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Analgesic Effect of Diprospan in Rats with Trigeminal Neuralgia*

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Summary: This study examined the analgesic effect of diprospan in rats with trigeminal neuralgia. Rat model of trigeminal neuralgic pain was established by loosely ligating the left infraorbital branch of the trigeminal nerve. After allodynia developed, the rats were randomly divided into 2 groups (n=20 in each): diprospan group, in which the rats received diprospan (7 mg/mL, 0.1 mL) injected to the left infraorbital foramen area; control group, in which saline (0.1 mL) was administered as the same manner as the diprospan group. The pain threshold (PT) in the left infraorbital area was measured before and 2, 6, and 8 weeks after the administration. The expression of neuropeptides [substance P, preprotachykinin A (PPTA), calcitonin gene-related peptide (CGRP)] in the trigeminal nerve was detected at the same time points as the PT measurement by immunohistochemistry or *in situ* hybridization method. The results showed that in the diprospan group, the PT was 10.65±1.26, 10.77±1.19 and 14.13±1.34 g 2, 6, and 8 weeks after the administration respectively, significantly higher than that before the administration (PT value: 0.36±0.11) (P<0.05 for each). In the saline group, the PT was 0.37±0.13, 0.66±0.09, 4.45±1.29 and 13.72±1.72 g before and 2, 6, and 8 weeks after the administration respectively with differences being significant between before and 6, 8 weeks after the administration (P<0.01). No significant difference existed in the PT between the diprospan group and the saline group at pre-administration (P>0.05). The PT in the diprospan group was significantly greater than that in the saline group 2 and 6 weeks post-administration (P<0.05). In the diprospan group, the expression levels of neuropeptides were significantly reduced as compared with those in the saline group 2 and 6 weeks post-administration (P<0.05). It was concluded that diprospan has an obvious analgesic effect on the trigeminal neuropathic pain partly by reducing the expression of neuropeptides in the trigeminal ganglia.
Key words: diprospan; betamethasone; trigeminal neuralgia; pain threshold; substance P; calcitonin gene-related peptide

Trigeminal neuralgia (TN) is an extreme form of neuropathic pain occurring in the region of the face. Recently, application of a long-acting anti-inflammatory drug diprospan in treating primary TN through local nerve cord injection has achieved satisfactory effects[1–4]. However, the action mechanism of this agent and the pathophysiological changes in the trigeminal nerve remain unclear. Some studies suggested that the anti-inflammatory, anti-rheumatic, and anti-anaphylactic properties of disprospan were involved[1, 2]. In this study, rat model of TN was established by inducing chronic constriction injury (CCI) to the left trigeminal nerve of the rats. By detecting the expression of neuropeptides [substance P, preprotachykinin A (PPTA), calcitonin gene-related peptide (CGRP)] in the trigeminal ganglia, the molecular mechanism by which diprospan relieves the TN pain was investigated.

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1 MATERIALS AND METHODS

1.1 Animal Grouping and Observation Items

Wistar male rats, weighing 200–250 g per rat, were supplied by the Shandong University Animal Center (China). The rats were housed at a ambient temperature of 23±2°C and subjected to a 12 h:12 h light-dark cycle. To determine the pain threshold (PT), the vibrissal pad in the rats was irritated by touching and holding 3 days before the model establishment. The rats showing intact vibrissa during the training were selected for the following experiments. After the PT was measured before the operation, the left infraorbital nerve (ION) was loosely ligated in all the rats for model establishment. A total of 40 rats which developed allodynia 2 weeks later were used and randomly divided into the diprospan group and the control group (n=20 in each group). For the diprospan group, 0.1 mL diprospan (7 mg/mL) was injected into the rat's left infraorbital foramen area. For the control group, 0.1 mL normal sodium instead of diprospan was injected. The PT in the rat's left vibrissal pad was determined before and 2, 6 and 8 weeks after the admini-

stration in each group. Every 5 rats were sacrificed at the above-mentioned time points. Immunohistochemical and *in situ* hybridization methods were used to detect the expression and distribution of neuropeptides (substance P, PPTA, CGRP) in the trigeminal ganglia.

The PT was measured by stimulating the vibrissal pad of the rats by using mechanical PT detector. The determination was based on the criteria formulated by Idänpään-Heikkilä *et al*[5]. The PT was defined as mechanical stimulating intensity which was recorded when the intensity was gradually increased and the rats developed any of the following behavior changes. These behaviors represented the maximum damage and scored highest according to the Vos behavior reaction scoring system[6]. The behaviors included: (1) withdrawal reaction: the rat moves its head briskly backward when the stimulation is applied; (2) escape/attack: the rat avoids further contact with the stimulus object, either passively by moving its body away from the stimulating object to assume a crouching position against the cage wall, or actively by attacking the stimulus object, making biting and grabbing movements; (3) asymmetric face grooming: the rat displays an uninterrupted series of at least three face-wash strokes directed toward the stimulated facial area. If the stimulating intensity was set as 26 g and the rats failed to present any of the behaviors mentioned above, the PT was regarded as 26 g. If the stimulating intensity of n+1 but not that of n could produce consistent behavior response in rats, the PT value was $2n + 1/2$.

1.2 Model Establishment

Rat model of TN pain was established by inducing chronic constriction injury to the left ION according to the method described by Imamura *et al*[7]. In brief, animals were fixed on a plank in a prone position after anesthesia with sodium pentobarbital (0.23 mL/100 g). A 1 cm longitudinal incision was made along the facio-nasal furrow of the rats on the left side of the face. The tissues were bluntly divided until the ION was exposed. Then, the ION was loosely ligated by using 2 chromic catguts (5-0) (2 mm apart). The ligation was tight enough to keep never conduction retarded while the blood supply still sufficient. The incision was sewed up with 3-0 silk. The entire operation was undertaken under the sterile condition.

1.3 Main Equipment and Experimental Agents

The mechanical response threshold detector was provided by the American North Sea Coast Limited Co., USA. The stimulating intensities were 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g. Diprospan consisting of betamethasone dipropionate and betamethasone sodium phosphate was produced by Schering-Plough Labo N.V., Belgium and subpackaged by Shanghai Schering-Plough Ltd. Co. (Batch No: 6BBKABDA02, China). SP, CGRP antibody, SABC, and DAB kits were bought from Wuhan Boster Bio-engineering Ltd. Co., China. PPTA was synthesized by TaKaRa Biotechnology (Dalian) Ltd. Co., China. The PPTA probe contained 35 bases with the sequence of 5'-GTT GGC ACC GAT TTC CTC TGC AAA CAG TTG AGT GG-3'. The CGRP was an oligonucleotide probe containing 32 bases with the sequence of 5'-GCTGCACTGG TGCAG AACTATATGC AGATG AA-3'. The 3'-end digolan marking method was used to mark the probe.

1.4 Specimen Preparation

The trigeminal ganglions (TG) on both sides were obtained from the animals sacrificed with a fatal dose of sodium pentobarbital. They were quickly frozen at –170°C by using liquid nitrogen. Serial sections at a thickness of 15 μm were prepared for the detection of the expression of SP, CGRP, PPTA and CGRP.

1.5 Immunohistochemistry

Immunohistochemistry of SABC method was performed according to the manufacturer's instruction provided in the kit. The blank control used normal rabbit serum instead of rabbit anti-SP and anti-CGRP antibodies to incubate the sections.

1.6 *In situ* Hybridization

The mRNA expression of PPTA and CGRP was detected by using *in situ* hybridization method. In the negative control, the hybridization solution of PPTA and CGRP oligonucleotide probe was replaced by distilled water.

1.7 Result Assessment

The positive results for mRNA expression of PPTA and CGRP, protein expression of SP and CGRP were defined as brown particles located in the cytoplasm of neurons in the TG under the light microscope.

1.8 Statistical Analysis

The TG sections with clear positive stains in the absence of non-special background stains were selected for quantitative analysis which was performed by using HPIAS-1000 color pathologic image analysis system. The absorbance of the positive cells was measured in 4 fields of view under the high-power microscope and averaged to reflect the mRNA expression of PPTA and CGRP, protein expression of SP and CGRP.

ANOVA analysis was used for comparison between the different time points in the same group with the aid of SPSS13.0. For the comparison between the two groups, t test was adopted. A P value less than 0.05 was considered to be statistically different.

2 RESULTS

2.1 PT Variation

The basic PT in the diprospan and control groups before the operation was 14.17±1.71 and 14.16±1.70 g, respectively. Animals developed allodynia, presenting with irritability 2 weeks post-operation. When the non-injury stimulating intensity was 0.36±0.11 and 0.37 ±0.13 g in the diprospan and control groups, the animals developed behaviors of escaping from attacks and constantly grooming. The PT in the diprospan group was gradually increased after the administration, reaching 10.65±1.26 g at 2 weeks. Meanwhile, allodynia disappeared. At 6 weeks, the PT was high up to 10.77±1.19 g, significantly higher than that before the administration or in the control group. At 8 weeks, the PT reached the preoperative level in the two groups (P>0.05) (table 1).

2.2 Protein Expression of SP and CGRP in the TG

As shown in the tables 2 and 3, the protein expression of SP and CGRP in the neurons of the TG in the diprospan group 2 and 6 weeks post-administration was significantly decreased as compared with that pre-administration and that in the control group (P<0.01

for each). There was no difference in the protein expression of SP and CGRP between the right and left TG in the diprospan group and between the diprospan and control groups in terms of the same side of TG 8 weeks post-administration (P>0.05 for each).

Table1 Comparison of PT at different time points in the two groups ($\bar{x}\pm s$, g)

Groups	Basic threshold	Pre-administration	2 weeks post-administration	6 weeks post-administration	8 weeks post-administration
Control (n=20)	14.16±1.70	0.37±0.09	0.66±0.09$^{\triangle\blacktriangle\bigstar}$	4.45±1.29$^{\triangle\blacktriangle\bigstar}$	13.72±1.72$^{\blacktriangle}$
Diprospan (n=20)	14.17±1.71	0.36±0.11$^{\triangle}$	10.65±1.26$^{\triangle\blacktriangle}$	10.77±1.19$^{\triangle\blacktriangle}$	14.13±1.34$^{\blacktriangle}$

$^{\triangle}P$<0.01 *vs.* basic threshold; $^{\blacktriangle}P$<0.01 *vs.* pre-administration; $^{\bigstar}P$<0.01 *vs.* diprospan group

Table 2 The protein expression of SP in the neurons of TG ($\bar{x}\pm s$, n=5)

Groups	Pre-administration	2 weeks post administration	6 weeks post administration	8 weeks post administration
Control				
Left TG	128.24±4.51	171.13±6.41	146.13±9.08	32.78±1.23
Right TG	42.97±1.28$^{\bigstar}$	63.97±1.29$^{\bigstar}$	52.97±1.33$^{\bigstar}$	32.24±1.29
Diprospan				
Left TG	128.25±4.42	32.67±1.22$^{\triangle\blacktriangle}$	31.29±1.28$^{\triangle\blacktriangle}$	31.19±1.31$^{\blacktriangle}$
Right TG	43.13±1.27$^{\bigstar}$	32.09±1.27$^{\triangle\blacktriangle}$	31.17±1.31$^{\triangle\blacktriangle}$	30.96±1.26$^{\blacktriangle}$

$^{\triangle}P$<0.01 as compared with the left TG in the control group; $^{\blacktriangle}P$<0.01 as compared with pre- administration in the diprospan group; $^{\bigstar}P$<0.01 as compared with the left TG in the same group

Table 3 The protein expression of CGRP in the neurons of TG ($\bar{x}\pm s$, n=5)

Groups	Pre-administration	2 weeks post- administration	6 weeks post- administration	8 weeks post- administration
Control				
Left TG	169.22±5.55	190.98±3.41	176.31±3.41	49.97±1.41
Right TG	63.39±2.47$^{\bigstar}$	80.27±3.21$^{\bigstar}$	71.29±2.26$^{\bigstar}$	49.76±2.27
Diprospan				
Left TG	169.24±5.57	48.91±2.87$^{\triangle\blacktriangle}$	49.29±3.32$^{\triangle\blacktriangle}$	49.29±4.19$^{\blacktriangle}$
Right TG	63.41±2.45$^{\bigstar}$	50.29±4.51$^{\triangle\blacktriangle}$	48.91±4.26$^{\triangle\blacktriangle}$	50.21±4.36$^{\blacktriangle}$

$^{\triangle}P$<0.01 as compared with the same side of the TG in the control group; $^{\blacktriangle}P$<0.01 as compared with pre-administration in the diprospan group; $^{\bigstar}P$<0.01 as compared with the left TG in the same group

2.3 mRNA Expressions of PPTA and CGRP in the Neurons of TG

The mRNA expression of PPTA and CGRP in the neurons of TG in the diprospan group was significantly decreased 2 and 6 weeks post-administration as compared with that in the same side of the control group and at pre-administration (P<0.01). The mRNA expression of PPTA and CGRP was not significantly different between the right and left TG in the diprospan group and between the right TG of diprospan and control groups 8 weeks post-administration (P>0.05) (table 4, 5).

Table 4 The mRNA expression of PPTA in the neurons of TG ($\bar{x}\pm s$, n=5)

Groups	Pre-administration	2 weeks post administration	6 weeks post administration	8 weeks post administration
Control				
Left TG	106.24±4.23	147.97±5.98	134.89±9.68	27.87±1.22
Right TG	35.21±2.69$^{\bigstar}$	44.31±2.33$^{\bigstar}$	39.29±2.26$^{\bigstar}$	27.38±2.15
Diprospan				
Left TG	106.25±4.24	29.17±3.02$^{\triangle\blacktriangle}$	27.31±2.87$^{\triangle\blacktriangle}$	26.99±3.11$^{\blacktriangle}$
Right TG	35.24±2.73$^{\bigstar}$	26.01±3.27$^{\triangle\blacktriangle}$	25.27±1.28$^{\triangle\blacktriangle}$	24.39±5.31$^{\blacktriangle}$

$^{\triangle}P$<0.01 as compared with the same side of TG in the control group; $^{\blacktriangle}P$<0.01 as compared with pre-administration in the diprospan group; $^{\bigstar}P$<0.01 as compared with the left TG in the same group

Table 5 The mRNA expression of CGRP in the neurons of TG ($\bar{x}$±*s*, *n*=5)

Groups	Pre-administration	2 weeks post administration	6 weeks post administration	8 weeks post administration
Control				
Left TG	147.72±5.50	169.53±5.49	148.69±3.18	45.69±4.51
Right TG	49.89±1.41★	57.64±4.69★	47.27±1.37★	44.32±4.24
Diprospan				
Left TG	147.76±5.49	42.51±2.24△▲	41.71±4.69△▲	43.81±4.52▲
Right TG	49.91±1.42★	43.12±3.16△▲	42.13±2.18△▲	44.16±3.24▲

△P<0.01 *vs.* the same side of TG in the control group; ▲P<0.01 *vs.* pre-administration in the diprospan group; ★P<0.01 *vs.* left TG in the same group

3 DISCUSSION

The present study demonstrated that diprospan injected to the infraorbital foramen area could significantly increase the PT of the vibrissal pad during the allodynia period (2 weeks postoperative) in the rat model of TN and the PT continued to rise with time (6 weeks postoperative), substantially higher than that in the control group. Moreover, the protein expression of SP and CGRP, and the mRNA expression of PPTA and CGRP were remarkably reduced in the TG of rat models after diprospan administration. These results suggested diprospan could exert an analgesic effect in TN and the mechanism may involve the down-regulation of some neuropeptides such as SP, CGRP and PPTA.

Previous studies had shown that conspicuous pathological changes occurred in the TG and its sensory root in a rat model of trigeminal neuropathic pain, including the disappearance of gangliocytes, infiltration of inflammatory cells and degeneration of the myelin sheath under the electron microscope[8]. Currently, it is believed that demyelinating changes are the important pathological basis for TN[9]. The ION-CCI rat model is a commonly used animal model for TN, in which the behavioral changes are most reminiscent of the clinical condition of trigeminal neuropathic pain and the pathogenesis is in accordance with TN peripheral neuropathy now generally believed[7]. In this study, the ION-CCI rat model was adopted and successfully established. The results showed that, 2 weeks after the operation, allodynia was developed in animals which showed hyper-responsiveness to innocuous mechanical stimulation.

Neuropeptides, a kind of bioactive polypeptide, play a crucial role in the information transmission. They functions not only as neurotransmitters or modulators but also as hormones. SP is the first ever discovered neuropeptide which contains 11 amino acids and is a member of the tachykinin family. CGRP is a neuropeptide that contains 36 amino acids [10–12]. They are excitatory transmitters of primary sensory neurons and are synthesized by the cell body of the first afferent neuron. Cells of TG can synthesize SP and CGRP and then transport them to the principal sensory nucleus of trigeminal nerve[13, 14] through central and peripheral processes. Previous study found that SP in the cerebrospinal fluid in patients with TN was significantly elevated[15]. SP and CGRP in the blood sample from the external jugular vein were also increased when the trigeminal nerve was stimulated by using radiofrequency thermocoagulation treatment in patients with TN and the similar results were obtained in cat models of TN when cat trigeminal nerve was stimulated [16]. The biochemical model of TN was discovered by Bouckoms *et al*[17], who proposed that SP and CGRP were increased at the onset of TN, but somatostatin decreased, lowering the activity of the monoamine transmitters and disturbing the purine and endogenous opioid peptides system. Studies by Marinković *et al*[18] indicated that SP expression was increased in the branch of the trigeminal nerve removed from a patient with TN. Other studies on the animal model of TN also demonstrated the increased SP and NK1 in the trigeminal nerve or brain tissues[19, 20].

Diprospan is comprised of betamethasone dipropionate and betamethasone sodium phosphate, which possesses high glucocorticoid and slight mineralocorticoid activity and has anti-inflammatory, anti-rheumatic, anti-anaphylactic, and immunosuppressive properties. This agent is clinically used to relieve acute neuropathic pain, such as cervical headache and acute posterior ganglionitis[21, 22]. In our study, after diprospan was injected to the rat's infraorbital foramen area during the allodynia period, the PT in the vibrissal pad was gradually increased. It was significantly higher than that in the control group 2 and 6 weeks after the administration. Moreover, the results showed that the protein expression of SP and CGRP, and the mRNA expression of PPTA and CGRP were significantly increased in the rat models of TN. After diprospan administration, with the PT increasing, the expression of these neuropeptides was greatly decreased and they returned to normal level at the same time as the PT, suggesting that diprospan can attenuate the trigeminal neuropathic pain by inhibiting the expression of neuropeptides.

In conclusion, our study demonstrated that diprospan could increase the PT of the vibrissal pad in the rat model of TN, partly by depressing the expression of neuropeptides. The results primarily explained the mechanism by which diprospan exerts an analgesic effect in TN. Other analgesic mechanisms may also be involved and in-depth studies will be warranted in the future.

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