Original article

Therapeutic effect of targeting Substance P on the progression of osteoarthritis

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The numbers of text pages and figure legends; 25

The numbers of tables and figures ; 7

KEY WORDS: NK1 receptor agonist, osteoarthritis, septide, substance P

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ABSTRACT

Objectives: Substance P (SP) modulates NK1 and has various functions such as pain, bone metabolism and angiogenesis, which are recognized as important factors in the osteoarthritis (OA). We aimed to evaluate the therapeutic effect of targeting SP on OA progression.

Methods: SP expression patterns were analyzed histologically in articular cartilage and subchondral bone of human knees from OA patients and autopsy donors as non-OA samples, and in mice articular cartilage. Moreover, to examine the effect of SP on the progression of OA, we administered drugs to mice following surgical destabilization of the medial meniscus: PBS, septide (NK1 receptor agonist), or aprepitant (NK1 receptor antagonist). Histological analysis and bone morphologic analysis using microCT was performed.

Results: In human analysis, the expression of SP in mild OA samples was significantly higher than that in severe OA, and that in healthy cartilage was significantly higher than that in OA. In mouse analysis, OARSI scores in the septide group were significantly lower than that in the control group. CT analysis showed that the subchondral bone's epiphysis in the control group had sclerotic change not present in the septide group.

Conclusions: The administration of septide ameliorates OA progression through preventing subchondral bone sclerosis.

INTRODUCTION

Osteoarthritis (OA) is a multifactorial degenerative disorder of the synovial joints that causes the irreversible destruction of the articular cartilage [1,2]. Although OA causes significant health and social problems, the precise mechanism of OA initiation and progression has not been completely elucidated [3]. Therefore, there are currently no effective preventative measures or treatments for OA. Various pathomechanisms of OA have been advocated, and among them is the theory that sensory and sympathetic joint innervation plays an important role in the perturbation of joint homeostasis in OA [4]. It has been reported that the peripheral nervous system is critically involved in bone metabolism [5,6]. Neuropeptides such as Substance P (SP), calcitonin gene-related peptide (CGRP), and vasoactive intestinal peptide (VIP), which are recognized as pain-related neurotransmitters, have roles in bone metabolism, angiogenesis, and inflammation. The expression of these neuropeptides is increased in the subchondral bone in OA, suggesting that sensory neuron-released neuropeptides are involved in joint homeostasis, and possibly the subsequent bone deformity, cartilage degeneration, and inflammation during OA progression [7,8]. If these neuropeptides play a crucial role in OA pathogenesis, drug therapy for OA using neuropeptide receptor agonists or antagonists may be promising.

SP, a neuropeptide composed of 11 amino acids, is a highly conserved member of the tachykinin peptide family that is widely distributed in both the central and peripheral nervous systems. SP preferentially activates the neurokinin-1 receptor (NK1R) and transmits nociceptive signals via primary

afferent fibers to spinal and brainstem second-order neurons [9]. SP is localized and distributed throughout the subchondral bone where it may contribute to OA pain [10-12]. SP has various biological functions other than regulating pain responses. NK1R is expressed by osteoblasts and osteoclast precursors and stimulates osteoblast and osteoclast differentiation and function [6,13]. Several animal studies have reported cartilage degeneration accompanying changes in subchondral bone condition such as in cases of bone sclerosis or absorption during the progression of OA [14-16]. SP regulates bone remodeling, which affects osteoblastic differentiation and osteoclastogenesis in the subchondral bone. Moreover, SP has roles in anti-inflammatory responses and tissue repair through the recruitment of mesenchymal stem cells (MSCs) [17-19]. Given these functions of SP, regulation of the SP expression can be beneficial or harmful in the OA pathogenesis. As SP expression is increased in OA, it may play an important role in the pathogenesis of OA. However, the precise expression pattern and role of SP as anabolic or catabolic factors in OA remains unclear. The study of SP in OA is necessary as it could serve as a potential therapeutic target to ameliorate OA symptoms. We hypothesized that the regulation of SP could inhibit OA progression through modulating bone remodeling of the subchondral bone, its anti-inflammatory effects and by inducing tissue repair through the recruitment of MSCs.

The purpose of this study to analyze changes in the subchondral bone and cartilage during OA progression, focusing on SP expression. Further, this study aimed to examine the therapeutic effect of SP on OA progression using a SP receptor agonist and antagonist in mice who underwent surgical

destabilization of the medial meniscus (DMM).

METHODS

Human samples

This study was reviewed and approved by the ethics committee of our institution, and informed consent was obtained from all patients. For analysis of SP expression patterns in OA patients, articular cartilage and bone samples were obtained from six patients who underwent total knee arthroplasty. This group included one male and five females, with a mean age of 73 years (range: 66 to 86 years). Knee OA was diagnosed based on standing radiographs using the American Rheumatism Association's criteria for OA, with Kellgren-Lawrence (KL) grades III (four cases) and IV (two cases) included in this group. Patients with a history of knee trauma and systemic diseases, such as rheumatoid arthritis, were excluded. The entire proximal tibia was obtained using standard surgical techniques for total knee arthroplasty, and was divided into six regions, as the degeneration of cartilage and subchondral bone in OA varies across the different regions of the proximal tibia. Specifically, the medial and lateral tibial plateau was subdivided into three regions each, with 30 samples obtained for analysis from each proximal tibia specimen. Six regions were not included because they did not contain the osteochondral unit due to severe deformity.

For the analysis of non-OA samples, human articular cartilages and subchondral bone from knee joints were obtained from six autopsy donors (two male and four females) with a mean age of 38 years (range: 23 to 48 years old) as healthy young samples and from seven donors (four male and three females)

with a mean age of 69 years (range: 62 to 76 years old) as healthy aged samples. Tissue collection was approved by the Scripps Human Subjects Committee. All samples were fixed in 4% PFA, decalcified in 20% ethylenediaminetetraacetic acid (EDTA) for 2 weeks, and subsequently embedded in paraffin. Coronal sections 4 μm in thickness were prepared for histological analysis.

Animal models

The study protocols involving animals were approved by the Ethics Committee for Experimental Animals of Hiroshima University and were performed in strict accordance with the committee guidelines. Male 10-week-old C57BL/6 mice were used in this study. The animals were provided free access to food and water and allowed unrestricted weight bearing prior to the surgical intervention. For the OA model, the medial meniscotibial ligament of right knee joint was resected (destabilization of the medial meniscus: DMM) according to the previous reports [20]. Other animals for the sham group underwent sham surgery which consisted of a skin incision for right knee joint [21]. To examine the effect of SP on the progression of OA, we administered the following drugs to animals of each group including DMM and sham mice through an intra-peritoneal injection immediately after surgery: Control group: PBS at a dose of 100 μl/animal, Aprepitant group: NK1R antagonist (Aprepitant; MK-0869, Adooq Bioscience, Irvine, CA, USA) dissolved in 100 μl distilled water containing 2.5% dimethyl sulfoxide (DMSO) at a dose of 3mg/kg [22], or Septide group: NK1R agonist (Septide; Bachem, Bubendorf, Switzerland) dissolved in 100 μl PBS at a dose of 10^{-8} mol/kg [23].

Animals were sacrificed at 2 days, 1 week, 4 weeks and 8 weeks after surgery or sham surgery by anesthesia (n=8 each time point). After fixation of the knee joints in 4% PFA for 24 hours, samples were analyzed using micro-computed tomography (m-CT). Then, they were decalcified in 20% EDTA for 10 days and embedded in paraffin. Knee joints sectioned (4.0 μm) in the sagittal plane through the central weight-bearing region of the medial femorotibial joint. The sections were stained with Safranin O/fast green and at least two different sections per sample were analyzed microscopically. All sections were graded by two independent observers, and median scores were determined for statistical analysis.

Micro-computed tomography

Samples were analyzed under high-resolution m-CT (Sky-Scan1176, Toyo Corporation, Tokyo, Japan) using the following parameters: source voltage 40 kV; source current 580 μA; pixel size 12.47 μm; and spatial resolution 9 μm. Images were reconstructed (NRecon, Toyo Corporation, Tokyo, Japan) for analysis (CT- analyzer, Toyo Corporation, Tokyo, Japan). In mice, the ratio of bone volume and total volume in the tibial plateau subchondral bone's epiphysis (BV/TV, %) measured as previously described [24]. In setting of the region of the interest for BV/TV, the subchondral bone plate, subchondral bone plate of growth plate and cortical bone were not included for the measurement [25]. In addition, the ratio of the medial and lateral height of the epiphysis at 8 weeks was calculated in the sham, control, aprepitant and septide groups according to the previous report [40].

Histological Analysis

Samples were stained using safranin-O fast-green and hematoxylin-eosin. The pathological changes in the tibial plateau for both human and mouse samples were scored using the Osteoarthritis Research Society International's (OARSI) scoring system for OA [26,27]. The average OARSI grade (0-6.5) were calculated. Human samples were classified into three groups, as follows: mild OA (OARSI grade: 1.0-2.5), moderate OA (OARSI grade: 3.0-4.5) and severe OA (OARSI grade: 5.0-6.5) groups according to the previous report [21]. Synovitis after injection for mouse models was evaluated at 1, 4 and 8 weeks using the established synovitis score for changes in synovial lining thickness and cellular density in the synovial stroma (0-3 points, maximum score: 6 points) [28]. Other sections were used for immunohistochemistry tests and tartrate-resistant acid phosphatase (TRAP) staining. Subchondral bone plate was identified as the region between the osteochondral junction and the bone marrow cavity and the thickness of the subchondral bone plate and BV/TV was measured by Image J (National Institution of Health) according to the previous report [14,25]. In addition, angiogenesis which was identified as two or more red blood cells in the luminal structure lined with endothelial cells in the subchondral bone was evaluated [25]. Then, subchondral bone changes were assessed using the histopathological scoring system by Nagira et al.[25]. This subchondral bone scoring system has three parameters, subchondral bone plate consisting of the combination of subchondral bone plate thickness and angiogenesis, BV/TV, and osteophytes in the horizontal plane. In our study, score for osteophyte formation was omitted because it was difficult to evaluate the osteophyte formation in the sagittal section. After quantifying each parameter except for osteophytes, they were graded

on a scale of subchondral bone plate 0-6; BV/TV 0-3. Minimum score is 0 points, and maximal score is 9.

Immunohistochemical Analysis

Each section was immunostained with an anti-SP antibody (1:100 dilution, Santa Cruz Biotechnology: sc-58591), anti-osteocalcin (1:100 dilution, Santa Cruz Biotechnology, Dallas, TX), anti-matrix metallopeptidase 13 (MMP 13) (1:20 dilution, Neomarkers, Fremont, CA), anti-A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5; 1:100 dilution, Gene Tex, Irvine, CA), or anti-type II collagen (1:10 dilution, The Developmental Studies Hybridoma Bank, IA), using a 3,3'-diaminobenzidine substrate according to the method of a previous report. [29,30,31]

SP positive cells in the articular cartilage and the subchondral bone were counted and the area was quantified using Image J. Three areas were randomly set in the articular cartilage and subchondral bone of the tibia which just below subchondral bone plate, and the cell number was counted and calculated mean values per unit area of 1 mm^2 for human and mouse samples was recorded. In addition, the percentage of SP positive cells to total chondrocytes in human articular cartilage was calculated. In human samples with loss of the articular cartilage layer, only subchondral bone was evaluated.

For mouse samples, TRAP staining was performed using a commercially available kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's protocol. TRAP-positive multinucleated cells containing more than three nuclei were recognized as osteoclasts and counted using

Image J in the subchondral bone with the cell count per unit area reported. To analyze bone formation, we counted the total number of osteocalcin-positive cells and osteoclasts in the subchondral bone per unit area.

For MMP-13 and ADAMTS-5 expression, three fields of the cartilage were randomly selected and positive cells in each area were counted at 200X magnification. The percentage of MMP-13- and ADAMTS-5- positive cells in each field was calculated.

Statistical Analysis

All data were repeated at least three times. All results in this study were expressed as the mean ± standard deviation (SD). Comparison among three or four groups was done using the Tukey–Kramer post hoc test, and the Mann–Whitney U-test was used for detection of the differences between the two groups using SPSS for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA). p values of less than 0.05 were considered to be statistically significant.

RESULTS

Expression pattern of Substance P in human

In the human tibia, SP is expressed in the articular cartilage and subchondral bone. In patients with mild OA whose bone marrow cavity was maintained, SP was expressed along the bone marrow cavities of the tibia. In severe cases of OA where the bone marrow cavity was decreased and bone volume was increased, SP expression decreased. The expression of SP in the mild OA group was significantly higher

than in the severe OA group in not only the subchondral bone but also in the cartilage. (Fig. 1). Furthermore, the expression of SP in the articular cartilage was significantly higher in samples from healthy individuals compared to those with moderate or severe OA (Fig. 2). Additionally, in mild OA samples, SP expression in the superficial cartilage **layer** decreased and was mainly expressed in the middle layer. Thus, SP is expressed at high levels in normal cartilage and decreases with the progression of OA. Percentage of SP positive cells to total chondrocytes in the healthy individuals was significantly higher than that in the OA group (Fig. 2).

The Effect of Aprepitant and Septide Administration on OA Progression

In mice from the control group who were injected with PBS following surgical destabilization of the medial meniscus, the degeneration of the articular cartilage progressed gradually over time. Even at 2 days post-surgery, loss of proteoglycan was observed. At 1 week, erosion, clefts, progressive proteoglycan loss were observed. In the subchondral bone of the tibia's epiphysis, the bone marrow cavity decreased and bone volume increased, which suggested sclerotic changes. The expression of SP was maximal on 2 days post-surgery and gradually decreased with the progression of OA (Fig.3), which was consistent with the expression patterns seen in humans. SP was expressed intensely in the cartilage on day 2 and decreased substantially particularly in the superficial cartilage layer. The expression pattern of SP in sham mice did not change over time.

To examine the effect of SP regulation on OA progression, an NK1R antagonist or agonist was

administered to mice following surgery (Fig. 4A). Like the control group, mice in the aprepitant group exhibited progressive degenerative OA over time, while mice in the septide group showed reduced cartilage degeneration. Although there was no significant difference in each at 2 days post-surgery, the OARSI score in the septide group was significantly lower than that in the control and aprepitant groups at 1 week, 4 weeks and 8 weeks post-surgery. There was no significant difference between of the OARSI score the control group and the aprepitant group at 4 weeks, but that in aprepitant group was higher than that in control at 8 weeks (Fig 4B.). There were no adverse events such as death following administration. To examine the effect of the aprepitant and septide on the normal architecture of the articular cartilage, subchondral bone and synovium, sham mice with administration of these drugs were evaluated. Any changes including cartilage degeneration and subchondral bone thickness or bone atrophy was observed. The synovitis score in the aprepitant group showed the highest and that of the septide group was significantly lower than PBS group at 1 week after surgery ($p<0.01$). There was no significant difference of synovitis score in all groups at 4 and 8 weeks (Fig. 4C). Subchondral bone score in the septide group at 8weeks showed significantly lowest among three groups ($p<0.01$). At 1 weeks, subchondral bone score in the apprepitant group was significantly lower than those in the control and septide groups ($p<0.05$) (Fig. 4D). The thickness of the subchondral bone plate in the control and aprepitant groups became thicker as OA progressed, while the subchondral bone thickness in the septide group did not increase at 8 weeks.

On m-CT analysis, the subchondral bone's epiphysis in the control group exhibited sclerotic changes.

The BV/TV of the subchondral bone's epiphysis of the septide group was significantly lower than that in the control group at 1 week, 4 weeks and 8 weeks post-surgery (Fig 5.). The BV/TV in the control, aprepitant and septide group was compared between day 2, 1, 4 and 8 weeks. In the control group, there were significant differences between day 2, 4 and 8 weeks ($p<0.05$ respectively), while apprepitant and septide groups did not exhibit any significant difference between each time point. The ratio of medial / lateral height of the epiphysis in the control was significantly lower than that in the sham and septide groups (sham: 79.3±4.5%, control: 65.6±9.4%, aprepitant: 70.2±10.9%, septide: 79.7±2.7%) at 8 weeks (Fig 5).

In the control group, osteocalcin was intensely expressed in the subchondral bone, while it was expressed at lower levels in the septide group (Fig. 6). Instead, the septide group showed abundant TRAP-positive cells in the subchondral bone (Fig. 6). The number of osteocalcin-positive cells in the control group was significantly higher than that in the septide group at 4 weeks and 8 weeks post-surgery, and the number of TRAP-positive cells in the control group was significantly lower than that in the septide group at 4 weeks post-surgery. Thus, SP seems to inhibit subchondral bone sclerosis through preventing osteoblast differentiation and increasing osteoclastogenesis.

Immunohistochemistry revealed that septide administration could suppress MMP-13 and ADAMTS-5 expression in the articular cartilage while the control OA mice exhibited MMP-13 and ADAMTS-5 expression intensely as cartilage degeneration progressed. The percentage of MMP-13-positive cells in the control group was significantly higher than that in the septide group at 4 weeks and 8

weeks post-surgery. Additionally, the percentage of ADAMTS-5 positive cells in the control group was significantly higher than that in the septide group at 4 weeks after surgery (Fig. 7).

DISCUSSION

This study showed that Substance P is expressed in the articular cartilage and subchondral bone, and its expression decreased in the cartilage with the progression of OA in both human OA and surgical mouse OA. The administration of an NK1R agonist could ameliorate OA progression through the inhibition of subchondral bone sclerosis, which suggests that SP regulates the balance of osteoblast and osteoclast differentiation in the subchondral bone.

We investigated the expression pattern of SP in the articular cartilage of the human tibia. Healthy cartilage does not contain blood vessels and is not innervated by nerve fibers. However, despite the lack of innervation, cartilage metabolism is modulated and influenced by neurotransmitters. Thus, evaluation of SP expression patterns in the articular cartilage might be useful to understand OA pathogenesis [8]. Nerves grow into joint structures through vascular channels from the subchondral bone and distribute toward the articular cartilage [4,10]. Pervious report showed that vascular channels invade to the articular cartilage with nerve fibers containing neuropeptide in the OA patients [21]. SP was reported to express in the chondrocytes of the middle and deep zones of normal human articular cartilage. However, it was not expressed in the superficial zone [32]. In our study, SP was expressed in the superficial zone of healthy young cartilage samples, but its expression decreased in the healthy aged sample. SP expression also

gradually decreased from the superficial zone as OA progressed. The percentage of the SP positive cells to total chondrocytes was decreased in the OA, which suggested that SP positive cells are decreasing, not total chondrocyte. In mouse samples, SP was expressed intensely during the initiation of cartilage degeneration, and then its expression gradually decreased. The rapid progression of OA in DMM mice may have resulted in differences in SP expression patterns in the early phase of OA, compared to humans. In a previous report, immunocytochemistry showed that the percentage of neurons expressing SP and CGRP increased with age [32]. These findings are consistent with the hypothesis that an age-related change in joint innervation may contribute to the development of OA [7]. Thus, decreased expression of SP might affect the progression of OA.

We also examined the role of SP on cartilage as an anabolic factor. We proved that septide treatment could suppress MMP-13 and ADAMTS-5 expression compared to control OA mice who exhibited high MMP-13 and ADAMTS-5 expression as cartilage degeneration progressed. Opolka et al. demonstrated that chondrocyte proliferation increases in both monolayer and micromass pellet cultures in a dose-dependent manner by the stimulation of exogeneous SP [33]. Moreover, there were several reports on the positive effect of SP on cartilage degeneration in vivo. Hong et al. showed that SP has a novel ability to mobilize mesenchymal stem cells (MSC) and modulate injury-mediated inflammation, while also ameliorating collagen II-induced arthritis in mice via suppression of the inflammatory response [34]. In an OA animal study, Kim et al. reported that intra-articular injection of SP coupled with self-assembled peptide

hydrogels markedly improved cartilage regeneration through the recruitment of MSCs [17]. SP also has the ability to mobilize endogenous stem cells from the bone marrow to injured sites and accelerate tissue repair [35-38]. Recent studies have noted that SP plays an important role in the wound-healing process by recruiting bone marrow stem cells to the injured tissue. Jiang et al. showed that SP treatment can enhance recovery from spinal cord injury (SCI) in rats through its function of stem cell mobilization and/or through the modulation of inflammation. [18]. These functions of SP may act together to prevent cartilage degeneration in OA. Besides, Muschter et al. demonstrated that DMM mice of Tac1 deficient mice exhibited significantly higher OARSI score than sham mice at 12 weeks [39]. They concluded that SP are required for bone and cartilage homeostasis, which suggests aprepitant treatment induced severe OA changes. These reports support our results which SP expression is important to maintain the homeostasis of the osteochondral unit in OA.

Previous reports showed that the administration of a CGRP receptor antagonist could ameliorate OA progression in DMM mice through the inhibition of subchondral bone sclerosis, which indicates the importance of the subchondral bone in OA progression [40]. Specifically, this report suggests that neuropeptides could be a therapeutic target for subchondral bone sclerosis prevention in OA. In this study, we focused on SP because SP has various functions for the tissue healing such as restoration of stem cells, anti-inflammation, and immune modulation in addition to the bone metabolism [34, 41,42,43,]. SP regulates bone remodeling where it stimulates osteoblast and osteoclast differentiation and function in vitro. SP

neurotransmitter release from sensory neurons could potentially regulate local bone turnover [6,13,44]. In our study, DMM mice administered SP exhibited the best subchondral bone score, which suggests that SP may regulate bone remodeling through the inhibition of increasing bone turnover. Besides, a single administration of septide could inhibit OA change compared to the control and aprepitant groups. Septide might be able to ameliorate the damage to the subchondral bone by DMM at initial phase, which could maintain the homeostasis of the osteochondral unit until 8 weeks. In the DMM model, loading stress at the medial compartment increases due to the destabilization of the medial meniscus as OA progression. Therefore, the height of the medial epiphysis decreases with subchondral bone sclerosis [40]. In our study, the ratio of the medial and lateral height of the epiphysis in the septide group at 8 weeks was almost same as that of the sham mice while control group exhibited significantly lower the medial and lateral height of the epiphysis, which means that administration of the septide could maintain the morphology of the epiphysis. Besides, synovitis score in the septide group was significantly lower than that in the control and aprepitant group at 1 weeks, although synovitis caused by DMM is usually stronger in an earlier phase. This is though to be due to the anti-inflammatory effect and immune modulation of SP, which supported that blocking SP by aprepitant induced the worst synovitis score at 1 week [41,42]. From these evidences, SP may function as the anabolic factor to OA pathogenesis. The reason why the expression of SP was highest after 2 days of DMM is thought to be that the stimulation of DMM increased the number of cells expressing SP in an attempt to enhance the anabolic effect. Various functions of SP may ameliorate the

progression of OA.

There are several limitations in this study. First, only a single dose of the NK1R agonist and antagonist was systemically administered to DMM mice. This OA model exhibited sudden OA onset by the severe instability of knee joint. There is a possibility that administration of multiple doses of the NK1R agonist may be more effective to prevent OA progression. In addition, it is required to investigate the effectiveness of the NK1R agonist administration in the aging OA model in the future. Second, the effect of the NK1R agonist on pain has not been evaluated. SP plays an important role in pain response and regulation [10]. Further studies to explore the influence of SP agonists on pain in relation to OA are necessary. Finally, the adverse effects of the NK1R agonist were not evaluated in detail, although there was no exacerbation of synovitis in this study. SP is expressed in various tissues and organs and has various function including inflammation and wound healing [45]. The impact of systemic administration of NK1R agonist on these tissues should be investigated, including determining the appropriate dose and method of administration. Intra-articular injection is possible another way to reduce the systemic adverse effects. There are two reasons why we chose systemic administration to DMM mice in this study. Since subchondral bone changes such as the sclerosis are quite important for the progression of OA, it is expected that drugs will reach the subchondral bone and work there by systemic administration. Another reason is that drugs will be administered orally in the future after the efficacy and safety of the drugs can be established. Further researches are needed to address these problems.

In conclusion, SP is expressed in the articular cartilage and subchondral bone and its expression decreases as OA progresses. An NK1R agonist ameliorates OA progression and cartilage degeneration through the inhibition of sclerotic change of the subchondral bone. SP might contribute to the homeostasis the articular cartilage and subchondral bone and could serve as a novel therapeutic target for OA.

ACKNOWLAGEMENT

We thank T. Miyata for her technical support. The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was partly supported by MEXT KAKENHI Grant-in-Aid for Scientific Research (C); Grant Number 18K09066 (T.N.). Research grant from the Nakatomi Foundation (T.N.).

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Figure 1

Expression pattern of SP in cartilage and SBP of OA patients. (A-D) Safranin O staining and immunohistochemistry of SP in the tibial plateau of moderate OA patients. The OARSI score is 3. SP is expressed in the articular cartilage and SCB. (E-H) Safranin O staining and immunohistochemistry of SP in the tibial plateau of severe OA patients. The OARSI grade is 5. Arrow indicates SP-positive cells. Scale bars represent 100 μm. (I) The number of SP-positive cells (/mm^2) in the articular cartilage. (J) The number of SP-positive cells (/mm^2) in the SCB. The number of SP-positive cells in the cartilage and SCB of mild OA samples were significantly higher than those in severe OA samples (I, J). SP: Substance P, SCB: subchondral bone, **$p < 0.01$, *$p < 0.05$.

Figure 2

Expression pattern of SP in healthy and OA samples. (A-I) Safranin O staining and immunohistochemistry of SP of the tibial plateau from autopsy donors and OA patients. Young healthy (A-C), aged healthy (D-F) and OA patient samples with 6.5 of the OARSI grade (G-I). Scale bars represent 100 μm. (J) The number of SP-positive cells in the articular cartilage. The number of SP-positive cells in the articular cartilage of young healthy samples was significantly higher than that of OA patients. (K) The percentage of the SP positive cells to total chondrocytes. The percentage of SP-positive cells in the articular cartilage of healthy individuals was significantly higher than that of OA patients. **$p < 0.01$, *$p < 0.05$.

Figure 3

Expression pattern of SP in the DMM mice. (A) Immunohistochemistry of SP in the knee joint of control DMM and sham mice. (B) The number of SP-positive cells in the articular cartilage of tibia of control DMM and sham mice. The expression of SP was maximal on 2 days post-surgery and gradually decreased with the progression of OA. Scale bars represent 100 μm. **p < 0.01, *p < 0.05.

Figure 4

The effect of aprepitant and septide administration on the OA progression in the DMM mice. (A) Safranin O staining of the knee joint of DMM and sham mice. Scale bars represent 100 μm. (B) OARSI score of DMM and sham mice. There was no significant difference between the 3 groups at 2 days post-surgery. The OARSI scores of the septide group was significantly lower than that of the control and aprepitant group at 1 week, 4 weeks and 8 weeks post-surgery. (C) Synovitis score of DMM and sham mice at **1,** 4 and 8 weeks. (D) Subchondral bone score at 1 week, 4 weeks and 8 weeks post-surgery. (E) The thickness of subchondral bone plate at 2days, 1, 4 and 8 weeks. **p < 0.01, *p < 0.05.

Figure 5

Micro-computed tomography (CT) analysis. (A) Micro-CT images of the knee joint from DMM mice. Broken line indicates measured area of BV/TV. (B) The ratio of bone volume and tissue volume in the subchondral bone's epiphysis. BV/TV of the septide group was significantly lower than that of the control group at 1 week, 4 weeks and 8 weeks post-surgery. **p < 0.01, *p < 0.05. BV: bone volume, TV: tissue volume. (C) Micro-computed tomography images of the knee joint in coronal images. Arrows indicate the

height of lateral (yellow) and medial (red) epiphysis. (D) The ratio of medial / lateral height of the epiphysis in the tibia plateau.

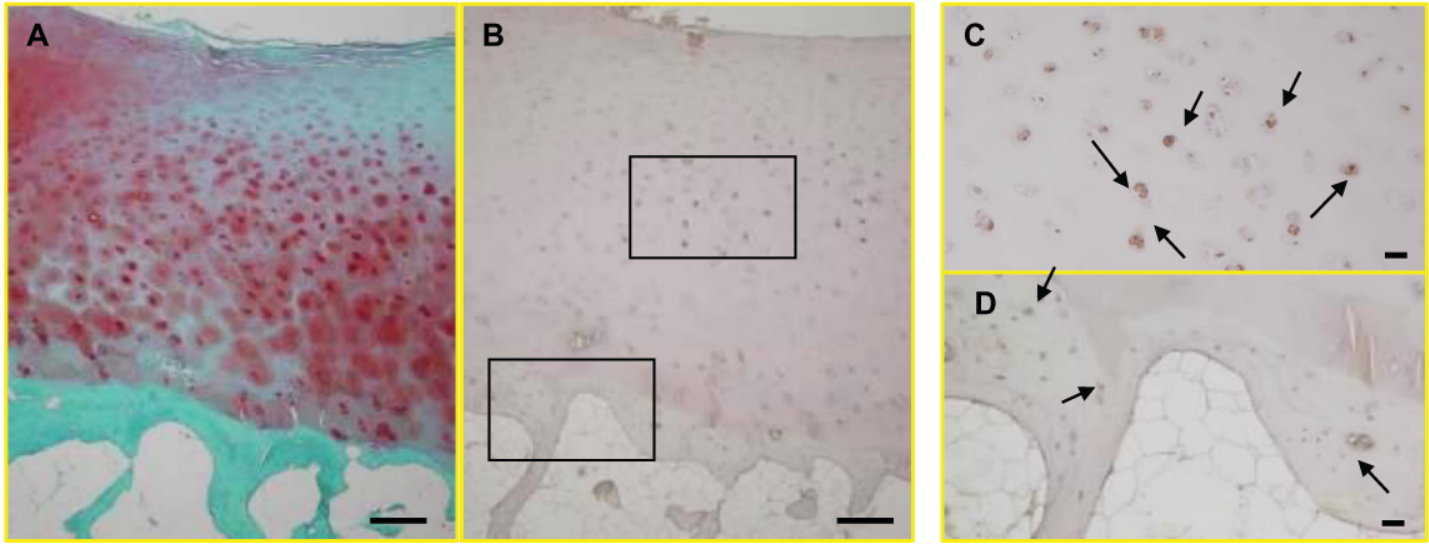
Figure 6

The expression pattern of the osteoblast and osteoclasts. (A) Immunohistochemistry of OC in DMM and sham mice. (B) Rectangles indicate the measured area for the cell number.(C) The number of OC-positive cells in the subchondral bone's epiphysis of tibia. The number of OC-positive cells in the control group was significantly higher than in the septide group at 4 weeks and 8 weeks post-surgery. Scale bars represent 100 μm. OC: osteocalcin. (D) TRAP staining of DMM and sham mice. (E) Rectangles indicate the measured area for the cell number.(F) The number of TRAP-positive cells in the subchondral bone's epiphysis of tibia. The number of TRAP-positive cells in the septide group was significantly higher than that in the control and aprepitant groups at 4 weeks post-surgery. There were no significant differences at 8 weeks. Scale bars represent 100 μm. $^{**}p < 0.01$, $^{*}p < 0.05$.

Figure 7

Expression analysis of catabolic factors in the articular cartilage. (A) Immunohistochemistry of SP of the knee joint from DMM mice. SP expression in the septide group was significantly higher than that in the control and aprepitant groups at 4 weeks and 8 weeks post-surgery. (B) (C) Immunohistochemistry of MMP13 and ADAMTS-5 in DMM mice, respectively. MMP13 expression in the septide group was significantly lower than that in the control group at 4 weeks and 8 weeks post-surgery. ADAMTS-5

expression was significantly lower in the septide group than in the control group at 4 weeks post-surgery, but there were no significant differences at 8 weeks. Scale bars represent 100 μm. **$p < 0.01$, *$p < 0.05$.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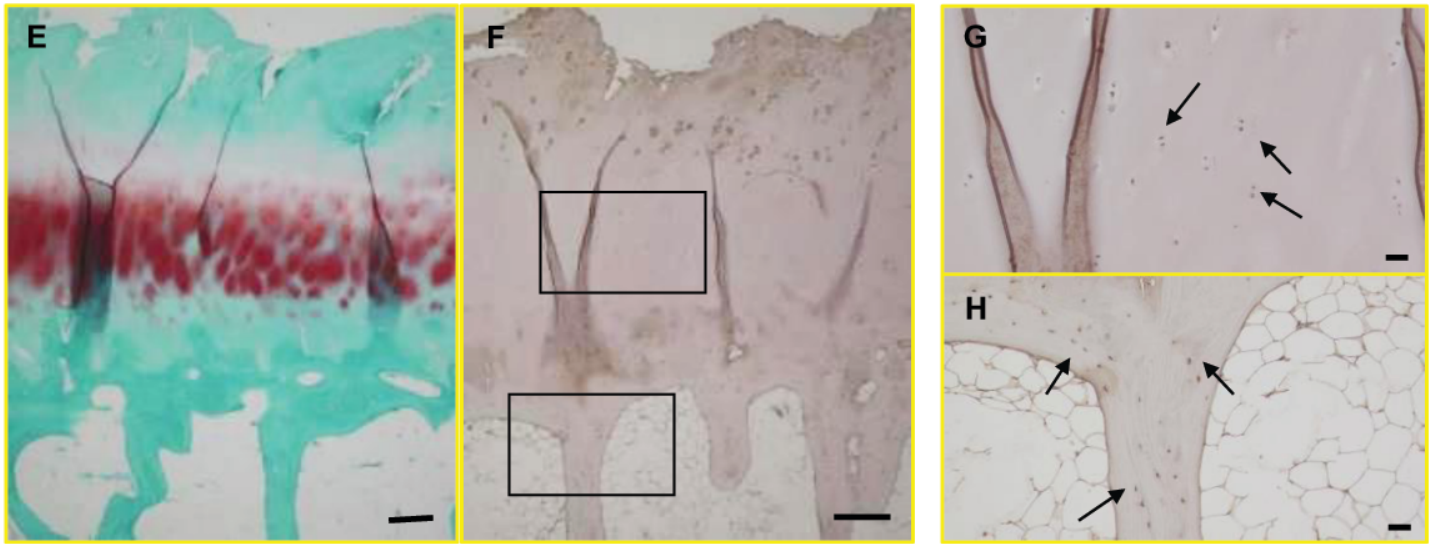
B
C
D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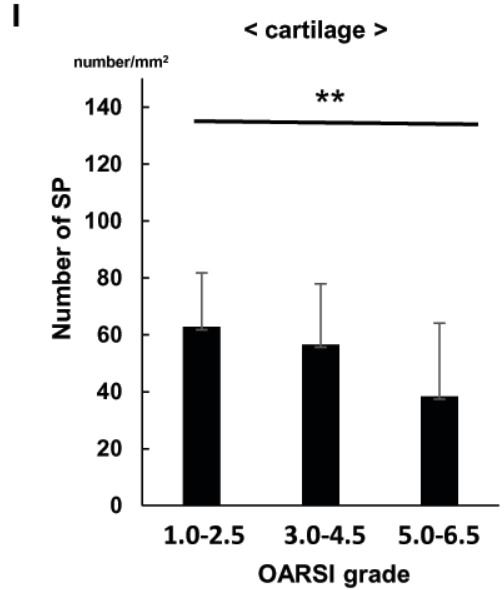
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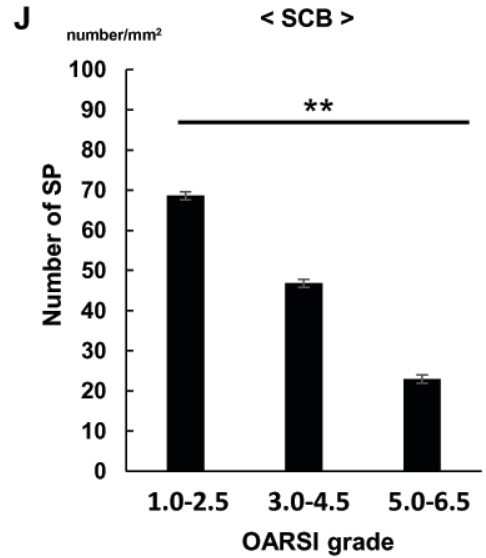
number/mm²
**
Number of SP
140
120
100
80
60
40
20
0
1.0-2.5
3.0-4.5
5.0-6.5
OARSI grade


J
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number/mm²
**
Number of SP
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90
80
70
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30
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10
0
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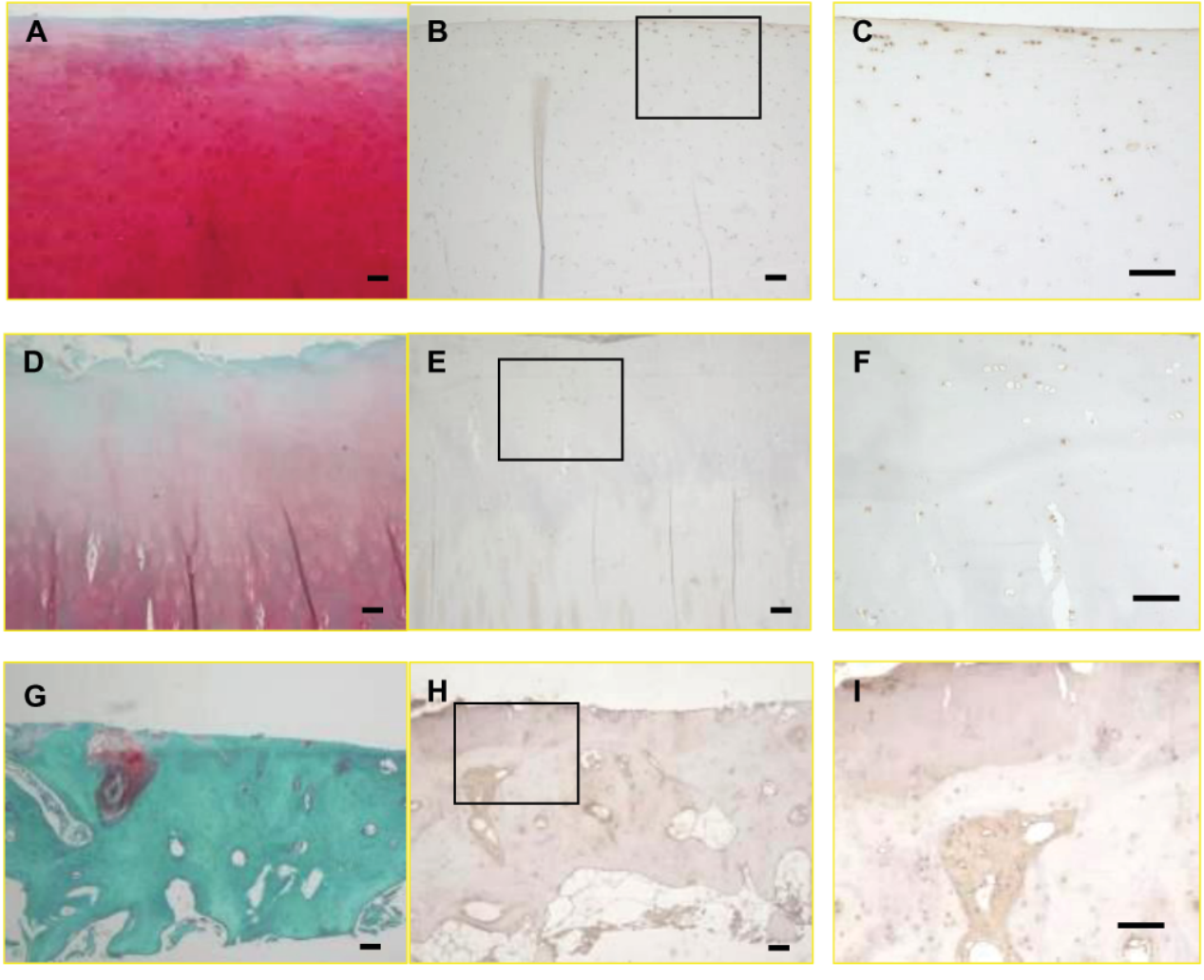
3.0-4.5
5.0-6.5
OARSI grade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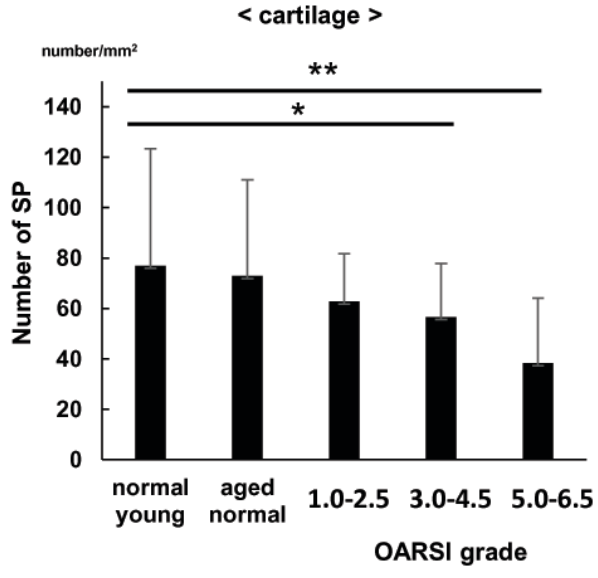
G
H
I

J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number/mm²
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*
140
120
100
80
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Number of SP
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aged normal
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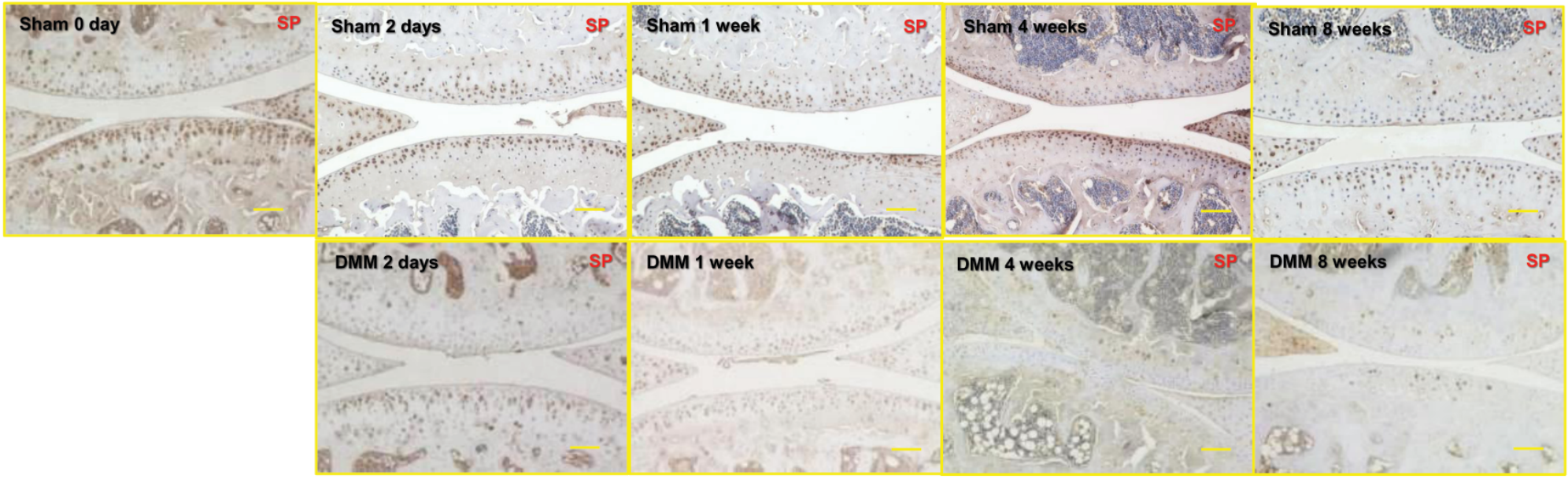
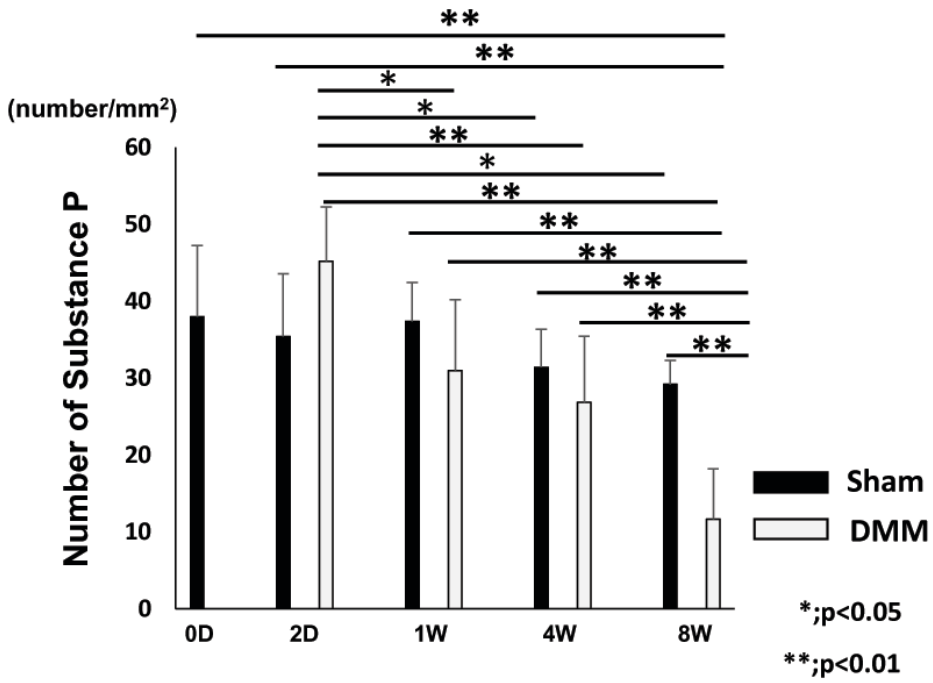
3.0-4.5
5.0-6.5
OARSI grade

A


Sham 0 day
SP
Sham 2 days
SP
Sham 1 week
SP
Sham 4 weeks
SP
Sham 8 weeks
SP
DMM 2 days
SP
DMM 1 week
SP
DMM 4 weeks
SP
DMM 8 weeks
SP

B


(number/mm²)
Number of Substance P
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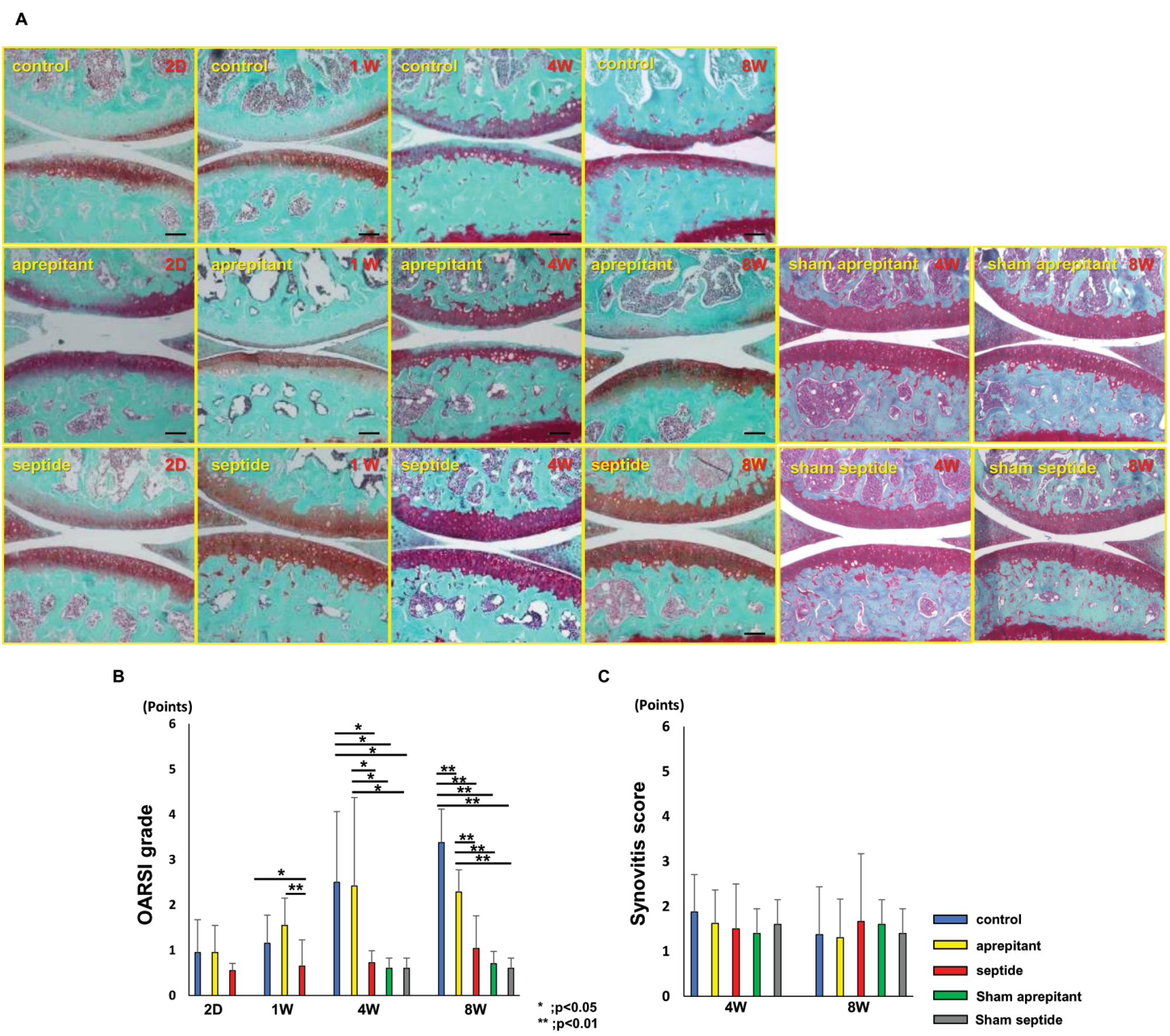
50
40
30
20
10
0
0D
2D
1W
4W
8W
**
**
*
*
**
*
**
**
**
**
**
**
Sham
DMM
*;p<0.05
**;p<0.01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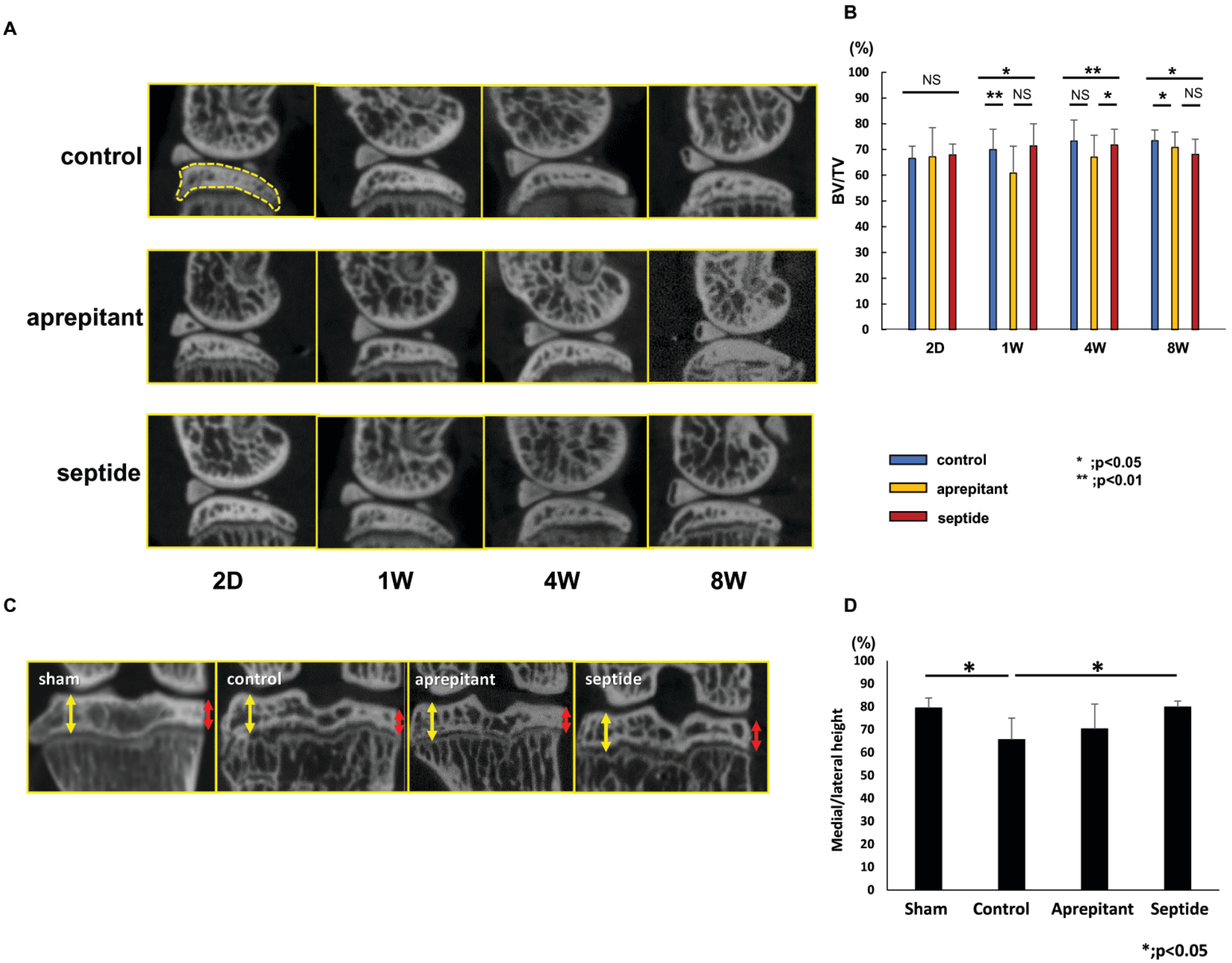
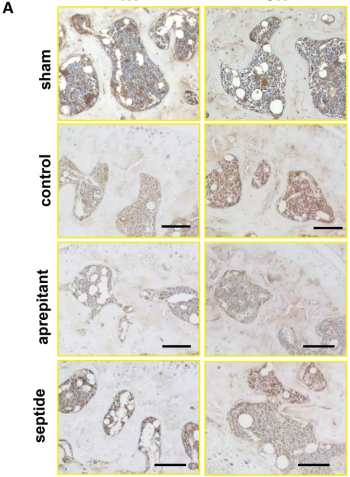
control 2D
control 1 W
control 4W
control 8W
aprepitant 2D
aprepitant 1 W
aprepitant 4W
aprepitant 8W
sham aprepitant 4W
sham aprepitant 8W
septide 2D
septide 1 W
septide 4W
septide 8W
sham septide 4W
sham septide 8W
B
(Points)
OARSI grade
2D
1W
4W
8W
* ;p<0.05
** ;p<0.01
C
(Points)
Synovitis score
4W
8W
control
aprepitant
septide
Sham aprepitant
Sham septide


A
control
aprepitant
septide
2D
1W
4W
8W
B
(%)
BV/TV
100
90
80
70
60
50
40
30
20
10
0
NS
*
**
NS
**
NS
NS
*
*
*
NS
2D
1W
4W
8W
control
aprepitant
septide
* ;p<0.05
** ;p<0.01
C
sham
control
aprepitant
septide
D
(%)
Medial/lateral height
100
90
80
70
60
50
40
30
20
10
0
*
*
Sham
Control
Aprepitant
Septide
*;p<0.05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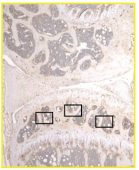
8W
sham
control
aprepitant
septide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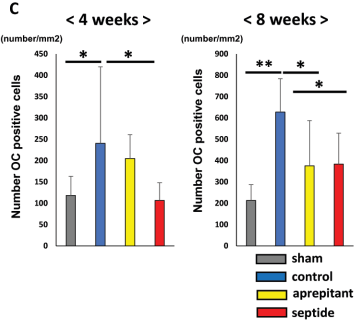
C
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(number/mm2)
Number OC positive cells
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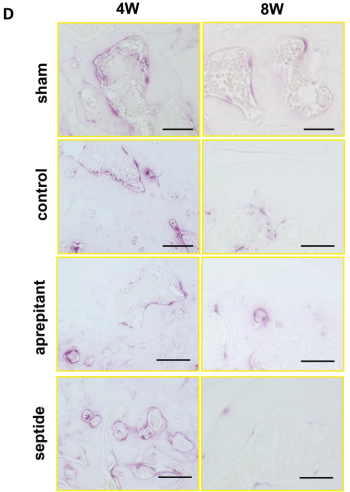
400
350
300
250
200
150
100
50
0
*
*
< 8 weeks >
(number/mm2)
Number OC positive cells
900
800
700
600
500
400
300
200
100
0
**
*
*
sham
control
aprepitant
septide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8W
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control
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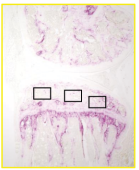
septide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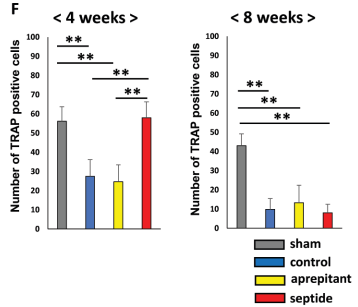
F
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Number of TRAP positive cells
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90
80
70
60
50
40
30
20
10
0
**
**
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< 8 weeks >
Number of TRAP positive cells
100
90
80
70
60
50
40
30
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10
0
**
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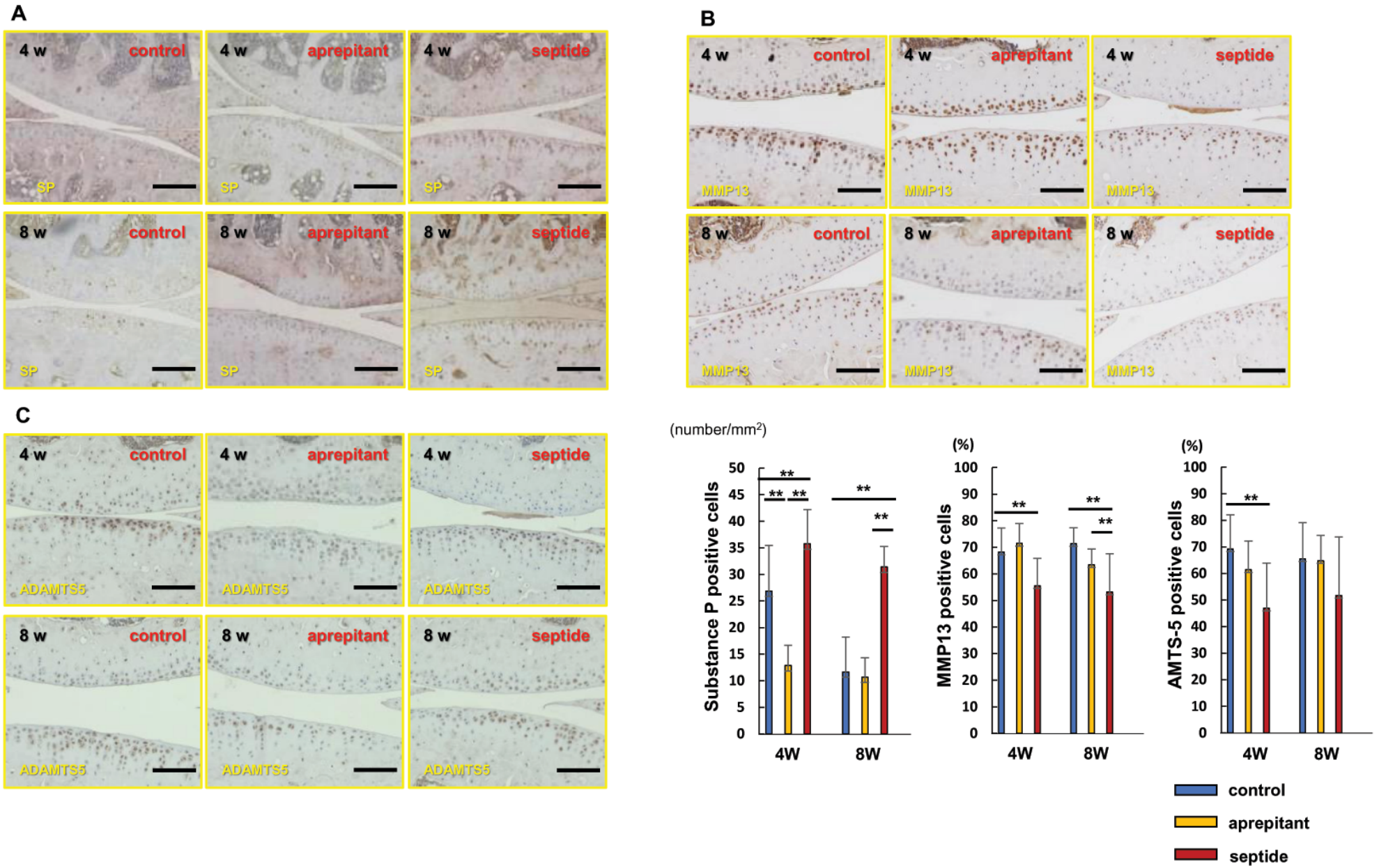
sham
control
aprepitant
septide


A
4 w control
4 w aprepitant
4 w septide
SP
8 w control
8 w aprepitant
8 w septide
B
MMP13
C
ADAMTS5
(number/mm2)
Substance P positive cells
(%)
MMP13 positive cells
AMTS-5 positive cells
4W
8W
control
aprepitant
septide