

Local Suppression Effect of Paclitaxel-Impregnated Hydroxyapatite/Collagen on Breast Cancer Bone Metastasis in a Rat Model

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Abstract:

Introduction: Breast cancer is one of the most frequent primary tumors that cause spinal metastases. Metastasis consequences impair both the patient's overall prognosis and quality of life. We previously developed a porous hydroxyapatite collagen composite (HAp/Col) as an osteoconductive scaffold. HAp/Col is a commercially available artificial bone that is frequently utilized in spinal fusion. Because of its high absorbance capacity, HAp/Col is regarded as a good chemical carrier.

Methods: This study investigated the effect of local administration of paclitaxel combined with HAp/Col scaffold on breast cancer metastasis. High-performance liquid chromatography was used to assess the in vitro release of paclitaxel from HAp/Col. In an in vivo rat model, the inhibitory effects of paclitaxel-impregnated scaffolds on local osteogenesis was examined, and then the local suppression effects on metastatic cancer were investigated.

Results: In vitro testing revealed that roughly 30% of the paclitaxel was released within 96 hours. Paclitaxel-impregnated HAp/Col inhibited local osteogenesis for the first 8 weeks in a rat femur. However, at 12 weeks following surgery, this negative effect appeared to have subsided. In the metastatic model, all rats in the control group reached the humane endpoint 14 days after surgery. On the other hand, the average time to the endpoint in the paclitaxel group was 26.5 days, which was substantially longer than that in the control group. Long-term survivors treated with paclitaxel had no remaining tumor cells in the femoral bone, and osteogenesis was seen surrounding the HAp/Col.

Conclusions: Paclitaxel-impregnated HAp/Col reduced local tumor development and extended the time to the target endpoint in rats with metastases from breast cancer. This study shows that local implantation of paclitaxel-impregnated HAp/Col may be a viable therapeutic option for the management of breast cancer metastases.

Keywords:

Breast cancer, Hydroxyapatite collagen, Paclitaxel, Metastasis, Osteoconductive, Local suppression, Survival, Tumor growth

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Introduction

After the lungs and liver, bone tissue is the third most common location of cancer metastasis¹⁾. The most prevalent location of bone metastases is the spinal column. Approximately 70% of bone metastasis occur at this place, implying that up to 10% of all patients with malignant tumors suffer from spinal metastases at some stage throughout their disease's progression^{1,2)}. Breast, lung, renal, and prostate cancers are the most frequent primary tumors that cause spinal

metastases³⁾. Pain, pathological fractures, and paralysis in situations of spinal metastases are all consequences of metastatic bone cancer⁴⁾. These problems impair the patient's overall prognosis as well as their quality of life⁵⁾.

Breast cancer is the most common primary cancer among females, and its prevalence is anticipated to rise further in the future. The most frequent disease that causes bone metastasis, including spinal metastasis, is breast cancer. It has been reported that bone metastases have been discovered in up to 75% of breast cancer deaths⁶⁾. Because breast cancer

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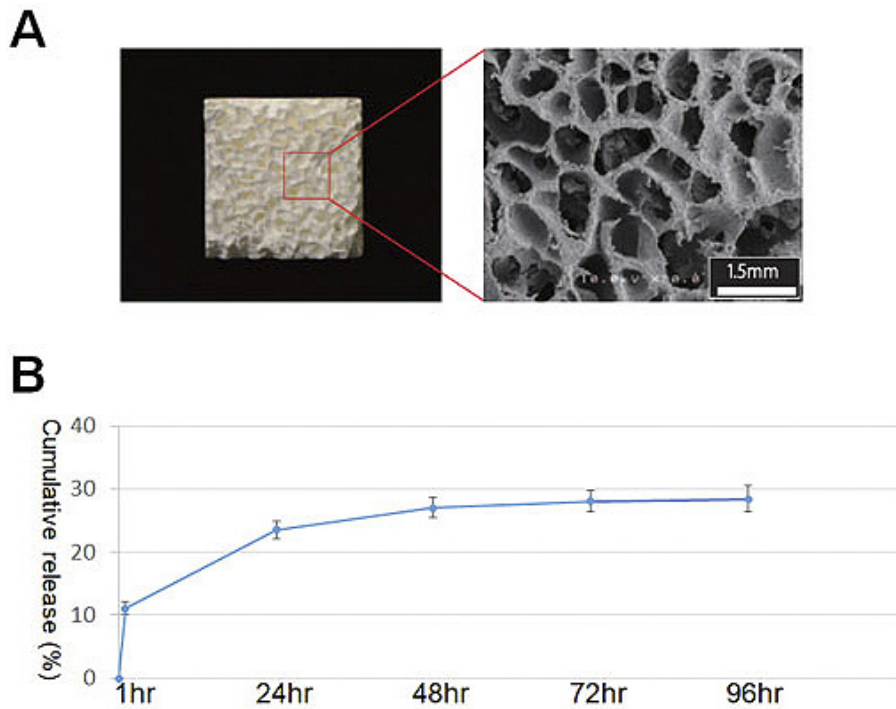


Figure 1.

(A) Structure and characteristic of HAp/Col. The scanning electron microscope image shows that HAp/Col is composed of collagen fibers and HAp nanocrystals deposited on the fibers.

(B) In vitro release experiment of HAp/Col impregnated with paclitaxel. The release of paclitaxel to phosphate-buffered saline was measured.

has a better prognosis than other solid tumors⁷), it is critical to avoid problems associated with bone metastases, such as fractures, discomfort, and paralysis. Chemotherapy is commonly used as a systemic therapy to directly destroy cancer cells in the treatment of bone metastases⁸, and is increasingly utilized in conjunction with radiation as adjuvant treatment. Radiotherapy has historically been the primary therapeutic option for spinal metastases. Even today, it is an important part of the entire therapy protocol. Furthermore, bone-modifying drugs such as bisphosphonates and denosumab are commonly used to avoid osteolysis⁹. Localized treatment, such as surgery, is also used to avoid fractures and paralysis caused by spinal metastases⁹. However, even if the leftover tumor tissue is modest, surgical therapy frequently results in tumor recurrence due to persistent tumors. Local chemotherapy for residual cancers would prevent local recurrence and enhance the patient's prognosis and quality of life.

We previously developed a porous hydroxyapatite collagen composite (HAp/Col) as a bone filler^{10,11}. HAp/Col is a commercially available artificial bone with high osteoconductivity that is frequently utilized in clinical practice, particularly in spinal fusion¹². Because of its high absorbance capacity, HAp/Col is regarded as a good chemical carrier¹³⁻¹⁵. We hypothesized that this HAp/Col might be utilized as an anticancer drug carrier for localized delivery to metastatic bone lesions and that it could limit metastatic

cancer progression while mending bone defects produced by metastatic lesion curettage. The purpose of this study is to confirm the effects of paclitaxel-containing HAp/Col on metastatic lesion management and bone regeneration in a breast cancer metastasis rat model.

Materials and Methods

We studied breast cancer because it is prone to bone metastasis, affects many patients, and has a relatively long prognosis⁶. We used paclitaxel, the preferred chemotherapeutic agent for treating breast cancer, as the target anticancer agent. HAp/Col is a highly porous scaffold (Fig. 1), which was used as the anticancer drug carrier. In this study, we utilized a rat femur bone metastatic model instead of a spine model because of the reproducibility of metastatic lesions and feasibility to evaluate bone regeneration inside the HAp/Col scaffold.

Animals

All animal experiments were performed after receiving approval from the Institutional Animal Experiment Committee, and all experiments were conducted according to the guidelines of our institution on the care and use of laboratory animals (A2017-026A, 18 L-R008). F344 rats were purchased from Sankyo Labo Service Corporation (Tokyo, Japan) and acclimatized for 1 week before the experiment.

Rats were housed in plastic cages with two animals in a cage at a constant temperature and humidity with a standard 12:12-h light/dark cycle. The animals were fed standard laboratory chow and sterile water *ad libitum*. Euthanasia criteria included weight loss of 25%, weakness, pus discharge, and/or fractures according to the institutional ethical guideline.

Cell line

Rat mammary adenocarcinoma (CRL-1666) was selected and purchased from the American Type Culture Collection. The cells, which were initially established at the EG&G Mason Research Institute, were cultured in Dulbecco Modified Eagle Medium containing 10% fetal bovine serum, 80.5 µg/mL of streptomycin, and maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Preparation of paclitaxel-impregnated HAp/Col

Paclitaxel was purchased from Wako Pure Chemical Industries. We used the standard dose of 2 mg/kg as the dose of paclitaxel in rats, which is slightly lower than that in humans (2.4 mg/kg). HAp/Col was moistened with distilled water, cut into 3×3×4-mm pieces, and then dried on a clean bench. We impregnated HAp/Col with 400 µg of paclitaxel. We prepared 25 mg/mL of paclitaxel in 100% ethanol and applied 16 µL to the slice of HAp/Col.

***In vitro* release experiment of HAp/Col impregnated with paclitaxel**

First, we examined the *in vitro* release profile of paclitaxel-impregnated HAp/Col. HAp/Col impregnated with 400 µg of paclitaxel was immersed in 2 mL of phosphate-buffered solution (PBS), and the concentration of paclitaxel in PBS was measured after 12, 24, 48, 72, and 96 hours (n=4). The concentration of paclitaxel in the eluted PBS was measured using high-performance liquid chromatography (HPLC).

Implantation of HAp/Col with paclitaxel into a normal femur

We examined the local and osteogenic effects of implanting paclitaxel-impregnated HAp/Col into the femurs of normal rats. F344 rats (20 males; age, 10 weeks; weight, 190-220 g) were anesthetized, and a 10-mm longitudinal incision was made on the lateral epicondyle of the bilateral femur after the legs were shaved and disinfected. The femur was then approached through the intermuscular space between the biceps femoris and vastus lateralis muscles, and the lateral epicondyle was exposed. A φ 3-mm monocortical hole was made on the lateral epicondyle with a 2.0-mm electrical drill, followed by flushing the bone dust with normal saline. Then, 400 µg of paclitaxel-impregnated HAp/Col was implanted on the right side, and physiological saline-impregnated HAp/Col was implanted into the bone on the left side to serve as a control. The rats were sacrificed at 4, 8, and 12 weeks after surgery. Imaging evaluations were

performed using micro-computed tomography (CT) (CosmoScan GX; Rigaku Co., Tokyo, Japan; FOV, 10 mm) for up to 12 weeks. We calculated bone volume (BV)/total volume (TV) using a previously reported method¹³⁾, in which new bone formation inside the material was measured as BV. After the micro-CT analysis (4, 8, and 12 weeks after surgery), the femurs were decalcified with 10% EDTA and assessed histologically as paraffin-embedded sections with hematoxylin and eosin staining.

Bone metastasis model in rats

We adjusted the number of cultured rat breast cancer cells (CRL-1666) to 100,000 with a cell counter. We then centrifuged and pelleted 100,000 CRL-1666 cells and implanted them under the back skin of F344 rats. Since CRL-1666 is a breast cancer cell line-derived from F344 rats, the cancer cells do not cause rejection upon implantation. CRL-1666 grew subcutaneously on the back of the animals and produced cancerous nodules. We removed the nodules, cut them into 1-mm³ pieces, and transplanted them into the femur through the 3-mm monocortical hole. Two weeks after the transplantation of the cancer nodule to the femur, the femur was examined by micro-CT and histology. After confirming the reproducibility in local growth of the transplanted cancer growth, this model was used as the bone metastasis rat model.

Local effects of paclitaxel-impregnated HAp/Col on bone metastasis

A total of 20 F344 rats (female; age, 10 weeks; weight, 180-240 g) were examined. The anesthesia, skin incision, and surgical approach were the same as stated above. Then, a φ 3-mm monocortical hole was made on the lateral side of the right femurs. The local suppression experiments against tumor growth using paclitaxel included the following two groups: the paclitaxel group, wherein HAp/Col was implanted into the femur infused with 400 µg of paclitaxel and a 1-mm-sized cancer graft (n=10), and the control group, wherein only HAp/Col and the cancer graft were implanted (n=10). Histological evaluations micro-CT images were performed until the humane endpoint was reached. According to the regulation of the institutional animal ethical committee, the humane endpoint was defined as a 25% weight loss from the preoperative weight or an estimated subcutaneous tumor diameter of more than 20 mm in the micro-CT findings.

Statistical analysis

Statistical analyses were performed using Stat Mate, Version 3 (Japan 3B scientific, Niigata, Japan). Disease-free and overall survivals were determined using Kaplan-Meier analysis. All data are presented as the means±standard error. The log-rank test was used to test the associations between the paclitaxel and control groups.

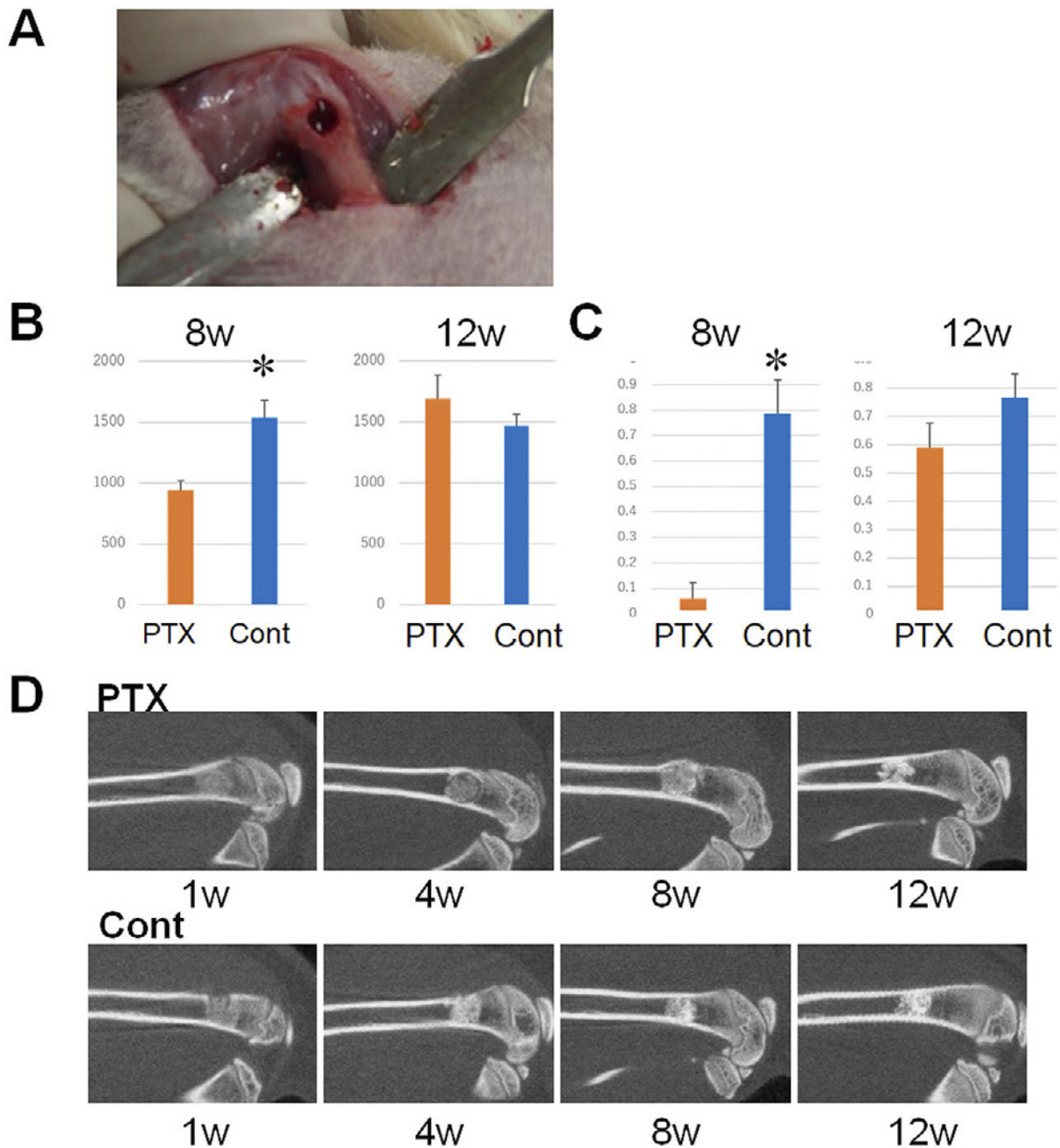


Figure 2. (A) In the experiment of implantation of paclitaxel (PTX)-impregnated HAp/Col to normal rat thigh bone. We created a 3-mm bone tunnel in the bilateral femur of 10-week-old F344 rats. (B) The mean CT value of the implantation region of HAp/Col with PTX was significantly lower than that in the control group up to 8 weeks after surgery. However, 12 weeks after the operation, no difference was found in the average CT value (Y-axis: HU). (C) Bone volume (BV) in the material per total volume (TV) was significantly lower in the PTX group than the control group 8 weeks after surgery (Y-axis: BV/ TV). (D) CT pictures of implanted region after implantation of HAp/Col with PTX or with saline (control).

Results

In vitro release experiment of HAp/Col impregnated with paclitaxel

According to the HPLC analyses, roughly 25% of the paclitaxel was discharged within 24 hours (Fig. 1B). Then,

the release profile became slower, i.e., an additional 5% of paclitaxel was released during the following 72 hours.

Implantation of HAp/Col with paclitaxel into a normal femur

HAp/Col with or without paclitaxel was implanted in a ϕ 3-mm monocortical defect (Fig. 2A). Bone formation in the

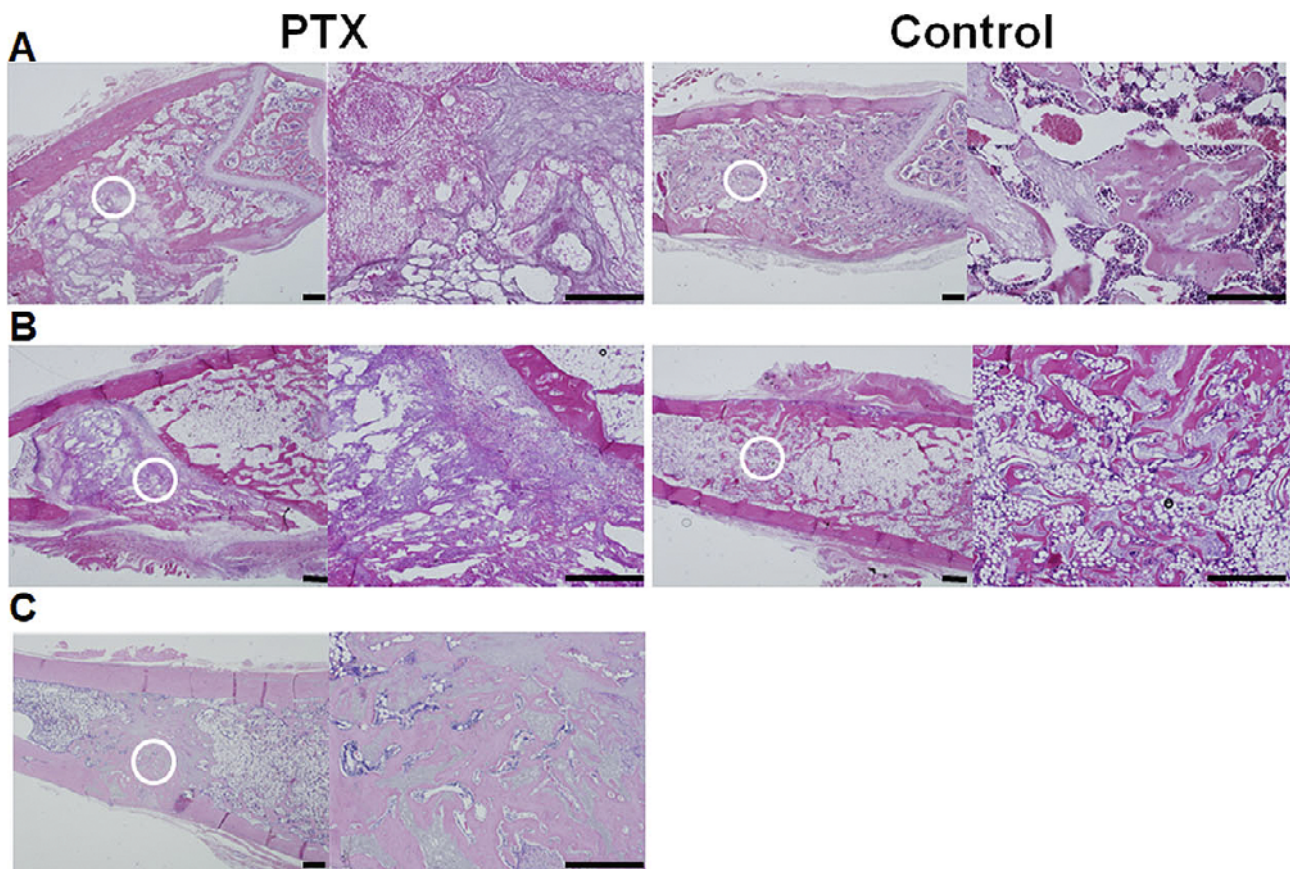


Figure 3.

(A) Histological findings at 4 weeks after the operation showed that HAp/Col was absorbed and internal osteogenesis was observed in the control group. Scale bar: 500 μ m.

(B) In the paclitaxel group, HAp/Col remained 8 weeks after the operation. No cell infiltration or bone formation was noted in HAp/Col, suggesting that the effect of paclitaxel (PTX) was sustained. Scale bar: 500 μ m.

(C) After 12 weeks of surgery, bone tissues were evident in and around HAp/Col in the PTX group. Scale bar: 500 μ m.

HAp/Col was evaluated using micro-CT images (Fig. 2B-D). On micro-CT 4 weeks after HAp/Col implantation, normal osteogenesis was detected in the implantation site; moreover, the internal Hounsfield unit (HU) values on the CT images increased (Fig. 2B). However, for up to 8 weeks following surgery, the mean CT value of the implanted region on the paclitaxel-treated side was substantially lower than that of the control group. At 12 weeks, however, there was no change in the mean CT value between the paclitaxel treatment side and the contralateral control side (Fig. 2B). In the paclitaxel group, BV/TV was significantly lower at 8 weeks postoperatively than in the control group, but there was no difference at 12 weeks postoperatively (Fig. 2C).

Histological data revealed that HAp/Col was absorbed, and internal osteogenesis was detected in the control side 4 weeks after the procedure (Fig. 3A). However, HAp/Col persisted 8 weeks after the procedure on the paclitaxel administration side, and no cell migration into the material was detected (Fig. 3B). However, bone tissue development surrounding the HAp/Col was detected 12 weeks following surgery (Fig. 3C).

Bone metastasis model in rats

Micro-CT revealed tumor development in the rat femur 2 weeks following the transplantation of the cancer nodule (Fig. 4A, B). Histological evaluation revealed that the cancer cells had grown into the bone marrow (Fig. 4C). Tumor development was recognized in all the rats that received the cancer nodule. In the gross observation at the time of sacrifice, we did not detect any metastatic lesions in the spine and other organs. No rat showed paralysis of the lower limbs.

Local effects of paclitaxel-impregnated HAp/Col on bone metastasis

All rats in the control group reached the humane endpoint 14 days following surgery. The average time to the target endpoint in the paclitaxel group was 26.5 days, which was substantially longer than that in the control group. Furthermore, 12 weeks after surgery, two rats in the paclitaxel group had not reached the endpoint (Fig. 5A). Micro-CT images revealed that local tumor development occurred in all instances in the control group 14 days following surgery. The size of the tumor mass was measured using CT images.

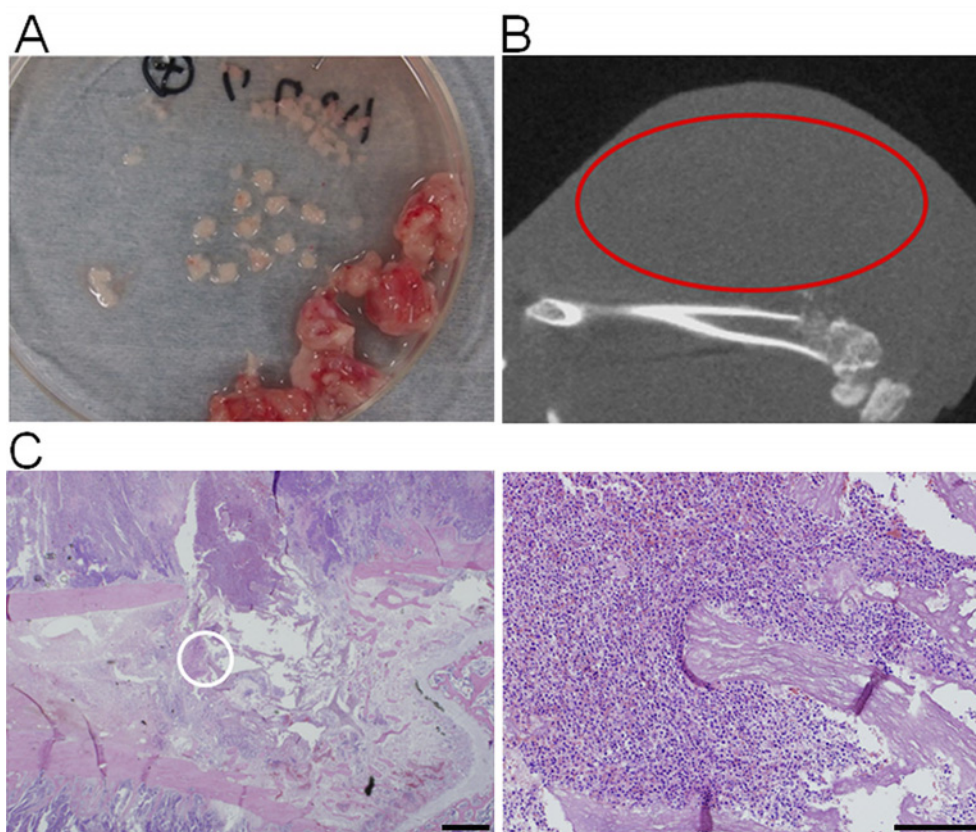


Figure 4.

(A) A rat breast cancer cell line called CRL-1666 was cultured and implanted subcutaneously into the rat's back to create a cancer nodule. Next, the nodule was cut to a size of 1 mm and then transplanted into the femur.
 (B) Two weeks after surgery, micro-CT showed the growth of the transplanted tumor.
 (C) Histological findings demonstrated the growth of subcutaneous cancer cells into the bone marrow. Scale bar: 500 μm .

In the control group, all rats reached the humane endpoint (>20 mm) at 2 weeks, whereas in the paclitaxel group, no rat reached the endpoint at 2 weeks and only 30% at 3 weeks (Fig. 5B).

Histological observations revealed marked tumor growth from femur to the subcutaneous tissue in rats that reached the humane endpoint (Fig. 6A). Long-term survivors, on the other hand, had no remaining tumor cells in the femoral bone, and osteogenesis was detected surrounding the HAp/Col in the paclitaxel group (Fig. 6B).

Discussion

This research investigated HAp/Col, which is presently one of the most often used artificial bones in spinal fusion, in combination with paclitaxel for the treatment of metastatic breast cancer. Approximately 30% of paclitaxel was released from HAp/Col within 96 hours in an *in vitro* release was assessed by HPLC. Paclitaxel is expected to be liberated from paclitaxel-impregnated HAp/Col by simple diffusion since it does not chemically bond to hydroxyapatite. Previous studies also reported that relatively slow release of antibiotics or growth factors from HAp/Col was ob-

served^{13,14}. However, it should be noted that the degradation of HAp/Col *in vivo* would result in a larger release of paclitaxel than that observed in this *in vitro* trial.

Paclitaxel is a vesicular medication that can cause skin necrosis and ulceration with even little extravasation of the drug¹⁶. In this trial, however, neither subcutaneous inflammation nor skin ulcers were detected at the thigh to which paclitaxel was given locally. We consider that topical paclitaxel delivery via HAp/Col does not affect skin and subcutaneous tissue when the paclitaxel-HAp/Col is implanted to the femur, which has rather substantial muscle coverings. Furthermore, no apparent systemic adverse effects were seen in the paclitaxel-treated group. While serum paclitaxel concentrations were not measured, we believe that locally implanted paclitaxel-impregnated HAp/Col had a minimal systemic deleterious impact. In this study, we set the dosage of paclitaxel at 2.0 mg/kg, which is slightly lower than that in humans (2.4 mg/kg). This dosage is estimated to be safe even if all the paclitaxel impregnated in the HAp/Col is absorbed into the bloodstream.

Paclitaxel-impregnated HAp/Col, on the other hand, had a negative influence on osteogenesis until at least 8 weeks following implantation. Paclitaxel is known to have an antipro-

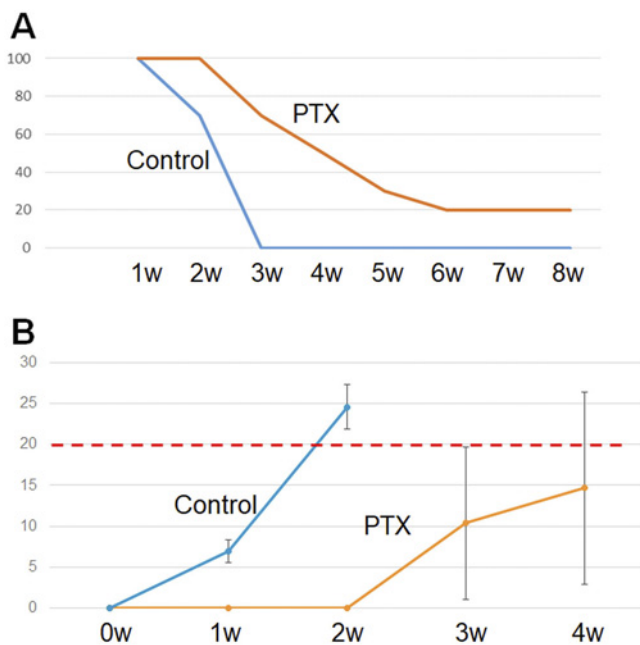


Figure 5.

(A) Kaplan–Meier analysis. In the control group, all rats reached the target endpoint at 14 days after surgery. On the other hand, in the paclitaxel (PTX) group, the average time to the target endpoint was 26.5 days ($p < 0.05$). Y-axis: rate of survival (%).

(B) Size of tumor (diameter: mm) on CT images. In the control group, all rats reached the humane endpoint (> 20 mm) at 2 weeks, whereas in the PTX group, none of the rats reached the endpoint at 2 weeks and only 30% at 3 weeks. Data from seven rats in the PTX group at 4 weeks are presented.

liferative and antiangiogenic impact¹⁶), which is beneficial in inhibiting tumor development and metastasis but affects local osteogenesis over the first 8 weeks. However, this negative effect appeared to fade after a while. Osteoblasts were identified surrounding the HAp/Col persisting in the bone marrow in the paclitaxel group 12 weeks following surgery. Paclitaxel-impregnated HAp/Col can be used as a scaffold to repair the bone defect once the paclitaxel effect has worn off. The contralateral side, where HAp/Col with physiological saline was implanted, showed a normal osteogenic response. Based on the results, it appears that the action of paclitaxel is restricted to the bone defect and its surroundings. The paclitaxel-impregnated HAp/Col, we believe, is unlikely to impede osteogenesis on the contralateral side.

Topical paclitaxel-impregnated HAp/Col reduced local tumor development while prolonging survival to the target endpoint. Suppression of cancer metastases increases survival, because it prevents cancer cells from spreading throughout the body¹⁷). Several chemotherapy drugs, including vincristine, doxorubicin, bleomycin, cisplatin, carmustine, 5-fluorouracil, methotrexate, and paclitaxel, have been studied for local administration. Paclitaxel, for example, has been found to have considerable local antitumor action in solid tumors such as breast cancer. Paclitaxel has well-balanced therapeutic effects when administered lo-

cally¹⁸). Furthermore, because radiation is frequently applied after surgery, the fact that it is radiosensitizing is advantageous for local tumor management. We think that paclitaxel-impregnated HAp/Col inhibits local tumorigenesis and improves prognosis by regulating residual micro-metastasis at the cellular level that surgery or radiation cannot eradicate. Furthermore, when the effect of paclitaxel was decreased, HAp/Col contributed to osteogenesis in long-term survivors, indicating the effectiveness of HAp/Col as a paclitaxel carrier as well as an osteoconductive scaffold.

Abe et al. used the spray-dry technique to produce paclitaxel-containing hydroxyapatite beads, which were subsequently implanted into the spine of a rat model with breast cancer bone metastases¹⁸). This therapy delayed the emergence of lower-limb paralysis. Thus, hydroxyapatite beads with paclitaxel are one of the most efficient treatments for breast cancer bone metastases. Nonetheless, their preparation is time-consuming and must be completed the day before the operation. However, because impregnation of paclitaxel to HAp/Col can be performed during surgery, we can readily prepare the paclitaxel-HAp/Col based on the size of the bone defect caused by the curettage of metastatic lesions. Furthermore, because HAp/Col has sponge-like characteristics, it clings to the defect location flexibly and eliminates dead space. This is thought to be advantageous in terms of promoting targeted paclitaxel administration and exerting a more osteogenic impact than hydroxyapatite beads alone.

This study has several limitations. The bone metastasis rat model was first generated by implanting a tumor graft. This is not the same as physiological bone metastases in people. Second, we employed a femur model rather than a spine model to assess the antitumor impact and osteoconductive capabilities of paclitaxel-impregnated HAp/Col. Furthermore, we must adjust the dosage of paclitaxel for clinical usage in terms of anticancer and osteogenesis inhibitory effects. We did not perform a detailed imaging evaluation of spinal lesions after transplantation of cancer nodules to the rat's femur. Despite these limitations, our study demonstrated that local implantation of paclitaxel-impregnated HAp/Col is a viable therapeutic option for management of breast cancer spinal metastases.

Conclusions

Paclitaxel-impregnated HAp/Col reduced local tumor development and extended the time to the target endpoint in rats with metastases from breast cancer. After the unfavorable impact of paclitaxel was reduced with time, paclitaxel-impregnated HAp/Col demonstrated osteoconductive capacity. This study shows that local implantation of paclitaxel-impregnated HAp/Col may be a viable therapeutic option for management of breast cancer spinal metastases.

Conflicts of Interest: The authors declare that there are no relevant conflicts of interest.

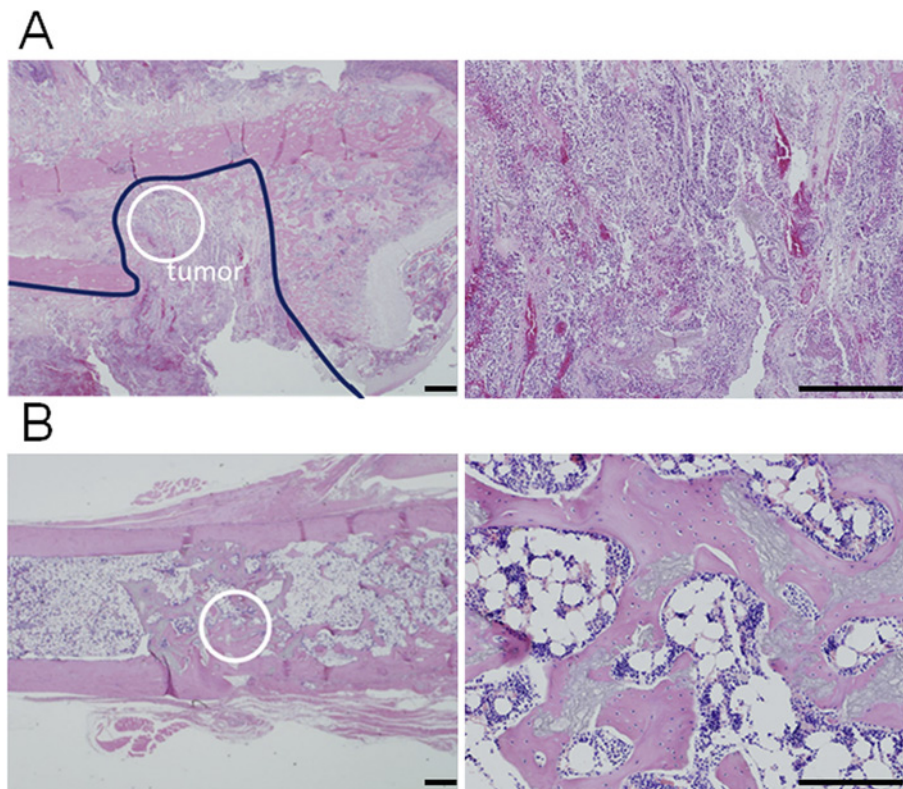


Figure 6.

(A) Histological findings showed tumor cell proliferation in cases that reached the target endpoint. Scale bar: 500 μ m.

(B) In a case of long-term survivors in the paclitaxel group, no residual tumor cells were noted in the femoral bone, and osteogenesis was observed around HAp/Col. Scale bar: 500 μ m.

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Author Contributions: Remppei Matsumoto: study design, experiments on animals, data analysis, drafting the manuscript

Toshitaka Yoshii: study design, data acquisition, interpretation of the data, critical revision of the manuscript

Satoru Egawa: data analysis, experiments on animals

Motonori Hashimoto: data analysis, experiments on animals

Takashi Hirai: data acquisition, data analysis

Yoto Oh: data acquisition, data analysis

Koji Fujita: data acquisition, data analysis

Hiroyuki Inose: data acquisition, data analysis, critical revision of the manuscript

Atsushi Okawa: interpretation of the data, critical revision of the manuscript

Shinichi Sotome: study design, data acquisition, interpretation of the data, critical revision of the manuscript

Ethical Approval: This study was approved by Tokyo Medical and Dental University Animal Experiment Committee Approval number A2017-033ca.

Informed Consent: Consent was not required because this study involved no human subject.

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