Single Intraperitoneal Injection of Low-Dose Ketamine Pretreatment Alleviates Mechanical Hyperalgesia and Downregulates the Expression of Inflammatory Cytokines in Spinal Dorsal Horn of Rats after Skin/Muscle Incision and Retraction

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**Background:** Various common surgeries, such as thoracotomy and inguinal hernia repair which involve essential prolonged tissue retraction, often evoke chronic postoperative pain (CPOP). They bring great pain to patients, and make the qualities of their lives lower. The concrete mechanism of CPOP has still been unclear, and lacks effective research methods. To solve this problem, a new rat model was developed, whereby skin/muscle incision and retraction (SMIR) in the medial thigh of rat evoked CPOP to simulate clinical scene. Many studies provided evidence that CPOP often is accompanied with a large number of inflammatory cytokines releasing, and these cytokines induce or facilitate inflammatory hyperalgesia. In the present study, we investigated the expression of inflammatory cytokines in a SMIR rat model and examined the effects of low-dose ketamine pretreatment.

**Methods:** Paw withdrawal mechanical threshold (PWMT) was used to assess mechanical hyperalgesia 1 day before and 1, 3, 7, 10, 14, 21, and 28 days after a SMIR operation. Western blotting was used to investigate the expression of interleukin (IL)-1, IL-6, IL-17, and tumor necrosis factor (TNF)-α at the spinal level.

**Results:** SMIR rats developed long-lasting mechanical hyperalgesia, accompanied with the upregulation of inflammatory cytokines at the spinal level. A single intraperitoneal injection of low-dose ketamine before SMIR surgery could alleviate mechanical hyperalgesia significantly and downregulate the expression of IL-1, IL-6, IL-17, and TNF-α at the spinal level.

**Conclusions:** Our data suggested that the expression of IL-1, IL-6, IL-17, and TNF-α at the spinal level in the rats of SMIR-induced CPOP were upregulated, and a single pre-intraperitoneal injection of low-dose ketamine could alleviate mechanical hyperalgesia and downregulate the expression of inflammatory cytokines in spinal dorsal horn. This might be a novel target for CPOP therapy.
Chronic postoperative pain (CPOP) is an important matter that both patients and doctors have shown great concern (1). It often occurs following various common surgical procedures including thoracotomy, inguinal hernia repair, coronary artery bypass surgery and caesarean section (2). Up to 60% of patients would develop CPOP following thoracotomy and inguinal hernia repair (3, 4). In recent years, CPOP comes to be a serious clinical condition for its widespread characteristics. Its pathogenic mechanism is still under debate, whether the origin of these pain syndromes are nociceptive from the skin incision, inflammatory from muscle damage, or neuropathic from surgical injury to peripheral nerves, is unclear. To investigate this kind of pain, a new rat model that uses skin/muscle incision and retraction (SMIR) in the rat’s thigh (5) is adopted. This animal model simulates the clinical surgical operation and has been a new method to explore the mechanism of CPOP.

Previous studies found that hypersensitivity to nociceptive input is a behavioral consequence of injury or inflammation (6, 7) and CPOP is usually accompanied by a large number of inflammatory cytokines release (8, 9). Inflammatory cytokines are the key points in trigger, enhancement and amplification of inflammatory reaction, including interleukin (IL)-1, IL-6, IL-17, tumor necrosis factor (TNF-α), and so on (10).

The researches of recent years demonstrated that despite the traditional functions of ketamine, such as anesthesia and analgesia, it also works as an anti-inflammatory drug. Ketamine could inhibit the release of inflammatory cytokines immediately (11) and attenuate hyperpathia even at a low dose (12).

In this study, we examined whether inflammatory cytokines participated in CPOP induced by SMIR operation on rats. Furthermore, we tested whether the pre-administration of low-dose ketamine could attenuate CPOP and its effects on inflammatory cytokines at the spinal level.

METHODS

Animals
All animal experiments were performed on adult male Sprague-Dawley rats weighing 180 to 220 g (provided by the Laboratory Animal Center of Drum Tower Hospital). The animal room was artificially lit from 7:00 AM to 7:00 PM. Rats were housed in individual cages with free access to water and food pellets. Room temperature was maintained at 24 °C. The experimental protocol was approved by our Institutional Animal Care and Use Committee.

SMIR Surgery
Aseptic techniques were used in all surgical procedures. The SMIR surgery was performed as Flatters described (5). Rats were anaesthetized with intraperitoneal (i.p.) pentobarbital (50 mg/kg) and the medial (inner) thigh, on one side, was shaved and sterilized with sterile alcohol wipes. A 1.5-2 cm incision was made in the skin of the medial thigh approximately 4 mm medial to the saphenous vein to reveal the muscle of the thigh. An incision (7-10 mm long) was then made in the superficial (gracilis) muscle layer of the thigh, approximately 4 mm medial to the saphenous nerve. The superficial muscle was then parted further with blunt scissors to allow the insertion of a micro-dissecting retractor (Cat. No. 13-1090, Biomedical Research Instruments Inc, Silver Spring, MD, USA). The retractor had 4 prongs spaced over an 8 mm distance and each prong was 4 mm deep. The retractor was inserted into the incision site, to position all prongs underneath the superficial layer of thigh muscle. The skin and superficial muscle of the thigh were then retracted by 2 cm, for 1 hours. During the retraction period, animals were closely monitored and if required, additional isoflurane anaesthesia was provided. The muscle and skin of the surgical site were closed separately with 4.0 nylon sutures. Sham operations were performed following the same surgical procedure but without skin/muscle retraction. Animals were treated with an i.p. injection of cefuroxime (DuPont-NEN, Boston, MA, USA) at the prophylactic dose of 20 mg/kg at an hours preoperatively and once daily for the next 2 days to prevent infection. Following recovery from anaesthesia, all animals could ambulate normally and rise up on their hindpaws to reach food and water.

Drug Preparation and I.P. Injection
Single i.p. injection of 10 mg/kg ketamine (ket-
amine hydrochloride injection, Jiangsu Hengrui Medicine Co., Ltd, China) was dissolved in 1 ml sterile saline. The dose of ketamine was selected as literature stated. Sham group received i.p. injection of saline 1 ml only. The timing of each injection was after rat anesthesia, and before SMIR operation.

Mechanical Allodynia Test
Animals were placed on an elevated wire mesh floor and confined underneath individual overturned plastic boxes. Mechanical allodynia was assessed using von Frey filaments (Touch-Test Sensory Evaluators, Stoeling Co., Wood Dale, IL, USA) as Chaplan et al. described (13). We used filaments to poke the mid-plantar area of the hindpaws encircled by tori/footpads vertically, and took lifting, avoiding or licking foot action for positive reaction and no foot lift even filament bended 90° for negative reaction. Evaluated the threshold based on up-down method and each filament was applied 5 times, with each interval of at least 1 minute. Calculated 50% response threshold by interpolation.

Tissue Collection
Rats were killed rapidly under pentobarbital sodium anesthesia. The L4-5 segment of spinal cord was removed into liquid nitrogen immediately and stored at -80℃.

Western Blot
Tissues were homogenized in a sodium dodecyl sulfate sample buffer containing a mixture of proteinase inhibitors. Quantification of the protein contents was performed using the bovine serum albumin method. Protein samples (40 μg) were separated on sodium salt-polyacrylamide gel (IL-1β, IL-6, IL-17 and TNF-α: 10% gradient gel) and transferred to polyvinylidene difluoride filters (Millipore, Darmstadt, Germany). The filters were blocked with 5% milk and immunoblotted using antibodies against IL-1β (ABcam, 1:500 dilution), IL-6 (ABcam, 1:500 dilution), IL-17 (ABcam, 1:500 dilution) and TNF-α (ABcam, 1:500 dilution). The blots were visualized in electrogenerated chemiluminescence solution (DuPont-NEN, Boston, MA, USA) for 1 minute and exposed to hyperfilms (Amersham Biosciences, Piscataway, Piscataway, NJ, USA) for 1 to 10 minutes. The density of specific bands was measured using a computer-assisted imaging analysis system and normalized against the corresponding loading control bands. β-actin (ABcam, United Kingdom, 1:1000 dilution) was used as the loading control.

Statistical Analysis
All data were expressed as means ± standard deviation (SD). Data from mechanical allodynia test were analyzed using repeated measures analysis of variance across testing time points to detect overall differences among the treatment groups. Data from western blot were analyzed by one-way analysis of variance to determine the differences among the experimental groups. When significant main effects were observed, the Bonferroni post hoc tests were performed to determine the sources of the differences. Statistical analysis was performed using SPSS 16.0 software (IBM Corporation, Armonk, NY, USA). The differences were considered statistically significant at the level of P<0.05.

RESULTS
SMIR-Induced Long-Term Mechanical Allodynia
Before the surgery (day -1), the baseline paw withdrawal mechanical threshold (PWMT) (20.045 ± 1.122 vs 20.273 ± 2.026, P>0.05) was similar in all groups. Compared with the sham group, SMIR surgery rats had decreased PWMT on days 3, 7, 10, 14, 21 and 28 (20.043 ±1.421 vs 11.201 ± 3.124, 21.877 ± 2.478 vs 9.333 ± 3.077, 20.877 ± 2.958 vs 7.833 ± 2.229, 20.418 ±2.867 vs 8.375 ±1.745, 20.273 ±2.937 vs 9.458 ± 3.018, 20.730 ± 2.065 vs 10.583 ± 2.654, P<0.05; Figure 1).

SMIR-Induced Upregulation of the Levels of IL-1β, IL-6, IL-17 and TNF-α at the Spinal Level
To test whether inflammatory cytokines are involved in SMIR processes, the expression of IL-1β, IL-6, IL-17 and TNF-α was measured using immunocytochemical approaches, and the level
was quantified using image analysis software. Our results indicated that compared with the sham group, the expression of TNF-α (2.728 ± 0.164, 3.071 ± 0.523, 3.794 ± 0.695, 4.592 ± 0.432, 5.16 ± 1.306, 4.42 ± 1.011, 3.413 ± 1.349, P< 0.05), IL-1β (2.662 ± 0.249, 3.372 ± 0.396, 3.794 ± 0.371, 5.058 ± 0.261, 5.509 ± 0.297, 3.681 ± 0.937, 3.446 ± 0.348, P< 0.05), IL-6 (2.695 ± 0.271, 4.005 ± 0.483, 4.127 ± 0.225, 4.258 ± 0.950, 4.419 ± 0.409, 3.861 ± 0.556, 3.046 ± 0.822, P<0.05), and IL-17 (1.825 ± 0.220, 3.172 ± 0.237, 3.400 ± 0.122, 3.592 ± 0.431, 3.447 ± 0.394, 3.194 ± 0.846, 2.626 ± 0.359, P< 0.05) was upregulated after surgery on day 1, 3, 7, 10, 14, 21 and 28 (Figure 2).

Effect of I.P. Injection of Ketamine on SMIR-Induced CPOP Behaviors

To further explore the effects of inflammatory cytokines at the spinal level in SMIR rats, we used low-dose ketamine to inhibit them. We treated rats with i.p. injection of low-dose ketamine before SMIR surgery, in order to simulate the clinical situation that is treating patients with administration before surgical operation. After administration of low-dose ketamine, compared with the levels in vehicle control group (11.000 ± 3.225, 8.500 ± 1.517, 7.417 ± 1.960, 8.042 ± 1.553, 10.042 ± 2.052, 10.962 ± 2.375), PWMTs were significantly increased on days 3, 7, 10, 14, 21 and 28 (18.310 ± 1.776, 18.230 ± 1.892, 18.893 ± 0.980, 19.275 ± 1.481, 20.808 ± 3.185, 20.585 ± 0.893, P<0.05). There were no significant differences between group sham + vehicle (20.273 ± 1.034, 20.732 ± 2.938, 20.043 ± 1.421, 21.190 ± 2.851, 20.877 ± 2.958, 20.563 ± 3.176, 20.502 ± 2.883, 20.730 ± 3.065) and group sham + ketamine (20.045 ± 1.122, 20.960 ± 3.096, 19.815 ± 1.443, 21.877 ± 3.478, 21.420 ± 2.559, 20.793 ± 3.307, 20.273 ± 2.937, 20.960 ± 2.83987, P>0.05), letting us know that the drug itself had no effect on pain. I.p. injection of low-dose ketamine reduced the enhancement of mechanical hyperalgesia induced by SMIR surgery (Figure 3).

I.P. Injection of Ketamine Inhibited the Expression of IL-1β, IL-6, IL-17 and TNF-α at the Spinal Level

14 days after low-dose ketamine pretreatment, the levels of inflammatory cytokines were 1.370 ± 0.507 (TNF-α), 1.440 ± 0.564 (IL-1β), 1.987 ± 1.439 (IL-6), and 1.785 ± 0.756 (IL-17), and the levels were significantly decreased compared with the vehicle group (3.150 ± 1.249, 2.186 ± 0.198, 4.970 ± 0.631, 4.089 ± 1.389, P<0.05; Figure 4).

DISCUSSION

CPOP is one kind of common clinical pathological procedure, the mechanism of which is complicated and still unclear. It is generally believed that central sensitization caused by sustained nociceptor excitement is one of the main reasons. In this study, the rat model of CPOP evoked by SMIR was used to stimulate this clinical procedure. SMIR-evoked mechanical hypersensitivity was observed since postoperative day 3, most prominent between postoperative days 10-14, and persisted at least three weeks. It has been reported that peripheral neuronal damages (5) and N-methyl-D-aspartate (NMDA) receptors activation (14) do not play direct roles in the maintenance of postoperative pain. Thus, we investigated the effects of inflammatory cytokines in CPOP.

Cytokines are necessary to conduct the inflammatory response at sites of infection and in-
jury, favoring proper wound healing. TNF-α has important functions both in inflammatory and neuropathic hyperalgesia. After a surgical procedure, trauma, or during infections, TNF-α is one of the earliest and potent mediators of the inflammatory response (15). Increasing amounts of evidence suggested an important role of TNF-α in the pathogenesis of pain (16), including neuropathic pain (17, 18), inflammatory pain (19), and cancer pain (20, 21). In addition, TNF-α participates in the activity of numerous ion channels, such as capsaicin receptor, Na⁺, Ca²⁺, and K⁺ channels (22-24), and induces spontaneous activity in primary sensory neurons (25). According to the previous studies, TNF-α induces the trafficking and surface expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (26, 27) and evokes increases in both spontaneous excitatory postsynaptic currents (sEPSC) frequencies and NMDA-induced currents in lamina II neurons in spinal cord slices (28).

IL-1β induces systemic inflammation through the activation of cyclooxygenase-2, and it also produces substance P (SP), nitric oxide and endothelia adhesion molecules (29-31). Recently, it has been suggested that IL-1β is important in the development and maintenance of CPOP (31, 32). Zhang et al. (33) have found that IL-1β receptor agonist could alleviate inflammatory hyperalgesia through preventing phosphorylation of NMDA receptor NR-1 subunit in rats. IL-1β is also found to reduce membrane currents of superficial dorsal horn neurons induced by the inhibitory neurotransmitters γ-aminobutyric acid
(GABA) and glycine (34).

IL-6 could activate astrocyte and microglia after injury, and it is considered as the most relevant marker of the degree of tissue damage during a surgical procedure in which excessive and prolonged expression is associated with high postoperative morbidity (30, 35). Moreover, TNF-α and IL-6 potentiate membrane currents of superficial dorsal horn neurons evoked by the excitatory neurotransmitters NMDA and/or AMPA (34).

IL-17 exhibits proinflammatory activities similar to those of innate immune cytokines such as IL-1β (36). Previous studies demonstrated that astrocytes release IL-17 during pain, and IL-17 significantly increases p-NR1 levels in the spinal cord and decreases PWML (37). Those data suggested that the enhancement of NR1 phosphorylation induced by IL-17 may contribute to hyperalgesia.

From our experiment, we found that the expression of proinflammatory cytokines IL-1β, IL-6, IL-17 and TNF-α were increased significantly in SMIR processing. After SMIR operation, the levels of these proinflammatory cytokines were upregulated with the passage of time which reached a peak at 10-14 days, and persisted up to 21 days. These results led us to consider the role of inflammatory cytokine in SMIR induced CPOP.

Central sensitization plays an important role in the development and maintenance of chronic pain (38, 39). It could be enhanced and maintained by activation of spinal cord glial cells such as microglia and astrocytes and release of proinflammatory cytokines, chemokines, or growth factor from these glial cells (40-42). They might promote central sensitization by increasing the release of glutamate from presynaptic terminals and the activity of NMDA receptor, which plays a crucial role in central sensitization (39). Therefore, at the level of the spinal cord, inflammatory mediators may either directly evoke neuronal activity or modulate it via disinhibition or potentiation of excitatory neurotransmission.

Ketamine is a usual clinical drug, and it was verified that ketamine could inhibit the production of proinflammatory cytokines immediately (43). We inhibited the overexpressed proinflammatory cytokines by i.p. injections of low-dose ketamine, and observed attenuation of mechanical allodynia and down-regulation of

![Figure 4. Expression of IL-1β, IL-6, IL-17 and TNF-α at the Spinal Level after I.P. Injection of Ketamine.](image-url)

A. Representative blot showing the downregulated SMIR-induced up-regulation of IL-1β, IL-6, IL-17 and TNF-α at the spinal level. Intraperitoneal low-dose ketamine pretreatment showed significant differences compared with the vehicle groups; B. Statistical analysis of the relative protein expression levels of IL-1β, IL-6, IL-17 and TNF-α (N=4 in each group). Data were expressed as the means ± SD. *P<0.05, **P<0.01, ***P<0.001 compared with the sham group; #P<0.05, ##P<0.01, ###P<0.001 compared with the vehicle group. One-way analysis of variance and Bonferroni post hoc tests.
inflammatory cytokines expression. This further confirmed the effects of ketamine at the spinal level in SMIR induced CPOP.

In addition, our experiment also found that the analgesia effect of ketamine was significantly longer than the duration of drug action. Ketamine could be combined with the benzene ring on the NMDA receptor, noncompetitively antagonizing glutamate combined with binding site, reducing the open time and frequency of calcium ion channel, and consequently impeded excitatory synaptic transmission, inhibited central sensitization (44). Long-acting analgesic mechanism of ketamine may be about reducing the degree of central sensitization. In addition, the timing of administration may be another factor. Synaptic long-term potentiation (LTP) is the cell model of central sensitization, and is associated with the degree of pain (45) and postoperative cognitive decline (46). Low dose of ketamine could prevent the development of long-term potentiation, but couldn't influence its maintenance (47). Therefore, in the present study, we chose the time course of drug administration before the SMIR operation, in another word, before the formation of CPOP.

Although the mechanism of CPOP is still unknown, the role of proinflammatory cytokines cannot be ignored. Our work suggested that the upregulation of IL-1β, IL-6, IL-17 and TNF-α expression was relatively synchronous with pain hyperalgesia in SMIR processing. A single i.p. injection of low-dose ketamine before SMIR operation could alleviate pain behaviors and decrease the expression of inflammatory cytokines. We could conclude that proinflammatory cytokines were involved in the formation and maintenance of CPOP, and this might be among the biological mechanisms of CPOP. At the same time, our findings may provide a new strategy for the treatment of CPOP.

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References