Evaluation of Bone Cancer Pain Induced by Different Doses of Walker 256 Mammary Gland Carcinoma Cells

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Background: Cancer pain is a complex medical syndrome. Understanding its underlying mechanisms relies on the use of animal models which can mimic the human condition. A crucial component of this model is the quantity of tumor cells; however, the exact relationship between the doses of tumor cells on bone cancer pain is yet unknown.

Objective: We explored the relationship of different doses of Walker 256 carcinoma cells using a bone cancer pain model in rats, and evaluated its success and stability.

Study Design: Experimental animal study using a comparative design.

Setting: Experimental Animal Center and Tumor Institute of Traditional Chinese Medicine.

Methods: We constructed the bone cancer pain model by implanting Walker 256 carcinoma cells into the right tibia of Sprague-Dawley (SD) rats (150 – 170 g). Spontaneous pain, mechanical threshold, and paw withdrawal latency (PWL) were measured and x-ray, bone mineral density (BMD), histological, interleukin-1 beta (IL-1β) mRNA, carboxyterminal telopeptide of type I collagen (ICTP), and bone alkaline phosphatase (BAP) were analyzed for bone pain model evaluation.

Results: The results showed that: (1) the 3 doses (3×10^5, 3.5×10^5, 4×10^5) of Walker 256 carcinoma cells can induce bone cancer pain from day 7 to day 21 after implantation into the right tibia of SD rats; (2) compared to the control group, 3×10^5, 3.5×10^5, and 4×10^5 Walker 256 carcinoma cells produced different pain manifestations, where the 3.5×10^5 dose of Walker 256 carcinoma cells resulted in the greatest bone cancer pain response; (3) the 3.5×10^5 dose induced the lowest mortality rate in rats; (4) Walker 256 carcinoma cells (3×10^5, 3.5×10^5, and 4×10^5) resulted in a significant decrease in the general condition and body weight of rats, where the 3.5×10^5 and 4×10^5 doses of carcinoma cells produced a greater effect than 3×10^5 dose of carcinoma cells; (5) progressive spontaneous pain, PWL, and mechanical threshold were exacerbated by 3.5×10^5 and 4×10^5 doses of carcinoma cells; (6) implantation of 3.5×10^5 and 4×10^5 doses of carcinoma cells induced progressive bone destruction and decrease in BMD; (7) ICTP and BAP were significantly increased following the implantation of 3.5×10^5 and 4×10^5 doses of carcinoma cells; (8) IL-1βmRNA was significantly up-regulated in the spinal cord of rats implanted with 3.5×10^5 and 4×10^5 doses of carcinoma cells.

Limitations: One limitation of this study was the small sample size; therefore, additional research is needed to provide better validation. Another limitation is the unavailability of small animal Micro computed tomography (CT), which is a more advanced and precise technique in determining bone marrow density than the x-ray imaging system we used. In addition, ethology experiments during late-stage tumor progression can be more objective.

Conclusion: This study provides evidence that implantation of 3.5×10^5 and 4×10^5 dose of Walker 256 carcinoma cells produced the greatest effects in relation to the bone cancer pain model in SD rats, and 3.5×10^5 dose induced the lowest mortality rate.

Key words: Bone cancer pain model, Walker 256 carcinoma cells, different doses

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Cancer pain is one of the most common clinical symptoms associated with malignant cancers (1,2). It is estimated that there will be 1.6 million new cancer cases in 2015 within the United States, and up to 50% of malignant cancers will eventually metastasize to bone (3,4). During the course of cancer progression, up to 90% of patients suffer from nociceptive or neuropathic pain, or both (5). Experiencing pain due to cancer can severely decrease the quality of life in patients, and providing pain relief has become a priority in oncology care. Cancer pain is associated with disease progression as well as during treatment; therefore managing pain in patients is essential from the onset of disease through to long-term treatments and terminal care (6). Although primary bone cancers are rare, malignant cancers originating from the breasts, lungs, and prostate often metastasize to the bone (7,8), and studies have shown that approximately 70% of patients suffering from advanced breast or prostate cancer will develop bone metastases (9). Cancer metastasis to bone causes several skeletal-related events (SREs) including pain, bone fractures, spinal cord compression, and hypercalcemia (10). The main objective in the treatment of bone cancer is preventing pain and improving the patient’s quality of life.

Bone cancer pain produces a unique mechanical and neurochemical onset that goes beyond the basic neuropathic and inflammatory pain of other cancers, and this unique characteristic makes bone cancer pain therapeutically intractable (11). Anti-inflammatory drugs and opiates have limited effectiveness in managing bone cancer pain, and increased doses often lead to side effects (12-14). Bisphosphonates are a class of drugs that are effective in the treatment of bone-related medical conditions, such as bone malignancy and osteoporosis. However, prolonged usage of bisphosphonates can also lead to severe side effects, such as bisphosphate-associated osteonecrosis of the jaw (BONJ), a rare but painful condition that severely affects the patient’s quality of life (15). Consequently, further research into the mechanisms involved in cancer pain would allow the development of better and more effective strategies in the management and treatment of cancer pain.

Animal models are often used to investigate the mechanisms of human diseases, including bone cancer. However, studies which focus on the effect of different doses of carcinoma cells on bone cancer pain are still lacking. Breast cancer is one of the most common forms of malignant cancers affecting women, which has a high rate of morbidity and mortality. Cancer metastasis to bone is the most common outcome in malignant breast cancers. In this study, different doses of breast cancer mammary gland carcinoma cells (Walker 256) were injected into the right tibia of Sprague-Dawley (SD) rats, and subsequent measurements of spontaneous pain, thermal hyperalgesia, mechanical hyperalgesia/allodynia, as well as bone x-ray, bone mineral density (BMD), pathology, carboxyterminal telopeptide of type I collagen (ICTP), bone alkaline phosphatase (BAP), and interleukin-1 beta (IL-1β) mRNA analyses were performed to determine the effectiveness and differences of varying doses of Walker 256 carcinoma cells on bone cancer pain.

**Methods**

**Animal Preparation**

Female 4 and 6 week old SD rats weighing (70 – 90 g and 150 – 170 g, respectively) were provided by Shanghai SLAC Laboratory Animal CO., Ltd (Shanghai, license NO. SCXK 2007-0005), and kept under controlled conditions (temperature 24 ± 0.5°C, relative humidity 50 – 60%, 12-hour alternating light-dark cycle, food and water ad libitum). Wood shavings were used as cage bedding to minimize the possibility of painful contact with a hard surface. All experiments were conducted in accordance with National Institute of Health (NIH) guidelines for the care and use of laboratory animals and the International Association for the Study of Pain (IASP) ethical standards and guidelines of animal pain research (16). It was also approved by Shanghai University of Traditional Chinese Medicine (China).

**Experimental Grouping**

Seventy-five female Sprague-Dawley rats weighing 150 – 170 g were randomly divided into 5 groups (n = 15 per group): (1) normal group, which received no injection, (2) sham bone cancer control group, which received vehicle (PBS) inoculation without carcinoma cells, (3) 3 x 10⁵ carcinoma cells group, which received inoculation of 3 x 10⁵ Walker-256 carcinoma cells, (4) 3.5 x 10⁵ carcinoma cells group, which received inoculation of 3.5 x 10⁵ Walker-256 carcinoma cells, (5) 4 x 10⁵ carcinoma cells group, which received inoculation of 4 x 10⁵ Walker-256 carcinoma cells. Prior to the experiment model, 2 rats from each group were selected and the right hind limb tibia tissue was removed for histology analysis.
Model reparation

Cell Preparation
Walker-256 rat mammary gland carcinoma cells were derived from SD rats and provided by Shanghai Laboratory Animal Research Center. Based on our previously reported method (17), 1×10^7 (cells/mL) carcinoma cells were injected into the abdominal cavities of the 4 week old rats (70 – 90 g), and the ascetic fluid was extracted after 6 – 7 days. After washing with PBS, the cells were collected and kept on ice until subsequent injection.

Model Preparation
The 6 week old rats (150 – 170 g) were anesthetized with 10% chloral hydrate (200 mg/kg) intraperitoneally (i.p) following shaving of the right knee joint. The bone cancer pain model was prepared according to the methods as previously described (18). The right hind limbs of the rats were shaved and disinfected with 75% v/v ethanol, and right lateral superficial incisions were made in the skin overlying the patella. A 23-gauge needle was first inserted at the site of intercondylar eminence 7 mm below the knee joint into the medullary cavity of the tibia. The needle was then removed and replaced with a 29-gauge needle attached to a 10 μl microinjection syringe, and 6 μl carcinoma cells (3×10^5, 3.5×10^5, or 4×10^5 doses) or PBS were slowly injected into the right tibia cavity. The injection site was closed using bone wax after syringe removal, and the wound was closed using a gelatin sponge. All animals were allowed to recover for 7 days following surgery, prior to further experimentation.

Spontaneous Pain
The rats were individually placed in a flat plastic observation box, measuring 1.2 m × 1.2 m × 0.45 m. The status and indicators of limping in the rat’s right tibia were observed, and a spontaneous pain scoring method was used based on the following criteria: normal walking as 0 points, mild limp as 1 point, severe limp as 3 points, complete loss of right hind limb function as 4 points. Spontaneous pain was measured on day 0, 5, 11, 17, and 21 following injection of Walker 256 carcinoma cells or PBS.

Thermal Hyperalgesia
The normal, sham, and carcinoma-injected rats were tested for sensitivity to mechanical stimuli. Hind paw withdrawal threshold was measured by von Frey filaments as previously described (20). Mechanical stimuli testing were performed on days 0, 5, 11, 17, and 21 following injection of Walker 256 carcinoma cells or PBS. The von Frey filaments with logarithmically incremental stiffness (0.4, 0.6 1.4, 2.0, 4.0, 6.0, 8.0, and 15.0 g; Stoelting, Wood Dale, Illinois, USA) were applied perpendicular to the mid-plantar surface, but avoiding the less sensitive plantar tori of the rat’s hind paw. Each von Frey filament was held for roughly 1 – 5 seconds, with a 10-minute interval before the next incremental stimuli. Each rat was first subjected to the application of the 2.0 g von Frey filament, whereby a positive response was defined as the withdrawal of the paw upon stimulus. When there was a positive response to a stimulus, a lower incremental von Frey filament was applied, and a negative response resulted in the application of a higher incremental von Frey filament. Each rat was subjected to 5 additional stimuli following the first response, and the pattern of response to mechanical stimuli was converted to 50% von Frey threshold.

X-ray and BMD
To assess the tumor-induced tibia bone destruction, radiographs, and BMD of the tibia bone were analyzed. Rats were individually placed on a clear plane Plexiglas and exposed to the Kodak In-Vivo Imaging System Fx Pro (Carestream Health CO, USA) following anesthesia with sodium pentobarbital (80 mg/kg, i.p), on day 0, 11, and 21 after inoculation of Walker 256 carcinoma cells or PBS. X-ray imaging was performed on the surgical side of the rat hind limbs to analyze the BMD and
observe any changes in the proximal tibia.

**Pathology**

Rats underwent deep anesthesia with sodium pentobarbital (80 mg/kg, i.p) at day 0 prior to experiment model, and day 21 post-cancer cell inoculation into the tibia, by transcardial perfusion with saline. The right tibial tissue was then removed and preserved in 10% neutral buffered formalin, and decalcified using 10% EDTA for 21 days. The preserved tissues were then embedded in paraffin, sectioned to 5-micron-thick (Leica RM 2165) and stained using standard Haematoxylin and Eosin (H&E) method.

**ICTP and BAP**

After inoculation of Walker 256 carcinoma cells or PBS on day 0, 5, 11, 17, and 21, blood samples (0.5 ~ 1 mL) of rats were drawn from the tail vein. ICTP and BAP concentrations in serum were subsequently measured using a commercial ELISA kit (Pierce Chemical, Rockford, IL, USA) according to the manufacturer’s instructions.

**Real-time PCR for IL-1β**

On day 21 following inoculation of Walker 256 carcinoma cells or PBS, rats were sacrificed and their spinal cords were removed. The dorsal horn was separated into contralateral and ipsilateral sections and stored immediately in liquid nitrogen. Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA using the standard RNA extraction protocol. The expression of IL-1β mRNA was measured using real time Polymerase Chain Reaction (PCR) (Step One Plus, ABI, Carlsbad, CA, USA). Primer sequences for IL-1β were RIL-1-S: 5’-cgagatgctgtgagatttg-3’, tm60.9, 54225, 172bp, NM-031512, Tm61.2 and RIL-1-A: 5’-gctatggcaactgtccctgaac-3’, tm60.9, 54-225, 172bp, NM-031512, Tm87.1; and GAPDH were RGAPDH-S: 5’-gtgccagcctcgtctcatag-3’ and RGAPDH-A: 5’-gttgaacttgccgtgggtag-3’, 192bp, Tm89.5.

**Real-Time PCR for IL-17 and IL-17RA**

Rat spinal cord RNA was extracted as mentioned above. The expression of IL-17 and IL-17RA was measured using real time PCR (Step One Plus, ABI, Carlsbad, CA, USA). Primer sequences for IL-17 were IL-17-S: 5’-gaagttggaccaccacatga-3’ and IL-17-A: 5’-tcccttcagaggtgccctac-3’; for hydroxymethylbilane synthase (Hmbs) as a housekeeping gene were Hmbs-S: 5’-tccctgaaggatgtgcctac-3’ and Hmbs-A: 5’-acaaggtttttccgttttg-3’; for hypoxanthine phosphoribosyltransferase minigenes (Hprt) also as a housekeeping gene were Hprt-S: 5’-ggtccattcctatgactgtagattt-3’ and Hprt-A 5’-caatcaagacgttctttccagtt-3’. The standard curve method was used to compare mRNA expression levels between groups. Similar results were obtained when IL-17 and IL-17RA expression levels were normalized to both endogenous control genes Hmbs and Hprt.

**Statistical Analysis**

The mean values and standard deviations (SD) were calculated for all collected behavioral data. Statistical significance was analyzed using one way ANOVA and repeated measures ANOVA using SPSS 16.0 statistical software. Criteria for statistical significance in all analyses were set as \( P < 0.05 \).

**Results**

**Changes in Body Weight**

There were no obvious changes in rat body weight from day 0 – 7 following inoculation of Walker 256 carcinoma cells or PBS among the 5 groups. However from day 11 – 21, the 3 groups which were inoculated with Walker 256 carcinoma cells showed significant weight loss compared to the normal and PBS injected groups (\( P = 0.0001 \)) (Fig. 1). A 2 × 2 × 5 repeated-measures ANOVA was used to determine the main effects of tumor cell implantation (\( F_{\text{intercept}} = 26938.94, P = 0.0001 \)) and time (\( F_{\text{GG}} = 129.28, P = 0.0001 \)), as well as the interaction between tumor cell implantation and time (\( F_{\text{GG}} = 198.64, P = 0.0001 \)). Post-hoc comparisons of means revealed that rats injected with Walker 256 carcinoma cells in the right tibia induced a significant (\( P = 0.0001 \)) decrease in body weight from day 11 to 21. The body weight of the rats in the 3×10^5 , 3.5×10^5 , and 4×10^5 groups decreased continuously from day 11, and in a dose dependent manner.

**Mortality Rate**

From day 7 after injection of Walker 256 carcinoma cells, there was a decrease in the general condition of rats, including weakened mental state and discoloration of body hair. Rat mortalities mainly occurred in the second week following injections. By the third week following injections, the bone cancer had progressed to the chronic stage, and thus the condition of the surviving rats became relatively stable. In the 4x10^5 group, there were 3 deaths which occurred on day 8, 9, and
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14 following injection; in the $3 \times 10^5$ group, there were 2 deaths which occurred on day 7 and 11 following injection; in the $3.5 \times 10^5$ group, there was only one death which occurred on day 9 following injection (Table 1). These results demonstrate that implantation of the $3.5 \times 10^5$ dose of Walker 256 carcinoma cells resulted in the lowest mortality rate in rats.

### Spontaneous Pain

There were no obvious differences in the function of the rat hind limb between the normal and sham PBS injected group. However, rats inoculated with Walker 256 carcinoma cells displayed an obvious limp in the affected hind limb from day 11 to 21 following injection (Fig. 2). In addition, rats which received $4 \times 10^5$ or $3.5 \times 10^5$ dose of carcinoma cells showed significant limping on day 11 ($P = 0.004$ vs normal, $P = 0.038$ vs PBS) and day 17 – 21 ($P = 0.001$) following injection. Whereas rats which received $3 \times 10^5$ dose of carcinoma cells showed only a slight limp on day 17 – 21 ($P = 0.0001$) following injection. A $2 \times 2 \times 5$ repeated-measures AVOVA was used to determine the main effects of tumor cell implantation ($F_{(intercept)} = 108.91, P = 0.0001$) and time ($F_{(GG)} = 48.25, P = 0.0001$), as well as the interaction between tumor cell implantation and time ($F_{(GG)} = 8.72, P = 0.0001$). Post-hoc comparisons of means revealed that rats injected with Walker 256 cells in the right tibia induced a significant increase in spontaneous pain on day 11 – 21 ($P = 0.0001$). These results demonstrate that breast cancer metastasis to the tibia caused progressive spontaneous pain, compared to the contralateral hind paw, as well as the normal and PBS-injected groups.

### Table 1. Mortality rate of rats after injection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days after injection</th>
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<tr>
<td>Normal</td>
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<td>PBS</td>
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<td>$3 \times 10^5$</td>
<td>7, 11</td>
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<td>$3.5 \times 10^5$</td>
<td>9</td>
<td>1</td>
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<td>$4 \times 10^5$</td>
<td>8, 9, 14</td>
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Fig. 1. Changes in body weight of normal rats and rats injected with PBS or different doses of Walker 256 carcinoma cells ($n = 10$). Body weight was measured on day 0 (prior to inoculation), 5, 11, 17, and 21 after injection. **$P < 0.001$ vs normal group; ##$P < 0.001$ vs PBS group.

Fig. 2. Changes of spontaneous pain score in normal rats and rats injected with Walker 256 carcinoma cells or PBS ($n = 10$). Score of spontaneous pain was tested on day 0 (prior to inoculation), 5, 11, 17, and 21 after injection. Carcinoma cells induced an obvious increase of spontaneous pain score in the right tibia of rats. *$P < 0.05$ vs normal group, #$P < 0.05$ vs PBS group; **$P < 0.001$ vs normal group, ##$P < 0.001$ vs PBS group.
Thermal Hyperalgesia

Rats inoculated with Walker 256 carcinoma cells exhibited significant changes in PWL on day 10 – 20, but not those injected with PBS or the normal un-injected groups (Fig. 3A). There was a significant decrease in the PWL of rats which received $4 \times 10^5$, $3.5 \times 10^5$, or $3 \times 10^5$ doses of carcinoma cells day 10 – 20 ($P = 0.0001$), which was more pronounced in the $3.5 \times 10^5$ and $4 \times 10^5$ dose groups compared to the $3 \times 10^5$ dose group on day 21 ($P = 0.001$). A $2 \times 2 \times 5$ repeated-measures ANOVA was used to determine the main effects of tumor cell implantation ($F_{\text{intercept}} = 12990.56$, $P = 0.001$) and time ($F_{\text{time}} = 103.55$, $P = 0.001$), as well as the interaction between tumor cell implantation and time ($F_{\text{interaction}} = 25.55$, $P = 0.001$). Post-hoc comparisons of means revealed that rats injected with Walker 256 cells in the right tibia induced a significant decrease in PWL on day 10 – 20 ($P = 0.001$). These results suggest that breast cancer metastasis to the tibia induced progressive thermal hyperalgesia, compared to the contralateral hind paw, as well as the normal and PBS-injected groups, which remained at the pre-injection level (Fig. 3B).

Mechanical Hyperalgesia/Allodynia

Rats inoculated with $3 \times 10^5$, $3.5 \times 10^5$, or $4 \times 10^5$ doses of Walker 256 carcinoma cells had a significant decrease in paw withdrawal threshold to von Frey filament stimulation in the right tibia on day 11 – 21 following injection ($P = 0.008$, $P = 0.022$, and $P = 0.0001$, respectively), but not rats injected with PBS or the normal un-injected groups (Fig. 4A). However, no changes were evident in either group from day 0 to 7 after injection. The decrease in paw withdrawal threshold was significantly more pronounced in the $3.5 \times 10^5$ and $4 \times 10^5$ high dose groups, compared to the normal and PBS groups on day 11 – 21 ($P = 0.001$), and the $3 \times 10^5$ low dose group on day 21 ($P = 0.0001$). A $2 \times 2 \times 5$ repeated-measures ANOVA was used to determine the main effects of tumor cell implantation ($F_{\text{intercept}} = 2327.40$, $P = 0.0001$) and time ($F_{\text{time}} = 36.18$, $P = 0.0001$), as well as the interaction between tumor cell implantation and time ($F_{\text{interaction}} = 7.72$, $P = 0.0001$). Post-hoc comparisons
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of means revealed that rats injected with Walker 256 cells in the right tibia induced a significant decrease in paw withdrawal threshold on day 12–21 ($P = 0.0001$). These results demonstrate that high doses of carcinoma cells resulted in a significant decrease of bone-cancer induced mechanical allodynia, compared to the contralateral hind paw, as well as the normal and PBS-injected groups, which remained at the pre-injection level (Fig. 4B).

X-ray

X-ray radiography was used to monitor bone destruction. At day 11 after injection of $3 \times 10^5$, $3.5 \times 10^5$, or $4 \times 10^5$ doses of Walker 256 carcinoma cells, radiolucent lesions in the proximal epiphysis indicating bone destruction was evident in right tibia of injected rats. Furthermore, at day 21 after injection of all 3 doses of Walker 256 carcinoma cells, there were increased destruction to bone marrow, as well as significant bone loss (Fig. 5). No radiological abnormalities were found in the normal and PBS-injected groups from day 0 to 21.

BMD

There was a significant decrease in BMD of the right tibia in rats at day 10, 16, and 20 following injection of $3 \times 10^5$ dose ($P = 0.023$, $P = 0.041$, $P = 0.0001$, respectively), $3.5 \times 10^5$ dose ($P = 0.005$, $P = 0.002$, $P = 0.0001$, respectively), or $4 \times 10^5$ dose ($P = 0.006$, $P = 0.003$, $P = 0.0001$, respectively) of Walker 256 carcinoma cells, compared to pre-injection levels (Fig. 6). However, there were no changes in BMD of normal and PBS-injected groups from day 0 to 20. A 2 × 2 × 5 repeated-measures AVOVA was used to determine the main effects of tumor cell implantation ($F_{\text{intercept}} = 24655.21$, $P = 0.0001$) and time ($F_{\text{SA}} = 33.74$, $P = 0.0001$), as well as the interaction between tumor cell implantation and time ($F_{\text{SA}} = 7.10$, $P = 0.0001$). Post-hoc comparisons of means revealed that rats injected with Walker 256 cells in the right tibia induced a significant decrease in BMD on day 10–20 ($P = 0.0001$). These results suggest that higher doses of carcinoma cells resulted in a greater reduction of BMD.

Pathology

Histological examination revealed that at day 21 following injection of Walker 256 carcinoma cells, significant tumor growth and bone destruction were evident compared to day 0, but there was no evidence of bone destruction in the normal and PBS-injected groups from day 0 to 21. X-ray radiography revealed significant bone destruction in rats injected with Walker 256 carcinoma cells in the right tibia, with radiolucent lesions evident in the proximal epiphysis indicating bone destruction. There were no radiological abnormalities found in the normal and PBS-injected groups.
Fig. 5. Radiographs of the tibia bone in normal rats and rats injected Walker 256 carcinoma cells or PBS (A: normal, B: PBS, C: 3×105, D: 3.5×105, E: 4×105, n = 10). Radiographs were measured on day 0 (prior to inoculation), 11 and 21 after injection. Inoculation of Walker 256 carcinoma cells induced apparent bone destruction, including radiolucent lesions in the proximal epiphysis, especially in the 3.5×105 and 4×105 groups.
groups (Fig. 7). Higher doses (4×10^5 and 3.5×10^5) of Walker 256 carcinoma cells also led to visibly increased bone destruction in the tibia of affected rats.

**Serum ICTP and BAP**

There was a significant increase of serum ICTP and BAP levels in rats injected with 3×10^5, 3.5×10^5, or 4×10^5 doses of Walker 256 cells from day 11 to day 21 (Fig. 8). However, there were no changes in serum ICTP and BAP levels in normal and PBS-injected groups from day 0 to day 21. A 2 × 2 × 5 repeated-measures AVOVA was used to determine the main effects of tumor cell implantation and time, as well as the interaction between tumor cell implantation and time. Post-hoc comparisons of means revealed that rats injected with Walker 256 cells in the right tibia induced a significant decrease of PWL on day 11 – 21 (P < 0.0001). These results suggest that higher doses (4×10^5 and 3.5×10^5) of Walker 256 carcinoma cells led to increases in serum ICTP and BAP.

**Real-time PCR for IL-1β, IL-17 and IL-17RA**

Real time PCR showed that the mRNA expression of IL-1β in rats injected with 3×10^5, 3.5×10^5, or 4×10^5 doses of Walker 256 cells was significantly increased (P = 0.0001) compared to the normal and PBS-injected groups (Fig. 9). In addition, 3.5×10^5 and 4×10^5 doses of Walker 256 carcinoma cells had significantly higher IL-1β mRNA expression compared to 3×10^5 dose (P = 0.0001). There were similar trends in the mRNA expression of IL-17 and IL-17RA (Figs. 10, 11). These results demonstrated that higher dose of carcinoma cells (4×10^5 and 3.5×10^5) resulted in increased IL-1β, IL-17, and IL-17RA mRNA expression.

**DISCUSSION**

Cancer pain is a complex medical syndrome, often caused by the tumor itself, tumor metastasis, or during treatment of cancer. Pain in terminal cancer patients often cannot be managed by systemic analgesics, and is commonly associated with adverse drug reactions to analgesics (21). However, the underlying mechanisms involved with cancer pain are still poorly understood.

Bone is one of the most common metastatic sites of cancer malignancy, which leads to possible side-effects including pain, hypercalcemia, and pathologic fracture (22). Pain is often the first symptom of cancer metastasis to bone (23). Conventional treatment requires a multidisciplinary approach, involving medical therapy, surgery, and radiation therapy (22). Proper pain management is a critical issue for patients with metastatic cancer (23). However, But treatment of bone cancer pain remains a clinical challenge due to limited understanding of the mechanisms involved (24). Bone cancer pain produces a unique mechanical and neurochemical
Fig. 7. Histological evaluation of bone destruction in normal rats and rats injected with Walker 256 carcinoma cells or PBS (A: normal, B: PBS, C: $3\times10^5$, D: $3.5\times10^5$, E: $4\times10^5$, n = 10). Bone tissue samples were obtained on day 0 (prior to inoculation, n = 2) and 21 (n = 10) after injection. On day 0, rats from all groups appeared normal. On day 21, rats from the normal and PBS groups exhibited normal cell morphology such as complete trabecular bone in neat arrangement, normal bone and cellular structure showing clear location of nuclei, and precise row arrangement of osteoblasts within trabecular bone. Rats injected with Walker 256 carcinoma cells showed significant cancer growth and progressive destruction of bone, as well as abnormal cell morphology such as blurring of trabecular bone edge, obvious bone destruction, irregular bone trabeculas, and tumor cells in bone marrow cavity growth. HE staining, original magnification: 400x.
onset that goes beyond typical inflammatory or neuropathic pain, and therefore needs to be considered as a separate entity (27). The use of animal models can accurately replicate the symptoms of bone cancer pain in humans, allowing a valid understanding of the clinical outcome of bone cancer pain (25).

Pre-clinical bone cancer pain models mimicking the human condition are required to provide a valid comparison to the clinical setting (26). Animal models of bone cancer pain in mice were developed by Schwei et al (27), where NCTC2472 cells were inoculated in the femur marrow cavity of C3H/HeJ mice. On this basis, various different carcinoma cells have been used on different animals as an extension of this model. This allows us to explore the mechanisms of cancer pain through observing the general condition and behavior of model animals, as well as understanding the cellular, pathological, and neurochemical changes related to cancer development and bone destruction. These animal models are of great significance for future research.

Fig. 8. Serum BAP and ICTP levels of normal rats and rats injected with Walker 256 carcinoma cells or PBS (A: BAP; B: ICTP, n = 10). Inoculation of Walker 256 carcinoma cells induced a significant increase of BAP and ICTP levels in rat serum, especially in the 3.5×10^5 and 4×10^5 groups. **P < 0.001 vs normal group, ##P < 0.001 vs PBS group, @P < 0.05 vs 3×10^5 group.
Due to physiological differences between rats and mice, many researchers have chosen the rat model in studying bone cancer pain. Research using the rat model of bone cancer pain was developed by Medhurst et al in 2002 (28), before a new rat model of bone cancer pain following prostate cancer cell inoculation of the tibia was established (29). Since then, the rat model using inoculation of mammary gland Walker 256 cells in the tibia of female Wistar rats was developed, and subsequent improvements to the model have been made, resulting in reduced mortality rates (30). Consistent with previous research (18,31,32), the quantity of carcinoma cells is a crucial aspect of the model, in respect to metastatic tumor progression, extent of ambulatory pain, and hind limb weight bearing ability. This study aimed to explore the relationship between different quantities of Walker 256 carcinoma cells on the cancer pain model's success and stability. We found that the $3.5 \times 10^5$ dose of Walker 256 carcinoma cells produced the greatest effect in relation to the bone cancer pain model, due to the lowest mortality rate, but also providing almost identical physiological and pathological features compared to the higher $4 \times 10^5$ dose of Walker 256 carcinoma cells.

Walker 256 mammary gland carcinoma cells can easily metastasize to different parts of the body (31), and often result in behavioral changes in the affected rats. The right tibia (ipsilateral) of rats inoculated with Walker 256 carcinoma cells displayed significant indicators of pain from day 10 to 21 following injection (Fig. 2, Fig. 3A, Fig. 4A), and x-ray radiography also displayed obvious bone destruction (Fig. 5), whereas no changes were evident in the contralateral hind limb (Fig. 3B, Fig. 4B). This demonstrated that the cancer cells did not metastasize to the left tibia during day 0 – 21.

BMD analysis was used to measure the degree of bone destruction following tumor metastasis. A previous study has shown that inoculation of syngeneic MRMT-1 mammary tumor cells into the tibia of female rats re-
sulted in tumor growth within the bone, leading to a reduction in BMD (33). A recent study demonstrated that transplantation of metastatic rat mammary tumor-1 cells into the tibia of 5D rats revealed characteristics of mixed metastasis, tumor development, and reduction of BMD (33). In this study, we found that the BMD of the 3.5x10^5 and 4x10^5 Walker 256 dose groups produced significantly decreased BMD in the right tibia of injected rats (Fig. 6).

Several models of bone cancer pain demonstrate that osteoclasts play an important role in bone destruction and cancer pain (34). Increased activity of osteoclasts can lead to bone destruction and decreased bone density, whereby pain receptors may be activated by bone trabeculas and cytokines (35). Osteoblasts may also impact the osteoblastic function. Tumor cells secrete various growth factors during bone metastasis in order to activate osteoclasts. Bone tissues also release large amounts of growth factors accompanying this process, in order to stimulate tumor growth and bone destruction (36). This generates a large protein and bone degradation matrix, which involves several closely related biochemical markers of bone metabolism (37). The most important markers of osteoclastic (bone resorption) and osteoblastic (bone formation) activity are ICTP and BAP (38-41). In this study, we found that the 3.5x10^5 and 4x10^5 dose groups produced the greatest increase in serum ICTP and BAP compared to the normal, PBS-injected, and 3x10^5 dose groups (Fig. 8). This demonstrated the occurrence of bone resorption and formation in the right tibia, as well as the likely activity of osteoclasts and osteoblasts in our cancer pain model.

Cytokines such as IL-1β play important roles in inflammatory (42) (Sun S, et al. 2012) and neuropathic pain (43) (Cha MH, et al. 2012). Intrathecal injection of IL-1β antagonist may alleviate thermal (44) (Sung, et al. 2004) and mechanical hyperalgesia (45) (Reeve et al. 2000). Therefore, cytokines including IL-1β, are likely to be implicated in the development and maintenance of bone cancer pain. A previous study showed that IL-1β levels increased significantly in the spinal cords of rats following injection of prostate cancer cells in the rat tibia (28). A separate study suggested that spinal IL-1β enhances NR1 phosphorylation to facilitate bone cancer pain (19). In this study, we showed that IL-1β mRNA expression of 3.5x10^5 and 4x10^5 dose groups significantly increased in the spinal cords of rats injected with Walker 256 mammary gland cells.

Interleukin-17 (IL-17) is a pro-inflammatory cytokine secreted by activated T-cells (46). Production of IL-17 is induced during infections, autoimmune diseases, and other inflammatory events. IL-17 is involved in host defense, but also in the destruction of inflammatory tissue (47). IL-17 has a limited role in the acute phase of nerve injury and pain, but is likely involved in later phases during the development of neuropathic pain (48). However the relationship between IL-17 and cancer pain is still unknown. This study showed that 3.5x10^5 and 4x10^5 dose groups had significantly increased expression of IL-17 mRNA and IL-17RA mRNA compared to the normal, PBS-injected and 3.5x10^5 dose groups in the spinal cords of rats injected with Walker 256 carcinoma cells. Therefore, IL-17 may be involved in the occurrence and development of cancer pain.

On the basis of previous studies, we found that the 3.5x10^5 dose is the optimal dose for studying the bone cancer pain model in SD rats (49-50). This dose achieved the consistent model effect of the 4x10^5 dose group, with the added advantage of decreased mortality rates. One limitation of this study was the small sample size; therefore, additional research is needed to provide better validation. Another limitation is the unavailability of small animal Micro computed tomography (CT), which is a more advanced and precise technique in determining bone marrow density than the x-ray imaging system we used. In addition, ethology experiments during late-stage tumor progression can be more objective.

**Conclusion**

In conclusion, the present study demonstrates that inoculation of 3.5x10^5 and 4x10^5 dose of Walker 256 mammary gland carcinoma cells into the tibia of rats induced the greatest destruction to bone. The 3.5x10^5 dose resulted in the lowest mortality rate, and progressively increased thermal and mechanical hyperalgesia. Bone cancer pain increased serum ICTP and BAP levels, which are associated with osteoclastic and osteoblastic factors, as well as spinal cord IL-1β mRNA, IL-17 mRNA, and IL-17RA mRNA expression levels, involved in the development and onset of cancer pain.

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bone cancer pain in the rat. 


