

Occurrence of substance P and neurokinin receptors during the early phase of spinal fusion

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Abstract. Spinal fusion is widely used for patients with spinal disorders; however, patients often suffer from back pain following fusion surgery. Substance P (SP) acts as a pain neurotransmitter via the sensory nerve afferent fibres up to the spinal cord, and is involved in the conduction and modulation of pain. The use of specific SP neurokinin receptor (NKR) antagonists may decrease postoperative pain. In the present study, the effects of alterations in the quantity of SP and NKRs in the early spinal fusion process were investigated. The results of the present study revealed that SP and NKRs began to appear 1 week post-surgery in fibrous tissues. The abundance of SP and NKRs peaked at 3 weeks post-surgery; the majority of SP and NKRs were distributed around the allograft and the new microvessels. In conclusion, SP and NKRs are involved in early spinal fusion, a finding that may facilitate the development of novel strategies to promote spinal fusion from a neurogenesis perspective.

Introduction

Spinal disorders, including traumatic vertebral fracture, spinal tumors and spinal deformities, are a leading cause of morbidity in orthopedics (1-3). Spinal fusion is an accepted

treatment approach for patients with spinal disorders. Numerous techniques and biomaterials have been developed to promote spinal fusion; however, the mechanisms involved in spinal fusion remain to be investigated (4,5). The role of neuronal mediators in fracture healing and bone regeneration has been previously highlighted, and neuropeptides have been reported to regulate fracture healing and local bone turnover (6,7).

Substance P (SP) is an 11-amino acid neuropeptide richly distributed in the peripheral and central nervous systems (8). In a variety of chronic pains, substance P acts as a pain neurotransmitter via the sensory nerve afferent fibers up to the spinal cord, and is involved in the conduction and modulation of pain. In addition to transmitting nociceptive information, SP also serves a role in the analgesic effect. The receptors of SP are known as neurokinin receptors (NKRs) (9), and the use of specific NKR antagonists may decrease postoperative pain (10-13). During the present study SP, NKRs were detected in operative areas, including bone fracture sites and inflammatory sites.

Evidence has demonstrated that SP-positive nerve fibers were active in osteogenic areas, including the bone marrow, periosteum and growth plate, in bone fractures (14,15). NKRs were also observed on endothelial cells during the process of angiogenesis (16). SP-positive nerve fibers and NKRs accompany the fracture process and angiogenesis; however, at present, studies mainly focus on their roles in pain sensation, with little investigation into the associations between SP, NKRs and spinal fusion. Therefore, the present study aimed to investigate the effects of alterations in the quantity of SP and NKRs during the spinal fusion process.

Materials and methods

Animals and surgery procedure. The Subcommittee on Animal Studies of The Second Military Medical University approved all experiments. A total of 20 adult male Sprague-Dawley rats (~16-weeks-old, ~400 g) were used in the present study. The rats were housed and maintained in a 26°C constant temperature environment with filtered air and 60% relative humidity under a 12-h light/dark cycle. Rats had free access to food and water, and a pair of rats was placed in an isolator cage.

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Anaesthesia was induced and maintained with isoflurane (xw266754671; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China; 0.5-2%) via inhalational oxygen. The back lumbar region and the hind limbs above the iliac crest were shaved. Rats were disinfected and a 3-cm dorsolateral incision was created over the lumbar 4/5 (L4/5) area, followed by blunt dissection of the longitudinal back muscles. Once the transverse process of the L4 vertebra was exposed, the transverse process was decorticated with an electric bur until shallow bleeding was observed. Then, a demineralized freeze-dried bone allograft (Aorui Biological Material Co., Ltd., Shanxi, China) was implanted in the decorticated fusion beds of the transverse process. Finally, fascia and skin underwent interrupted suturing layer by layer. Postoperative antibiotics were administered intramuscularly for 2 consecutive days (cefuroxime; cat. no. YB-8342; Shanghai Yu Bo Biological Technology Co., Ltd., Shanghai, China; 0.5 mg/kg). Animals were sacrificed by excessive anaesthesia and euthanized at 1, 2, 3 and 4 weeks post-surgery (n=5/week). The specimens, allograft and the fused transverse process were obtained and frozen at -80°C until further analysis.

Histological analysis. Harvested specimens were fixed in 4% paraformaldehyde for 24 h at 26°C, and then decalcified with 5% nitric acid at room temperature for 72 h. Subsequently, the specimens were washed in distilled water three times and then embedded in paraffin. A series of sections (5-μm thickness) were obtained from the midline of the transverse processes. The sections were stained with hematoxylin & eosin (hematoxylin for 5 min and with eosin for 3 min, both at 26°C) and viewed under the bright field of an Eclipse 80i microscopy (Nikon Corporation, Shanghai, China).

Immunohistochemical staining. SP, NK1R, and NK2R were immunostained. The area of interest included regions around the transverse process and the allograft. The 5-μm thin sections were blocked at 4°C for 12 h with 5% bovine serum (cat. no. E661003; Sangon Biotech Co., Ltd., Shanghai, China) and 0.3% Triton X-100 (cat. no. P0096; Beyotime Institute of Biotechnology, Shanghai, China). Then, the specimens were incubated with a rabbit anti-rat polyclonal primary antibody (1:1,000; Substance P antibody: Cat. no. sc-58591; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; NK-1R antibody: Cat. no. sc-365091; Santa Cruz; Biotechnology, Inc.; NK-2R antibody: Cat. no. 25270-1-AP; Wuhan Sanying Biotechnology, Wuhan, China) at 4°C for 24 h, followed by a fluorescein isothiocyanate-conjugated donkey anti-rabbit secondary antibody (1:200; cat. no. sc-2090; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Images were captured with an Eclipse 80i fluorescent microscope (Nikon Corporation). ImageJ software v.1.51 (National Institutes of Health, Bethesda, MD, USA) was used to calculate the content of the immunostained areas; the densities of SP, NK1R and NK2R were measured as follows: (Positive area/total image area) x 100%.

Statistical analysis. The statistical data were analysed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS v.22.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant

Table I. Mean density of SP in the fusion site.

Variable (%)	Week 1	Week 2	Week 3	Week 4
MD of SP	0.99±0.65	5.54±1.18	10.32±1.23 ^a	7.87±0.35

Values are presented as the mean ± standard deviation. ^aP<0.01. SP, substance P; MD, mean density.

difference. The densities of SP, NK1R, and NK2R at different stages post-surgery were repeatedly measured and compared with one-way with analysis of variance (ANOVA) and SNK was used as a post hoc test. All immunohistochemical staining results are presented as the mean ± standard deviation.

Results

Histological osteogenesis. At 1 week post-surgery, a spot of deeply stained cells were detected in the fusion site, and few fibrous tissues were observed in the gap between the allograft and transverse process. At 2 weeks post-surgery, chondrocytes were detected at the fusion site. The quantity of deeply stained cells increased compared with the deeply stained cells at 1 week post-surgery and a layer of osteoblasts spread along the interlayer of the allograft and the newly formed fibrous tissues. In comparison with those at 2 weeks post-surgery, at 3 weeks post-surgery, the number of chondrocytes and fibrous tissues increased continuously, while the number of deeply stained cells decreased. Chondrocytes were mainly located on the allograft meshwork; novel cartilage formed surrounding the allograft. Additionally, osteoblasts were observed in the interface of the allograft. At 4 weeks post-surgery, the deeply stained cells were detected at a normal level; more osteoblasts in the in the interface of the allograft, and of several layers were also observed (Fig. 1).

Occurrence of SP in the fusion site. The immunohistochemical analysis quantified and demonstrated the alterations and specific localizations of SP-positive nerves (Fig. 2). At 1 week post-surgery, few SP-positive nerve fibers were detected at the fusion site. The number of SP-positive nerve fibers increased continuously at week 2 post-surgery. The peak in SP abundance was observed at 3 weeks post-surgery, which was as high as 10.33±1.23% of the fusion site (Fig. 2; Table I). At 4 weeks post-surgery, the density of SP decreased to a lower level of 7.87±0.35%. One-way ANOVA statistical analysis revealed that the density of SP at 3 weeks post-surgery was higher than at all other weeks (*P<0.01).

Occurrence of NKRs at the fusion site. At 1 week post-surgery, there were only a few NKRs at the fusion site. At 2 weeks post-surgery, the density of NKRs had increased; some NKRs appeared surrounding the allograft and microvessels. Additionally, NKRs were observed around the chondrocytes in the cartilage areas. At 3 weeks post-surgery, numerous NKRs were detected at the fusion site. Most NK1R and NK2R were distributed within the endothelium of the microvessels and the interface of the allograft. The density of NK1R and NK2R

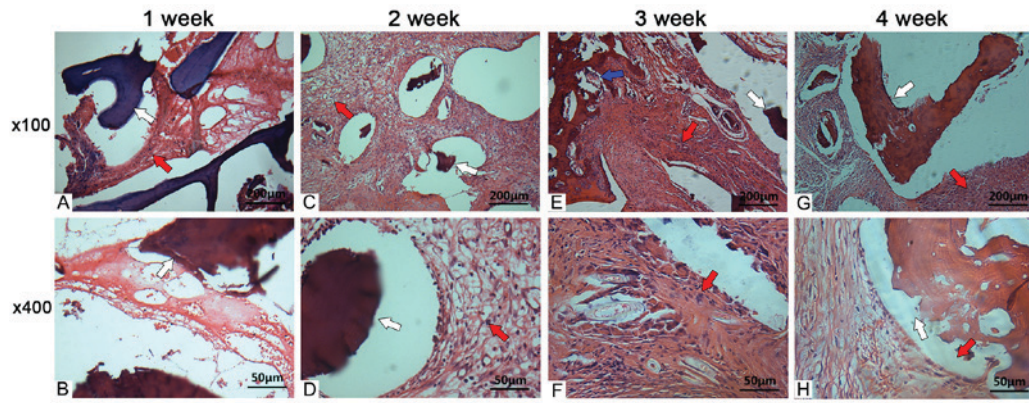


Figure 1. Photomicrograph of the fusion mass at (A and B) 1 week, (C and D) 2 weeks, (E and F) 3 weeks and (G and H) 4 weeks post-surgery. Red arrows indicated deeply stained cells. White arrows indicated the allograft. Blue arrows revealed newly formed bone.

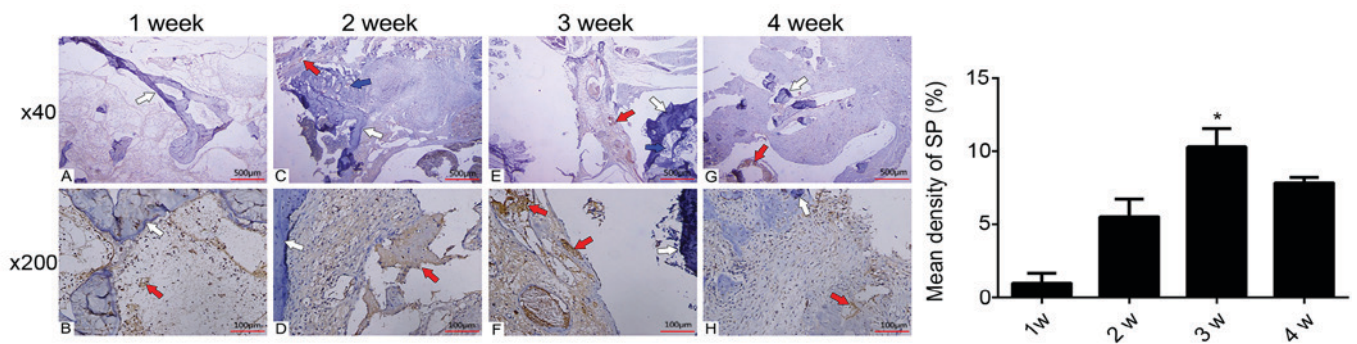


Figure 2. Expression of SP at the (A and B) 1 week, (C and D) 2 weeks, (E and F) 3 weeks and (G and H) 4 weeks post-surgery. At 1 week post-surgery, few SPs were detected in the visual fields. SP abundance increased at 2 weeks post-surgery. SP reached a peak at 3 weeks post-surgery temporally, and was mainly distributed in the newly formed microvessels and the surrounding chondrocytes spatially. At 4 weeks post-surgery, the density of SP decreased, but remained higher than that at 2 weeks post-surgery. Red arrows, SP; white arrows, allograft; and blue arrows, newly formed bone. * $P < 0.01$. SP, substance P.

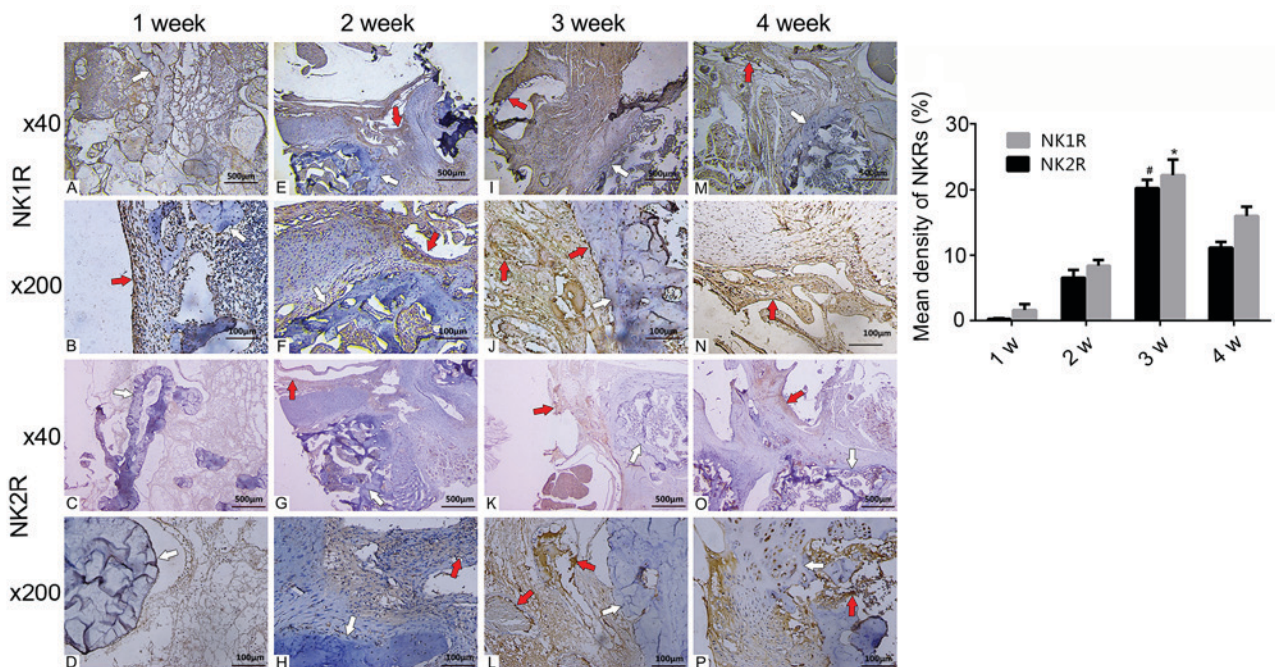


Figure 3. Expression of NKR1R at (A-D) 1, (E-H) 2, (I-L) 3 and (M-P) 4 weeks post-surgery. NKR1Rs increased and reached a peak at 3 weeks post-surgery with a distribution surrounding the allograft and endothelial cells of the microvessels. Fewer NK2Rs were present than NK1Rs, but were similarly distributed. Density of NK1Rs and NK2Rs was significantly higher at 3 weeks post-surgery compared to other time points (weeks 1, 2 and 3). Red arrows, NK1R/2R; white arrows, allograft. * $P < 0.01$ vs. NK1R, * $P < 0.01$ vs. NK2R. NKR, neurokinin receptors; NK1R, neurokinin 1 receptor; NK2R, neurokinin 2 receptor.

Table II. Mean density of NKRs in the fusion site.

Variable (%)	Week 1	Week 2	Week 3	Week 4
MD of NK1R	0.26±0.10	6.58±1.18	20.26±1.25 ^a	11.13±0.87
MD of NK2R	1.57±0.91	8.39±0.87	22.21±2.36 ^b	15.95±1.38

Values are presented as the mean ± standard deviation. The mean density of NK1Rs and NK2Rs was significantly higher at 3 weeks post-surgery compared to other time points (week 1, 2 and 3). ^aP<0.01 vs. NK2R ^bP<0.01 vs. NK1R. NKRs, neurokinin receptors; MD, mean density.

was as high as 20.26±1.25 and 22.21±2.36%, respectively, and peaked at 3 weeks post-surgery. At 4 weeks post-surgery, NKRs numbers began to decrease but were higher compared with in specimens analysed at 2 weeks post-surgery. NK2R numbers were lower than those of NK1R, but their respective distributions were similar (Fig. 3; Table II).

Discussion

The present study investigated the occurrence and enhancement of SP and NKR expression in allograft spinal fusion. The results demonstrated that SP and NKRs were increased in the early phase of spinal fusion; a close association was noted between alterations in the quantity of SP and NKRs, and the various stages of histological healing. Nerve fibers detected post-surgery may be necessary for the transportation of various neuronal mediators, such as SP, in order to regulate the fusion process. In addition, elevated levels of NKRs were determined to be essential for the function of SP. It has also been reported that SP has a lower affinity for NK2R than NK1R, but that it can stimulate NK2R in some peripheral nerve fibers (17).

SP-positive nerve fibers and NKRs appeared at 1 week post-surgery. Haematoma usually occurred at ~8 h following injury, and was gradually replaced by fibrous tissue in the subsequent 2-3 weeks (18). Previous studies demonstrated that SP-positive nerve fibers may be observed in the first week post fracture healing; these peaked at day 21, suggesting the possible roles of SP in hematoma absorption and in the inflammatory process (19,20). As a type of wound modulatory peptide of the tachykinin family, circulatory SP was increased by 10-fold within 24 h of the bone fracture, with a marked effect on microvessel dilation and increasing vascular permeability (18,21,22). Eglezos *et al* (23) revealed that, via the activation of NK1R in endothelium cells, SP increased the permeability of microvessels and further caused plasma extravasation and oedema (23). Therefore, the ingrowth of SP at the fusion sites may be involved in the inflammatory response at 1 week post-surgery.

At 2 weeks post-surgery, distinct SP and NKRs were observed at the fusion site. Additionally, chondrocytes and newly formed microvessels were detected at the fusion site. The synchronization between SP and chondrocytes in the present study reflected the inducible association between SP and bone formation as previously reported (24). At 3 weeks post-surgery, the density of both SP and NKRs peaked with the newly formed bone callus and fewer deeply stained cells were observed. At 4 weeks post-surgery, the density of SP and

NKRs both began to decrease in the fusion site. It is possible that SP may have promoted spinal fusion in the early bone formation phase via NKRs, particularly in the endochondral ossification stage, but not in the later bone mechanical modification stage. Spatially, the distribution alterations of SP were observed surrounding the allograft and in the fibrous tissues; previous evidence demonstrated that SP may stimulate the proliferation of fibroblasts (25). Furthermore, previous studies reported that NK1R expression levels were increased post-fracture, and that they began to return to normal levels gradually at 4 weeks post-fracture (11). Located on chondrocytes, osteoblasts and osteoclasts, NKRs were demonstrated to influence the bone remodeling process *in vivo* (26,27). *In vitro* experiments revealed that the increase in SP-positive nerve fibers during fracture healing accelerated the bone formation process compared with the control number of SP-positive nerve fibres (9,28). In addition, animals with neuropathies or peripheral nerve resection exhibited reduced SP levels and decreased bone mechanical characteristics (17). Similar to the increase in SP abundance, NK1R was also increased in the early phase of spinal fusion. Compared with NK2R, NK1R numbers were relatively more selective for SP and were distributed on osteoblasts, particularly in areas with active osteogenesis, such as the interface of the allograft, as observed in the present study. Thus, SP may exert effects on spinal fusion by promoting bone formation via NKRs in bone.

As aforementioned, circulatory SP levels increased ≥10-fold within 24 h of the bone fracture (21). As one of the released angiogenic factors post-fracture, SP served a significant role in the primary process of angiogenesis (29). SP may induce the migration of endothelium progenitor cells that expressed NK1R and promote the proliferation of endothelial cells, thus accelerating reparative angiogenesis (29,30). Additionally, SP may recruit granulocytes to the injured site and bind specific receptors on granulocytes, promoting these cells to release angiogenic cytokines, including vascular endothelial growth factor, basic fibroblast growth factor and angiopoietin-2 (31-33). *In vivo* experiment results revealed that NK1R agonists induced endothelial cell proliferation and enhanced angiogenesis (34). However, NKR antagonists inhibited SP-induced proliferation and angiogenesis (13,35). *In vitro* studies indicated that angiogenesis was reduced at both the arteriolar and capillary levels in NK1R-KO mice, compared with in wild type mice (29). The newly formed microvessels carry oxygen, stem cells and various growth factors (36,37). In the present study, SP and NKRs were distributed around the microvessels and peaked in abundance at 3 weeks post surgery. Previous studies also reported

that SP reached a peak at approximately 21 days post injury (19,38). Collectively, the results of the present study and prior evidence indicated that SP may be involved in the positive regulatory process of angiogenesis during spinal fusion.

Numerous limitations existed in the present study. The *in vivo* analysis merely provided a morphological and immunohistochemical analysis of SP and NKR occurrence without reporting a causal association with spinal fusion. Furthermore, regarding morphological observations, the rat spines were limited to a small size, thus the fusion mass was not measured. Therefore, the regulatory roles of SP and NKRs during spinal fusion, and interventional *in vivo* experiments permitting investigations into specific cell types during a different phase of spinal fusion, should be conducted in the future.

In summary, the present study explored the alterations in the quantity of SP and NKRs during allograft spinal fusion. SP and NKRs were detected 1 week post surgery in the fibrous tissues; the majority of SP and NKRs surrounded the allograft and the newly formed microvessels. These results highlighted the role of SP and NKRs in the processes of bone metabolism and new microvessel formation during the early phase of spinal fusion, which may present a novel strategy for promoting spinal fusion from a neurogenesis perspective.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

TX and JS designed the study, and SW, XX and YZ performed the experiments. PL and KS assisted with surgical operations, and YZ conducted the statistical analysis. SW and XX wrote the manuscript, and TX and JS revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Subcommittee on Animal Studies of the Second Military Medical University approved all experiments.

Consent for publication

Not applicable.

Competing interests

All authors declared that they have no competing interests.

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