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Original Article

Mechanism and antagonism of naloxone hydrochloride on neuropathic pain in rats

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Abstract: Objective: To study the antagonistic effect of naloxone hydrochloride on neuropathic pain in rats and explore mechanisms of action. Methods: Forty-eight male SD rats were randomized into normal control (N group, n=8), sham operation (S group, n=8), pain model (M group, n=8), and naloxone hydrochloride (K group, n=24) groups. Results: There was no difference in the pre-surgery levels of paw withdrawal latency (PWL), paw withdrawal threshold (PWT), TNF-α and IL-6 among the four groups (all P>0.05). After surgery, the PWL of rats in the M and K groups decreased, and the levels of PWT, TNF-α and IL-6 increased (all P<0.05). Post-drug PWL level was higher in the K group compared to that on days 7 and 14 after surgery and was lower than the pre-surgery value (all P<0.05). The levels of post-drug PWT, TNF-α, and IL-6 were lower than those on days 7 and 14 after surgery and were higher than the pre-surgery values (all P<0.05). There was no difference between the post-drug levels of PWT, TNF-α and IL-6 and the values on day 1 post-surgery (all P>0.05). Logistical regression analysis showed that TNF-α and IL-6 levels were correlated with the degree of neuropathic pain in chronic constrictive injury (CCI) rats (both P<0.05). Conclusion: Naloxone hydrochloride can inhibit neuropathic pain in CCI rats and may play an antagonistic role by reducing the expression of TNF-α and IL-6.

Keywords: Naloxone hydrochloride, TNF-α, IL-6

Introduction

Neuropathic pain is a complex pathological change, which is triggered by primary damage and dysfunction of the nervous system. It is mainly caused by the plasticity changes of the peripheral nervous system and the central nervous system [1, 2]. The pathogenesis of neuropathic pain is not yet fully understood. Pain sensitization of primary sensory neurons caused by neurological impairment, and enhancement of excitatory synaptic conduction in the spinal cord, brain stem and cortex leading to chronic pain are the currently accepted etiologies [3, 4]. Clinical treatment of neuropathic pain is challenging due to its complex etiology, and only a few specific drugs are available for treating neuropathic pain. Therefore, it is important to discover new therapeutic drugs to alleviate this condition.

Naloxone hydrochloride is a non-selective opioid receptor antagonist. It competitively antagonizes the binding of endogenous opioid peptides such as β-endorphin to peripheral or central nervous system receptors, stabilizes neuronal intracellular Ca^{2+} and Mg^{2+} levels, enhances cell membrane stability and cerebral perfusion pressure, and relieves inflammatory reactions: thereby protecting neurological functions [5-7]. Currently, naloxone hydrochloride is widely used in the treatment of cranio-cerebral injury, and studies have shown that it can effectively repair nerve function in rats and reduce mortality [8, 9]. However, naloxone hydrochloride has not been used to treat neuropathic pain.

Therefore, we established a rat sciatic nerve CCI neuropathic pain model by sciatic nerve ligation to investigate whether naloxone hydrochloride has an antagonistic effect on rat neuropathic pain, and elucidated its mechanism of action.

Materials and methods

Experimental animals and establishment of neuropathic pain model

This study was approved by the Ethics Committee of Guangdong Provincial People's Hos-

pital, Guangdong Academy of Medical Sciences. Fifty SPF mature male SD rats were purchased from Guangdong Medical Experimental Animal Center and were all fed SPF-grade nutritionally fortified rat feed (Jiangsu Province Synergistic Pharmaceutical Bio-Engineering Co., Ltd.). The age of the rats were 42-50 days (average 46.2 ± 3.4 days), and they weighed 216-250 g (average body weight 233.6 ± 4.8 g). The housing conditions were maintained at 22 ± 3°C and 45-60% humidity with fluorescent lighting, and the animals had access to food and water *ad libitum*. Forty-eight male SD rats were randomized into the normal control (N group, n=8), sham operation (S group, n=8), pain model (M group, n=8), and naloxone hydrochloride (K group, n=24) groups.

Rats in the designated M and K groups were operated on as per the method of Chen to produce a CCI neuropathic pain model of the sciatic nerve [10]. The animals were fixed on a sterile operation table and intraperitoneally injected with 10% chloral hydrate (Wuhan Yuancheng Science and Technology Development Co., Ltd.) at 300 μg/g body weight for anesthetization. The skin was cut and the sciatic nerve was bluntly separated. A 4-0 chrome catgut (Henan Zeyuan Medical Devices Sales Co., Ltd.) was used to ligate the sciatic nerve trunk 3 times at intervals of ≤ 2 mm. The incisions were sutured one-by-one with a Johnson-absorbable suture (Shanghai Hanfei Medical Devices Co., Ltd.), and the contralateral lower limb was injected intramuscularly with lincomycin hydrochloride (0.2 mL, 10 mg/mL) to reduce inflammation. Ligature can guarantee nerve compression within 7 days, without affecting the epineurial blood transport. Mild tremors were seen in the calf muscles during ligation. After successful operation, the animals were randomized into the untreated (M) and naloxone hydrochloride treated (K) groups. S group rats underwent the same surgery but without ligation.

On the 15th day after surgery, all rats were anesthetized with pentobarbital sodium (50 mg/kg) intraperitoneally, and a needle (Nanjing Jiancheng Bioengineering Institute) was used puncture between the lumbar 5 and lumbar 6 disc space. The puncture was successful when the rats appeared to swing sideways, or the cerebrospinal fluid seeped from the end of the needle. A 50 μL microsyringe was used to draw physiological saline (10 μg/g) and 1 μL of air, followed by either naloxone hydrochloride (Hubei Yuancheng Saichuang Science and Technology Co., Ltd.; 10 μg/g) or the same amount of physiological saline. The loaded microsyringe was connected to the puncture needle, and the contents were slowly and evenly injected into the spinal canal. The K group was given naloxone hydrochloride, and S and M groups were given physiological saline [11].

Paw withdrawal latency

PWL was measured by Hargreaves method on the day before surgery, on days 1, 7 and 14 after surgery, and 10 minutes after drug administration. To detect the latent period of thermal pain threshold, the rats were placed in observation cages, and after they had calmed down, a concentrated beam of light was focused on the bottom of the middle toe [12]. To avoid burns, the upper limit of PWL was set to 20 s. The duration from the start of irradiation to the time when the rat lifted the leg or escaped was the latent period of thermal pain threshold. The process was repeated 3 times with intervals of 10 min, and the average of the three values was calculated. The PWL measured on the day before the establishment of the model was used as the baseline value.

Mechanical pain threshold measurement

VonFrey filaments were used to measure the mechanically stimulated PWT [13]. The rat foot was stimulated with different forces of vonFrey filaments and the highest threshold was set at 26 g. A rapid contraction reaction occurred immediately during the stimulation time or immediately after removal of the filaments, and the withdrawal was considered a positive reaction. A minimum of 3 positive reactions with 5 consecutive measurements, spaced at 10 s intervals, with the same vonFrey filament was used to measure the PWT threshold.

Enzyme-linked immunosorbent assay

Serum levels of TNF-α and IL-6 were measured by ELISA (Shanghai Jing Kang Biological Engineering Co., Ltd.) according to the manufacturer's instructions. Briefly, 50 μL of each sample was added per well to an ELISA microplate, and 2 positive and negative control wells were

set up. One drop of positive control was added to each well and 1 well of blank control was set. A drop of the enzyme conjugate was added to each well, mixed well, and the sealed plate was incubated at 37°C for 30 minutes. After discarding the supernatant, the wells were filled with the washing solution and spin dried, and the process was repeated 5 times. To each well, a drop each of reagent A and reagent B were added and mixed thoroughly, and the plate was incubated at 37°C for 15 minutes. The reaction was stopped with a drop of stop solution per well. Absorbance was measured at 450 nm using a microplate reader, and the OD of the blank well was used to normalize the sample values.

Statistical analysis

SPSS 22.0 software was used for statistical analysis. The measurement data are expressed as $\bar{x} \pm$ sd. Multiple groups were compared using variance analysis, and comparison between two groups was analyzed using the LSD test. Analysis at different time points within one group was based on variance analysis of repeated measurements. Logistical regression was used to determine the correlation between TNF-α and IL-6 levels and neuropathic pain in CCI rats. $P<0.05$ is considered statistically significant.

Results

Establishment of the neuropathic pain model

CCI was successfully established in 48 rats and the remaining 2 died, resulting in a success rate of 96%. The CCI rats were in good condition and had uniform body hair color. On the third day after surgery, the rats showed claudication in their gait, curling of the toes, ectropion of the feet, and atrophy of the leg muscles. When the feet were stimulated, the rats either licked and sucked the area, or showed abnormally painful behavior of the hind limbs. Toe autophagy was not observed.

PWL test results

There was no difference in the pre-surgery, day 1 post-surgery and post-drug PWL values among the four groups (all $P>0.05$), but the PWL on days 7 and 14 post-surgery were different among the groups (both $P<0.05$).

Pairwise comparison between four groups showed that the pre-surgery PWL had no difference ($P>0.05$). The post-surgery (all time points) and post-drug PWL was significantly lower in the M and K groups compared to those of the N and S groups (all $P<0.05$). There was no difference in PWL between the N and S groups (all $P>0.05$). Although pre- and post-surgery PWL values were similar in the M and K groups (all $P>0.05$), the post-drug PWL was significantly higher in the K group ($P<0.05$).

Intragroup comparison showed that the post-surgery PWL (all time points) in M and K groups were significantly lower than their respective pre-surgery values (all $P<0.05$). In addition, the PWL on days 7 and 14 post-surgery were lower compared to that on day 1 post-surgery (all $P<0.05$), and the day 14 PWL was lower than that on day 7 post-surgery (all $P<0.05$) in both groups. Intragroup comparison in groups N and S did not reveal any difference in the PWLs between different pairs of time points (all $P>0.05$).

Post-drug PWL of group N and S were not significantly different from the 4 time points before drug administration (all $P>0.05$). Post-drug PWL of M group was similar to that of day 14 post-surgery ($P>0.05$), but was lower than the pre-surgery, and days 1 and 7 post-surgery values ($P<0.05$). The post-drug PWL of K group rats was higher than that on the 7 and 14 days post-surgery, lower than the pre-surgery PWL (all $P<0.05$), and similar to that of day 1 post-surgery ($P>0.05$; **Figure 1**).

PWT test results

There was no difference in the pre-surgery, post-surgery, and post-drug PWT among the four groups (all $P>0.05$). Pairwise comparison of groups showed no difference in pre-surgery PWT between any two groups ($P>0.05$). The post-surgery (all time points) and post-drug PWT of groups M and K were higher than those of groups N and S (all $P<0.05$). PWT of groups N and S were similar (all $P>0.05$), as were the pre-drug (all four time points) PWT of the M and K groups (all $P>0.05$). The post-drug PWT in the K group was lower than that in the M group ($P<0.05$).

Post-surgery (all time points) PWT in the M and K groups were higher than respective pre-sur-

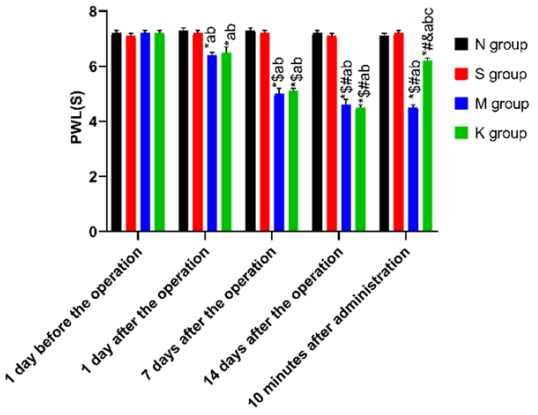

Figure 1. PWL test results. *P<0.05, compared with the same group 1 day before surgery; $P<0.05, compared with the same group 1 day after surgery; #P<0.05, compared with the same group 7 days after surgery; &P<0.05, compared with the same group 14 days after surgery; aP<0.05, compared with the N group at the same time point; bP<0.05, compared with the S group at the same time point; cP<0.05, compared with the M group at the same time point. PWL, paw withdrawal latency.

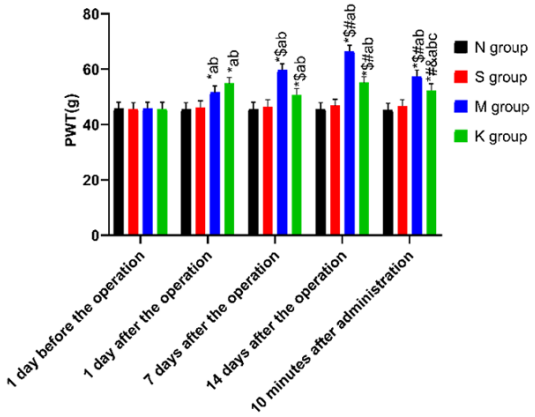

Figure 2. PWT test results. *P<0.05, compared with the same group 1 day before surgery; $P<0.05, compared with the same group 1 day after surgery; #P<0.05, compared with the same group 7 days after surgery; &P<0.05, compared with the same group 14 days after surgery; aP<0.05, compared with the N group at the same time point; bP<0.05, compared with the S group at the same time point; cP<0.05, compared with the M group at the same time point. PWT, paw withdrawal threshold.

gery values (all P<0.05). The PWT on days 7 and 14 post-surgery was higher than that of day 1 post-surgery (all P<0.05), and day 14 post-surgery PWT was higher than that on day 7 post-surgery (both P<0.05), respectively in both groups. Intragroup comparison in groups N and S did not reveal any difference in the PWTs between different pairs of time points (all P>0.05).

Post-drug PWT in group N and S was similar to pre-drug values (all four time points; all P>0.05). In group M, post-drug PWT was similar to that on day 14 post-surgery (P>0.05), and was higher than the PWTs on days 1 and 7 post-surgery (both P<0.05). The post-drug PWT in K group rats was lower than that on the days 7 and 14 post-surgery, higher than pre-surgery PWT (all P<0.05), and similar to that on day 1 post-surgery (P>0.05; **Figure 2**).

Serum levels of TNF-α and IL-6

There was no difference in the pre-surgery levels of either TNF-α or IL-6 among the four groups (all P>0.05). However, the TNF-α or IL-6 level post-surgery and post-drug were different among them (all P<0.05).

Pairwise comparison between different groups pre-surgery did not show any significant differences in the levels of either cytokine (all P>0.05). The post-surgery (all time points) and post-drug levels of TNF-α and IL-6 were significantly higher in the M and K groups compared to the N and S groups (all P<0.05), and those between N and S groups were different (all P<0.05). Pre-drug cytokine levels were similar between the M and K groups (all P>0.05). Post-drug levels of both TNF-α and IL-6 were lower in the K group compared to M group (P<0.05).

The levels of both cytokines increased after surgery in the S, M and K groups compared to their respective pre-surgery levels. Intragroup comparison showed that the levels of TNF-α and IL-6 on days 1, 7 and 14 post-surgery were higher than the pre-surgery levels in S, M and K groups (all P<0.05). Levels of both cytokines in groups S, M and K were higher on days 7 and 14 post-surgery compared to the respective values on day 1 post-surgery (all P<0.05), and those on day 14 were higher compared to day 7 post-surgery (all P<0.05). Pairwise comparison of different time points showed no difference in either TNF-α or IL-6 levels in the N (all P>0.05).

Post-drug cytokine levels were similar to the pre-drug levels (all four time points) in the N (all P>0.05). In the S and M group, post-drug TNF-α and IL-6 levels were similar to that on day 14 post-surgery (all P>0.05) but were higher than

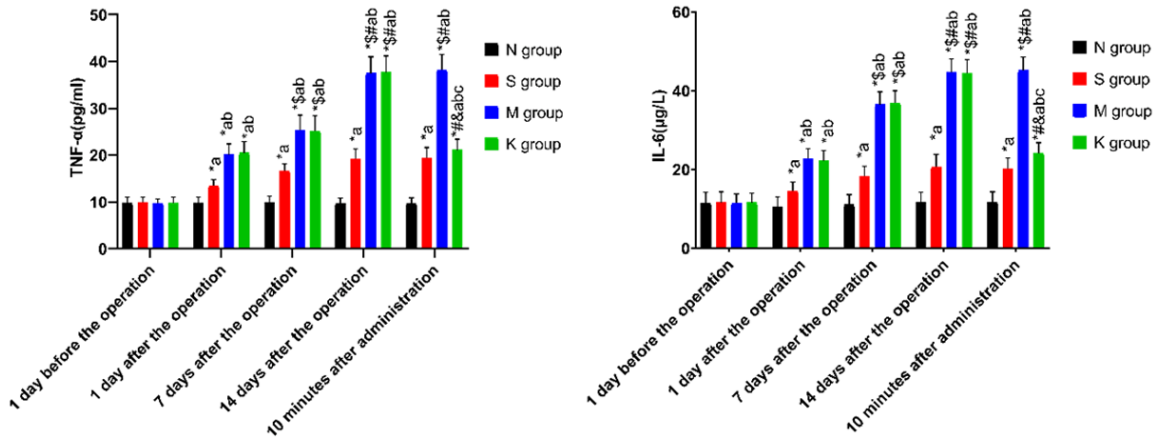

Figure 3. Serum levels of TNF-α and IL-6. *P<0.05, compared with the same group 1 day before surgery; $P<0.05, compared with the same group 1 day after surgery; #P<0.05, compared with the same group 7 days after surgery; &P<0.05, compared with the same group 14 days after surgery; aP<0.05, compared with the N group at the same time point; bP<0.05, compared with the S group at the same time point; cP<0.05, compared with the M group at the same time point. TNF, tumor necrosis factor; IL, interleukin.

Table 1. Correlative analysis of TNF-α, IL-6 levels and neuropathic pain in CCI rats

	TNF-α		IL-6	
	R	P	R	P
PWL	-0.712	0.012	-0.733	0.015
PWT	0.725	0.013	0.717	0.012

Note: TNF, tumor necrosis factor; IL, interleukin; CCI, chronic constrictive injury; PWL, paw withdrawal latency; PWT, paw withdrawal threshold.

the pre-surgery, and days 1 and 7 post-surgery levels (all P<0.05). In the K group, post-drug levels of TNF-α and IL-6 were lower than the days 7 and 14 post-surgery levels, and higher than the pre-surgery levels (all P<0.05), and similar to day 1 post-surgery levels (both P>0.05; **Figure 3**).

Correlative analysis of TNF-α, IL-6 levels and neuropathic pain in CCI rats

Cytokine levels were negatively correlated with PWL, and positively correlated with PWT, thus were positively correlated with the degree of neuropathic pain in CCI rats (**Table 1**).

Discussion

Neuropathic pain is a very complex pain syndrome, affecting about 1.5% of the world's population with different symptoms. The incidence of neuropathic pain in China is also increasing annually. Therefore, it is important to design an effective treatment regimen for neuropathic pain [14, 15]. We established a rat model of CCI to simulate neuropathic pain and explore the mechanistic basis of naloxone hydrochloride antagonism on neuropathic pain. The CCI rat model showed neuropathic pain about 24 hours after operation, and the symptoms lasted for about 10 weeks, which is similar to the clinical manifestations of neuropathic pain, thus validating the animal model [16, 17].

PWL was significantly lower, and PWT was higher in the CCI rats on days 1, 7 and 14 after surgery compared to normal controls and sham-operated rats. The successful simulation of neuropathic pain in the CCI model was manifested as the abnormal licking or shaking of the stimulated hind paw. Naloxone hydrochloride is rarely used to treat neuropathic pain, although it shows considerable neuroprotection in patients with brain injury [18, 19]. In addition, neuropathic pain is also caused by impaired nerve function [1-4]. Therefore, we treated the CCI rats with naloxone hydrochloride to observe its effects on neuropathic pain. The post drug PWL of the K group was significantly higher and PWT was significantly lower compared to the pre-drug values on the 7th and 14th days after surgery. Both PWL and PWT levels were restored to the day 1 post-surgery levels after naloxone hydrochloride injection indicating its good therapeutic effect on neuropathic pain.

Studies have shown that naloxone hydrochloride can reduce the level of TNF-α in a rat model of traumatic brain injury, protect nerve function

and relieve the brain injury [20]. Studies also show that naloxone hydrochloride can effectively reduce the level of IL-6 in patients with traumatic shock and exert an anti-shock effect [21]. We hypothesized that naloxone hydrochloride may relieve neuropathic pain in rats by improving the levels of TNF-α and IL-6 in CCI rats. Therefore, we examined the serum levels of TNF-α and IL-6 as the possible mechanism of naloxone hydrochloride neuroprotection. The levels of TNF-α and IL-6 were significantly higher in the CCI rats compared to the normal controls and sham-operated rats on days 1, 7 and 14 after surgery, suggesting that TNF-α and IL-6 levels may be associated with neuropathic pain. Logistic regression analysis found a positive correlation between the degree of neuropathic pain and TNF-α and IL-6 levels. Therefore, TNF-α and IL-6 may participate in the development of neuropathic pain in CCI rats. After the CCI rats were treated with naloxone hydrochloride, the levels of TNF-α and IL-6 were lower than the pre-treatment levels, which corresponded to a reduction in neuropathic pain.

In summary, naloxone hydrochloride inhibits neuropathic pain in CCI rats, most likely by reducing the expression of TNF-α and IL-6. Therefore, our results provide a theoretical basis for the use of naloxone hydrochloride in treating neuropathic pain, and can be validated by clinical trials once its safety is confirmed.

Disclosure of conflict of interest

None.

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