcified with Morse’s solution (10 % sodium citrate and 22.5 % formic acid)$^{23, 24}$, cut along the
collateral ligament in the frontal plane, dehydrated and embedded in the paraffin block. Sections with a thickness of 5 μm were cut from each paraffin block using 200 μm steps and then stained with safranin-O/fast green, to evaluate cartilage degeneration\textsuperscript{25}.

Articular cartilage degeneration was evaluated using the OARSI cartilage pathology assessment system. This method produces the OARSI score with a range of 0 (normal) to 24 (severe) based on six grades (depth of the lesion) and four stages (extent of involvement)\textsuperscript{26}. The most severe score in all sections from each knee was determined as the OARSI score\textsuperscript{27}. The sections were scored blindly without any knowledge of the experimental groups.

**Mast cell staining**

Mast cells were reported to have association with the pathophysiology of OA\textsuperscript{28}. And, previous studies reported that the administration of PTH improved bone healing via the regulation of mast cell accumulation at the bone healing site\textsuperscript{29, 30}. To evaluate the influence of SBF and PTH on the accumulation of mast cells in the SB, we measured the number of mast cells in the SB. To visualize mast cells, deparaffinized sections were stained with 0.05% toluidine blue, pH 2.5, for 15 minutes at room temperature. The sections were then dehydrated and mounted for viewing. The number of mast cells in
the SB was evaluated from standardized rectangular fields (500μm deep and 500μm long) within the SB of the middle region in the medial tibia. The images were analyzed using BZ-analyzer (Keyence Co., Osaka, Japan).

Immunohistochemistry

Immunohistochemical staining of matrix metalloproteinase-13 (MMP-13) and type X collagen was performed in deparaffinized sections collected at 6 weeks after the surgery. The sections were treated with hyaluronidase (Sigma-Aldrich Co., St Louis, MO, USA) for 45 min at 37 °C. Subsequently, the sections were treated with 3 % hydrogen peroxide in methyl alcohol for 15 min at room temperature. After blocking with Cas Block (Invitrogen Co., Carlsbad, CA, USA), the sections were incubated with an anti-MMP-13 rabbit polyclonal antibody (1:100, Abcam Co., Cambridge, MA, USA) and an anti-type X collagen rabbit polyclonal antibody (1:500, LSL Co., Tokyo, Japan) overnight at 4 °C. The primary antibody was omitted in the negative controls. The sections were incubated with a polymeric secondary antibody (SuperPicTure Polymer Detection Kit, Invitrogen Co.) and diaminobenzidine solution, followed by counterstaining with hematoxylin. We defined the expression of MMP-13 and type X collagen in the articular chondrocytes as the percentage of MMP-13-positive...
chondrocytes and type X collagen-positive chondrocytes observed within the middle region of the medial tibia with a mediolateral width of 0.5 mm. The images were analyzed using BZ-analyzer (Keyence Co., Osaka, Japan)

Serum biochemistry

To investigate the effect of PTH on bone metabolism, we analyzed osteocalcin as a bone-formation marker and type I collagen C-telopeptide (CTX-I) as a bone-resorption marker in rat serum. In the MMT+SBF+saline and MMT+SBF+PTH groups, blood samples were obtained by abdominal aortic puncture performed under anesthesia at the time of sacrifice. All blood samples were from animals that had been fasting for at least 6 hours prior to sampling. Serum samples were stored at –80 °C until use. We used the Osteocalcin Rat ELISA System (GE Healthcare Bio-Science, Piscataway, NJ) and Rat Laps ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark). All assays were performed according to the manufacturers’ directions.

Statistical analysis

For statistical analysis, JMP 11 software (SAS Institute, Cary, NC, USA) was used. All results are reported as the mean ± standard deviation (SD). An analysis of variance was performed.
used to analyze the data from the micro-CT scanning, safranin O/fast green staining, toluidine blue staining, immunohistochemistry and serum biochemistry, followed by the post-hoc Tukey–Kramer HSD test. In analyzing the data from the MMT+SBF+PTH and MMT+SBF+saline groups, we used the data from the Sham group at 6 weeks after surgery as a normal control data. In all cases, $P$ values less than 0.05 were considered significant.

Results

Effects of SBF on SB microarchitecture

Representative micro-CT images of the knee joints are shown in Fig. 2A. In the SBF and MMT+SBF groups, trabecular SB defects were partially observed 3 weeks after the surgery, but these defects had resolved by 6 weeks after the surgery. In the SBF group, SB microarchitecture was slightly changed, and slightly increased Tb.Th and slightly decreased Tb.N was observed at 3 and 6 weeks after the surgery, compared with the Sham group. In the MMT+SBF group, apparently abnormal SB microarchitecture in the load-bearing lesion of medial tibial plateau was observed at 6 weeks after the surgery. In this group, higher BV/TV, higher Tb.Th and lower Tb.N were observed at 3 weeks after
the surgery, compared with the MMT group, and these differences increased furthermore at 6 weeks after the surgery (Fig. 2).

**Effects of SBF on articular cartilage degeneration**

In the SBF group, the surface of the articular cartilage was smooth and similar to that of the Sham group at all time points. In the MMT and MMT+SBF groups, the loss of articular cartilage increased in a time-dependent manner. Compared with the MMT group, the articular cartilage degeneration observed in the MMT+SBF group was more severe and the OARSI score of the MMT+SBF group was significantly higher at 6 weeks after the surgery. This value tended to be higher at 3 weeks after the surgery, but the difference was not statistically significant (Fig. 3).

**Effect of SBF on the accumulation of mast cells in the SB**

In the Sham group, mast cells were rarely found in the SB. In the SBF and MMT groups, only a small number of mast cells were detected in the SB. However, the combination of SBF and MMT dramatically increased the number of mast cells in the SB compared with other groups, and the number increased in a time-dependent manner.

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The difference between the number of mast cells in the SBF+MMT group and that of other groups was statistically significant (Fig. 4).

Effects of SBF on MMP-13 and type X collagen expression in the articular cartilage

In the Sham and SBF groups, the expression of MMP-13 and type X collagen in the articular cartilage was detectable, and no significant difference was observed in the ratio of MMP-13-positive and type X collagen-positive cells in the articular cartilage. In the MMT and MMT+SBF groups, MMP-13-positive and type X collagen-positive chondrocytes were prevalent in the articular cartilage. The expression levels of MMP-13 and type X collagen were increased in the MMT+SBF group, compared with the MMT group. The difference in the ratio of MMP-13-positive and type X collagen-positive cells between the MMT and the MMT+SBF groups was statistically significant (Fig. 5).
Effects of PTH treatment on bone metabolism

Subcutaneous injection of PTH for 6 weeks significantly increased osteocalcin in the serum of rats, compared with subcutaneous injection of saline control. It slightly decreased CTX-1, but the difference between the MMT+SBF+saline and MMT+SBF+PTH groups was not statistically significant (Fig. 6).

Effects of PTH treatment on SB microarchitecture alterations and articular cartilage degeneration

Fig. 7A shows representative micro-CT images of the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. In the MMT+SBF+saline group, apparently abnormal trabecular in the load-bearing lesion of the medial tibial plateau were observed, whereas in the MMT+SBF+PTH group, the microstructure of subchondral trabecular bone was kept comparatively normal at 6 weeks after the surgery. In the MMT+SBF+saline group, abnormally high BV/TV, high Tb.Th and low Tb.N were shown, but subcutaneous injection of PTH for 6 weeks improved these parameters (Fig. 7B). Furthermore, as indicated by safranin-O/fast green staining, the degenerative changes in the articular cartilage observed in the MMT+SBF+PTH group were milder compared with those observed in the MMT+SBF+saline group (Fig. 7C). The OARSI
score of the MMT+SBF+PTH group was significantly lower than that of the MMT+SBF+saline group (Fig. 7D). The administration of PTH also strikingly decreased the number of mast cells in the SB compared with saline control (Fig. 7E), and the difference of the mast cell number between PTH and saline control was statistically significant (Fig. 7F).

Discussion

In the present study, we first developed a novel rat model of OA with medial meniscal tear and SBF. Then we found that SBF alone did not produce articular cartilage degeneration, but SBF in combination with meniscal tears led to the development of articular cartilage degeneration. Moreover, SBF with meniscal tear increased the number of mast cells in the SB, and induced abnormally remodeled SB microstructure in the load-bearing lesion of the medial tibial plateau. The administration of a bone-forming agent, PTH, decreased the number of mast cells in the SB, kept the microarchitecture of SB comparatively normal and delayed the progression of articular cartilage degeneration. To our knowledge, this is the first study to directly demonstrate that SBF in combination with meniscal tear leads to the development of articular
cartilage degeneration and that treatment of SBF via the administration of PTH delays
the progression of articular cartilage degeneration.

Several recent studies suggested that SBF affected articular cartilage degeneration
In a surgically-induced canine OA model, decreased bone volume fraction in the
medial proximal tibia and worsened cartilage degradation were confirmed at the same
time point. And in a surgery-induced rat OA model, SB resorption was associated with
eyearly development of cartilage degeneration. Our finding that an increase in bone
formation and improvement of SB microarchitecture via administration of PTH delayed
the progression of articular cartilage degeneration also supports the hypothesis that SBF
affects the development of articular cartilage degeneration.

Menisci play a significant role in shock absorption and load distribution within knee
joints. A knee without a meniscus experiences a peak pressure with loading twice as
high as the intact knee. Therefore, impairment of menisci increases the risk for
ipsicompartmental articular cartilage degeneration and leads to SB alterations such as
subchondral cysts, alteration of bone mineral density, and bone marrow lesions.

In recent studies that used finite element modeling, models that contained SB defects
experienced an increase in contact stress within the adjacent bone. Moreover, both
focally increased bone turnover and altered bone microstructure were reported within

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the bone adjacent to the SB cysts\textsuperscript{41}. These effects of meniscal damage and SB defects on SB alterations might have synergistically caused the apparently abnormal SB microarchitecture observed in the MMT+SBF group at 6week. In that group, BV/TV and Tb.Th were dramatically increased and Tb.N was dramatically decreased in a time-dependent manner compared with other groups. That result indicated that the SB became much stiffer via the meniscal damage and SBF. SB has been shown to have important shock absorbing capacity in normal joint\textsuperscript{42}, but once SB became stiffer, the capacity decreased and the contact pressure on the articular cartilage possibly increased\textsuperscript{39}. Considering that SBF led to the development of articular cartilage degeneration only in combination with meniscal tear, we can presume that SBF is one of the factors that enhance the development of articular cartilage degeneration in knees with high stress distribution, such as those of individuals who are overweight or have meniscal damage, ligament impairment, and repetitive knee contusions.

The aberrant maturation of articular chondrocytes along a pathway that resembles endochondral ossification has been reported to play a crucial role in the development of OA\textsuperscript{43, 44}. This process starts with the hypertrophic differentiation of chondrocytes that express type X collagen, followed by cartilage degradation by proteases such as MMP-13. The hypoxia-inducible factor 2\(\alpha\) (HIF-2\(\alpha\)) and RUNX-2 are pivotal transcription factors.
factors for type X collagen expression. Heterozygous HIF-2α and RUNX-2 knockout mice show decreased expression of type X collagen and MMP-13 in the articular cartilage, and much milder articular cartilage degradation compared with wild type mice. In vivo studies have shown that abnormal mechanical stress leads to the overexpression of type X collagen and MMP-13, as well as the development of cartilage degeneration. In the present study, we found that the expression of type X collagen and MMP-13 was increased and that cartilage degeneration was more severe in the MMT+SBF group compared with the MMT group. These results suggest that SBF with meniscal tear increases mechanical stress in the articular cartilage, and increases the number of aberrantly maturated chondrocytes, followed by the development of cartilage degeneration.

Several studies have revealed that targeting SB with bone-modifying agents such as bisphosphonates, strontium ranelate, and PTH has protective effects on the articular cartilage in OA. Therapeutic effects of bone-modifying agents on articular cartilage were described not only in osteoporosis OA models, but also in surgery induced instability OA models. In surgery induced instability OA models, increased SB resorption was detected in early stage of OA. It was suggested that bone-modifying agents might protect articular cartilage by suppressing this SB resorption in

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early stage of OA in surgery induced instability OA models\textsuperscript{49}. We previously reported that alendronate prevented periarticular bone loss and cartilage degeneration in a rabbit anterior cruciate ligament transection model\textsuperscript{14}. The British Study of Risedronate in Structure and Symptoms of Knee OA trial demonstrated that bisphosphonate risedronate therapy decreased the levels of type II collagen degradation products in urine samples from patients with OA\textsuperscript{50}. Strontium ranelate improved SB microarchitecture indices and decreased chondrocyte apoptosis in a rat MMT model\textsuperscript{13}. The Strontium Ranelate Efficacy in Knee Osteoarthritis trial showed that treatment with strontium ranelate was associated with a less severe degradation in joint space width compared with a placebo in patients with OA\textsuperscript{51}. PTH alters bone metabolism depending on dosage and administration frequency\textsuperscript{52, 53}. Furthermore, new bone formation induced by the administration of PTH is enhanced at sites with a high mechanical stress distribution\textsuperscript{54}. In the present study, we administered 30 μg/kg/day of PTH 3 days/week, which resulted in an increase of about 20 % in bone formation markers and a slight decrease in bone resorption markers compared with the saline group. This effect on bone metabolism was similar to that reported in a previous human study, in which PTH was administered once a week\textsuperscript{53}. In the MMT+SBF+PTH group, BV/TV and Tb.Th were lower and Tb.N was higher compared with those in the MMT+SBF+saline group.
but BV/TV and Tb.Th were still higher and Tb.N was still lower compared with those in the Sham group. These results indicated that the administration of PTH prevented SB from becoming too stiff and improved its microstructures and shock absorbing capacity. The administration of PTH also decreased articular cartilage degeneration in our experimental rat model, similarly to that reported previously\(^{11,12}\). Our results support the hypothesis that treatment of SBF via the administration of bone-modifying agents can delay the progression of articular cartilage degeneration.

Mast cells are bone marrow-derived leukocytes which release cytokine and chemokine and evoke local inflammation\(^{55}\). They were reported to have association with pathophysiology of OA\(^{28,56}\). Previously it was also suggested that mast cell influenced osteoblast and osteoclast, and changed bone turnover\(^{55,57}\). Moreover, recent studies reported that the administration of PTH had effect on bone healing via the regulation of the mast cell accumulation at the bone healing site\(^{29,30,58}\). In the present study, only the small number of mast cells were detected in the SB in the SBF group, but the combination of meniscal tears and SBF strikingly increased the number of mast cells in the SB. And the administration of PTH for six weeks significantly decreased the number of mast cells in the SB compared with the saline control. The results of this study indicated that repetitive excessive mechanical load and SBF increased the
accommodation of mast cells in the SB, induced the too stiffened SB and worsened the
degeneration of articular cartilage. Moreover, it was shown that PTH decreased the
accumulation of mast cells in the SB, prevented the SB from becoming too stiff and
delayed the degeneration of the articular cartilage. This is the first study reporting about
the association between the progression of OA and the accumulation of mast cells in the
SB. A further study to investigate pathophysiological association of OA progression and
mast cell should be conducted.

This study had some limitations. First, we developed a novel rat model, by directly
creating SB defects of 0.5 mm in seven directions. SB defects with a different size and
position may yield other results. Second, this model creates temporary SBF, which may
not be the same SBF seen in human OA. Third, we administered PTH at 30 μg/kg/day 3
days/week after surgery for 6 weeks, but we did not confirm other dosages, frequency,
or durations of treatment. Lastly, we used an experimental rat model. Because bone
metabolism and mechanical loading differ between rat and human knees, our results
might not translate directly to humans.

In conclusion, SBF increased articular cartilage degeneration in the knees of rats that
also carried meniscal tears, and the administration of a bone-forming agent: PTH
delayed OA progression in our experimental rat OA model. These results suggest that
SBF is one of the factors underlying the development of articular cartilage degeneration, especially in knees with traumatic OA, and that the administration of PTH is a potential therapeutic treatment for the prevention of OA progression. Further studies are required to develop ideal therapeutic interventions that can counteract OA progression.
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References


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Figure 1. Surgical procedure for subchondral bone fragility. In the SBF and MMT+SBF groups, we inserted microdrill through three points (1, 2, 3). These three points were a center point of antero-posterior width in medial aspect of proximal tibia, and 1mm anterior and posterior points from the center point. We microdrilled the subchondral bone in 2mm depth between the articular cartilage (red line) and growth plate (blue line), and the direction of drilling was parallel to the surface of articular cartilage in frontal plane. PT = patella tendon, MCL = medial collateral ligament

Figure 2. Effect of subchondral bone fragility on subchondral bone microarchitecture. (A) Representative two-dimensional micro-CT images of subchondral bone (SB) in Sham, SBF, MMT and MMT+SBF groups. In the SBF and MMT+SBF groups, SB defects (white arrowhead) were still partially observed at 3weeks, but they were resolved at 6weeks. And, abnormally remodeled subchondral trabecular bone was observed in the medial tibia in the MMT+SBF group at 6weeks. (B) Morphometric parameters of SB in the Sham, SBF, MMT and MMT+SBF groups. In the MMT+SBF group, BV/TV and Tb.Th were significantly higher and Tb.N was significantly lower, compared with the MMT group at 3weeks and 6 weeks. n = 5 per group, Mean ± SD, *p < 0.01, **p < 0.05

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Figure 3. Effect of subchondral bone fragility on articular cartilage degeneration. (A) Historical findings of Safranin-O/fast green staining in Sham, SBF, MMT and MMT+SBF groups. Scale bars = 100μm. (B) OARSI score of Sham, SBF, MMT and MMT+SBF groups. The OARSI score of the MMT+SBF group was significantly higher at 6 weeks, compared with the MMT group. n = 5 per group, Mean ± SD, *p < 0.01, **p < 0.05.

Figure 4. Effect of subchondral bone fragility on the accumulation of the mast cells in the SB. (A) Representative medial tibial SB sections stained with toluidine blue in Sham, SBF, MMT and MMT+SBF groups. Black arrowhead indicates mast cells (purple cells). Scale bars = 100μm. (B) The number of mast cells from standardized rectangular fields (500μm deep and 500μm long) within the SB of the middle region in the medial tibia. The number of mast cells of the MMT+SBF group was significantly higher, compared with other groups. n = 5 per group, Mean ± SD, *p < 0.01, **p < 0.05.
Figure 5. Effect of subchondral bone fragility on MMP-13 and type X collagen expression in articular chondrocytes. Immunohistochemical staining and positive cell ratios of MMP-13- and type X collagen-positive chondrocytes (brown) in Sham, SBF, MMT and MMT+SBF groups. The expression levels of MMP-13 and type X collagen significantly increased in the MMT+SBF group, compared with the MMT group. Scale bars = 100 μm, n = 5 per group, Mean ± SD, *p < 0.01, **p < 0.05

Figure 6. Effect of PTH on bone metabolism. Serum concentration of osteocalcin (A) and CTX-1 (B) in the MMT+SBF+saline and the MMT+SBF+PTH groups. Subcutaneous injection of PTH for 6 weeks significantly increased osteocalcin in rat serum, compared with subcutaneous injection of saline solution. n = 5 per group, Mean ± SD, *p < 0.01

Figure 7. Effect of PTH [1-34] on subchondral bone microarchitecture, articular cartilage degeneration and the accumulation of the mast cells in the SB. (A) Representative images of SB in the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. (B) Morphometric parameters of SB in the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. Administration of PTH significantly improved
BV/TV, Tb.Th and Tb.N, compared with the administration of saline (C) Histological findings of Safranin-O/fast green staining in the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. Scale bars = 100μm. (D) OARSI score of the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. The OARSI score of the MMT+SBF+PTH group was significantly lower at 6 weeks, compared with the MMT+SBF+saline group. (E) Representative medial tibial SB sections stained with toluidine blue in the MMT+SBF+saline, MMT+SBF+PTH and Sham groups. Black arrowhead indicates mast cells (purple cells). Scale bars = 100μm. (F) The number of mast cells from standardized rectangular fields (500μm deep and 500μm long) within the SB of the middle region in the medial tibia. The number of mast cells of the MMT+SBF+PTH group was significantly lower at 6 weeks, compared with the MMT+SBF+saline group.

n = 5 per group, Mean ± SD *p < 0.01, **p < 0.05
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