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Effect of bushen tongluo decoction on the ERK/c-Fos signal transduction pathway in rats with bone cancer pain

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Abstract: Bone cancer pain could lead to pain sensitization. Traditional Chinese Medicine could relieve bone cancer pain (BCP). This study aimed to investigate the analgesic effect of Bushen Tongluo decoction on rats with BCP and its impact on ERK/c-fos pathway in spinal dorsal horn. Cancer cells were injected to induce bone cancer pain rats. Inflammatory factors in serum were determined using enzyme-linked immunosorbent. ERK/c-Fos in the spinal dorsal horn were detected using western blotting and RT-qPCR.Thermal hyperalgesia and mechanical allodynia were observed in BCP rats. The ERK/c-Fos pathway activation was observed in the spinal dorsal horn and the expression of inflammatory cytokines increased in the serum. Bushen Tongluo decoction alleviated inflammatory cytokines and reduced the ERK/c-Fos pathway. We provided evidence that Bushen Tongluo decoction exhibits a potential and beneficial effect on inflammatory cytokines, effectively alleviating allodynia and hyperalgesia in rats with bone cancer. This effect may be attributed to down-regulation of the ERK/c-Fos pathway in spinal dorsal horn and serum inflammatory cytokines.

Keywords: Bone cancer pain, pain sensitization, traditional Chinese medicine, central sensitization, ERK/c-fos pathway.

INTRODUCTION

Bone cancer pain (BCP) is a persistent and intractable condition caused by tumor invasion of bone, soft tissue and surrounding areas, which leads to significant challenges in pain relief and adversely affecting the mental well-being of patients (Takei *et al*., 2023). A prominent feature of this situation is pain sensitization. Its characteristics include increased pain sensitivity and increased pain response. The three-step analgesic therapy recommended by the World Health Organization exhibits a high incidence of drug resistance, ranging from 37% to 57% and prolonged use of opioids can exacerbate pain sensitization (Fallon *et al*., 2022).

The complex nature of bone cancer pain involves diverse interactions among tumor cells, osteoclasts, inflammatorcells and activated bone neurons. These interactions occur at multiple levels, including local tissues, nerves, spinal cord and the brain (Zajaczkowska *et al*., 2019). Studies have shown that persistent activation of osteoclasts can induce BCP (Andriessen *et al*., 2021, De Clauser *et al*., 2021). Mature osteoclasts form closed grooves tightly attached to the bone surface and promote bone resorption by releasing a large amount of H^+. The H^+ in these resorption groove can stimulate sensory nerve fiber on the periosteal surface, thereby driving the initiation and progression of cancer-related bone pain. The interaction between peripheral tumors and nerve fiber endings transmits pain signals to the spinal dorsal horn, leading to central sensitization and further exacerbating cancer pain (Romero-Morelos *et al*., 2021). Consequently, the likelihood of central response to low low-threshold injury information increases, resulting in central sensitization (Urch *et al*., 2003).

In recent years, numerous diverse models of cancer pain have contributed to our comprehension of pain associated with cancer. Tumor and related cells in the tumor microenvironment release numerous peripheral mediators, such as inflammatory factors, tumor necrosis factor, endothelin and nerve growth factor. These mediators activate sensitize peripheral and central neurons, leading to the clinical manifestations of cancer pain (Mazaki *et al*., 2022, Han *et al*., 2018, Zhang *et al*., 2023). Scientific investigations have revealed that the prevention of central sensitization induced by pain is achievable through the antagonism of inflammatory cells using tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), as well as interleukin-6 (IL-6) (Liu *et al*., 2019). The activation of neurons in the dorsal horn of the spinal cord plays a crucial role in the process of pain sensitization. As it increases the sensitivity of these neurons, resulting in persistent pain. Inhibiting relevant pain pathways in spinal dorsal horn neurons can alleviate hypersensitivity in cancer-related bone pain (Yuan *et al*., 2022). The activation of ERK (extracellular signal-regulated Kinase) in postsynaptic neurons, triggered by continuous pain input, play a role in

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pain sensitization (Kondo *et al*., 2020). The expression of c-Fos, a transcription factor, rapidly occurs in central neurons following traumatic stimulation, often serving as an indicator neuronal excitability. The initiation of pain sensitization is ultimately caused by the activation of the ERK/c-Fos pathway occurring in neurons located in the spinal dorsal horn (Ruan *et al*., 2019).

Bushen Tongluo decoction is composed of six traditional Chinese medicines, namely, Cortex eucommiae, Rhizoma drynariae, Scorpio, Aspongopus, Radix curcumae, Rhodiola sacra. Our research group has been actively involved in the diagnosis and treatment of cancer pain, employing an integrated approach that combine traditional Chinese and Western medicine. Through clinical practice, we observed that the use of Bushen Tongluo decoction can reduce dosage of opioids, prolong the interval between opioids administration and delay the need for escalated medication. The objective of this research aimed to examine the impact of BCP by implementing Bushen Tongluo concoction, evaluate its effect on ERK/c-Fos signaling pathway *and* explore its mechanism in alleviating BCP.

MATERIALS AND METHODS

Preparation of Bushen Tongluo decoction solution

Bushen Tongluo decoction (Jiangyin Jiangtian Pharmaceutical Co., Ltd., Suzhou, China) is prepared from Suzhou Hospital of Integrated Traditional Chinese and Western Medicine. The main ingredients are Cortex eucommiae (10g), Rhizoma drynariae (10g), Scorpio (6g), Aspongopus (6g), Radix curcumae (10g) *and* Rhodiola sacra (10g) in total of 6 Chinese medicine ingredients. The decoction of each component of Bushen Tongluo decoction (52g in total) was fully dissolved in 250mL of saline and prepared into a suspension of Bushen Tongluo decoction with a concentration of 20.8mg/mL for later use.

Animals

The study utilized Wistar rats (120g-150g, Qinglongshan, Nanjing, China). These rats were individually placed in cages, providing them with controlled conditions for food and water access. The experiments were performed with the animals under isoflurane anesthesia (RWD R510-22, Shenzhen, China).T

Cancer cell preparation

An 80-90g female Wistar rat received an injection into its abdominal cavity with a 1 ml suspension containing Walker 256 cells. After 5-7 days, rats with noticeable ascites were selected. Ascites was then extracted from the abdominal cavity and centrifuged at 1500r/min for 5min to discard the supernatant. The cell concentration was adjusted and cells with a concentration of $1x10^5$ cells/ml was prepared using normal saline and stored in an ice box for later use.

Establishment of experimental bone cancer pain model

Under sevoflurane inhalation anesthesia, the rats' right hind limbs were sheared, supine on the operating table. The skin of the right tibia was disinfected and a one cm incision was made to expose the surface of the upper tibia carefully, ensuring no damage to the surrounding blood vessels and nerves. A drilling point was selected 0.2 cm horizontally parallel to the medial condyle of the tibia. The syringe needle was drilled vertically into the bone surface until a sense of resistance was felt *and* then the syringe plunger was inserted obliquely at a 30 angle to the bone cavity surface, creating a fistula. Using a 10 ml microsyringe, 5 ml saline cell suspension containing cancer cells was slowly injected into the bone marrow cavity.

The contents of the syringe should be injected slowly into the tibial cavity. Keep the needle in place for 2 minutes to enhance cell diffusion. Subsequently, extract the needle while concurrently sealing the crevice using bone wax. For the purpose of averting the leakage of tumor cells, it is imperative to seal the bone surface using dental bone cement at the site of injection. After the injection, the wound was cleaned with a sterile alcohol cotton ball *and* the wound was sutured. In the case of the Sham group, after drilling, an equivalent amount of sterile saline was introduced via injection.

Administration of bBushen tongluo decoction

Wistar rats were randomly divided into six groups: Sham group (injection of physiological saline only), BCP group (injection of 5ml saline cell suspension only), High dose group (H-D) (inject 5 ml prepared cell mixture to establish the model and then gavage 12.5g/kg of Bushen Tongluo decoction), Medium dose group (M-D) (inject 5ml prepared cell mixture to establish the model and then gavage 6.25 g/kg of Bushen Tongluo decoction), Low dose group (L-D) (inject 5ml prepared cell mixture to establish the model and then gavage 3.125g/kg of Bushen Tongluo decoction) and Tramadol group (inject 5 ml prepared cell mixture to establish the model and then gavage 10mg/kg of tramadol decoction). Bushen Tongluo decoction were given to rats intragastrically once daily for 14 consecutive days after the experimental BCP model is successfully established.

Behavioral assays

Behavioral evaluation of mechanical allodynia

In this study, rats were tested before, during, and after day zero, seven, fourteen, and twenty-eight for their mechanical pain thresholds. The individual rats were placed in a glass cage with a metal mesh floor beneath it. Vertically, the Von Frey fibers were applied to the hind paw sole of the rat until withdrawal of the paw occurred. Each stimulus lasted approximately 1-2 s, with at least 5 minutes between the two

stimuli. The paw withdrawal threshold (PWT) was recorded and the average value of 4 consecutive tests was calculated.

Behavioral evaluation of thermal hyperalgesia

The thermistor threshold of rats was measured before and on day 0, 7, 14, 21 and 28. The hot plate was set at temperature of 55±0.2º. The rat was placed on the hot plate instrument *and* adapt for a half hour. When the rat retracted its paw, the time button was pressed. The time indicates the paw retraction latency (PWL) for thermal stimulation. The PWL was recorded and the mean value was calculated from three measurements, with a minimum 5-minute interval between each measurement.

Bone histology analysis

Rats from each group were anesthetized by sevoflurane inhalation *and* the right tibia was removed and fixed in 4% PFA for 48 hours. For 3-4 weeks. 10% EDTA was used to decalcify. The bones were prepared for analysis by rinsing, dehydrating, embedding in paraffin, and sectioning into 10mm slices using a rotary wheel microtome. The paraffin sections underwent dehydration, staining with hematoxylin and eosin, transparent seal dehydration, and subsequent scanning using a scanner (OLYMPUS VS200, Japan).

X-ray

On the 7th, 14th, 21st and 28th days after modeling, the rats in each group were anesthetized by sevoflurane inhalation *and* X-ray images were taken to observe the bone condition.

ELISA assay

Serum levels of IL-6, TNF-á and IL-1â were determined using enzyme-linked immunosorbent (ELISA) assay (EK382, EK306, EK301B MULTISCIENCES, China). Blood samples were collected from each rat on day 28 and inflammatory mediator levels were measured according the manufacturer instructions.

RNA extraction and quantitative real-time PCR analysis (RT-qPCR)

Total RNA was extracted from L4 and L5 spinal dorsal horn using TRIzol reagent. Reverse transcription was performed on 1ìg of RNA using HiScript III All-in-one RT SuperMix Perfect for qPCR (R333-01 Vazyme, China). A total of 20µl of final volume was used for PCR, PCR was performed in triplicate *and* the reaction mixture contained 10µl of SYBR RT-qPCR Master Mix (Medicalbio, MR0321), 1µl of diluted cDNA products, 0.4 µM of each paired primers and 8.2µl of deionized water. According to the manufacturer's instructions, ERK and c-Fos expression levels were normalized to GAPDH and quantified. The primer sequences are provided in table 1.

Western blot analysis

The rats were sacrificed and the lumbar L4-L5 spinal dorsal horns were harvested and pooled. The harvested tissues were lysed and the supernatant was centrifuged at 4 ℃ for 15 minutes at 12,000 r/min. The total protein concentration was determined. On 10% polyacrylamide gels, equal amounts of proteins were separated. To transfer liquid onto a PVDF membrane, a dilute tris glycine 1 x buffer and 20% methanol were used for 80 minutes at 200 mA. PVDF membranes were blocked with 5% BSA mixed with Tris-buffered saline for one hour. The primary antibodies, including phosphorylated ERK1/2 (ZENBIO 301245, 1:750); total ERK1/2 (ZENBIO 343830, 1:750); GAPDH (MedicalbioAB03231,1:7500), c-Fos (ZENBI340249, 1:750); were diluted in TBST containing 5% BSA and incubated with the membranes overnight at 4□. The membranes should be washed four times with TBST for five minutes each time, HRP -conjugated secondary antibodies (ZENBIO 511203, 1:7500) were incubated with the membranes at room temperature for one hour. Finally, the membrane was imaged using a Tanon scan imager after visualization with an ECL reagent (Medicalbio, PT01001, China). Protein expression was then quantified using GAPDH as a standard for normalization.

Ethical approval

A detailed guide for the care and management of laboratory animals was used to carry out the experiments, and an ethical approval was obtained through Reference No.2022013 by the Ethics Committee of Suzhou Hospital of Integrated Chinese and Western Medicine.

STATISTICAL ANALYSIS

Unless otherwise specified, all data are expressed as mean±SD from at least three independent experiments. For statistical analysis, GraphPad Prism 8 software (USA) was used.In order to analyze the difference between the two groups, Student's t-test was used. When p value<0.05, the difference was considered statistically significant.

RESULTS

X-ray change

On the 7th and 14th day after modeling, X-ray scans were conducted for the rats' right hind limbs. The tibia imaging on the 7th day showed no notable alterations. However, on the 14th day, we observed an increase in tibial light transmittance, along with 2-3 defects in the cortex, indicating cortical destruction and successful modeling. Subsequent imaging on the 21st and 28th day revealed further bone tissues destruction, with severe cortical damage and erosion of bone trabeculae. Tumor cells observed protruding from the bone cortex and invading surrounding tissues.

Table1: The primer sequences used in RT-qPCR

Gene	primer	primer sequence [5'-3']
GAPDH	Forward	GACATGCCGCCTGGAGAAAC
	Reverse	AGCCCAGGATGCCCTTTAGT
ERK	Forward	AGACCATGTCAGGCGGCAGAG
	Reverse	GTCAGCTCCCTCCTCCGATTCC
c-Fos	Forward	AACTCGCCTGGTCTTGCTTTCTG
	Reverse	GCTTTCGTGGTGCTGAGGTACTG

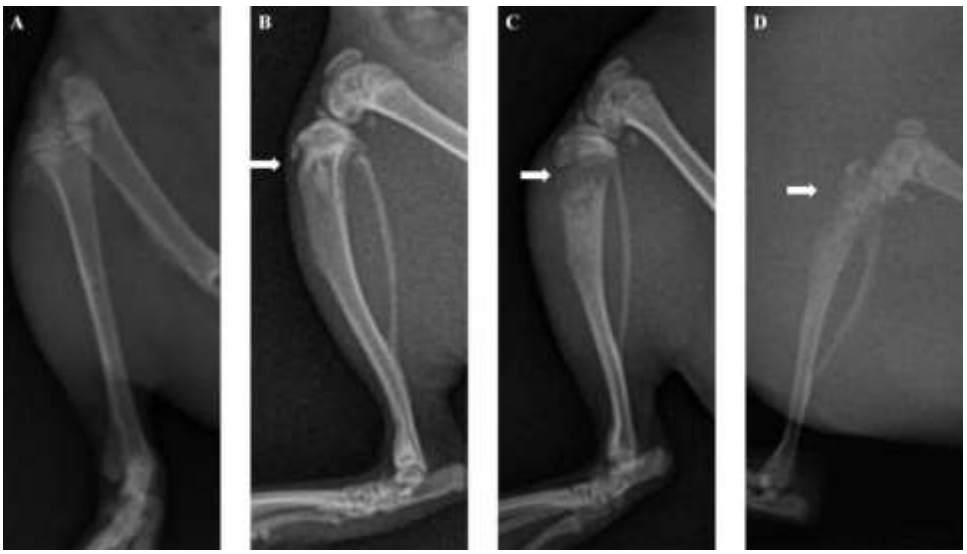

Fig. 1: (A) Represents the 7th day after modeling, showing intact Cortical integrity and uniform bone density. (B) Represents the 14th day after modeling, showing mild cortical damage. (C) Represents the 21th day after modeling, indicating incomplete cortex. (D) Represents the 28th day after modeling, displaying severe damaged cortex, bone invasion by tumors, and visible Swelling of the surrounding tissue. The white arrow indicates the structural destruction in rats with BCP.

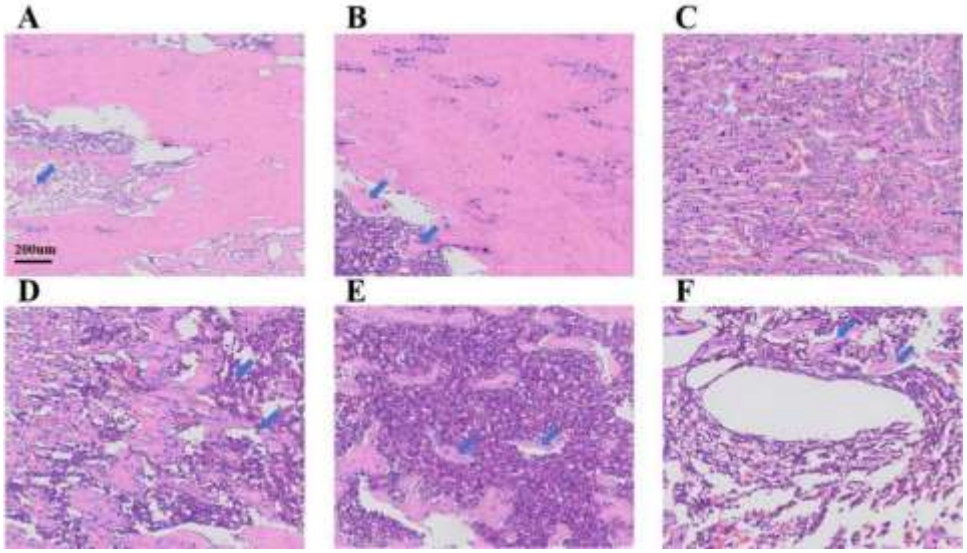

Fig. 2: H&E staining pathological pictures of tibia of rats in each group H&E staining staining of tibia from Tramadol group. (B) H&E staining of tibia from BCP group. (C) H&E staining of tibia from Sham group. (D) H&E staining of tibia from H-D group. (E) H&E staining of tibia from M-D group. (F) H&E staining of tibia from L-D group. The blue arrow indicates a discontinuous trabecular structure. Scale bars 200 μm.

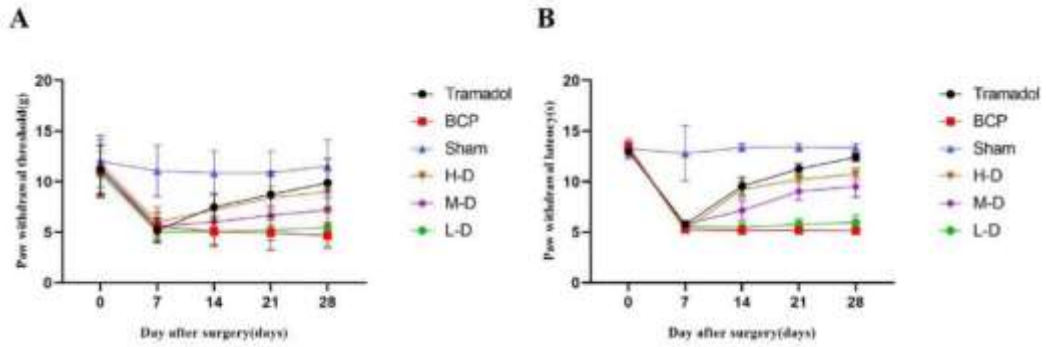

Fig. 3: (A) Paw withdrawal mechanical threshold (PWT) and (B) paw withdrawal thermal latency (PWL) for pain behavior test in sham, BCP, L-D, M-D, H-D and Tramadol group. H-D group was compared with BCP group, $P<0.01$.

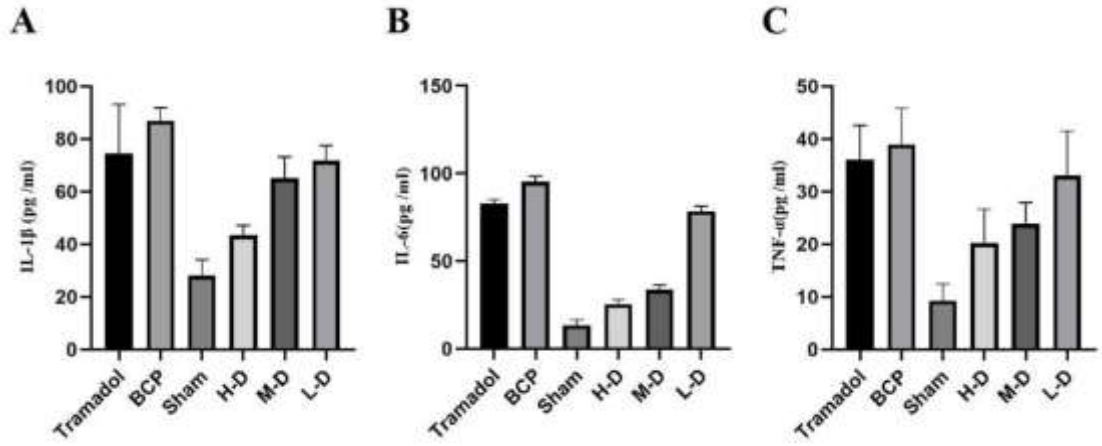

Fig. 4: (A) The level of IL-1β was determined by ELISA. (B) The level of IL-6 was determined by ELISA. (C) The level of TNF-α was determined by ELISA. H-D group group were compared with BCP group, $P<0.01$

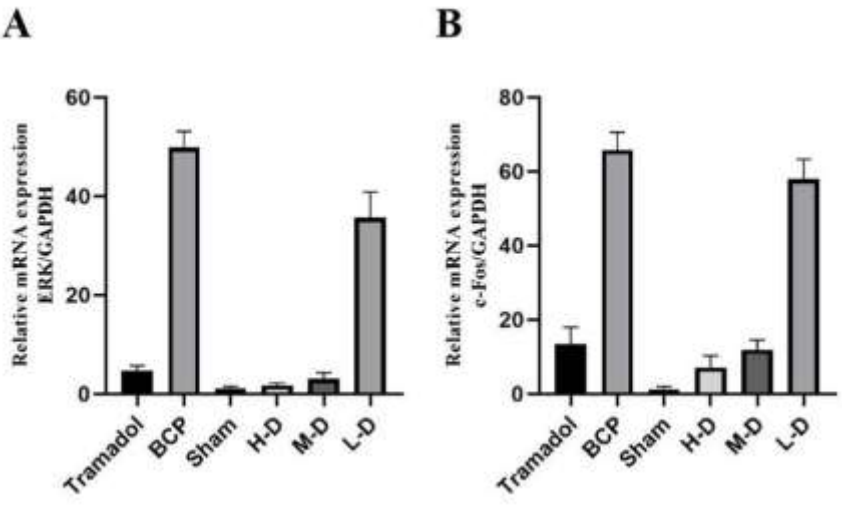

Fig. 5: (A) Represents the ERK expression. (B) Represents c-Fos expression. Tramadol group refers to BCP rat treated with 10 mg/kg/day tramadol for 2 weeks. The BCP group refers to BCP rat treated with placebo (saline). H-D group refers to BCP rat treated with 12.5g/kg/day Bushen Tongluo decoction for 2 weeks. M-D group refers to BCP rats treated with 6.25g/kg/day Bushen Tongluo decoction for 2 weeks. L-D group refers to BCP rats treated with 3.125 g/kg/day Bushen Tongluo decoction for 2 weeks. H-D group group were compared with BCP group, $P<0.01$.

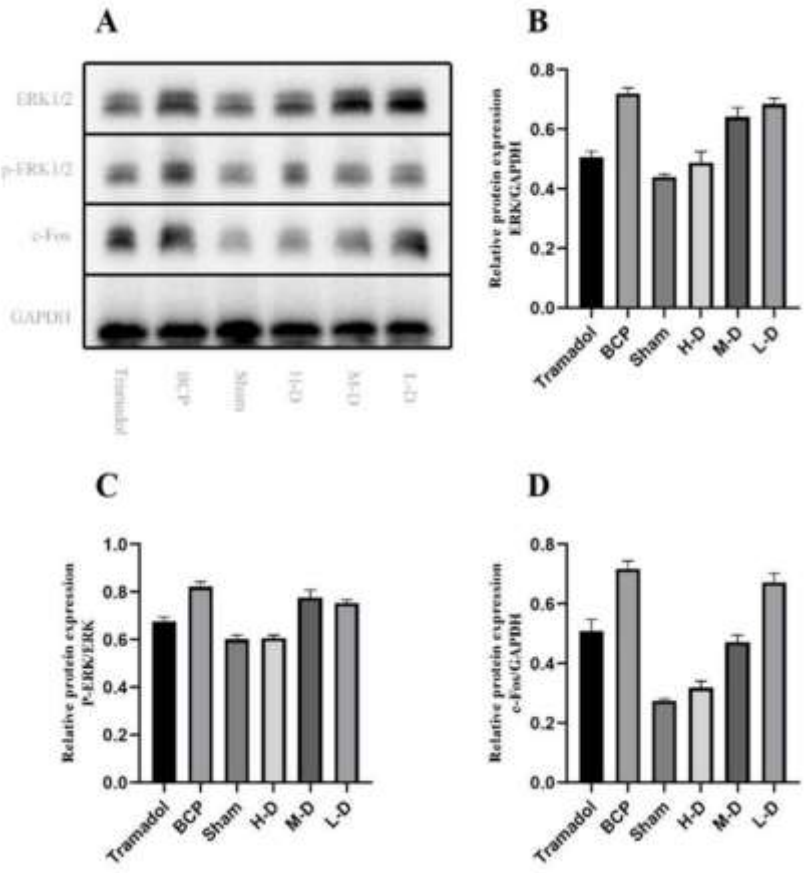

Fig. 6: (A)Represents the protein bands, (B)Represents the ERK expression, (C) Represents the p-ERK expression, (D) Represents the c-Fos expression. Tramadol group refers to BCP rats treated with 10mg/kg/day tramadol for 2 weeks. BCP group refers to BCP rats treated with placebo (saline). The H-D group refers to BCP rats treated with 12.5 g/kg/day Bushen Tongluo decoction for 2 weeks. The M-D group refers to BCP rats treated with 6.25 g/kg/day Bushen Tongluo decoction for 2 weeks. The L-D group refers to BCP rats treated with 3.125 g/kg/day Bushen Tongluo decoction for 2 weeks. H-D group was compared with BCP group, $P< 0.01$.

Pathological evaluation

We further evaluated the disruption of bone in rats after cancer cell injection. Hematoxylin and eosin (H&E) staining of the tibia from each rat revealed intact cortical bone in the sham operation group (fig. 2). Moreover, BCP rats exhibited a severe damage to the trabeculae of the tibia (fig. 2B). On the other hand, the damage to the trabeculae of the tibia was rescued by intragatrically injection of Bushen Tongluo decoction in a dose-dependent manner (fig. 2D-F). These results revealed that Bushen Tongluo decoction could dose-dependently protect bone disruption in rats after cancer cell injection.

Pain responses to mechanical and thermal stimuli

We next investigated the mechanical allodynia and thermal hyperalgesia in rats after cancer cell injection. After 7 days of cancer cell injection, the mechanical threshold (fig. 3A) and paw retraction latency for thermal stimulation response (fig. 3B) were significantly reduced *and* a constant sensitivity was maintained in rats for 28 days. On the other hand, Bushen Tongluo decoction dose-dependently alleviated either mechanical allodynia ($P<0.01$, fig. 3A) and thermal hyperalgesia ($P<0.01$, fig. 3B) in rats after cancer cell injection. Moreover, Tramadol, as well-known analgesics, dramatically elevated either mechanical threshold (fig. 3A) and paw withdrawal latency in response to heat stimulation (fig. 3B) in BCP rats as well. In addition, the results also demonstrated that the analgesic effect of Bushen Tongluo decoction in high dose is comparable with Tramadol.

Change of IL-1∝, IL-6 and TNF-á after Bushen Tongluo decoction Treatment Detected by ELISA

ELISA analysis demonstrated that the expression levels of IL-1β ($P<0.01$, fig. 4A), IL-6 ($P<0.01$, fig. 4B) and TNF-á ($P<0.01$, fig. 4C) in serum of rats in the BCP group were significantly up-regulated compared to the group Sham. After 14 days of continuous intragastric administration of Bushen Tongluo decoction, the expression levels of IL-1β ($P <0.01$, fig. 4A), IL-6 ($P<0.01$, fig. 4B) and TNF-á ($P< 0.01$, fig. 4C) in the serum of rats in H-D group were significantly

decreased compared to those in the BCP group. These data suggested that Bushen Tongluo decoction may also relieve BCP through suppressing the levels of IL-1β, IL-6 and TNF-α.

Change in mRNA Expression of ERK and c-Fos after Bushen Tongluo decoction Treatment Detected by RT-qPCR

RT-qPCR analysis was performed to measure the mRNA expression of ERK and c-Fos in the spinal dorsal horn of BCP rats with Bushen Tongluo decoction treatment. The findings suggested that BCP induced significant upregulation of ERK and c-Fos ($P<0.01$, fig. 5A-B). In the spinal dorsal horn of BCP rats, the treatment with Bushen Tongluo decoction resulted in the down-regulation of mRNA expression for ERK and c-Fos ($P<0.01$, fig. 5A-B).

Change in Protein Expression of ERK, p-ERK and c-Fos after Bushen Tongluo decoction Treatment detected by Western Blotting

To delve deeper into the matter, we conducted an analysis of the protein expression levels of ERK and c-Fos in the dorsal horn of the spinal cord in rats with BCP after being treated with Bushen Tongluo decoction. Our findings unequivocally revealed a notable augmentation in the protein bands pertaining to ERK, p-ERK, and c-Fos as a direct consequence of BCP, whereas Bushen Tongluo decoction treatment significantly suppressed the increases of ERK, p-ERK and c-Fos protein bands (fig. 6A). Statistical analysis results revealed that BCP induced significantly up-regulation of the protein expression of ERK ($P<0.01$, fig. 6B), p-ERK ($P<0.01$, fig. 6C) *and* c-Fos ($P<0.01$, fig. 6D). In addition, Bushen Tongluo decoction treatment significantly suppressed the up-regulation of ERK ($P< 0.01$, fig. 6B), p-ERK ($P<0.01$, fig. 6C) *and* c-Fos ($P< 0.01$, fig. 6D). Taken together, these results suggested that Bushen Tongluo decoction may alleviate BCP through suppression of the spinal neuron activation in BCP rats.

DISCUSSION

BCP is one of the main complications of cancer pain. This is usually caused by bone metastases from lung, prostate and breast cancers (Buehlmann *et al*., 2019). The BCP is mostly moderate to severe pain. Moreever, BCP has a substantial impact on patients' quality of life. Progressive persistent pain, burst pain and touch-induced pain are the main clinical manifestations of BCP. Nowadays, the incidence of BCP has been increasing, whcih makes pain relief more difficult (Yang *et al*., 2023, Lou *et al*., 2022). Consequently, patients diagnosed with BCP experience a significant decline in their overall life quality, ultimately resulting in an increased rate of mortality (Kapoor *et al*., 2021). Long-term and repeated using of morphine can lead to the excitation of spinal dorsal horn neurons, promoting enhanced transmission efficiency between these neurons. This excitation can result in sensitization of nociceptors, leading to the development of drug tolerance (Wang *et al*., 2020).

NMDA (N-methyl-D-aspartate) receptor is a crucial excitatory glutamate receptor found in the central nervous system. Its significance extends to the spinal cord, where it plays a critical role (Leonardon *et al*., 2022). The activation of presynaptic NMDA receptors occurs after prolonged using of opioids. On the other hand, the enhancement of spinal dorsal horn neurons' glutaminergic input results from the activation of NMDA receptors on the presynaptic terminals. Such activation increases the injurious input from the primary afferent nerve to the spinal dorsal horn neurons, which may lead to hyperalgesia and opioid tolerance. Furthermore, the activation of NMDA receptor in morphine-tolerant states is mediated through MAPK related signaling pathways (Deng *et al*., 2019). Among these pathways, ERK1/2 is involved in NMDA receptor activity regulation and mediates pain sensitization due to morphine tolerance (Deng *et al*., 2019, Zhong *et al*., 2020). Studies have shown that morphine tolerance-induced pain sensitization is associated with increased of ERK phosphorylation and c-Fos protein expression in the spinal cord. Inhibiting NMDA receptor function can alleviate pain sensitization induced by morphine tolerance *and* downregulate the expression of ERK and c-Fos protein (Xu *et al*., 2020, Gao *et al*., 2020).

The findings from our study show that the H-D and M-D dose groups of Bushen Tongluo decoction significantly improve the PWT and PWL in rats with BCP. The analgesic effect of Bushen Tongluo decoction in H-D and M-D groups was similar to Tramadol group. In addition, The Bushen Tongluo decoction can suppress the expression of IL□1â, TNF□á and IL□6 in the serum of BCP rats. The occurrence of pain can be improved through inhibiting the expression levels of IL□1â, TNF□á and IL□6 in bone metastasis group (Wang A *et al*., 2021, Sliepen Shj *et al*., 2021). Aligned with our findings in the current investigation, we observed a substantial reduction in inflammatory cytokine levels in the serum of BCP rats following treatment with Bushen Tongluo decoction. These collective findings imply that Bushen Tongluo decoction might possess remarkable analgesic properties in BCP.

CONCLUSION

In summary, Bushen Tongluo decoction exhibits a potential and beneficial effect on BCP rats. On the other hand, Bushen Tongluo decoction can alleviate pain sensitization in BCP rats effectively. This effect may be attributed to the downregulation of IL□1â, TNF□á and IL□6. Moreover, Bushen Tongluo decoctio can downregulate the expression of ERK in the spinal dorsal horn. Therefore, Bushen

Tongluo decoction demonstrates promising analgesic effect in a rat model of BCP.

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