

Evidence of the Involvement of Spinal EZH2 in the Development of Bone Cancer Pain in Rats

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Introduction: Bone cancer pain (BCP) seriously affects the quality of life of patients with advanced cancer, but effective treatment methods are lacking. This study mainly investigates the role of EZH2 in a well-established BCP model induced by Walker 256 breast cancer cells in rats.

Methods: Female Sprague–Dawley rats of the same age weighing approximately 160 g were selected for the experiment. The BCP model was established by injecting inactivated Walker 256 breast cancer cells into the tibia. von Frey filaments were used to measure the paw withdrawal threshold, and bone destruction in the rat was observed using x-ray. The spinal EZH2 and H3K27Tm levels were measured using Western blotting and RT–qPCR analysis. Intrathecal injection of an EZH2 inhibitor was performed to examine the role of EZH2 in trigeminal BCP.

Results: Experimental results showed that injecting Walker 256 breast cancer cells into the tibia induced bone cancer pain. Spinal EZH2 and H3K27Tm levels were significantly increased over time in BCP rats. An intrathecal injection of 3-deazaneplanocin A (DZNep), a selective EZH2 inhibitor, downregulated the expression of EZH2 and attenuate the BCP-induced mechanical allodynia state.

Conclusion: Intrathecal injection of DZNep relieve bone cancer pain in rats. EZH2 expressed in spinal cord tissue may be involved in the process of bone cancer pain in rats.

Keywords: enhancer of zeste homologue 2, bone cancer pain, histone methyltransferase, 3 deazaneplanocin A, intrathecal injection

Introduction

Cancer-related pain is the main factor contributing to a poor quality of life of patients. Based on related studies, bone is the most vulnerable tissue to cancer metastasis and readily experiences pain.^{1,2} In addition, bone cancer pain (BCP) is the most severe and uncontrollable pain compared with other types of cancer pain. Currently, the molecular mechanism of BCP remains unclear, and suitable therapeutic drugs are lacking. Common clinical therapeutic drugs for BCP cannot be used in large quantities or long-term due to serious adverse reactions and addiction.³ Therefore, a better understanding of the molecular mechanism of BCP is necessary.

Zeste gene enhancer homologue 2 (EZH2) is human homologue 2 of the *Drosophila* zeste gene enhancer.⁴ The structure of EZH2 is complex and mainly includes the following four highly conserved domains: I, II and cysteine regions and the SET domain composed of the Su3-9, enhancer of zeste and trithorax.⁵ EZH2 is an important member of the polycomb-group (PcG) protein family.⁶ The PcG family

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includes two complexes, PRC1 and PRC2, which maintain gene suppression and gene silencing, respectively.⁷ PRC2 mainly methylates lysines 9 and 27 of histone 3 through the SET domain of EZH2, thereby silencing a series of downstream target genes, including genes involved in cell differentiation, pain and a series of related genes. Therefore, EZH2 is one of the three subunits comprising PRC2.

Based on accumulating evidence, levels of EZH2 and trimethylation of lysine 27 in histone 3 (H3K27 trimethylation, H3K27Tm) are increased in the spinal dorsal horn of rats with neuropathic pain. Intrathecal injection of DZNep, an inhibitor of EZH2, attenuates the development of neuropathic pain.⁸ These findings indicated that EZH2 may be involved in neuropathic pain. However, BCP is not completely equivalent to neuropathic pain. BCP includes some common characteristics of inflammatory pain and neuropathic pain, as well as its own characteristics.⁹ At present, few studies have assessed whether EZH2 is involved in the process of BCP. In this study, we verified the important role of EZH2 in the spinal cord of BCP rats and provided a possible theoretical basis for the development of new drugs to treat BCP.

Materials and Methods

Animals

Eighty-eight female Sprague–Dawley rats weighing 160 to 180 g were obtained from the Zhao Yan Limited Research Center in Suzhou, China. Before starting the experiment, all animals were acclimated to the environment for 3 days, housed at a temperature of approximately 21 ± 3 °C, and maintained on a photoperiod of 07:00–19:00, with food freely available. The animal experiments were approved by the Institutional Animal Care and Use Committee of Jiangsu University (DJS-LAER-AP-2018030635) and performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 80–23, revised 1996).

Bone Cancer Pain Model

Walker 256 breast cancer cells were kindly provided by Soochow University, and the use of the cell line was approved by the Institutional Animal Care and Use Committee of Jiangsu University and the Ethics Committee of Kunshan First People's Hospital. Rats were anaesthetized with an intraperitoneal injection of 5% chloral hydrate (400 mg/kg). The skin was cut longitudinally at the middle and lower 1/3 of the left tibia to

expose the tibia, and a 25G sterile needle attached to a syringe was used to drill a hole perpendicular to the tibia.¹⁰ The bone marrow cavity of rats in the BCP group was injected with Walker 256 cells (approximately 1×10^5 cells, 10 μ L), and rats in the sham group were injected with heat-killed Walker 256 cells. Cells were injected slowly, the tibial canal was sealed with bone wax, and the wound was sealed with tissue glue to prevent leakage of cells outside the bone.¹¹ Rats with obvious swelling and wound redness were excluded.

Intrathecal Catheterization

Rats were anaesthetized with an intraperitoneal injection of 5% chloral hydrate (400 mg/kg). A longitudinal incision approximately 1 cm in length was made at L4–5 of the spine. After puncturing the ligamentum flavum and dura mater with a 25G needle attached to a syringe, clear cerebrospinal fluid flowed out. The PE capillary catheter (1.5 cm) was inserted through the ruptured spinal membrane and then fixed at the back of the neck.¹² Finally, the incision was sutured carefully. After an intrathecal injection of 10 μ L of 1% lidocaine, paralysis of the lower limbs on both sides of the rat confirmed the successful catheterization. Rats with nerve injury, paralysis, and poor wound healing were excluded. Usually, the intrathecal dose was 10 μ L, followed by 10 μ L of saline to flush the tube.¹³

Pain Behaviour Analysis

We measured the ambulatory pain score (AS) and paw withdrawal threshold (PWT) of eight rats in each group to assess changes in behaviour before and after inoculation. Briefly, on the day of the measurement, the rats were placed in a transparent Plexiglas box (15×15×30 cm), and the box was placed on a metal mesh frame. The rats could walk freely in the box. After 30 minutes of acclimation, the score was recorded based on the degree of lameness observed during walking. 0 point: rats can move freely and walk normally, 1 point: rats walking speed decreased but could still walk normally, 2 points: obvious discomfort with a degree ranging from 1–3 points, 3 points: rats were severely restricted in walking and the walking time is significantly reduced, and 4 points: the rat's hind limbs did not touch the ground. Scores were recorded based on these criteria, and finally the AS was calculated from the values of three consecutive tests separated by intervals of 30 s.¹⁴

For the PWT measurement, rats were allowed to acclimate for 30 minutes before the experiment. The calibrated von Frey filaments were applied perpendicularly to the left plantar

posterior of the rat. The PWT was determined by the size of von Frey filaments applied when the rat quickly retracted or raised the leg. The mean PWT was calculated from the values of three consecutive tests separated by intervals of 60 s.¹⁵

X-Ray Detection of Bone

After the administration of 5% chloral hydrate (400 mg/kg) anaesthesia, the rats were placed in the prone position and an X-ray was captured (47 Kv, 0.5 mA, Siemens, Germany).

Real-Time Quantitative PCR and Western Blotting

Rats were anaesthetized with an intraperitoneal injection of 5% chloral hydrate (400 mg/kg). Spinal L4-6 tissues were dissected from rats on ice and stored at -80°C until use. Spinal cDNAs were fully obtained through reverse transcription. The expression of the EZH2 mRNA relative to β -actin was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

For Western blot analysis, 50 μg of protein from the spinal homogenate was obtained, separated on an SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk for at least 1 h and incubated with polyclonal rabbit anti-EZH2 (1:500, Abcam), anti-H3K27Tm (1:500, Abcam) or anti- β -actin (1:2000, Abcam) primary antibodies overnight at 4°C ; an HRP-conjugated goat antirabbit IgG H&L (1:5000, Abcam) secondary antibody was subsequently incubated with the membrane. The resulting bands were analysed using ImageJ software.

Statistical Analysis

All data are presented as the means $\pm$ SEM and were analysed using SPSS 21.0 software. Two-way ANOVA followed by a Bonferroni post hoc analysis was used to analyse the behavioural data. One-way ANOVA was used to analyse qPCR and Western blot data. P values less than 0.05 were considered statistically significant.

Results

Inoculation of Walker 256 Cells into the Tibia Induced Hyperalgesia in Rats

During the 12-day observation period, the rats were in good health. No significant difference in the baseline PWT was observed between rats in the normal group and the sham group. The AS value of rats in the BCP group increased gradually from the normal level of 0 ± 0.0 to 3.38 ± 0.70 on the 12th day (Figure 1A, $F_{23}=81.0$, $N=8$, $P<0.01$), and the PWT value decreased from the normal level of 34.00 ± 4.00 to 6.25 ± 1.56 on the 12th day (Figure 1B, $F_{23}=170.0$, $N=8$, $P<0.01$, S-Table 1).

Bone X-Ray

X-rays of the normal group and sham group showed that the left tibia bone was not damaged, the cortical bone was smooth, and no bone tumour growth occurred. On the 6th day after inoculation in the BCP group, the tibial bone density began to decrease, and the bone of the epiphysis appeared to be slightly damaged. Bone density

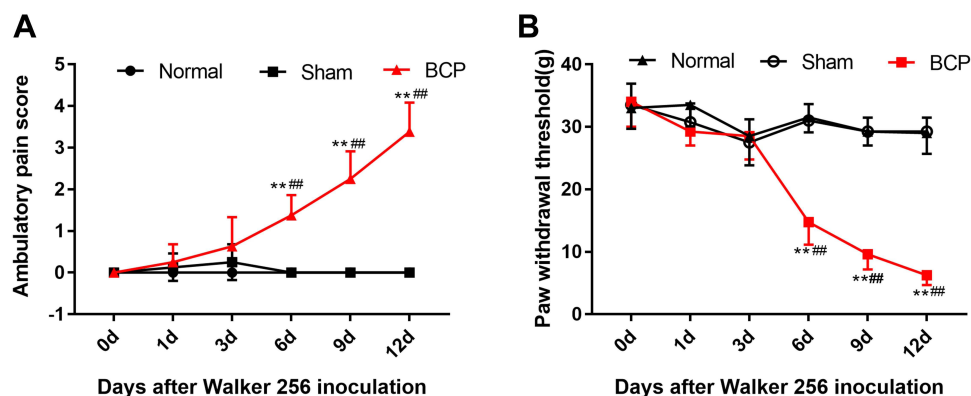


Figure 1 Inoculation of Walker 256 cells into the tibia induced hyperalgesia in rats. During the 12-day observation period, (A) the AS value of the BCP group gradually increased compared with the normal group and sham group. (B) The PWT value gradually decreased compared with the normal group and sham group. Data are presented as the means $\pm$ SEM for 8 rats. Compared with the normal group, $**P<0.01$; compared with the sham group, $###P<0.01$.

Abbreviations: AS, ambulatory pain score; PWT, paw withdrawal threshold; BCP, bone cancer pain.

was further reduced, and the range of bone destruction increased on the 9th day. Finally, the cortical bone morphology began to change on the 12th day (Figure 2).

Increased Spinal EZH2 and H3K27Tm Levels in BCP Rats

Compared with the normal group and the sham group, the qPCR results showed significantly increased expression of the EZH2 mRNA on Days 6, 9, and 12 in BCP rats, and the highest expression level was detected on the 6th day (Figure 3A, $F_{11}=140.3$, $N=4$, $P<0.01$). Western blot results showed significantly higher levels of the EZH2 and H3K27Tm proteins in the BCP group than those in the normal and sham groups, and the highest levels were observed on the 9th day (Figures 3B and C). These results suggest that increased levels of EZH2 and HK27Tm may contribute to BCP in rats.

Intrathecal Injection of the EZH2 Inhibitor DZNep Relieved BCP and Reduced EZH2 Expression

The EZH2 inhibitor DZNep (20 nM, Selleck, USA) was injected intrathecally into BCP rats from Day 3 to Day 5 (once a day for 3 days) after inoculation to further study the role of EZH2 in the spines of BCP rats. The AS and PWT of rats in the BCP+DZNep group improved from Days 6 to 12 after inoculation (Figure 4A and B, $F_{31}=26.97$, $N=8$, $P<0.01$ and 4C, $F_{31}=114.92$, $N=8$, $P<0.01$, S-Table 2). Western blot results showed significantly lower EZH2 expression in the BCP+DZNep group than in the BCP group, but the levels were still higher than those in the sham group (Figure 4C). Thus, an intrathecal injection of DZNep relieves BCP and reduces EZH2 expression in rats.

Discussion

A classic BCP model was established by administering an intratibial injection of Walker 256 breast cancer cells. Previous studies have shown that female rats are more sensitive to pain, and thus behavioural changes are easier to observe. Furthermore, Walker 256 breast cancer cells featuring simple reproduction and strong carcinogenicity better simulate the pathophysiological process of breast cancer bone metastasis.¹⁶ Cancer cells destroy bone and produce inflammatory factors in the bone marrow cavity.¹⁷ These inflammatory factors or neuropeptides bind to the corresponding receptors on neurons and transmit

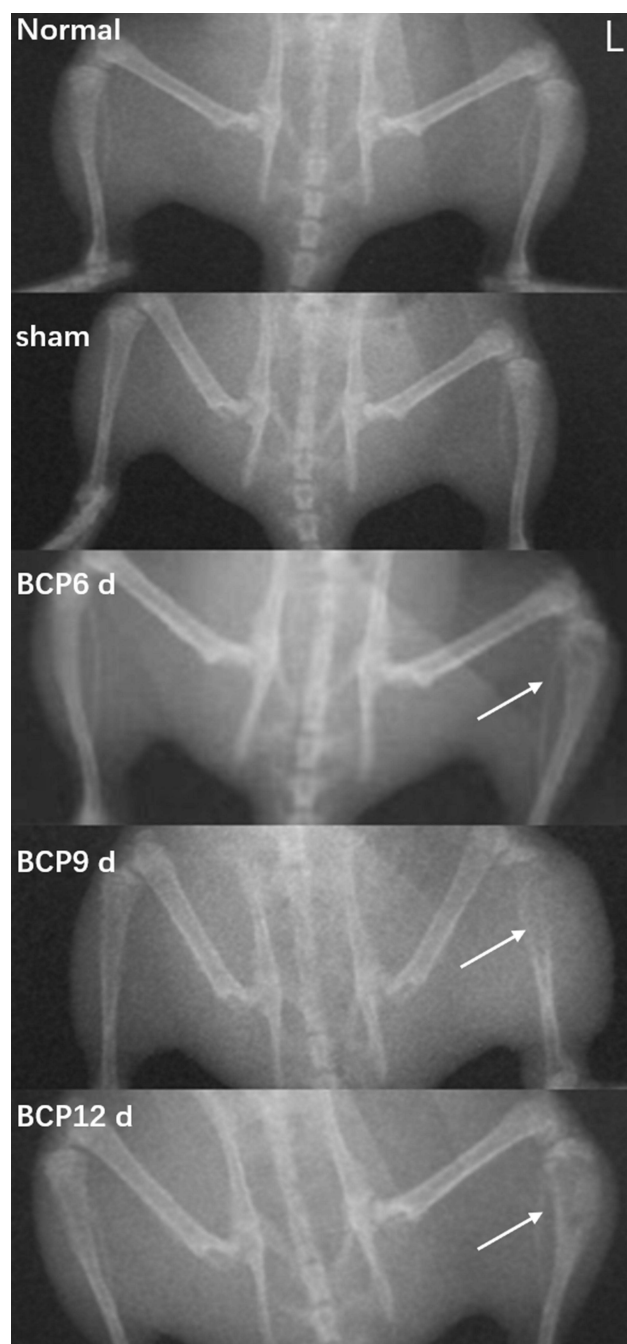


Figure 2 X-rays of the rat tibia. The bones of rats in the normal group and the sham group were not damaged. The tibial bone density began to decrease, and the bone of the epiphysis appeared to be slightly damaged on Day 6 after inoculation. Bone density was further reduced, and the range of bone destruction increased on Day 9 after inoculation. The cortical bone morphology began to change on Day 12 after inoculation. The arrow indicates a significant decrease in bone density and trabecular bone defects in the left tibial metaphysis of BCP rats.

nociceptive signals to the rat spinal lumbar enlargement through axonal reverse transmission, leading to the continuous excitement of spinal neurons.¹⁸ With the input of persistent pain signals, the primary afferent central

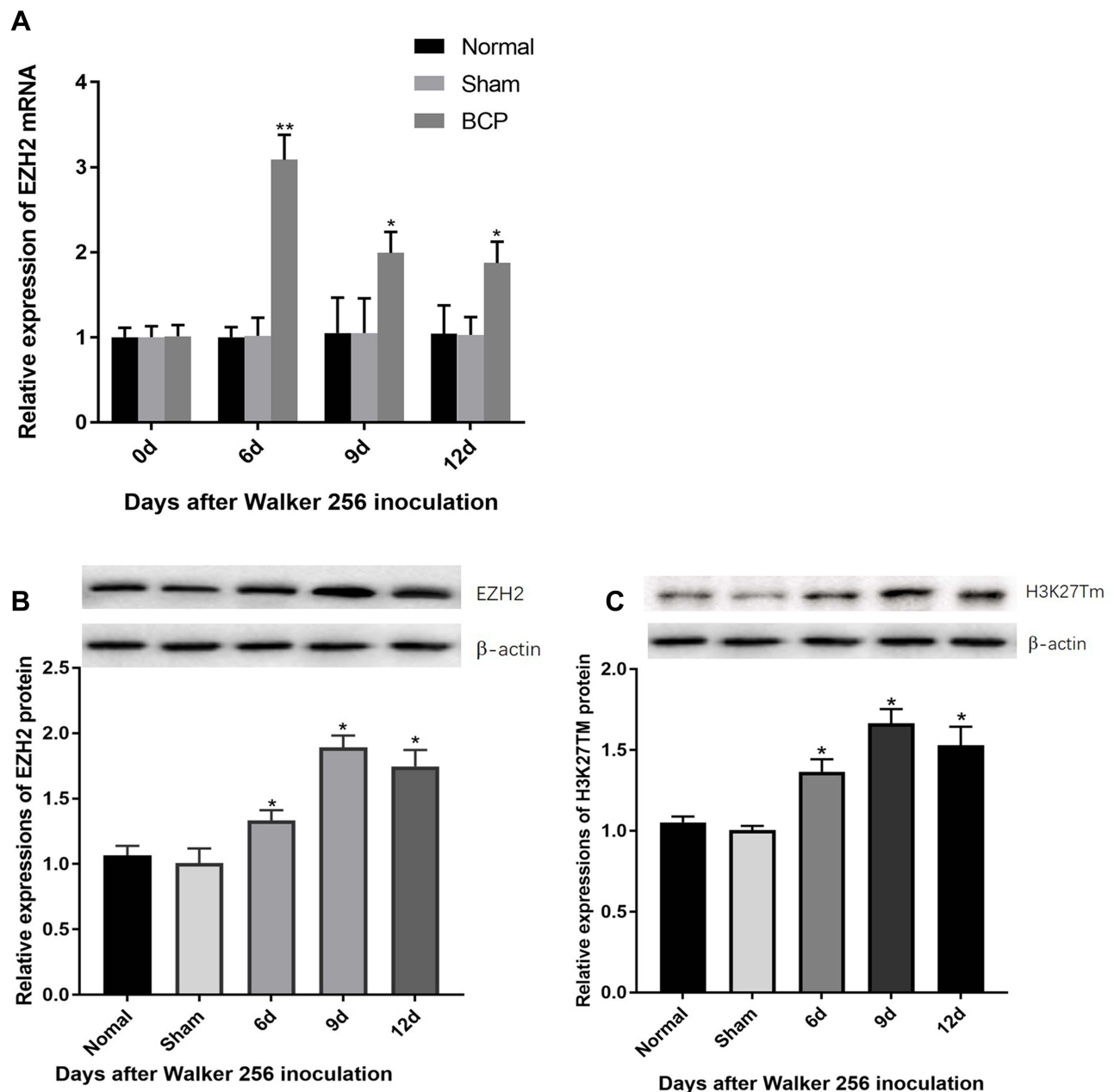


Figure 3 Increased spinal EZH2 and H3K27Tm levels in BCP rats. **(A)** The qPCR results showed significantly upregulated expression of the EZH2 mRNA in the spine of the BCP group. **(B and C)** Western blot results showed significantly higher levels of the EZH2 and H3K27Tm proteins in the BCP group than those in the normal and sham groups. Data are presented as the means $\pm$ SEM for 4 rats. Compared with the normal group, ** $P < 0.01$ and * $P < 0.05$.

Abbreviations: EZH2, enhancer of zeste homologue 2; H3K27Tm, trimethylation of lysine 27 in histone 3; BCP, bone cancer pain.

terminal releases more neurotransmitters (glutamate, CGRP, and BDNF). These neurotransmitters produce a state of overactive and overexcited neurons in the spine called central sensitization that eventually causes persistent bone cancer pain.^{19,20} According to the present work and previous studies, foot withdrawal and limp were observed on the 6th day after inoculation and gradually worsened, peaking on the 12th day. Therefore, we chose

a 12-day observation period and completed the intrathecal injection before the 6th day.

As one of the three subunits constituting PRC2, EZH2 functions mainly by generating a trimethylation mark on H3K27 when PRC2 is recruited to chromatin, preventing RNA polymerase II-dependent transcription elongation and then silencing downstream genes.^{21,22} The levels of EZH2 and H3K27Tm were significantly upregulated in the

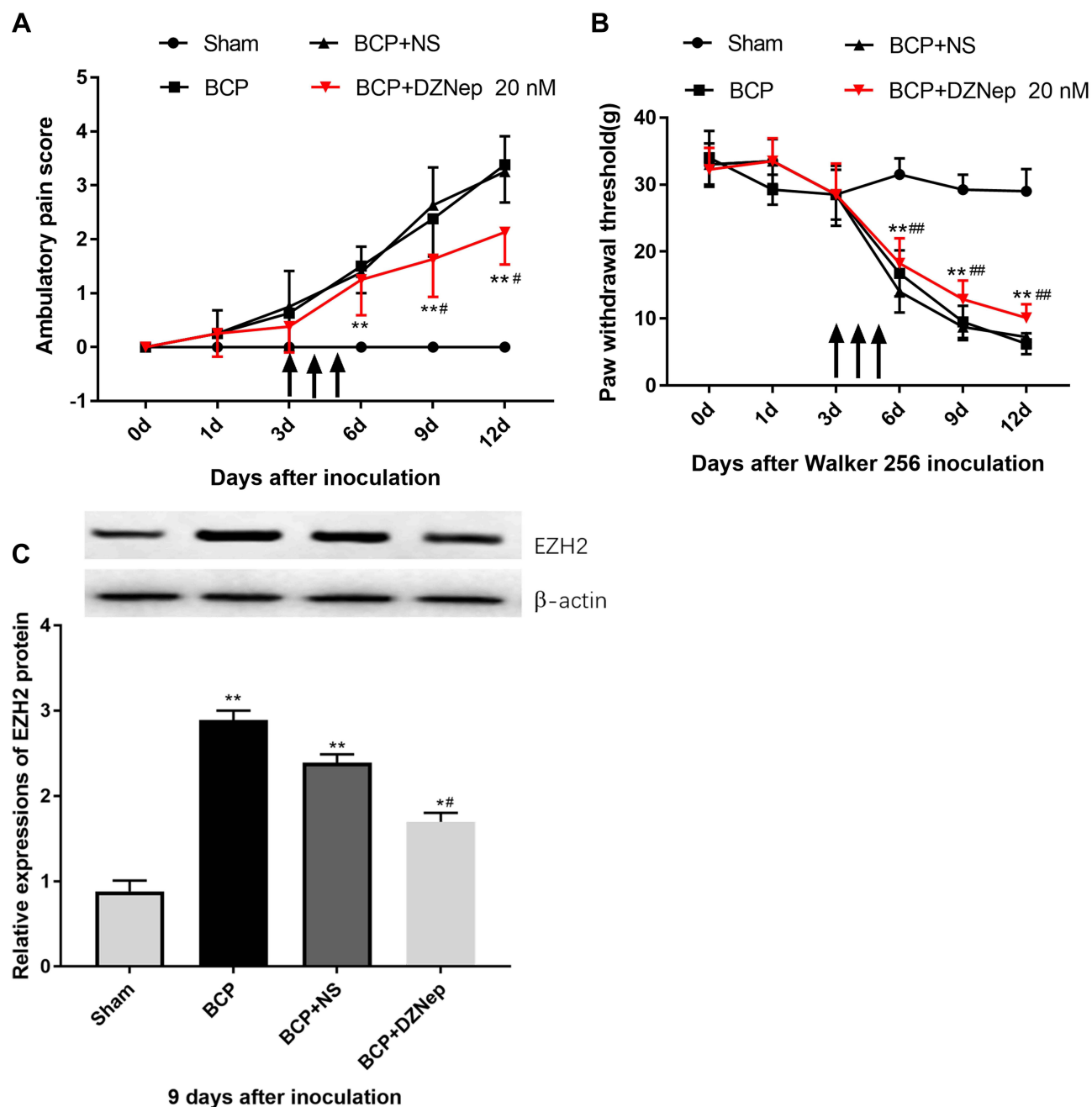


Figure 4 Intrathecal injection of the EZH2 inhibitor DZNep relieves BCP and reduces EZH2 expression. **(A and B)** The AS and PWT of rats in the BCP+DZNep group improved from 6 to 12 days after inoculation. Data are presented as the means $\pm$ SEM for 8 rats, compared with the sham group, ** $P < 0.01$, compared with the BCP group, ### $P < 0.01$ and # $P < 0.05$. The arrow indicates the intrathecal injection. **(C)** Western blot results showed lower EZH2 expression in the BCP+DZNep group than in the BCP group, but the levels were still higher than those in the sham group. Data are presented as the means $\pm$ SEM for 4 rats, compared with the sham group, ** $P < 0.01$ and * $P < 0.05$, compared with the BCP group, # $P < 0.05$.

Abbreviations: BCP, bone cancer pain; DZNep, 3-deazaneplanocin A.

spines of BCP rats on Days 6, 9 and 12, indicating that EZH2 may be involved in the BCP process by promoting the methylation modification. However, this experiment still has limitations due to the limited experimental conditions; for example, paraformaldehyde was not used for perfusion when the spinal cord was removed. Therefore,

the results do exclude the interference of changes in the expression of the target gene in tissues such as blood, stroma, and glia. The highest expression of the EZH2 mRNA was observed on Day 6, while the highest protein expression level was detected on Day 9. The difference in mRNA and protein expression may be related to the

mRNA secondary structure. Several studies have confirmed that a greater number of base pairs in the secondary structure of the stem increases the free energy that must be overcome by dissociation, resulting in a slower translation rate.²³ Generally, the translation process is completed within 72 hours.²⁴ As a result, the prolonged translation time may be due to the presence of numerous base pairs in the stem of the EZH2 mRNA. However, the specific mechanism must be investigated in future studies.

Intrathecal administration is the most suitable method to assess spine function.²⁵ It not only directly affects spinal tissue but also has little effect on the whole body.²⁶ DZNep is an inhibitor of S-adenosyl-L homocysteine hydrolase that decreases the expression levels of the EZH2 protein and H3K27me3.²⁷ The results of preliminary studies indicate no statistically significant difference in PWT values of BCP rats intrathecally injected with 5 nM DZNep. The intrathecal administration of 40 nM DZNep increased the autonomous activity of BCP rats (see [S-Table 3](#) and [S-Figure 1](#)). As a result, the application of 20 nM DZNep in this study was reasonable.

Based on our results, an intrathecal injection of DZNep relieved BCP and reduced the levels of EZH2 expression in BCP rats. Similarly, in a rat chronic constriction injury (CCI) model, intrathecal administration of CGRP8-37 (a CGRP antagonist) and GSK126 (an inhibitor of EZH2) attenuated pain hypersensitivity and decreased the levels of EZH2 and H3K27me3 in the spinal dorsal horn.²⁸ Yadav et al⁸ found that an intrathecal injection of DZNep prevents the development of neuropathic pain, and its analgesic effects are associated with suppression of glial activation and the production of TNF- α , IL-1 β , and MCP-1. Zhang et al²⁹ reported that EZH2 overexpression abrogates the effects of miR-124-3p on neuroinflammation and neuropathic pain. According to these studies, a potential mechanism of EZH2 in neuropathic pain may be to suppress neuroinflammatory responses. However, BCP includes some common characteristics of inflammatory pain and neuropathic pain. Therefore, one of the underlying mechanisms of EZH2 in BCP may be to suppress neuroinflammation. However, the exact mechanisms must be clarified in models of this condition.

Conclusion

Taken together, the current study suggests that spinal EZH2 may be involved in the process of bone cancer pain in rats.

Abbreviations

AS, ambulatory score; BCP, bone cancer pain; DZNep, 3-deazaneplanocin A; EZH2, enhancer of zeste homolog 2; H3K27Tm, trimethylation of 27th lysin of histone H3; PcG, polycomb-group proteins; PRC, polycomb repressive complex; PVDF, polyvinylidene fluoride; PWTpaw withdrawal threshold; RT-qPCR, real-time quantitative PCR; WB, Western blot.

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Disclosure

All authors have no conflicts of interest.

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