

## Original Article

# Ondansetron attenuates bone cancer pain through the inhibition of Fos in the descending facilitation related nuclei

Zhang-Xiang Huang<sup>1,2\*</sup>, Cai-Yang Chen<sup>1\*</sup>, Yan He<sup>1\*</sup>, Wei-Qing Ma<sup>3</sup>, Fei-Xiang Wu<sup>1</sup>, Zhi-Jie Lu<sup>1</sup>, Wei-Feng Yu<sup>1</sup>

<sup>1</sup>Department of Anesthesia and Intensive Care, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, Shanghai, China; <sup>2</sup>Department of Pain Medicine, The First Affiliated Hospital of Kunming Medical University, Yunnan, China; <sup>3</sup>Department of Anesthesiology, Kunming General Hospital of Chengdu Military Command, Yunnan, China. \*Equal contributors.

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**Abstract:** Most cancer patients with bone metastasis experience severe pain. However, the treatment for this type of pain is limited by our understanding of its underlying mechanism. A large number of studies have shown that the activation of descending facilitatory system is critical in the maintenance of many chronic pain states. We used Walker 256 cell to establish a rat bone cancer model. Immunohistochemistry was employed to investigate the expression of Fos in the spinal cord and descending facilitation related nuclei (ACC, PAG, RVM) of rats at different stages of cancer development. Our results indicated that Fos expressions in these sites were all significantly upregulated in bone cancer rats. Furthermore, intrathecal injection of ondansetron attenuated bone cancer induced pain behavior as well as the expression of Fos in ACC, PAG and RVM. These data suggest that the descending facilitatory system plays an important part in the maintenance of bone cancer pain, and 5-HT<sub>3</sub> receptor antagonist might be a possible non-opioid pain reliever in the management of bone cancer pain.

**Keywords:** Ondansetron, fos, descending facilitation system, bone cancer pain, rat model

## Introduction

Cancer induced bone pain (CIBP) is a clinically common and very severe pain status. It greatly affects the quality of life of those late stage cancer patients [1, 2]. Currently, there is no effective treatment for this type of pain. Since the establishment of mouse model of bone cancer pain by Manthly and colleagues, a lot have been learned in the research of CIBP [3]. However, the mechanism of CIBP is still not fully understood. Cancer pain is distinct from inflammatory and neuropathic pain status [2]. The descending facilitatory system started to catch researchers' attention since 1990s, and it was shown to be involved in some pathological pain status [4]. A large number of projection neurons amass in the superficial of spinal dorsal horn (Lamina I). About 80% of them express NK1 receptor. These NK1 positive neurons are found to have projections to many brain regions, such as hypothalamus, periaqueductal gray (PAG), parabrachial nuclei (PB) and rostral ven-

tromedial medulla (RVM), and then further relay to higher central regions like anterior cingulate cortex (ACC). Activated central regions then project back through RVM to the spinal dorsal horn. Suzuki and colleagues reported that, selectively damaging NK1 positive cells in Lamina I-III using substance P-saporin (SP-SAP) reduced the excitability of wide dynamic range (WDR) neurons in deep laminae of dorsal horn. This result indicated that the predominant descending control was facilitation. The effect of SP-SAP can be repeated using 5-HT<sub>3</sub> receptor antagonist Ondansetron, suggesting that the descending facilitation is mediated by 5-HT<sub>3</sub> receptors. This descending facilitatory 5-HT pathway regulates the spinal nociceptive transmission [5, 6]. c-fos is an immediate early gene in normal neuron nucleus. Multiple types of stimulations can induce its expression. The Fos expression is closely related to the central and peripheral response to the nociceptive stimuli. Peripheral nociceptive stimuli can induce Fos expression in many brain regions [7].

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In the present study, Fos activation in the descending facilitatory system related nuclei was investigated. In addition, we also antagonized 5-HT3 receptor to examine its effect on pain behavior and Fos expression of the rat model of cancer pain.

### Materials and methods

#### *CIBP modeling*

Adult female Sprague Dawley rats, 6-8 weeks old, weighing 150-180 g (The Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were used in this study. Animals were housed under a 12/12 h light/dark cycle at a room temperature of  $24 \pm 0.5^\circ\text{C}$  with food and water available ad libitum according to NIH guideline of laboratory animal care and use. Animals were placed in the testing equipment to habituate the environment before the start of experiments. All experiments followed the policies issued by International Association for the Study of Pain on the use of laboratory animals.

#### *Preparation of walker 256 cells*

Walker 256 carcinosarcoma breast cancer cells were kindly provided by the Shanghai Institute of the Pharmaceutical Industry. They were prepared as previously described [8]. Walker 256 tumor cells were obtained from an ascitic tumor-bearing rat, washed and suspended in PBS solution to achieve a final injection concentration of  $2 \times 10^5$  cells in a volume of 10  $\mu\text{l}$  and kept on ice. For the sham group, Walker 256 cells were prepared at the same final concentrations for injection and boiled for 30 min [8].

#### *Induction of bone cancer*

All animals were anesthetized with 10% chloral hydrate (i.p. 300 mg/kg). A small incision was made over the left tibia and a hole bored through the periosteum. Ten microlitres of Walker 256 cells ( $2 \times 10^5$  cells) or boiled cells were injected into the intramedullary cavity. After a 2-min delay to allow cells to fill the bone cavity, the syringe was removed and the hole plugged with bone wax. Distilled water was used to wash the incision (kill the leakage carcinosarcoma cell outside the hole). The wound was closed using 1-0 silk threads and dusted with penicillin powder.

#### *Drug administration*

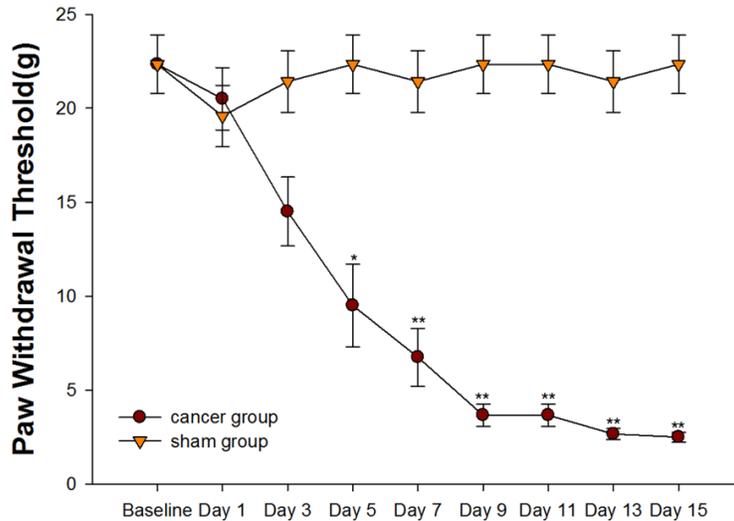
Intrathecal drug applications were performed on day 14 when the hyperalgesia of cancer rats reached its peak. Experimental animals were randomly assigned to following treating groups: 1) sham + ondansetron group; 2) sham + saline group; 3) cancer + saline group; 4) cancer + 1  $\mu\text{g}$  ondansetron group; 5) cancer + 10  $\mu\text{g}$  ondansetron group; 6) cancer + 100  $\mu\text{g}$  ondansetron group. In ondansetron treatment groups, ondansetron (Ningbo Tianheng Pharmaceutical) was diluted with saline to the final concentration of 1  $\mu\text{g}/30 \text{ ul}$ , 10  $\mu\text{g}/30 \text{ ul}$  and 100  $\mu\text{g}/30 \text{ ul}$ . The diluted ondansetron and saline were delivered into the subarachnoid space using a microsyringe. Behavioral test was then performed 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h after the intrathecal injection.

In order to minimize the injury of intrathecal surgery and the inflammatory response triggered by implanted PE catheter, drugs or vehicles were delivered into the spinal space via lumbar puncture (LP) made with a 30 gauge needle between L5 and L6 vertebrae as described in previous study [9]. A brisk tail flick was considered as an indicator of the accuracy of each injection.

#### *Mechanical allodynia test*

Animals were placed in a plastic cage ( $26 \times 20 \times 14 \text{ cm}^3$ ) with a  $2 \times 2 \text{ mm}$  wire-mesh grid floor. After 30 min accommodation, mechanical allodynia was measured as the hind paw withdrawal response to von Frey hair stimulation according to the up-down method described by Dixon. Testing was blind with respect to groups. A series of von Frey hairs (0.40, 0.60, 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g) (Stoelting, Wood Dale, Illinois, USA) were applied through to the grid floor to the ventral surface of the hind paw of each rat. Each von Frey hair was held about 5 s or until the animal withdrew their hind paw without ambulating. A trial began with the application of the 2.0 g von Frey hair. Whenever a positive response to a stimulus occurred, the next lower von Frey hair was applied, and whenever a negative response occurred, the next higher hair was applied. The testing consisted of five more stimuli after the first change in response occurred, and the pattern of response was converted to a 50% von Frey threshold using the method described by Dixon.

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**Figure 1.** Time course of mechanical pain behavior in sham and cancer groups. Paw withdrawal threshold was markedly decreased in the cancer group, compared to sham group (two-way ANOVA, \* $P < 0.05$ , \*\* $P < 0.01$ ,  $n = 12$ ).

### Immunohistochemistry

At 1 day, 7 day, 14 day after the surgery, rats were deeply anesthetized with urethane (0.6 mg/100 g). Then the rats were perfused via aorta with 0.9% saline followed by ice-cooled 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS, pH 7.4). The spines and brains were removed, post-fixed in the same fixative for 24 h at 4°C, and immersed from 10% to 30% gradient sucrose in PBS for 24-48 h at 4°C for cryoprotection. The spines and brains were then frozen and cut in the coronal plane (6 series of 35  $\mu\text{m}$ -thick sections) on a microtome (CM-1900; Leica Instruments, Nussloch, Germany) and stored at -20°C in antifreeze solution.

Following several rinses in PBS, sections were soaked in blocking solution (4% normal goat serum, 0.04% Triton X-100 and 1% BSA in PBS) for 1 h and then incubated with the primary antibody Fos (rabbit, 1:1500 in 0.02% Triton X-100, 1% BSA in PBS; Oncogen Research Products, Cambridge, MA, USA), for 40 h at 4°C. Additional sections were incubated without the primary antibody as a control for background labeling. Sections were then rinsed several times in PBS, incubated with a biotinylated secondary antibody (1:1500 in 0.02% Triton X-100 and 1% BSA in PBS; Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature and subsequently reacted with avidin-bio-

tin complex (Elite ABC Kit; Vector Laboratories) for 90 min at room temperature. The reaction was visualized using diaminobenzidine-nickel, and stopped in acetateimidazole buffer (175 mM and 10 mM, respectively). Sections were mounted on poly-L-lysine-coated slides, and dehydrated in a series of graded alcohol. Immunoreactive primary antibodies in PBS with 1% normal goat serum and 0.3% Triton X-100 for 48 h at 4°C.

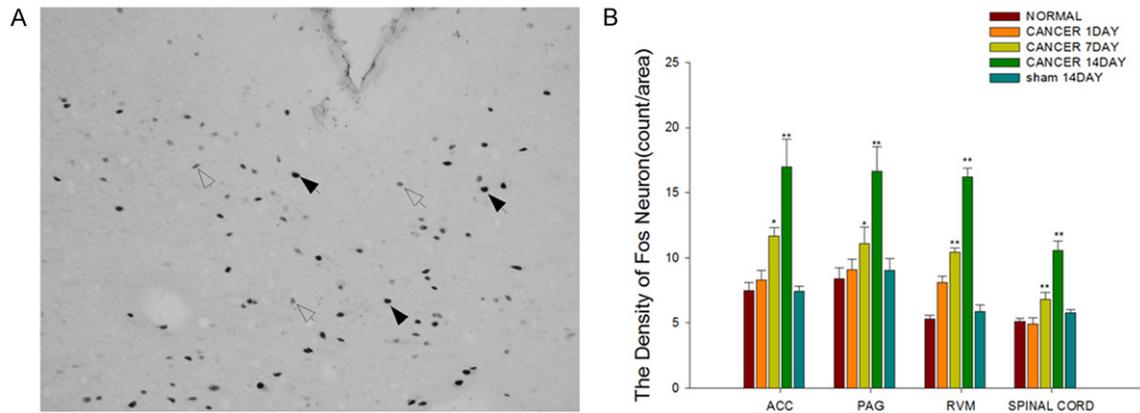
### Counting of Fos positive neurons

The two sections were separated by at least 0.6 mm. Images of the spinal, RVM, PAG and ACC were captured on a computer from a CCD color video camera (MTV-7480ND, Mintron, Taiwan) attached to a microscope (Axioskop, ZEISS, Germany) by a researcher observer 'blind' to the experimental conditions, and then all microphotographs were numbered at random. Counts of Fos protein immunoreactive neurons (Fos-IRs) were performed by another researcher using Scion Image computer software (Scion Corporation, Frederick, MA). Because the diameters of the Fos immunoreactive nuclei were generally in range of 5-15  $\mu\text{m}$ , the appropriate sizes were inputted into the software with the scale beforehand. After selection of the territories (lamina I/II and III/IV) of the spinal dorsal horn and the brain nucleus with reference to the atlas of Paxinos and Watson, the area sizes were calculated. Next, threshold levels for detection of Fos-IRs in comparison of background level were decided visually. The numbers of Fos-IRs were evaluated as the numbers of Fos-IRs per area (counts/ $\text{mm}^2$ ) and the results from two sections averaged.

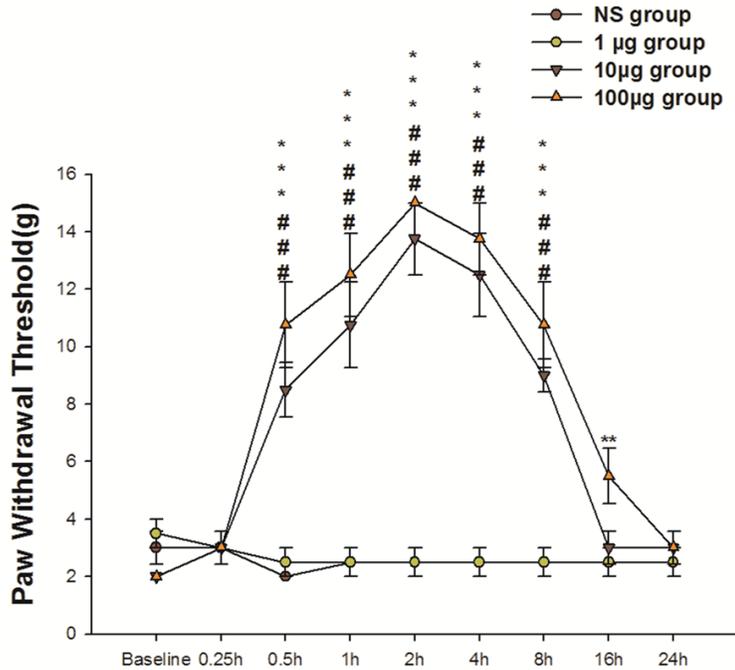
### Statistical analysis

All data were expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) or Student's t-test. The behavioral results between different groups were performed using two-way ANOVA.  $P < 0.05$  was considered statistically significant.

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**Figure 2.** A. Histological staining for indications of positive nuclei. Black solid arrows indicate gradation threshold value of positive cells, referred to as positive cells. Hollow arrows indicate gradation threshold value of negative cells, not to be referred to as positive cells. B. The density of Fos positive neurons in the spinal cord and the brain nucleus. (\* $P < 0.05$  compared to normal group,  $n = 12$ ; \*\* $P < 0.01$  compared to normal group,  $n = 12$ ).



**Figure 3.** Time course of mechanical pain threshold in bone cancer rats after intrathecal injection ondansetron. Paw withdrawal threshold was significantly increased in the 10 µg and 100 µg groups, compared to the NS group (two-way ANOVA, \*\*\* $P < 0.01$ ,  $n = 12$ ; ### $P < 0.01$ ,  $n = 12$ ; \*\* $P < 0.05$ ,  $n = 12$ ).

### Results

#### Activation of descending facilitation related nuclei in bone cancer pain model

Bone cancer pain model rats exhibited significant mechanical hyperalgesia from Day 5 to Day 15 (Figure 1). The calculation of Fos positive neurons showed significant difference on Day 7 and Day 14 but not Day 1 in RVM, PAG,

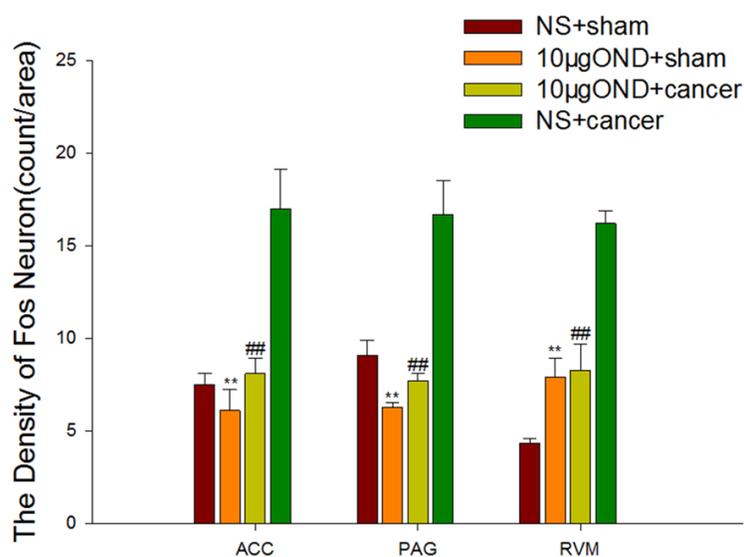
ACC and the spinal cord. The number of Fos positive neurons is significantly higher in modeling group ( $n = 8$ ) compared with sham group ( $n = 8$ ) (Figure 2).

*Intrathecal application of ondansetron can effectively inhibit pain behavior of rats and the activation of the neurons of the descending facilitatory system related nuclei*

On day 14, 1 µg ondansetron application in cancer group induced no significant behavioral change compared with saline treatment. Mechanical pain threshold was significantly increased by 10 µg ondansetron 0.5 h after the application. This effect lasted for 8 h. The pain threshold was also markedly increased by 100 µg ondansetron compared with saline treatment and this effect lasted for 16 h (Figure 3).

On day 14, intrathecal application of 10 µg ondansetron reduced the number of Fos positive neurons in RVM, PAG, ACC in cancer rats compared with saline treatment (Figure 4).

As can be seen from the above results, after bone cancer pain model was established successfully, the number of Fos positive neurons is significantly higher on Day 7 and Day 14 in



**Figure 4.** The density of positive Fos neurons in the brain nucleus. (## $P < 0.05$  compared to NS + sham group,  $n = 12$ ; \*\* $P < 0.01$  compared to NS + sham group,  $n = 12$ ).

RVM, PAG, ACC and the spinal cord of modeling group compared with sham group. Intrathecal administration of ondansetron can significantly inhibit mechanical pain behavior of rats in the period of mechanical hyperalgesia and the Fos activation of the descending facilitatory system related nuclei.

### Discussion

The study concerning descending facilitation of spinal nociceptive transmission was established by Gebhart and Zhuo in the early 90s of last century [10]. Anatomic and pharmacological evidence has shown that descending facilitatory pathway and descending inhibitory pathway are independent, and they are simultaneously activated by acute nociceptive stimuli. However, this balance can be altered. Continuous nociceptive stimuli can induce change in neuronal plasticity in RVM and thus lead to the prolonged facilitation and amplification of pain [11, 12]. Rats with dermorphin-saporin treatment displayed no altered behavioral response to normal nociceptive stimuli. However, these rats showed no sign of neuropathic pain behavior after SNL surgery, which demonstrated that descending facilitatory effect is eliminated by the damage of on cells. Therefore, the continuous transmission of nerve injury is similar to the activation of C-fiber which enhances the descending facilitatory effect of RVM via upreg-

ulation of on cell activity [13, 14].

Descending facilitation was suppressed after Midcollicular decerebration and soma-selective lesions in the brainstem, which demonstrated the critical role of a forebrain loop in the descending facilitatory system [13]. Few studies have been conducted to investigate the facilitatory effect of supra-RVM cortex. Recent research indicated that electrical or chemical stimulation of ACC enhanced the tail flick response of rats. There are direct projection from ACC to PAG, and indirect connections between ACC and RVM. Intra-RVM application of Lidocaine inhibited facilitatory effect of

ACC [15, 16]. Therefore, RVM is critical link in the descending facilitatory system but not the origin of the effect. This present study investigated 2 supra-RVM regions, ACC and PAG.

C-fos is immediate early gene and a molecular marker of pain. Its expression indicates the neuronal activation by nociceptive transmission [7, 17]. Mounting evidence has shown that both neuropathic and inflammatory pain activated descending facilitatory system. Local application of lidocaine in RVM blocked behavioral and electrophysiological responses to pain. RVM lesion by administration of excitotoxic neurotoxin and ibotenic acid can alleviated hyperalgesia and Fos-like reaction [18, 19]. CIBP is an intense chronic pain, which is also possible to activate descending facilitatory system. In our study, after implantation of walker 256 cell, the rats showed a progressively aggravating mechanical hyperalgesia. The Fos expression in descending facilitation related brain regions, including ACC, PAG, RVM, was also progressively upregulated over time. Statistical significance was observed since day 7 and the upregulation reached peak at day 14. Our results suggested that these brain regions were activated by the aggravation of bone cancer pain. This may underlie the supra-spinal involvement in the maintenance of bone cancer pain status.

## 5-HT3 receptor involved in bone cancer pain

Many studies have shown that 5HT is a major transmitter in the descending facilitation. Stimulation of RVM resulted in the facilitation of nociceptive transmission through the release of 5HT in dorsal column. This effect is mediated by 5-HT3 receptors [19, 20]. 5-HT3 receptors are abundant in the terminals of small diameter neurons, mostly in the superficial laminae of the spinal dorsal horn. Electrophysiological and behavioral data indicated that this type of receptor enhances nociceptive transmission probably by increasing the transmitter release in the nerve terminal [21, 22]. However, it is also reported that this receptor has anti-nociception effects. Spinal application of 5-HT3 receptor selective antagonist ondansetron inhibited the reaction of spinal WDR neurons as well as mechanical allodynia in inflammatory pain, neuropathic pain and cancer pain models [23-25]. In our study, intrathecal ondansetron alleviated mechanical pain behavior, indicating the activation of descending facilitatory system in the maintenance of bone cancer pain.

Ondansetron is used clinically as an antiemetic. However, some clinical researches shed light on its anti-nociceptive effect [26, 27]. In this study, our results indicated that it may antagonize nociceptive transmission by inhibition of the descending facilitatory system. This mechanism is distinct from that of most analgesics. In addition, ondansetron has milder side effects compared with opioid analgesics, anticonvulsant and anti-depressants. Therefore, our study suggests that ondansetron may have a promising supportive action in the management of pain, especially in the post-surgery Patient-controlled epidural analgesia (PCEA).

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Wei-Feng Yu and Zhi-Jie Lu, Department of Anesthesia and Intensive Care, Eastern Hepatobiliary Surgery Hospital, The Second Military Medical University, 225 Changhai Road, Shanghai 200438, China. Tel: +862181875231; Fax: +862181875231; E-mail: ywf808@sohu.com (WFY); lzjwxyz@163.com (ZJL)

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