Abstract. Several materials have been used as drug delivery systems to maintain a high concentration of anticancer drugs at localized sites. The feasibility of using doxorubicin-loaded calcium phosphate cement (CPC) as a new material, which can release the drug as well as fill a postoperative bony defect, was investigated. The mechanical strength of cylinders of doxorubicin-loaded CPC was not lower than that of CPC alone. Culture medium incubated with doxorubicin-loaded CPC from 1 to 7 days suppressed the proliferation of RMT-1 E4 rat breast cancer cells. In rabbits with doxorubicin-loaded CPC in their femurs, histological examinations showed diffuse edematous changes in the medullary space, but neither fracture nor skin necrosis occurred. Doxorubicin-loaded CPC markedly inhibited the proliferation of sarcoma 180 cells in the mouse air-pouch model. These results indicate that doxorubicin-loaded CPC may be useful in the local treatment of malignant bone and soft tissue tumors.

Chemotherapy plays an important role in eradicating malignant bone tumors and bone metastases. Although several anticancer drugs can be intravenously administered, the systemic delivery of these drugs also exerts high invasive activity on other organs. The management of bone metastases includes the removal of gross disease or internal fixation after curettage of the tumor, followed by chemotherapy or radiation. However, this is not always sufficient for local control. Therefore, a bone cement, to maintain local structural strength and a high concentration of an anticancer drug at a local site, would be desirable.

CPC (calcium phosphate cement, Biopex-R®) is now widely used in orthopedic surgery in the treatment of fractures (1,2). It consists of a powder and solution which are stable at room temperature. During surgery, it can be injected through a small incision since it remains in gel form for several minutes after the powder and solution have been mixed. In addition, bone formations gradually permeate into CPC, and then replace it. In this experiment, the feasibility of using doxorubicin-loaded CPC as a new material which can release the drug, as well as fill a postoperative bony defect, was investigated.

Materials and Methods

Mechanical properties. CPC was kindly supplied by PENTAX (Tokyo, Japan) in the form of 10 g of powder comprising 75% tribasic calcium phosphate, 20% tetra basic calcium phosphate and 5% dibasic calcium phosphate cement, and 3.6 ml of a solution consisting of 5% chondroitin sulfate, 12% sodium succinate and 83% water. In the following experiments, 5 or 10 mg doxorubicin powder (Kyowa Hakko, Tokyo, Japan) and 10 g of CPC powder were mixed, and then the solution was added. One milliliter of gel-form CPC contained 0.85 or 1.7 mg of doxorubicin. Cylindrical CPC specimens were made by introducing the mixture into an acrylic tube 5 mm in diameter and 14 mm in height while still in gel form, and then maintaining for 60 minutes at room temperature to permit the consolidation of the CPC. The specimens were immersed in 10 ml of phosphate-buffered saline for 7 days, and then their compressive strengths were measured using an Instron Autograph AG-25TD (Tokyo, Japan) at a cross-head speed of 0.5 mm/min at room temperature (n=5). The results were statistically analyzed using ANOVA, and statistical significance was considered when the p-value was less than 0.05.

In vitro study of sustained release (3). Cells of the RMT-1 E4 rat mammary carcinoma cell line were obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in medium containing 50% Ham’s F12 medium, 50% Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, CA, USA), 10 mM HEPES (Nakarai, Tokyo, Japan), 1 mM EGTA (Sigma, St. Louis, MO, USA), 1 μg/ml insulin (Sigma), 20 nM sodium selenite (Sigma) and 1 nM estradiol (Sigma). Two types of cylindrical CPCs, which contained only CPC (control) or CPC with 10 mg of doxorubicin, were incubated with 10 ml of medium (n=3). The medium was changed every 24 hours for 14 days, and each volume was collected and frozen at −80°C (Figure 1). The RMT-1 E4 cells...
were seeded at 4x10^5 cells / well in 24 wells of a multi-well plate and left for 36 hours to permit good adhesion onto the substrate. The medium was then replaced by fresh medium or with the medium collected after incubation with the cylinders of CPC. After culturing for 48 hours, the medium was removed and the cultures were incubated with medium loaded with 1 mg/ml MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) (Sigma) for 2 hours at 37°C. The medium was then discarded and 500 μl acid-isopropanol (0.04 N HCl in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes, which convert MTT to an insoluble purple formazan in living cells. The plates were then agitated at room temperature for 15 to 20 minutes, and the level of the colored formazan derivative was determined on a Benchmark multiscan reader, (BIO-RAD, Tokyo, Japan) at a wavelength of 540 nm with a reference wavelength of 630 nm. The degree of toxicity was expressed as the percentage of surviving cells compared with the cylinders which only contained CPC (control). The results were then statistically analyzed using the Student’s t-test, with p<0.05 being considered statistically significant.

Histological study. Eighteen male Japanese white rabbits, weighing 3-4 kg, were used. The leg of each rabbit was draped, and a 20-mm skin incision was made just medial to the distal femur. A hole of 5-mm diameter was then made in the bone using a drill, and 1ml gel-form CPC loaded with 1.7 mg of doxorubicin was implanted into the right femur, while only CPC was implanted into the left femur. The distal femurs were removed from 6 rabbits at 1 week, from 6 at 4 weeks, and from 6 at 24 weeks after implantation. They were then fixed in 10% formalin solution for 24 hours, before being decalcified for 7 days in EDTA solution. Finally, the specimens were sectioned into 3 to 4-μm-thick slices and examined under a light microscope after hematoxylin and eosin staining.

In vivo study of anticancer effects. The ascitic Sarcoma 180 cell line was used for these experiments. A suspension of Sarcoma 180 cells was intraperitoneally injected into 7-week-old ICR female mice (Shizuoka Laboratory Co., Shizuoka, Japan). After 7-10 days, 1.0 to 2.0 ml of ascitic fluid were collected and diluted in phosphate-buffered saline so that the cell density was 1x10^6 / ml. A mouse air-pouch was made in the back subcutaneous tissue of ICR mice by injecting 3 ml of air twice a week (4). For the last air injection, 1x10^6 Sarcoma 180 cells were injected into the air pouch. The following day, 1.0 ml of gel-form CPC loaded with 1.7 mg doxorubicin (n=16) or CPC only (n=16) was injected into the air pouch. The major axes of the tumors in the two groups were measured using calipers every week for 16 weeks. Comparisons of proportional survival were made using the Kaplan-Meier method, and the condition of the skin was observed. The results were statistically analyzed using the Student’s t-test and the log-rank test, with p<0.05 being considered statistically significant. Four weeks after the injection of CPC, histological studies were performed by excising tumors that arose in the backs of the mice. The tumors were fixed in 10% formalin solution for 24 hours, specimens were sectioned into 3 to 4-μm-thick slices, and these were examined under a light microscope after hematoxylin and eosin staining. The protocols for animal experimentation described in this paper had been previously approved by the Animal Research Committee of Akita University, Japan, and all subsequent animal experiments adhered to the "Guidelines for Animal Experimentation" of the University.

Results

Mechanical properties. The compressive strength of cylindrical CPC was 29.2±3.5 (mean±SD) MPa in the group of CPC alone, 28.9±2.6 MPa in the group of CPC loaded with 5 mg doxorubicin and 31.2±2.3 MPa in the group of CPC loaded with 10 mg doxorubicin (Figure 2). There were no significant differences among these three groups (p=0.32, ANOVA).

Figure 1. Two types of cylindrical CPCs, which contained only CPC (control) or 10 mg of doxorubicin, were incubated with 10 ml of medium, and the medium was changed every 24 hours for 14 days (n=3). At each time-point, the medium was collected and frozen at −80°C. The RMT-1 E4 cells were incubated with these media and the rate of cell proliferation was measured by the MTT method.

Figure 2. The compressive strengths of the CPC cylinders showed no significant difference among the three groups (p=0.32, ANOVA). The data is shown as mean±S.D., (n=5).
In vitro study of sustained release. When the medium collected after 24 hours was added to the cells, the cell proliferation rate was 8.9±3.0% compared with medium used for CPC only (p=0.0017). The cell proliferation rate in the medium after 48 hours, 3 days and 7 days was 26.2±5.5%, 32.1±2.2% and 35.6±2.7% compared with the control, respectively (p=0.0017, p=0.0185, p=0.02). After 14 days, the cell proliferation rate was not significantly different from that of the control (76.4±15.0%, p=0.0537; Figure 3).

Histological study. In the femurs with CPC alone (control), histological examination showed that mild edematous changes occurred in the medullary space 1 week after implantation. On the other hand, the doxorubicin-loaded CPC group showed diffuse edematous changes (Figure 4A). Four weeks after implantation, reactive zones encompassing new bone formation were observed along the border between the CPC and the medullary space in the controls (Figure 4B), while the doxorubicin-loaded CPC group showed extensive fibrosis and a small focus of new bone formation. Twenty-four weeks after implantation, large bone formations were detected on the surface and within the CPC in the control and doxorubicin-loaded CPC groups (Figure 4C). During the entire period, there was no observable necrosis of cartilaginous tissue and all skin healed without undergoing necrosis.
In vivo study of anticancer effects. The mean major axis of the tumors in the air-pouch gradually increased from 1 week after tumor injection in the controls, reaching 40 mm after 4 weeks. On the other hand, in the doxorubicin-loaded CPC group, the mean major axis of the tumor did not change, but appreciated relative to the size of the solid CPC (Figure 5A and B). Histological findings showed proliferation of the tumor cells extending into the subcutaneous tissue in the controls (upper right). Small tumor masses were observed around the remaining CPC in the doxorubicin-loaded CPC group (lower right). C) In the doxorubicin-loaded CPC group, 12 of the 16 mice (75%) survived for 16 weeks. In contrast, only 2 of the 16 mice (12.5%) survived in the control group. The doxorubicin-loaded CPC group had a significantly better survival rate (p<0.0001).
control group. In contrast, small tumor masses were observed around the remaining CPC in the doxorubicin-loaded CPC group (Figure 5B). In the doxorubicin-loaded CPC group, 12 of the 16 mice (75%) survived for 16 weeks. In contrast, only 2 of the 16 mice (12.5%) survived in the control group. The doxorubicin-loaded CPC group had a significantly better survival rate ($p<0.0001$; Figure 5C). During the entire period, no skin necrosis or redness was observed in the doxorubicin-loaded CPC group.

**Discussion**

The possibility of adding anticancer drugs such as methotrexate (MTX), cis-diaminedichloroplatinum (CDDP) and doxorubicin to hydroxyapatite cement or acrylic cement for the treatment of bone tumor or bone metastasis has been previously reported (5-14). In 1990, Wu et al. (6) first reported on the sustained release of CDDP and doxorubicin from hydroxyapatite, and showed tumor growth suppression when using doxorubicin-impregnated bone cement and ceramic implants in a rat sarcoma model. After this, several reports regarding drug delivery systems for anticancer drugs loaded into hydroxyapatite were published. Uchida et al. (7) and Shinto et al. (8) reported on the sealing of CDDP in hydroxyapatite, while Wang et al. (9, 10) measured the in vitro diffusion of MTX and the in vivo effect of MTX in rabbits. Also, Itokazu et al. (11, 12) reported on the sustained release of doxorubicin from doxorubicin-loaded hydroxyapatite blocks using centrifugation methods. With regard to acrylic cement, Rosa et al. (3) examined the sustained release of doxorubicin, CDDP and MTX.

Recently, CPC has been widely used in orthopedic surgery in cases with fractures (1,2). CPC has several characteristics, including: i) the gel form of CPC can be used to fill bone defects by injection through a small incision; ii) bone formations gradually permeate into CPC, and then replace it; and iii) it is chemically stable at room temperature. In this experiment, the feasibility of adding anticancer drugs such as doxorubicin, which is widely used to treat malignant bone soft tissue tumors, to CPC to develop a new material which can release the drug, as well as fill in postoperative bony defects, was investigated.

In the treatment of primary or secondary bone neoplasms, complete excision with reliable reconstruction is an essential procedure. Since our data showed that the compressive strengths of cylinders of CPC did not decrease when loaded with doxorubicin, doxorubicin-loaded CPC should be useful as a defect filling material. However, it should be noted that bone formation around the doxorubicin-loaded CPC occurred more slowly than that around pure CPC. Similarly, bone formations permeating into CPC may occur at a later stage in doxorubicin-loaded CPC.

The sustained release of doxorubicin from CPC was studied using the methods described by Rosa et al. (3). The direct measurement of the concentration of doxorubicin is unreliable, but medium incubated with doxorubicin-loaded CPC showed a continuous toxic effect for at least 7 days. When 3 antiblastics drugs were included in acrylic cement, the duration of the doxorubicin effect was longer, lasting for 15 days (3). Only doxorubicin particles on the surface of CPC might be released, meaning that if the degradation of CPC can be induced, the local concentration of doxorubicin can be maintained longer. In contrast to the controls, the doxorubicin-loaded CPC group showed edematous changes in the medullary space 1 week after implantation. Since various studies reported that hydroxyapatite and CPC do not inhibit bone formation (14), this may be indicative of a cytotoxic effect of doxorubicin in the medullary space.

The sustained release of anticancer drugs at a local site may reduce systemic side-effects. On the other hand, the local administration of anticancer drugs may induce severe damage to the surrounding tissue including the skin. In particular, diffuse skin damage due to extravasation may occur (15). The current study on the sustained release of doxorubicin did not reveal the development of skin necrosis, even for subcutaneous injection in the air-pouch models.

**References**


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