# Effect of systemic delivery of Substance P on experimental tooth movement in rats

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**Introduction:** The purpose of this study was to investigate the effect of systemic delivery of Substance P (SP) on experimental tooth movement. **Methods:** Forty-eight adult Sprague-Dawley rats were randomly divided into 2 groups and their maxillary first molars were mesially moved with the use of closed-coil springs. The experiment group received systemic injection of SP and the control group received phosphate-buffered saline solution. Transportation distances of first molars were measured. Hematoxylin and eosin staining, tartrate-resistant acid phosphatase staining, and immunohistochemistry staining were performed to evaluate alveolar bone remodeling. Then the interferon (IFN) $\gamma$ and tumor necrosis factor (TNF) $\alpha$ concentrations in peripheral blood and local periodontal tissue were measured. Finally, the effects of SP on bone marrow–derived stem cell (BMSC) proliferation and migration were tested in vitro. **Results:** Systemic delivery of SP significantly increased the distance of tooth movement and stimulated both osteoclast and osteoblast activities. The concentrations of IFN-$\gamma$ and TNF-$\alpha$ increased in peripheral blood at early phases of the experiment and decreased in periodontal tissue at late phases. In vitro, the proliferation and migration of BMSCs were promoted by SP. **Conclusions:** Systemic delivery of SP can accelerate orthodontic tooth movement and promote alveolar bone remodeling potentially through immunomodulation and mobilizing endogenous mesenchymal stem cells. (Am J Orthod Dentofacial Orthop 2019;155:642-9)

Orthodontic tooth movement is a process that involves force-induced bone metabolism and aseptic inflammation, which is accompanied by minor reversible injury to the tooth-supporting tissue.[1] Sufficient studies have confirmed that inflammatory cytokines, such as tumor necrosis factor (TNF) $\alpha$ and interferon (IFN) $\gamma$, play important roles in the process of orthodontically induced tooth movement.[2-6] There have been lesser known mediators, such as Substance P (SP), an undecapeptide produced from peripheral nerves and immune cells, which regulates immune cell functions through autocrine and paracrine mechanisms via the high-affinity neurokinin 1 receptor.[7] Studies have shown increased levels of SP in periodontal tissues during orthodontic tooth movement, indicating that SP may be associated in some way with the altered inflammatory microenvironment and consequently influence tooth movement.[8,9] SP acts as a chemoattractant for monocytes and lymphocytes and can enhance lymphocyte proliferation and immunoglobulin production.[10-15] In a recent study, researchers demonstrated that SP acted as a systemic messenger of injury and that systemic administration of SP could successfully mobilize mesenchymal stem cells (MSCs) into peripheral blood in both injured and uninjured animals.[16] Because these mobilized cells possess pluripotent capacities for bone remodeling and immunomodulation, how they would affect orthodontic tooth movement remains unpredictable and untested.[17-19] Therefore, we conducted the present study to investigate the effect of systemic delivery of SP on experimental orthodontic tooth movement in rats, which is probably one of the first studies to test the systemic role of SP in orthodontic treatment.

## MATERIAL AND METHODS

All experimental procedures performed in this study were based on a protocols approved by the Animal

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Experiment Ethics Committee of the State Key Laboratory of Oral Diseases of Sichuan University in China. All animals were obtained from the Sichuan University's experimental animal center. Six-week-old Sprague-Dawley rats were used for cell culture and 10-week-old Sprague-Dawley rats were used for establishing the animal model of experimental tooth movement.

Forty-eight male Sprague-Dawley rats (10 weeks old, 200 g) were randomly divided into 2 groups: the SP group and the control group. A nickel-titanium closed-coil spring (Grikin Advanced Materials, Beijing, China) was fixed between the maxillary first molars and incisors. The maxillary first molars were moved mesially with an orthodontic force of 40 g. The appliances were activated immediately after insertion, and no reactivation was performed in the following experiment period. The rats in the SP group received 3 intravenous injections of SP (5 nmol/kg; Sigma, St Louis, Mo) respectively at 0, 24, and 48 hours after the installation of the orthodontic appliance. Correspondingly, the rats in control group received 3 intravenous injection of phosphate-buffered saline solution. All rats were able to survive during the experiment and maintain appropriate body weight.

Eight rats from each group were killed on days 3, 7, and 14 after application of orthodontic forces, and 1-1.5 mL peripheral blood was taken from each killed rat. Then the maxilla was dissected and divided into halves.

The tooth movement was determined by measuring the distance between the first and second maxillary molars with the use of a feeler gauge on the dissected maxillas from days 3, 7, and 14.

For histologic staining, the left half of the maxilla of each rat was fixed in 4% paraformaldehyde for 24 hours and decalcified in 10% EDTA solution (pH 7.4) at 4°C for at least 4 weeks. After dehydration, the hemimaxillas were paraffin embedded and cut into 7-μm serial sections in a mesiodistal direction parallel to the long axis of the distal root of the first molar. Selected sections were investigated with hematoxylin and eosin staining, tartrate-resistant acid phosphatase (TRAP) staining, and immunohistochemical staining. The primary antibody for osteocalcin (Santa Cruz Biotechnology, Santa Cruz, Calif) was used at a dilution of 1:100.

For Western blots, the maxillary first molar together with its periodontal tissue was collected from the right half of the maxilla. The collected tissue was immediately frozen with the use of liquid nitrogen and then ground with mortars and pestles. The tissue powder was lysed with ice-cold lysis buffer (Keygen Biotech, Nanjing, China) and then centrifuged at 10,000 rpm for 20 minutes at 4°C before quantification. Equal amounts of protein samples were separated by means of 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membrane. After blocking with 5% nonfat dry milk, the membrane was incubated with primary antibodies for TNF-α and INF-γ (Abcam, Cambridge, U.K.) followed by incubation with secondary antibody (1:5000; ZSGB-Bio, Beijing, China). The signals were then visualized by means of chemiluminescence detection.

For enzyme-linked immunosorbent assay (ELISA) analysis, peripheral blood samples harvested from rats killed on days 3, 7, and 14 were used for evaluating serum concentrations of TNF-α and IFN-γ with the use of ELISA kits (R&D Systems, Minneapolis, Minn). The samples were set stable for 30 minutes. After centrifugation at 3000 rpm at 4°C for 10 minutes, the supernate was collected and assayed by means of ELISA for IFN-γ and TNF-α (Abcam).

For cell counting, bone marrow cells were flushed out from bone cavity of femurs and tibias of 6-week-old Sprague-Dawley rats. A single-cell suspension was obtained by passing all bone marrow cells through a 70-μm cell strainer (BD Bioscience, Franklin Lakes, NJ). All of the single cells were seeded at $1 \times 10^6$ onto 100-mm culture dishes (Corning, Inc., Corning, NY) and incubated in α-MEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco, Grand Island, NY) at 37°C and 5% $CO_2$. Medium was changed every 3 days.

We seeded $1 \times 10^6$ bone marrow–derived stem cells (BMSCs) on 100-mm cell culture plates and cocultured them with different doses of SP (0 mol/L, $1 \times 10^{-10}$ mol/L, $1 \times 10^{-8}$ mol/L, and $1 \times 10^{-6}$ mol/L). After 48 hours of treatment, cell counting was performed.

Cell Counting Kit 8 (CCK-8) was also carried out. We seeded $4 \times 10^3$ BMSCs in each well of a 96-well plate in 100 μL culture medium and preincubated the plate for 24 hours at 37°C and 5% $CO_2$. Then the cells were treated with $1 \times 10^{-10}$ mol/L, $1 \times 10^{-8}$ mol/L, and $1 \times 10^{-6}$ mol/L SP in 3 experiment groups. After 48 hours of treatment, 10 μL CCK-8 solution (Dojindo, Tabaru, Japan) was added to each well of the plate. After 2-hour incubation at 37°C and 5% $CO_2$, the optical density value of each well was measured at 450 nm with the use of a microplate reader (Bio-Rad, Hercules, Calif).

Cell migration was evaluated with the use of a scratch assay. We seeded the third passage of BMSCs in 100-mm plates and incubated the plates at 37°C and 5% $CO_2$. When cell confluence reached 90%, a scratch was created with the use of a P200 pipette tip in all groups. In 3 experiment groups, the cells were treated with $1 \times 10^{-10}$ mol/L, $1 \times 10^{-8}$ mol/L, and $1 \times 10^{-6}$ mol/L of SP. At 0, 9, and 18 hours after the treatment, the cells in the 4 groups

were photographed with the use of a phase-contrast microscope.

### Statistical analysis

SPSS 11.5 software (IBM, Chicago, Ill) was used for the statistical analyses. Significance was assessed with the use of 1-way analysis of variance, least square difference test, and $t$ test. $P < 0.05$ was considered to be significant.

## RESULTS

Substance P accelerated orthodontic tooth movement. During the first 3 days, the tooth movement under orthodontic forces was limited in both SP and control groups (Fig 1, *C*). In addition, our data showed that during the early phase of the experiment, there was no significant difference of total tooth movement between groups ($P > 0.05$; Fig 1, *C*). However, during the late phase, the differences became significant (Fig 1). On Days 7 and 14, systemic delivery of SP increased total tooth movement by, respectively, 1.3-fold ($P < 0.01$) and 1.5-fold ($P < 0.01$) compared with the control group (Fig 1, *C*).

Systemic delivery of SP promoted alveolar bone remodeling during tooth movement. Histologic staining showed that after application of orthodontic forces, the periodontal ligament on the compression side of the first molar became compressed whereas on the tension side the tissue expanded (Fig 2, *A*). Multinucleated clastic cells were observed on the compression side, and these cells formed resorption lacunae around themselves on the surface of alveolar bone (Fig 2, *B*). Meanwhile, new bone deposition and many cuboidal osteoblasts were detected on the surface of alveolar bone on the tension side (Fig 2, *C*).

According to TRAP staining, TRAP-positive osteoclasts appeared on day 3 and increased on days 7 and 14 (Fig 2, *D*). In addition, quantitative analyses revealed that there were more osteoclasts in the periodontal tissue of the SP group than the control group on days 7 and 14 ($P < 0.05$; Fig 2, *D*).

Immunohistochemical staining was performed to investigate expression of osteocalcin to evaluate new alveolar bone formation. The signals of osteocalcin were detected mainly in periodontal ligament cells, osteoblast-like cells, and extracelluar matrix (Fig 2, *E*). Statistical analyses have shown that the expression of osteocalcin within each group increased on days 7 and 14 compared with day 3 (Fig 2, *E*). In addition, according to quantitative analyses the SP group showed a significantly higher expression of osteocalcin than the control group at each time point ($P < 0.05$; Fig 2, *E*).

Systemic delivery of SP altered both local and systemic inflammatory states during orthodontic tooth movement. As described earlier, orthodontic tooth movement is accompanied by aseptic inflammation, and proinflammatory cytokines such as TNF-$\alpha$ and IFN-$\gamma$ are important in alveolar bone remodeling during tooth movement. We assessed serum concentrations of IFN-$\gamma$ and TNF-$\alpha$ in peripheral blood. ELISA showed that the serum concentrations of proinflammatory cytokines increased after systemic delivery of SP (Figs 3, *A* and *B*). On days 3 and 7, IFN-$\gamma$ was 1.4-fold greater in the SP group than in the control group ($P < 0.01$), and TNF-$\alpha$ was 1.3-fold greater in the SP group ($P < 0.05$; Figs 3, *A* and *B*). Then we evaluated local inflammatory states of periodontal tissue by means of Western blot analysis. The data suggest that systemic delivery of SP could reduce the IFN-$\gamma$ and TNF-$\alpha$ expression in periodontal tissues, especially on days 7 and 14 ($P < 0.05$; Fig 3, *C*).

Substance P promoted the proliferation and migration of rat BMSCs in a dose-dependent manner. To find out the effects of SP on the proliferation of BMSCs, we seeded BMSCs in 100-mm plates and treated them with $1 \times 10^{-10}$ mol/L, $1 \times 10^{-8}$ mol/L, and $1 \times 10^{-6}$ mol/L of SP respectively in three experiment groups. After 24 hours and 48 hours, the experiment group under treatment with $1 \times 10^{-6}$ mol/L SP reached higher cell confluences than the control group (Fig 4, *A*). And viable cell counting assay showed that the group with $1 \times 10^{-6}$ mol/L SP showed the largest number of viable cells ($P < 0.01$) whereas the other 2 SP groups ($1 \times 10^{-8}$ mol/L and $1 \times 10^{-10}$ mol/L) had similar numbers of viable cells to the control group ($P > 0.05$; Fig 4, *B*), which was consistent with the results of CCK-8 assay (Fig 4, *C*).

Then we applied a wound scratch assay to determine the effects of SP on migration of BMSCs. The results showed that SP promoted BMSC migration in a dose-dependent manner after 9 hours and 18 hours of treatment (Fig 4, *D*).

## DISCUSSION

Various approaches have been reported to accelerate the orthodontic tooth movement, such as laser therapy, corticotomy, infusion of parathyroid hormone, and local receptor activator of nuclear factor κB ligand gene transfer.[20-22] Acceleration of efficient tooth movement has become the focus of orthodontic research.[22] These techniques will shorten the time of orthodontic treatment sharply and subsequently benefit both patients and orthodontists.

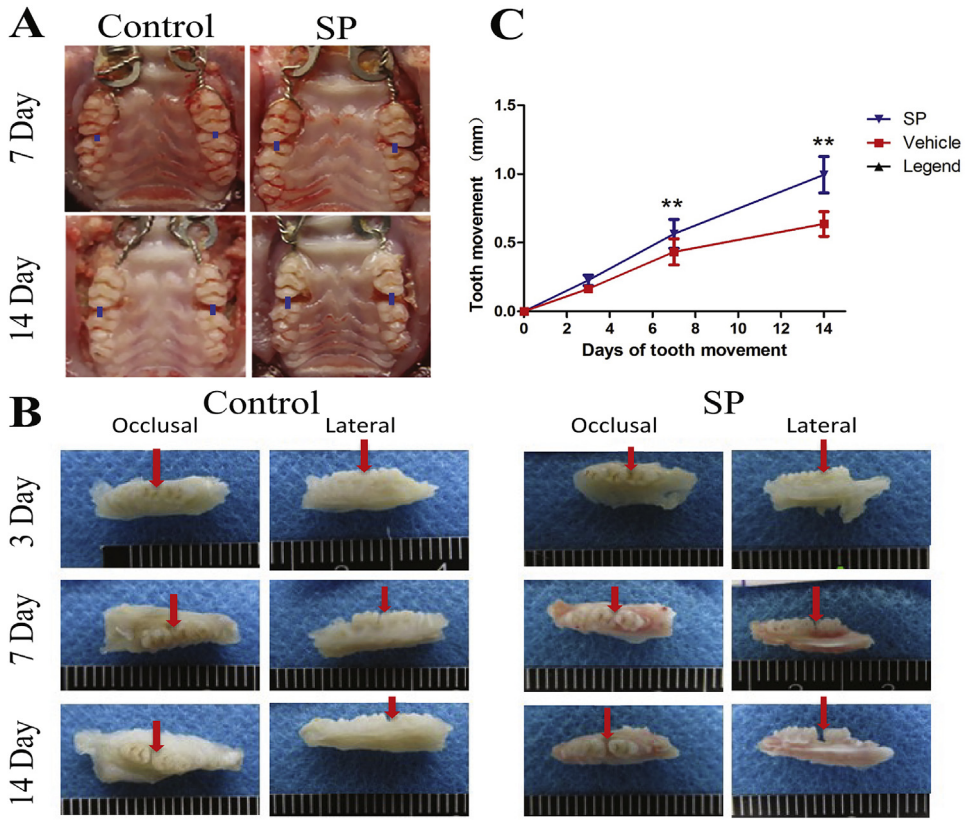


**Fig 1.** Effects of systemic delivery of substance P on orthodontically induced tooth movement in rats. **A,** Representative pictures of intraoral molar movement for both groups on days 7 and 14 (occlusal view of maxilla). The short blue lines indicate the spaces between the first and second molars which was created by the mesial movement of maxillary first molar. **B,** Representative pictures of molar movement on each side of the maxilla with a feeler gauge on days 3, 7, and 14 (occlusal and lateral views of maxilla). The red arrows point to the spaces between first and second molars created by mesial movement of the first molar. **C,** Time course of orthodontic tooth movement in rats treated with phosphate-buffered saline solution (Vehicle) injection and substance P (SP) injection mean ± SD. $^{**}P < 0.01$. Each group at each time point: n = 8.

Substance P, as a neuropeptide, can be released from both nervous system and immune cells.[7] Its receptor neurokinin 1 receptor can be found on both osteoclast and immune cells, both of which are involved in alveolar bone remodeling during orthodontically induced tooth movement.[23,24] In addition, some studies have shown that SP, as an injury-inducible factor, increased in periodontal tissue during orthodontic treatment and had the potential to regulate inflammation during this process.[8,9] To find out whether SP could also be a potential target for accelerating orthodontically induced tooth movement, we used the animal model of experimental tooth movement to test the systemic role of SP. This study is probably one of the first studies to investigate the effects of SP on orthodontic tooth movement.

The results regarding transportation distances of first molars showed that SP facilitated tooth movement at the late stages of orthodontic treatment compared with control (Fig 1, *A-C*). In addition, the number of TRAP-positive osteoclasts on the alveolar bone surface of the compression side increased significantly in the SP group (Fig 2, *D*), which was consistent with some previous in vitro studies.[25] These results indicate that SP could enhance osteoclast activities, which were associated with alveolar bone remodeling during tooth movement. In addition, osteocalcin levels increased on the tension side of alveolar bone after systemic administration of SP, suggesting the potential capacity of SP in osteoblast stimulation (Fig 2, *E*). Some previous researchers have shown similar results although other researchers have reported that SP could inhibit osteogenetic activities.[26,27] This discrepancy could be explained by the different inflammatory states within different experiment models. In a periodontal disease model, SP together

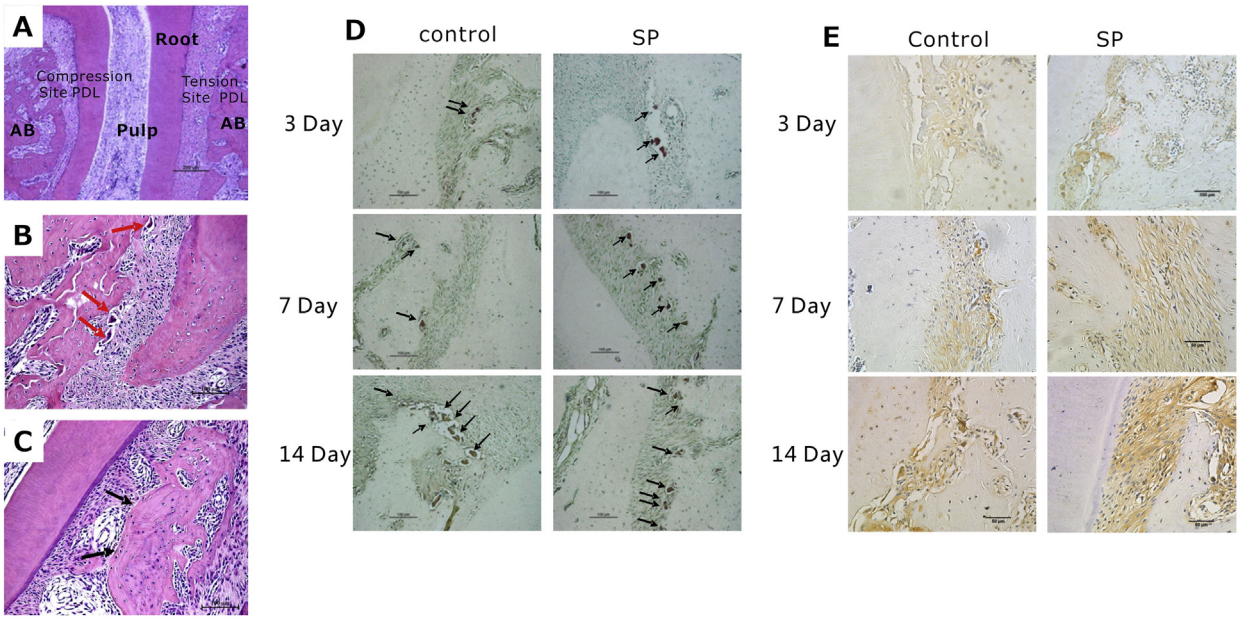


**Fig 2.** Effects of systemic delivery of substance P on alveolar bone (AB) remodeling during orthodontic tooth movement. **A,** Hematoxylin and eosin staining of periodontal tissue of maxillary first molar. *PDL*, periodontal ligament. Scale bar = 200 μm. **B,** Magnification of the compression side of the distal root of maxillary first molar. The red arrows indicate the osteoclasts and the resorption lacunae around them. Scale bar = 100 μm. **C,** Magnification of the tension side of the distal root of maxillary first molar. The black arrows indicate the cuboidal osteoblast. Scale bar = 100 μm. **D,** TRAP staining of periodontal tissue of maxillary first molar on days 3, 7, and 14 for both groups. The black arrows indicate the TRAP-positive osteoclasts. Scale bar = 100 μm. **E,** Immunohistochemical staining for osteocalcin of periodontal tissue of maxillary first molar on days 3, 7, and 14 for both groups. Scale bar = 50 μm.

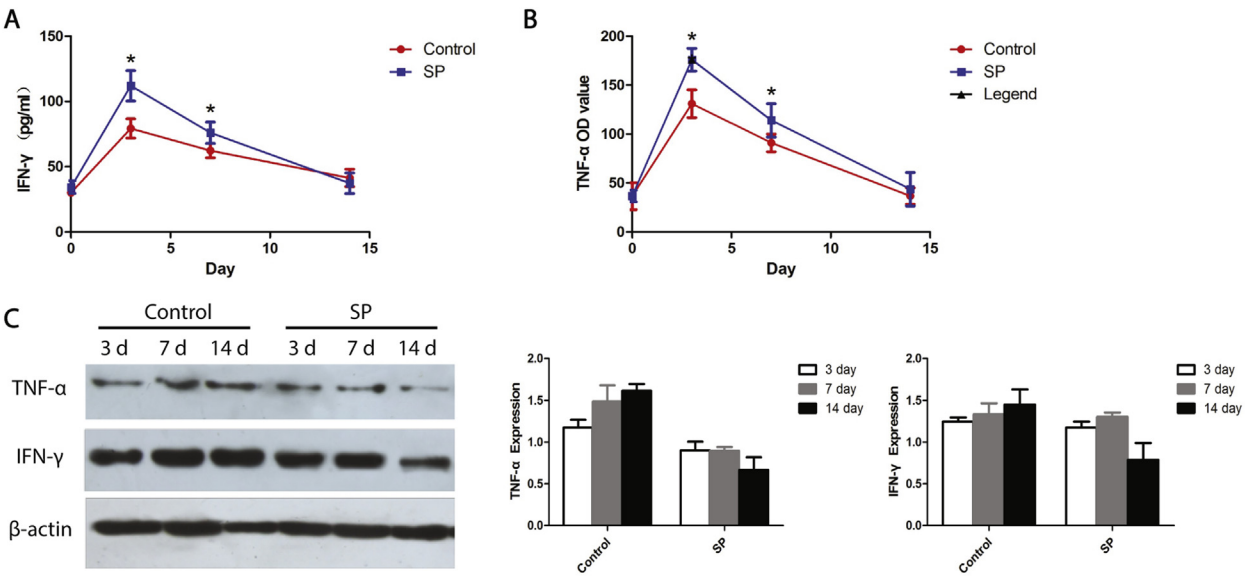


**Fig 3.** Effects of systemic delivery of substance P on local and systemic inflammatory states during orthodontic tooth movement. **A, B,** Time course of proinflammatory cytokine levels in peripheral blood of rats treated with phosphate-buffered saline solution (Vehicle) injection and substance P (SP) injection respectively, mean ± SD. **P* <0.05. Each group at each time point: n = 8. **C,** Western blot analysis of IFN-γ and TNF-α in periodontal tissue of maxillary first molar on days 3, 7, and 14.

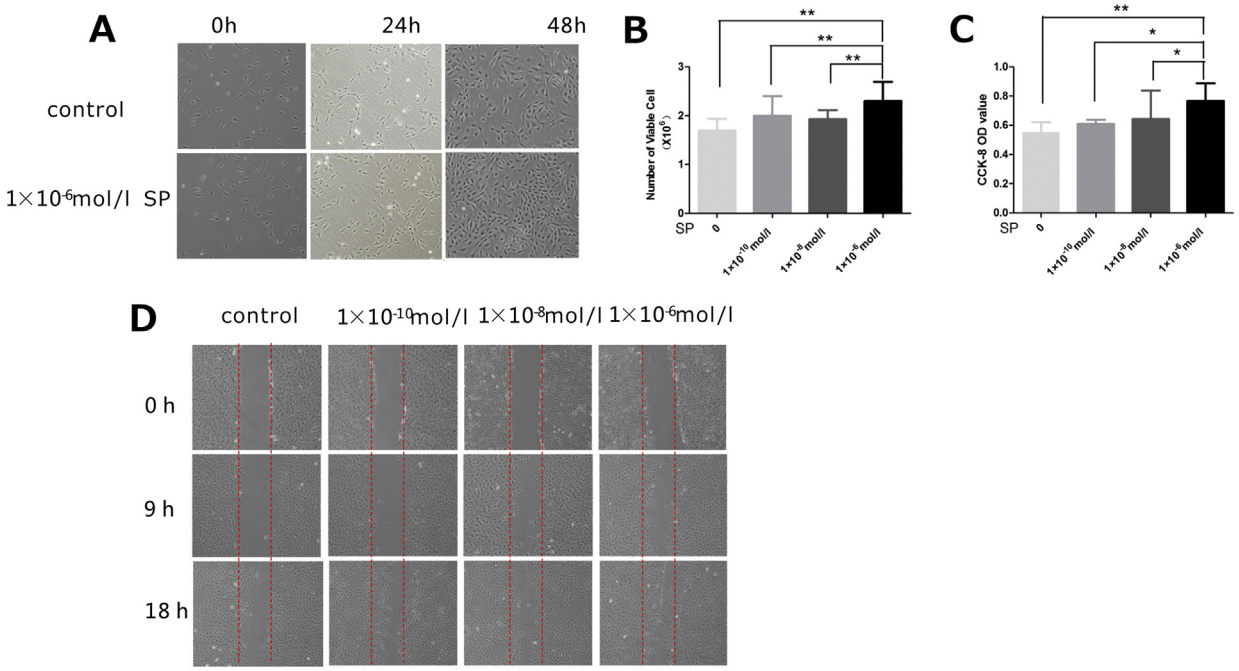


**Fig 4.** Effects of substance P on the proliferation and migration of rat BMSCs. **A,** Representative pictures of cell counting assay for control group and SP group ($1 \times 10^{-6}$ mol/L) at 0, 24, and 48 hours. **B,** Statistical analysis of viable cell numbers within 4 groups after 48-hour treatment. $^{**}P < 0.01$. **C,** CCK-8 analysis of 4 groups after 48-hour treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. **D,** Migration assay of 4 groups at 0, 9, and 18 hours. The space between the two red dotted lines indicates the initial scratch created with the use of a P200 pipette tip.

with lipopolysaccharide (LPS) could potentially inhibit osteoblast differentiation.[26] However, in our orthodontic aseptic inflammatory model without LPS, SP could potentially promote osteoblast activities.[27] Furthermore, considering the concept that mechanical loading may alter the effects of proinflammatory substances on resident cells, the mechanobiology of osteoblasts might be changed to another situation during orthodontic treatment, in which osteoblasts might increase their responses to the stimulation of SP.[28] Further studies need be conducted to find out the exact effects of SP on osteoblasts under different inflammatory and mechanical conditions.

In addition to the direct roles of SP on osteoclasts and osteoblasts during orthodontically induced tooth movement, our studies also demonstrated its capacity for immunomodulation. The reduced levels of proinflammatory factors such as TNF-α and IFN-γ suggest that SP could also alter the inflammatory state during the orthodontic treatment (Fig 3, *C*). It is worth noting that inflammation is "a double-eged sword": It can promote alveolar bone remodeling and accelerate the tooth movement, and it may also have a destructive effect on periodontal tissue as well as tooth roots if not well controlled.[4,29] At early stages of orthodontically induced tooth movement, TNF-α and IFN-γ can be essential triggers of the remodeling of alveolar bone.[3] However, it has also been demonstrated that long-lasting inflammation can impair tooth movement, damage the periodontal tissue and even lead to unfavorable root resorption.[4] In this study, we showed that local inflammation can be efficiently controlled with systemic delivery of SP from day 7 after surgery (Fig 3, *C*). Correspondingly, tooth movement increased from day 7 after surgery (Fig 1). Taking all of this together, SP could indirectly promote tooth movement through reducing inflammatory states at late stages of orthodontic treatment.

In our studies, SP altered not only the local inflammatory states but also systemic inflammation (Fig 3). By days 3 and 7 after surgery, the serum concentrations of IFN-γ and TNF-α were significantly greater in the SP group than in the control group (Fig 3, *A* and *B*). This phenomenon could be associated with the proinflammatory roles of SP.[7] However, the increased concentrations of these proinflammatory cytokines in the circulatory

system were restricted to early stages of orthodontic treatment, and within 2 weeks after the treatment the inflammation had been brought back to levels similar to that of control group (Fig 3, *A* and *B*), which suggests that systemic delivery of SP is safe for the overall health of the animals.

Recent researchers have reported that systemic delivery of SP could mobilize MSCs from the bone marrow and accelerate wound healing.[16] Considering this capacity of SP, it is possible that SP could accelerate rat tooth movement through mobilizing endogenous MSCs. Endogenous MSCs mobilized by systemic delivery of SP might contribute to speeding tooth movement by differentiating into osteoblasts and then participating in alveolar bone remodeling.[16,17] These cells could not only have multipotent differentiation ability but also play an important role in immunoregulation. Considering the reduced expression of TNF-$\alpha$ and IFN-$\gamma$ (Fig 3, *C*), it is also possible that recruited MSCs together with SP help to control the local inflammatory state and subsequently promote tooth movement. In this way, the role of systemic delivery of SP may be attributed to the concept of in situ tissue regeneration, which means using bioactive molecules to recruit endogenous stem cell homing to the site of injury.[30,31]

To find out the effects of SP on BMSCs, we carried out in vitro culture of rat BMSCs and administered different concentrations of SP. The results showed that SP could stimulate BMSC proliferation and migration, which further indicated that SP might promote tooth movement through mobilizing endogenous MSCs in this experimental tooth movement model (Fig 4).

## CONCLUSIONS

Our results show that systemic delivery of SP can potentially promote orthodontic tooth movement and stimulate alveolar bone remodeling. This phenomenon could be explained in part by the potential roles of SP in increasing osteoclast proliferation initially and controlling inflammation at later stages of orthodontic tooth movement. In addition, our results suggest that SP can promote tooth movement through mobilizing endogenous MSCs, which may differentiate into osteoblasts and then contribute to alveolar bone remodeling.

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