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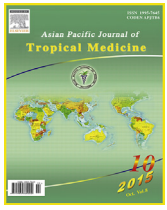

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Effect of preemptive local injection of ropivocaine with dexmedetomidine on mirror pain in rats and its mechanism

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ABSTRACT

Objective: To observe the effect of preemptive local injection of ropivocaine with dexmedetomidine on activation of glial cells and on the mirror pain in rats and its mechanism.

Methods: A total of 48 adult male Sprague-Dawley rats (weighing 180 g–220 g) were included in the study and randomized into 3 groups, Group S, Group R, and Group RD_1. A rat model of persistent postoperative pain evoked by skin/muscle incision and retraction was established in the three groups. Before procedures and nerve extraction, Group S (n = 16) was injected 0.9% saline locally; Group R (n = 16) was injected 0.5% ropivocaine locally, and Group RD_1 (n = 16) was injected 0.5% ropivocaine in combined with 1 μg dexmedetomidine locally. After the model being established in the three groups, 8 rats were used for behavior test until 28 d, and dorsal root ganglions (DRGs) of the other 8 rats were harvested on the 3rd day after surgery. Immunofluorescent and transmission electron microscopy were used to observe the activation of glial cells in DRG, and the behavior test results in the three groups were compared.

Results: The results showed that mechanical pain threshold in ipsilateral hind-paws of the Group S, Group R, Group RD_1 animals dropped to (3.640 ± 1.963) g, (5.827 ± 1.204) g, (7.482) ± 1.412 g at 3 d respectively; while in contralateral paws dropped to (7.100 ± 1.789) g, (17.687 ± 1.112) g, (16.213 ± 1.345) g on the 3 d respectively. Immunofluorescent showed that the glial cells were activated in bilateral side DRG after surgery in 3 groups, but ipsilateral paws expressed more active glial cells than contralateral paws. Transmission electron microscopy showed that mitochondria swelling/vacuolization and lysosomes were more obvious in ipsilateral paws than contralateral paws, but Group RD_1 formula could reduce glial cells activity, mitochondria swelling/vacuolization and the amount of lysosomes.

Conclusions: Local injection of ropivocaine and/or dexmedetomidine can effectively inhibit the activation of glial cells in DRG, mitigate the pathological changes of neuron in DRG and reduce mirror image pain.

1. Introduction

Open inguinal hernia repair consisting of tissue extraction is one of the most painful procedures in the clinical surgery. Skin/muscle incision and retraction (SMIR) model, akin to a common clinical procedure, is one rat model of persistent postoperative pain [1]. However, when simulating this model, we found that unilateral nerve extraction can evoke bilateral pain. A growing body of evidence indicates that unilateral nerve injury results in bilateral cellular and molecular changes in the nerve structure and pain sensitivity. This phenomenon is known as mirror image pain (MIP) [2–4]. To date, the mechanism of MIP is still unclear. Although poorly understood, a lot of researchers released findings of bilateral nociceptive-related molecular changes in the

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nervous system of unilateral pain models. It may be related to humoral immunity, central sensitization, and/or cortical downstream regulation. Surprisingly, evidence of changes in primary neurons and glial cells in regards to MIP in SMIR model is lacking [5]. It is possible that glials in the contralateral dorsal root ganglion (DRG) may play a role in primary neuronal sensitization [6,7]. They become activated and proliferate after nerve injury or inflammation [8]. Based on the close proximity of the glails cells and their ability to affect primary neurons [9], we hypothesize that glail cells activation in the contralateral DRG following unilateral peripheral nerve injury leads to increased excitability of contralateral DRG neurons and thus, MIP [10–16]. In order to observe the effect of preemptive local injection of ropivocaine with dexmedetomidine in inhibiting the activation of glial cells and reducing the mirror pain in rats and its mechanism, male SD rats were included, SMIR model was prepared, ropivocaine and dexmedetomidine were preemptively injected before model preparation. The study is reported as following.

2. Materials and methods

2.1. Experimental animals

A total of 48 SPF male SD rats, weighing (180–220) g, were included in the study. The animals were housed in a 12 h light/dark cycle and given adequate food and water. All procedures were carried out in compliance with the guidance suggestion of Animal Care Committee of Southern Medical University and the International Association for the Study of Pain [17].

2.2. Instruments and reagents

Nikon (Tokyo, Japan) fluorescence microscope, ropivacaine hydrochloride injection (Trade name: Naropin, AstraZeneca Pty Ltd, Registration No. H20100083), dexmedetomidine hydrochloride injection (produced by Jiangsu Enhua Pharmaceutical Ltd., Approval No. H20090248), goat anti-rabbit TRITC (1:1 000, Beijing Zhongshan Golden Bridge Biotechnology Co. Beijing, China) and goat-antimouse FITC (1:1 000, Beijing Zhongshan Golden Bridge Biotechnology Co. Beijing, China) were used in the study.

2.3. Model preparation

According to the method reported by Sarah J. L. Flatters [1], SMIR model was established. Rats were intraperitoneally injected with 10% chloral hydrate at a dose of 400 mg/kg. After anesthesia, a supine position was taken. Rat's back and medial thigh on one side was shaved and then swabbed with sterile alcohol wipes to sterilize the area for visualization of the saphenous vein. An incision with a length of about 7–10 mm was made in the superficial muscle. Until the muscle was separated to the adductor tendon fascia, a microscopic retractor was placed to expose the adductor tendon fascia for a persistent traction for 1 h. During the traction period, the incision was covered with sterile gauze. After traction, the muscle was sutured. Antibiotics were applied after operation for infection prevention.

2.4. Animal grouping

The experimental rats were randomly divided into 3 groups, Group S, Group R, and Group RD_1. A rat model of persistent postoperative pain evoked by SMIR was established in the three groups. Before procedures and nerve extraction, Group S (n = 16) was injected 0.9% saline locally; before procedures and nerve extraction, Group R (n = 16) was injected 0.5% ropivocaine locally; before procedures and nerve extraction, Group RD_1 (n = 16) was injected 0.5% ropivocaine in combined with 1 μg dexmedetomidine locally. After the model being established in the three groups, 8 rats were used for behavior test until 28 d, and DRGs of the other 8 rats were harvested on the 3rd day after surgery. Immunofluorescent and transmission electron microscopy were used to observe the activation of glial cells in DRG, and the behavior test results in the three groups were compared.

2.5. Observation of indicators

Behavioral testing mechanical sensitivity was assessed using the up-down method described in a previous study [18] and a set of von Frey hairs (Ugo Basile, Italy) was used to apply logarithmically increasing stiffnesses ranging from 3.61 (0.41 g) to 5.18 (15.14 g). Quick withdrawal in response to the stimulus was considered to be a positive response. Immunofluorescent and transmission electron microscopy were used to observe the activation of glial cells in DRG.

2.6. Statistical analysis

SPSS19.0 software was used for statistical analysis. The measurement data were expressed as mean ± SD, and t test was used. $P < 0.05$ was regarded as statistically significant difference.

3. Results

3.1. Changes in behavior test in rats

Unilateral SMIR model rats, Group S, exhibited noticeable bilateral pain. Compared with the basic value, the ipsilateral paw pain thresholds and mechanical hyperalgesia were significantly increased at 1–21 d following operation ($P < 0.05$) (Figure 1A). Mechanical tests of ipsilateral paws showed the paw withdrawal threshold dropped from (20.300 ± 1.204) g before surgery to (3.640 ± 1.963) g, (4.52 ± 1.89) g, (3.89 ± 1.963) g at 1 d, 3 d and 5 d after surgery. The contralateral paw pain thresholds for mechanical hyperalgesia were increased at 3–7 d following surgery as well ($P < 0.05$) (Figure 1A). Mechanical tests showed the contralateral paw withdrawal threshold dropped to (7.100 ± 1.789) g from (20.010 ± 1.412) g at 3 d post surgery.

Group R's ipsilateral side of the paw withdrawal threshold was markedly lower than contralateral side by the value of (5.827 ± 1.204) g *vs.* (17.687 ± 1.112) g at day 3 ($P < 0.05$) (Figure 1B). But from day 5 to day 7, contralateral side expressed more significant hypersensitivity than operational side. After day 14, two sides had similar trend to each other, but ipsilateral side still had lower value than contralateral side.

In Group RD_1, at day 1, both paws expressed similar value of withdrawal threshold with the value of ipsilateral (7.482 ± 1.412) g *vs.* contralateral (6.204 ± 1.963) g. At day 3 and day 5, ipsilateral side of Group R expressed lower threshold than contralateral side with the value of (11.881 ± 1.141) g *vs.* (16.213 ± 1.345) g, (7.869 ± 1.251) g *vs.* (11.549 ± 1.773) g,

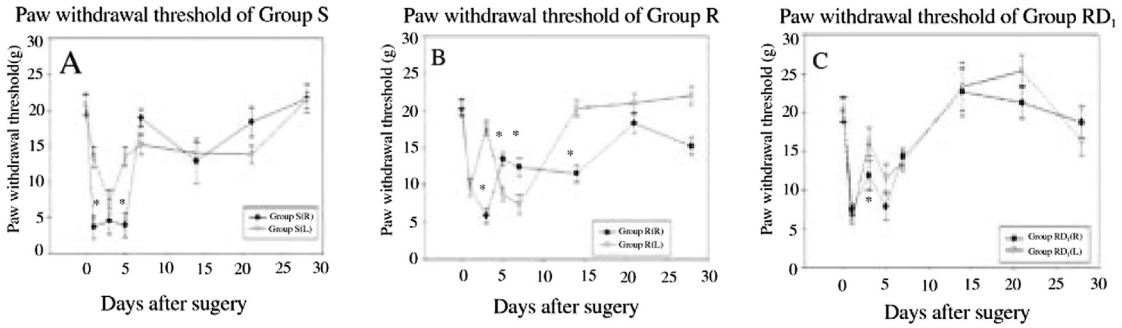

Figure 1. Behavioral changes after SMIR model surgery in rats of 3 groups.
A: Mechanical hyperalgesia after SMIR model surgery in rats. *$P < 0.05$ *vs*. contralateral paws; $n = 8$; B: After injection of ropivocaine before surgery, *$P < 0.05$ *vs*. contralateral paws; C: after injection of ropivocaine with dexmedetomedine before surgery, *$P < 0.05$ *vs*. contralateral paws.

after day 7, both sides expressed the same value and trend which was close to the basic value (Figure 1C).

3.2. Changes of ultrastructure in DRG of 3 groups

Mitochondria swelling or vacuolization were seen in ipsilateral and contralateral paws in Group S and Group R, and ipsilateral paws mitochondria swelling or vacuolization were severer than contralateral paws (Figure 2A, B). Group RD_1 mitochondria swelling or vacuolization was rare in both pawsk, but interstitial edema was more obvious than contralateral DRG (Figure 2C). In Group S and Group R, lysosomes of ipsilateral DRG were more than contralateral sides, while in Group RD_1 lysosomes were seldom seen in DRG of both paws.

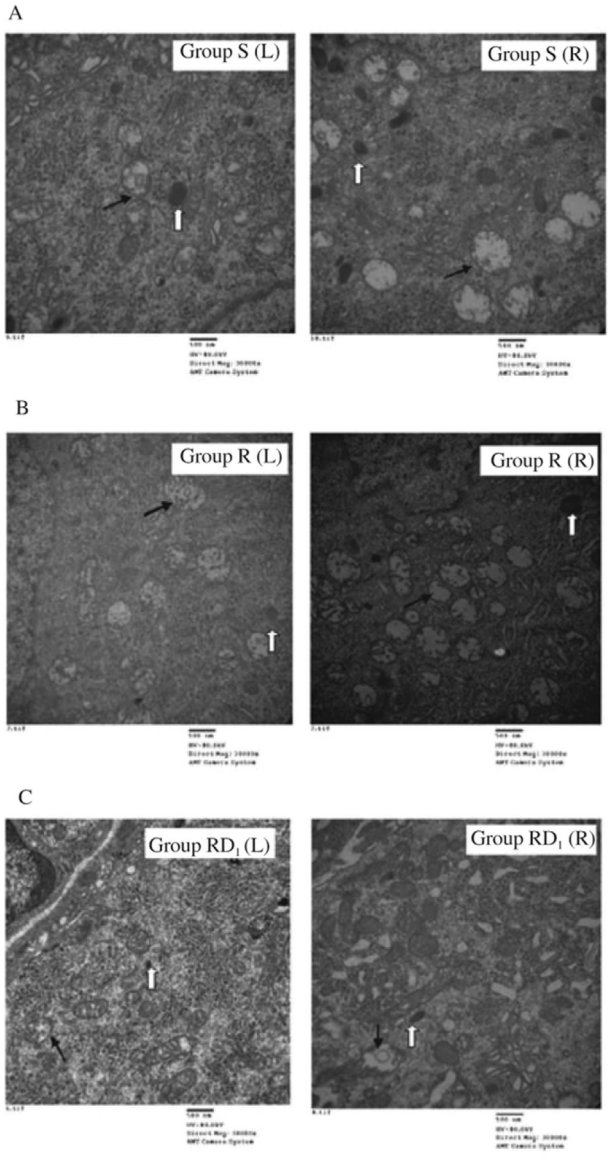

Figure 2. Ultrastructure of DRG of SMIR model surgery in rats (30000×).

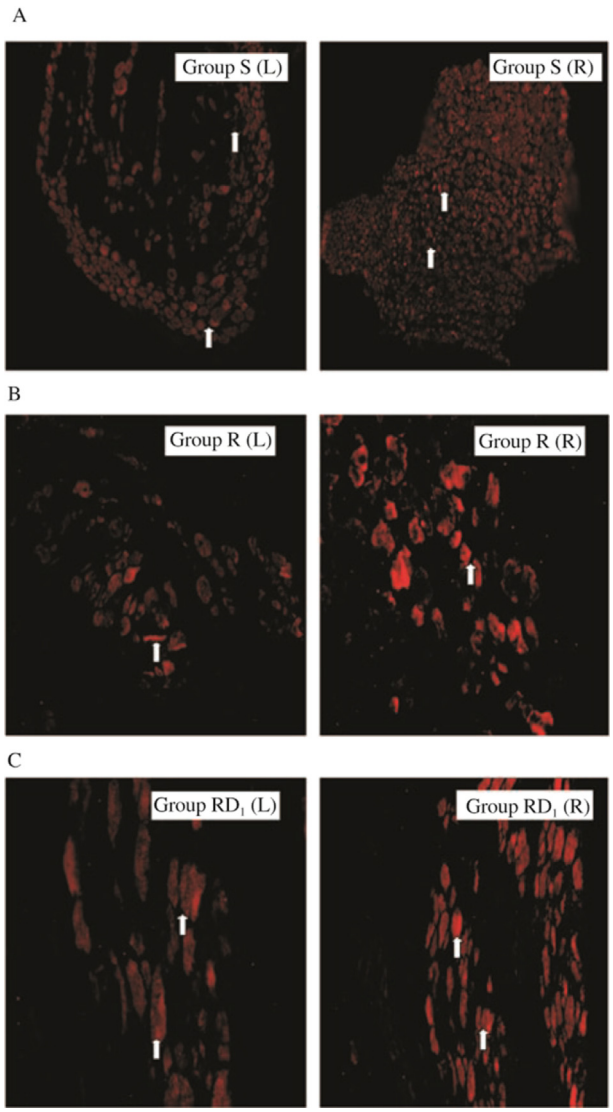

Figure 3. Immunofluorescence (OX-42) of DRG of SMIR model surgery in rats (400×).

Nine scopes in each group were randomly selected and the number of swelling mitochondria of each scope was counted. The number of ipsilateral paws' swelling mitochondria of Group S and Group R per scope was significantly more than Group RD_1 with the number 257.2 ± 60.9 and 291.6 ± 82.1 *vs.* 97.2 ± 33.3 ($P < 0.05$) (Figure 2). Contralateral paws were 247.2 ± 50.9 and 261.6 ± 52.1 *vs.* 88.2 ± 21.2 in each group respectively. Contralateral paws of each group revealed minor pathological changes comparing with ipsilateral paws ($P > 0.05$) (Figure 2).

3.3. Immunofluorescence (OX-42, GFAP) of DRG of SMIR model surgery in rats

Immunofluorescence experiment revealed microglia-OX-42-positive cells in the DRG of Group S, especially in the ipsilateral DRG paws. Comparing with contralateral paws, ipsilateral DRG of Group S, Group R and Group RD_1 expressed obvious immunofluorescence (Figure 3). Comparing with Group S, Group R and Group RD_1 expressed minor OX-42-positive immunofluorescence.

Immunofluorescence experiment revealed GFAP-positive cells in the DRG of Group S, especially in the ipsilateral DRG paws. Comparing with contralateral paws, ipsilateral DRG of Group S, Group R and Group RD_1 expressed obvious immunofluorescence (Figure 4). Group RD_1 expressed less GFAP-positive cells than the other 2 groups.

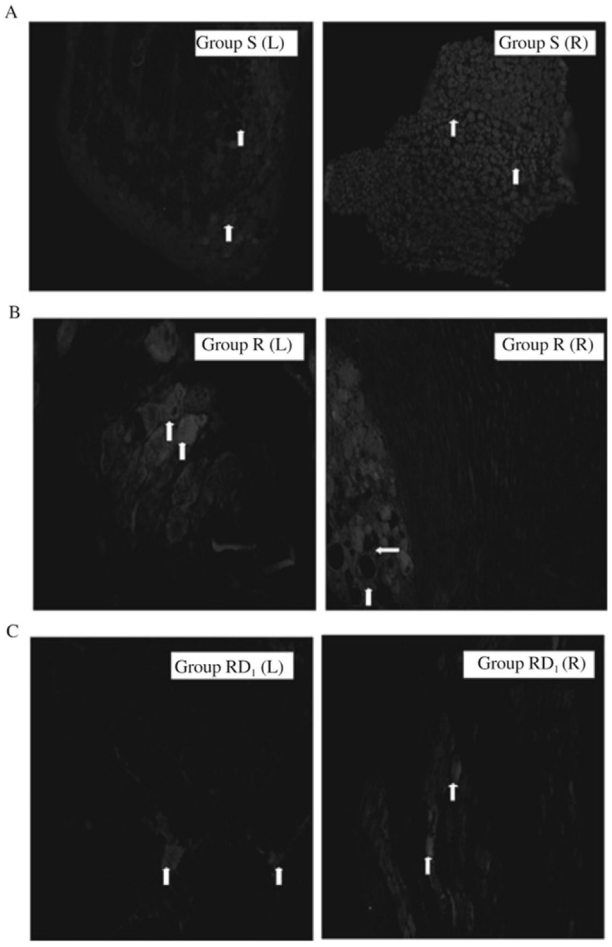

Figure 4. Immunofluorescence (GFAP) of DRG of SMIR model surgery in rats (400×).

4. Discussion

After unilateral nerve extraction, pain and increased pain sensitivity are evident on both sides of the body. This phenomenon is known as MIP [19]. Although not all unilateral nerve injury in clinical settings will appear as mirror pain, the contralateral side often displays hyperalgesia. So in our experiment, we did SMIR model to detect mechanical hyperalgesia. We found that unilateral nerve extraction exhibited noticeable bilateral mechanical pain. Following SMIR model surgery, both the ipsilateral and contralateral hind paw thresholds for mechanical hyperalgesia were significantly increased.

We injected local anesthetic in the surgery site to block sodium channels which is the symbol of neuronal excitation [20]. The relief of the mechanical pain hyperalgesia within the first 3 d of both sides can suggest local anesthetic injection is one temporarily hyperalgesia treatment for MIP/hyperalgesia. At the same time, the unchanged ultrastructure and the excitation of glial cells (OX-42-positive cells and GFAP-positive cells) are also enhanced, showing that a simple injection of local anesthetic can not suppress the neuronal injury in SMIR model.

At the same time, we injected local anesthetic combined with one novel α_2-adrenoceptor agonist-dexmedetomidine in the surgery site. This treatment not only prolonged duration and enhanced the analgesic effect of the local anesthetic, but also prevented the neuronal damage from mitochondria swelling and proliferation of ipsilateral and contralateral sides. MIP of both sides of the paws mechanical pain was reduced significantly, and the ultrastructure of DRG is revealed minor pathological changes (endoplasmic reticulum edema).

After unilateral nerve extraction, how did the contralateral side neuron be exited? A number of studies showed that glial cells can profoundly affect the genesis and/or maintenance of pain; based on this background, we focus on the glial cells of the peripheral nervous system: satellite glial cells, astrocytes and microglia. When they become activated, they may proliferate and release substances that act as messengers to excite DRG neuron. Satellite glial cell activation occurs after nerve injury or inflammation [21,22]. Within our experiment, we arranged immunofluorescence to show the activated glial cells in DRG (OX-42-positive cells and GFAP-positive cells).

After unilateral extraction without treatment, mitochondria swelling or vacuolization was increased in both sides of DRG. Therefore, we imagine mitochondria swelling or vacuolization involved in the mechanism of MIP after nerves extraction. And the changes of DRG can be inhibited by local injection of local anesthetic combined with one novel α_2-adrenoceptor agonist-dexmedetomidine. Then after the injection, we tested mechanical and thermal sensitivity, our study showed that combined formular reversed mechanical allodynia not only the ipsilateral but also the contralateral paws.

Regardless of whether this is the explanation, the significance of our results is that an alteration of the activation of glial cells (OX-42-positive cells and GFAP-positive cells) can have significant behavioral consequences. So we can draw an conclusion from this study that after SMIR, glial cells became activated and may release some substances, these substances may be the important mediators of chronic pain, as well as this experiment showed that the enhance GFAP and OX-42 expression in glial cells in bilateral DRG following nerve extraction.

How the signal passed from the ipsilateral side to the contralateral side still remains to be answered. The answer may

lie in the following two mechanisms: 1) after neuronal injury, the pain signal will quickly reach the contralateral side glial cells from the ipsilateral side [9,23]. The limitation of our study is that we should do more about the underlying mechanisms. 2) Glial cells activation will enhance primary neuron excitability in the form of paracrine release in the contralateral primary neurons, thus the contralateral pain sensitivity will increase.

Because mitochondrial swelling/vacuolization and proliferation is the symbol of neuronal excited or hypoxia damage [24]. Our results showed that mitochondrial swelling/vacuolization and proliferation as well as glial cells activation appeared bilaterally after unilateral injury. Then, after locally injection of ropivocaine or ropivocaine with dexmedetomedine, glial cells activation decreased significantly in the bilateral sides. Therefore, this means that inhibition of glial cells activation in DRG also suppressed the excitability of neurons and pain sensitivity; these findings suggest that glial cells play an important role in the mirror pain mechanism. Based on the above studies, we can draw the following conclusion: After unilateral peripheral nerve extraction, glial cells become activated, leading to an increase mitochondrial swelling/vacuolization and proliferation in bilateral DRG, thus producing mirror pain.

In conclusion, we discovered that SMIR model rats can induce MIP. Unilateral nerve extraction can lead to glial cells activation, increased neuronal swelling mitochondrial and proliferation in both sides of peripheral ganglia, which may be the glial cells signal transduction between the same spinal segment. Locally injection ropivocaine and/or dexmedetomidine can inhibit the activation of glial cells in spinal cord and DRG and reduce MIP.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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