

## An Experimental Study on the Therapeutic Effect of Consecutive Low-dose Cisplatin with Caffeine in Sarcoma-bearing Mice

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**Abstract.** *The aims of this study were to investigate the anti-tumor effects of consecutive low-dose cisplatin (LD-CDDP) in comparison with single high-dose CDDP (HD-CDDP) in combination with caffeine. Materials and Methods: Human fibrosarcoma cells (HT-1080) were transplanted in BALB/C-nu mice. According to the administration of CDDP and caffeine, 5 groups were defined: HD-CDDP, LD-CDDP, HD-CDDP + caffeine, LD-CDDP + caffeine and control group. The total dose of CDDP was 3.5 or 6 mg/kg. CDDP was injected i.p. bolus (HD-CDDP) or divided into 5 days (LD-CDDP). Caffeine was injected i.p. at a dose of 60 mg/kg twice a day for 4 days. Results: Significant inhibition of tumor growth and prolongation of survival time were recognized in the HD- and LD-CDDP groups with and without caffeine compared to the control group. The caffeine-assisted groups had no advantage compared to the CDDP alone groups. Conclusion: The effects of LD-CDDP were similar to the effect of HD-CDDP.*

CDDP is a platinum analog, which covalently binds to DNA bases and disrupts DNA function (1-3). The restriction factors of CDDP administration are renal insufficiency, nausea and vomiting (1, 4-7). In malignant bone and soft tissue tumors, CDDP is administered by intra-arterial (*i.a.*) or intravenous (*i.v.*) injection at high dose (100-120 mg/m<sup>2</sup>). It is possible to increase the maximum blood concentration (C<sub>max</sub>) of CDDP by bolus administration. The bolus administration of high-

dose CDDP to increase C<sub>max</sub> is a popular administration method in malignant bone and soft tissue tumors.

The anti-tumor effects of CDDP depend on the area under the concentration-time curve (AUC) as well as C<sub>max</sub>, treatment duration and dose (8, 9). Administration of consecutive low doses of CDDP increases the total AUC compared to a single bolus dose (10, 11). In addition, since there is a correlation between the prognosis and the dose intensity (DI) of CDDP within a certain range, the amount of total doses administered during a certain period should be more important than the amount of an individual dose (12, 13). These basic data suggested the efficacy of a consecutive low-dose CDDP, and this administration method has generally been adopted in cancer treatment (14). In addition, dose-limiting toxicities such as renal insufficiency and peripheral neuropathy can be reduced by consecutive low-dose CDDP.

Caffeine, a xanthine analog, enhances the cytotoxic and growth inhibitory effects of DNA-damaging agents such as radiation, ultraviolet light and anti-cancer agents on tumor cells and mammalian cells, due to its DNA repair inhibiting effects (15-18). Caffeine permits cell cycle progression and mitosis before DNA repair is completed, thus leading to mitotic failure and cell death, while tumor cells with damaged DNA delay the G<sub>2</sub>/M- or S-phase in order to repair their damaged DNA (19, 20). In bone and soft tissue tumors, it has been reported that caffeine-assisted CDDP therapy can reinforce a good local control of tumors and clinical prognosis (21, 22). Especially in patients with osteosarcoma, the results of caffeine-assisted chemotherapy in our institute have shown an advantage over those in other institutions (23).

We have previously studied the inhibitory effects on pulmonary metastases of a single high bolus dose and consecutive low doses of CDDP in sarcoma-bearing mice.

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These mice were used as an experimental metastatic model with sarcoma cells injected *via* the tail vein. In that study, the total amount of CDDP was 10 mg/kg. In the single high-bolus-dose group, *i.p.* administration of 10 mg/kg was given in one shot, while the consecutive low-dose group received *i.p.* administration of 2 mg/kg/day for 5 days. As a result, consecutive low-dose CDDP was confirmed to be as effective as the single high-bolus-dose CDDP regarding reduction of the number of pulmonary metastases (24). This result suggested that the consecutive low-dose CDDP chemotherapy might be effective in malignant bone and soft tissue tumors as post-operative chemotherapy performed to reduce metastases. On the other hand, the aims of pre-operative chemotherapy are reduction of the primary lesion as well as of metastasis. Therefore, it was necessary to confirm the anti-tumor effect of consecutive low-dose CDDP on the primary lesion. In addition, it was also examined whether caffeine could have an effect on consecutive low-dose CDDP.

## Materials and Methods

**Test animals.** In this study, slc.ddy mice (Crea Japan, Tokyo, Japan) were used for measurement of pulmonary CDDP concentration and plasma caffeine concentration, as well as for the investigation of adverse drug reactions based on biochemical findings. Male nude mice of the BALB/c-nu (Clear Japan, Tokyo, Japan) were used as tumor-bearing mice. The mice were kept under specific pathogen-free conditions, maintained at a room temperature of 25°C, and fed radiosterilized water and chow. Six-week-old mice were ordered, and the experiment started when the mice were seven weeks old (23-25 g body weight).

**Preparation of model.** Human fibrosarcoma (HT-1080) cells were cultivated as monolayers in RPMI1640 medium with 10% heat-inactivated fetal bovine serum (HyClone, Utah, USA) and 1% penicillin-streptomycin solution (Gibco, New York, USA), with 5% CO<sub>2</sub> at 37°C in culture flasks. Subcultures were made twice a week. Cell suspensions for examination were prepared as follows. Log-phase cells were rinsed once with 0.16 M phosphate-buffered saline (PBS) and a 0.5 ml mixture of 0.1% trypsin (Sigma Chemical Co., St. Louis, USA) and 0.2 mM EDTA was added. The culture was kept at room temperature for 1 to 2 minutes to allow cells to detach and subsequently, 1 to 2 ml DMEN medium with 10% FBS was added, and a homogeneous suspension was obtained. The existence of more than 90% live cells was verified through a 0.3% trypan blue dye exclusion test. HT-1080 cells (5x10<sup>5</sup> cells/ animal) were transplanted on the back of the mice.

**Administration method of CDDP and caffeine.** CDDP dissolved in saline (Randa, Nippon Kayaku, Co, Tokyo, Japan) was used. About one month after transplantation of HT-1080, when the tumor transplanted on the back had grown to be approximately 1 cm in diameter, administration of CDDP started. The total dose of CDDP was 3.5mg/kg or 6 mg/kg. The mice in the single high bolus dose group were injected *i.p.* with 3.5 or 6 mg/kg of CDDP. The mice in the consecutive low dose group were administered *i.p.* with

a daily dose of 0.7 or 1.2 mg/kg/day for 5 days. The mice in the control group received *i.p.* injection of saline only. A solution of 20% caffeine in sodium benzoate (Annaka, Fuso Pharmaceutical Industries, Osaka, Japan) was used. The total dose of caffeine was the dose usually used in a clinical study. Two hours after CDDP injection, caffeine at a dose of 60 mg/kg was injected *i.p.* into mice twice a day for 4 days. According to the dose levels of CDDP and the co-administration of caffeine, the mice were divided into the following 5 groups; the control group received only saline, the high-dose CDDP (HD-CDDP) group received a bolus dose of CDDP, the high-dose CDDP and caffeine (HD-CDDP + caffeine) group received caffeine and bolus dose of CDDP, the low-dose CDDP (LD-CDDP) group received consecutive low-doses of CDDP, and the low-dose CDDP and caffeine (LD-CDDP + caffeine) group received caffeine and consecutive low-doses of CDDP (Figure 1).

**Adverse drug reactions.** The body weights of slc.ddy mice in the control, HD-CDDP + caffeine and LD-CDDP + caffeine groups were measured 1, 2 and 3 weeks after the administration of CDDP (or saline) (n=6). Three weeks after the administration of CDDP (or saline), blood cell counts (erythrocyte, leukocyte and platelet) were measured as an index of myelosuppression, and blood urea nitrogen (BUN) and creatinine were measured as an index of renal insufficiency.

**Concentrations of CDDP in lung.** The mice were sacrificed and the lungs were delivered 24, 48, 72, 96 and 120 hours after the administration of CDDP (or saline). The extracted lungs were decomposed at 80°C for 5 hours. After cooling, liquid components were withdrawn, transferred to 3.5 ml of 40% NaOH, 2.5 ml of Na<sub>2</sub>CO<sub>3</sub> and 300 mg of diethylcarbamide citrate, and then warmed at 80°C for 1 hour. The coat of chloroform was removed. The scum from the coat of chloroform was concentrated, dried and dissolved in methanol. In this material, the concentration of CDDP was measured by absorptiometric analysis (n=3).

**Plasma caffeine concentrations.** Blood was collected 2, 4, 6, 8 and 10 hours after administration of caffeine. Samples were prepared with 0.1 ml of plasma, 0.05 ml IS and 0.1ml H<sub>2</sub>O. After solid-phase sampling by high-performance liquid chromatography, plasma concentrations were measured by absorption spectrophotometry (n=3).

**Tumor growth curve.** Tumor volume transplanted on the back was measured once a week from the start of chemotherapy. The long axis (a) and short axis (b) of the tumor were measured. Reduced tumor weight (RW) was measured according to the formula of RW (mg) = a<sup>2</sup> x b/2, and then a tumor growth curve was obtained.

**Survival rate.** Survival were counted days from the start of CDDP administration and the survival rate was calculated by the Kaplan-Meier method.

**Histological response to chemotherapy.** Three weeks after chemotherapy was started, the mice in each of the 5 groups that were administered 6 mg/kg of CDDP were sacrificed and tumors were fixed in 10% formaldehyde solution (Wako, Tokyo, Japan) for 3 days. The fixed tumors were embedded in paraffin and 4-μm tissue sections were prepared. After hematoxylin and eosin staining, the largest sections were observed under the optical microscope. According to the percentage of viable tumor cells, histological

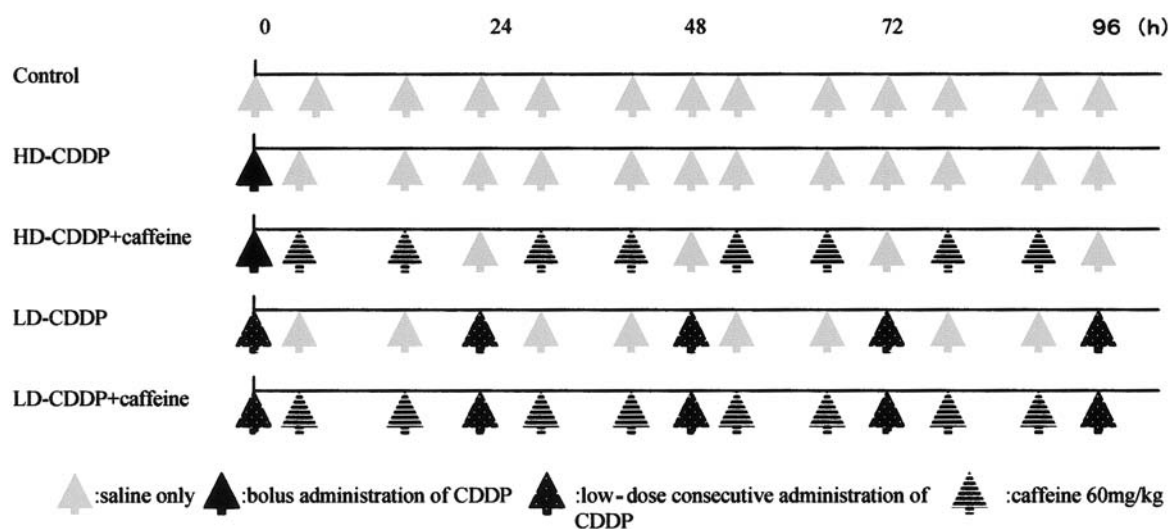


Figure 1. Administration schedule of CDDP and caffeine.

response was evaluated with the grading system of Rosen *et al.* as follows: Grade 1 indicated that little or no effect of chemotherapy was observed; Grade 2 that some areas of histologically viable tumor cells, as well as areas of acellular tumor, necrotic and/or fibrotic material, were observed; Grade 3 that improvement of predominant areas attributed to chemotherapy were observed with only scattered viable tumor cell foci or viable tumor cells; and Grade 4 that no evidence of viable tumor cells was seen in extensive sampling.

**Evaluation of lung metastasis.** Lungs were extracted when the mice died due to the tumor. Large sections of 5 lobes were made, processed for hematoxylin and eosin staining and observed under the optical microscope. The number of pulmonary metastases was counted.

**Statistical analysis.** Repeated measure ANOVA was used for the comparison of changes in body weight. A two-group *t*-test was used for comparison of pre- and post-dose biochemical data which were used as indices of adverse drug reaction. Repeated measure ANOVA and *post hoc* test were used to investigate the changes of tumor volume. The Kruskal-Wallis test was used to count the number of pulmonary metastases. Survival rate was calculated by the Kaplan-Meier method.

## Results

**Adverse drug reactions.** No significant differences were observed in changes of body weight among control, HD-CDDP + caffeine, and LD-CDDP + caffeine groups when the total dose of CDDP was 3.5 or 6 mg/kg (Figures 2-A, 2-B). No significant differences were observed in blood cell counts among control, HD-CDDP + caffeine and LD-CDDP + caffeine groups when the total dose of CDDP was 6 mg/kg (Figures 3-A, 3-B, 3-C). No significant differences were observed in BUN or creatinine among control, HD-CDDP + caffeine and LD-CDDP + caffeine groups when the total dose of CDDP was 6 mg/kg (Figures 4-A, 4-B) ( $n=6$ ).

**Concentrations of CDDP in the lung.** When the total dose of CDDP was 3.5 mg/kg, pulmonary CDDP concentrations were higher in the HD-CDDP group than in the LD-CDDP group until 72 hours after administration. Afterwards, the concentrations became the same in these two groups (Figure 5-A) ( $n=3$ ). When the total dose of CDDP was 6 mg/kg, pulmonary CDDP concentrations were higher in the HD-CDDP group than in the LD-CDDP group until 48 hours after the administration. Afterwards, the concentrations became the same in these two groups (Figure 5-B) ( $n=3$ ).

**Concentration of caffeine in plasma.** Two hours after caffeine was administered, the plasma caffeine concentration rose to be about 60  $\mu\text{l/ml}$ , and decreased afterwards (Figure 6) ( $n=3$ ).

**Growth curve of tumor volume.** Because the skin covering the tumor was ruptured in several mice from 5 weeks after the start of administration of CDDP, the measurement of RW could not be performed in these mice. Therefore, only RW measured until 4 weeks after the start of chemotherapy was subject to analysis. When the total dose of CDDP was 3.5 mg/kg, a significant inhibition of tumor growth was observed in the LD-CDDP + caffeine ( $p=0.038$ ) and HD-CDDP + caffeine ( $p=0.049$ ) groups compared to the control group. However, the difference of the HD-CDDP ( $p=0.50$ ) and LD-CDDP ( $p=0.38$ ) groups from the control group was not significant (Figure 7-A) ( $n=6$ ). When the total dose of CDDP was 6 mg/kg, a significant inhibition of tumor growth was observed in the HD-CDDP ( $p=0.03$ ), HD-CDDP + caffeine ( $p=0.002$ ), LD-CDDP ( $p=0.003$ ), and LD-CDDP + caffeine ( $p<0.001$ ) groups compared to the control group. The difference of the HD-CDDP + caffeine and LD-CDDP + caffeine groups from the

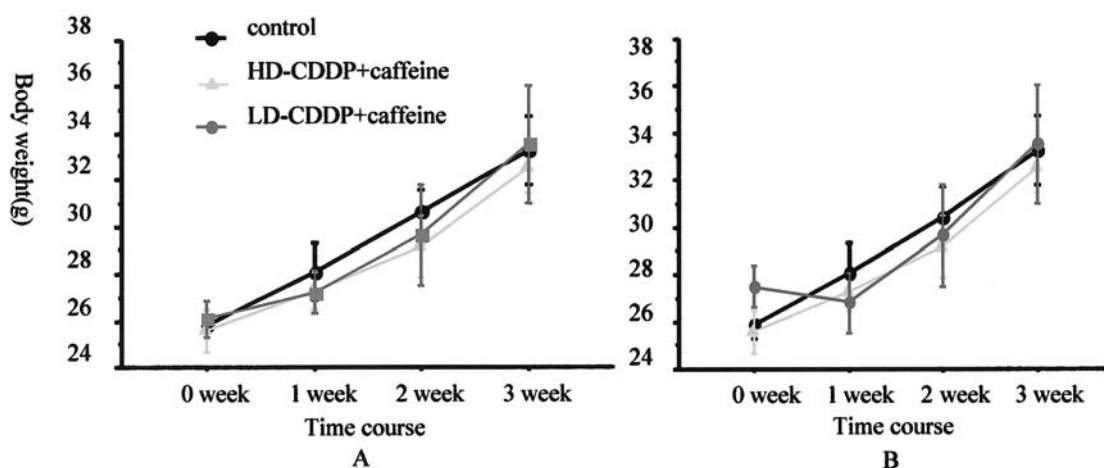


Figure 2. Changes of body weight after administration of CDDP. Each point represents the mean  $\pm$  SD of 6 mice. (A) The total dose of CDDP was 3.5 mg/kg. (B) The total dose of CDDP was 6 mg/kg. At each dose, no significant difference was observed among control, HD-CDDP + caffeine and LD-CDDP + caffeine groups.

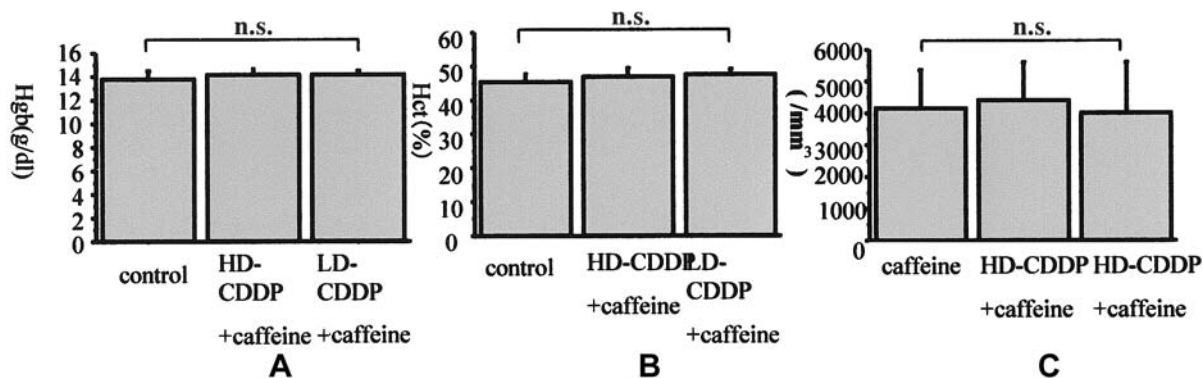


Figure 3. Blood cell counts 3 weeks after administration of CDDP (total dose of CDDP 6 mg/kg) (A). Hemoglobin, (B) Hematocrit, (C) Leukocyte. Not significant difference was observed among control, HD-CDDP + caffeine and LD-CDDP + caffeine groups.

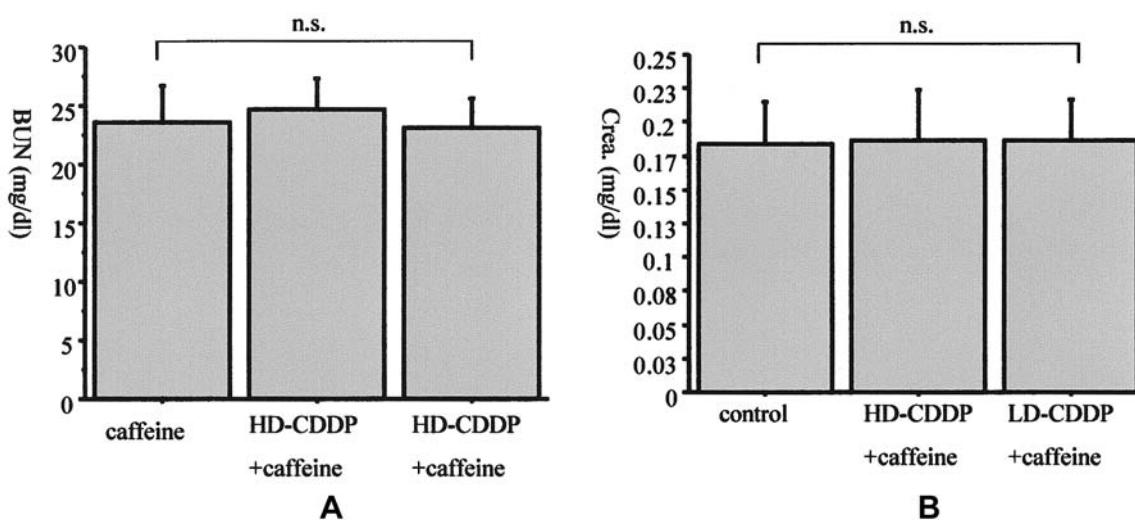


Figure 4. (A) BUN and (B) creatinine 3 weeks after administration of CDDP (total dose of CDDP 6 mg/kg). No significant difference was observed among control, HD-CDDP + caffeine and LD-CDDP + caffeine groups.

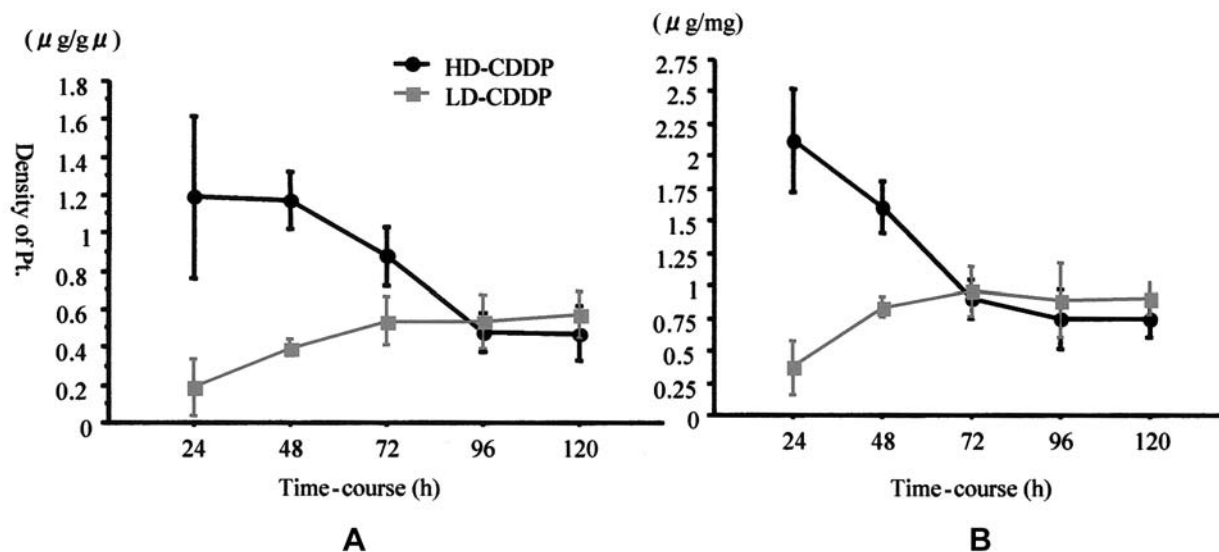


Figure 5. Time-course concentration of CDDP in the lung. (A) The total dose of CDDP was 3.5 mg/kg. (B) The total dose of CDDP was 6 mg/kg.

HD-CDDP ( $p=0.18$ ) and LD-CDDP ( $p=0.37$ ) groups, respectively, was not significant (Figure 7-B) ( $n=6$ ). In short, administration of CDDP assisted with caffeine had no advantage compared with administration of CDDP alone.

**Survival rate.** When the total dose of CDDP was 3.5 mg/kg, no significant difference in survival time was observed among the control, HD-CDDP, HD-CDDP + caffeine, LD-CDDP and LD-CDDP + caffeine groups (Figure 8-A) ( $n=5$ ). When the total dose of CDDP was 6 mg/kg, prolongation of survival time was noted in tumor-bearing mice administered CDDP by either high bolus dose or consecutive low-dose regimens, regardless of caffeine administration, as compared to the control group. The caffeine-assisted chemotherapy (LD-CDDP + caffeine, HD-CDDP + caffeine) groups had no advantage in survival as compared to the CDDP (HD-, and LD-CDDP) groups (Figure 8-B) ( $n=5$ ).

**Histological response to chemotherapy.** Viable tumor cells were still alive in the diffuse area in the control group and the histological response was judged to be grade 1. Tumor necrosis was conspicuous, but viable tumor cells were alive in several areas in the other 4 groups. Therefore, the histological response was judged to be grade 2 in the HD-CDDP, HD-CDDP + caffeine, LD-CDDP and LD-CDDP + caffeine groups (Figures 9-A, 9-B).

**Evaluation of lung metastasis.** When the total dose of CDDP was 3.5 mg/kg, 4.6 nodules of lung metastases on average were observed in 5 out of 6 cases in the control group. On the other hand, only 1 nodule of lung metastases was observed in 1 out

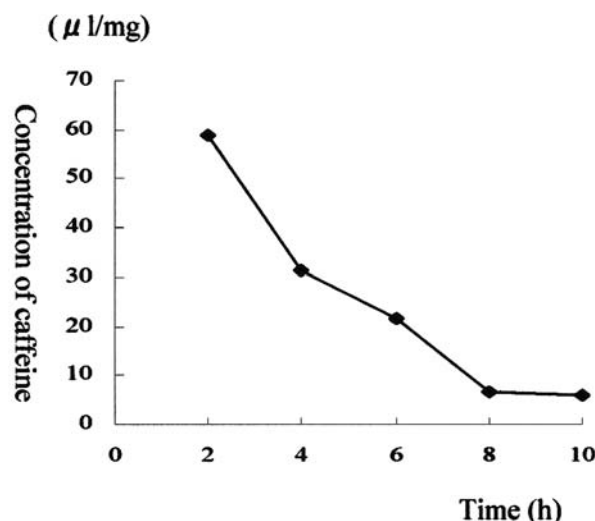


Figure 6. Time-course concentration of plasma caffeine.

of 6 cases in the HD-CDDP, HD-CDDP + caffeine and LD-CDDP groups. No nodule was observed in the LD-CDDP + caffeine group ( $n=6$ ). A significant decrement of lung metastases was observed in the CDDP-administered groups compared with the control group ( $p<0.0001$ ). The difference between HD-CDDP + caffeine and LD-CDDP + caffeine groups and the HD-CDDP ( $p=0.86$ ) and LD-CDDP ( $p=0.82$ ) groups, respectively, was not significant. In short, administration of CDDP assisted with caffeine had no advantage compared with administration of CDDP alone.

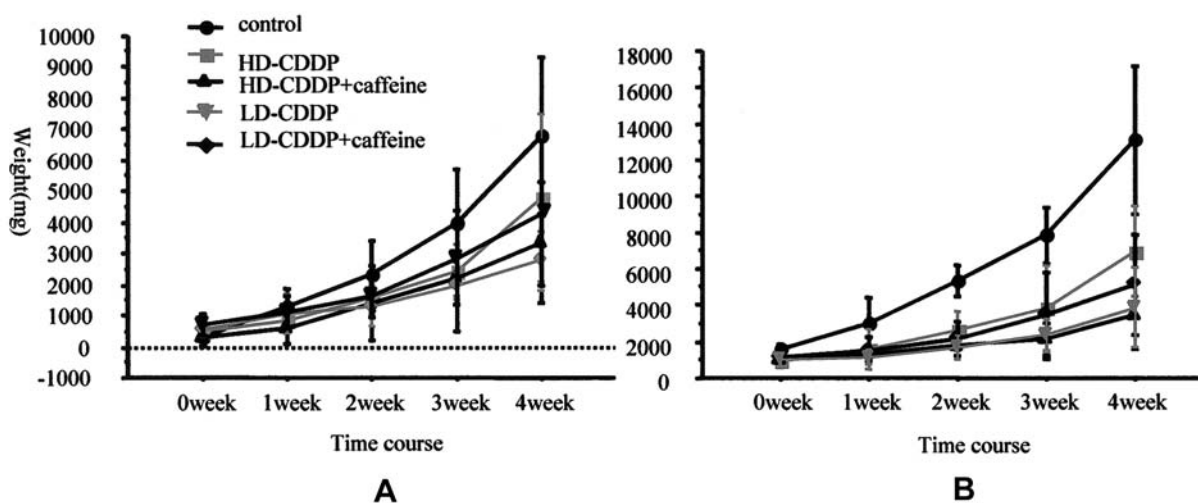


Figure 7. Growth curve of tumor volume. (A) Total dose of CDDP: 3.5 mg/kg. Significant inhibition of tumor growth was observed in LD-CDDP + caffeine ( $p=0.038$ ) and HD-CDDP + caffeine ( $p=0.049$ ) groups compared to the control group. (B) Total dose of CDDP: 6 mg/kg. Significant inhibition of tumor growth was recognized in HD-CDDP ( $p=0.03$ ), HD-CDDP + caffeine ( $p=0.002$ ), LD-CDDP ( $p=0.003$ ) and LD-CDDP + caffeine ( $p<0.001$ ) groups. The difference of HD-CDDP + caffeine and LD-CDDP + caffeine groups and HD-CDDP ( $p=0.18$ ) and LD-CDDP ( $p=0.37$ ) groups was not significant.

## Discussion

Pulmonary CDDP concentrations in the HD-CDDP group were the same as those in the LD-CDDP group from 72 or 96 hours after administration of CDDP. This might be the reason why the effect of the single high-bolus-dose CDDP was the same as that of the consecutive low-dose CDDP. In addition, previous reports have indicated that plasma and tissue concentrations of CDDP are equal regardless of administration methods (*i.e.*, *i.p.* or *i.v.*). Therefore, similar results might be obtained if *i.v.* administration were performed.

This study suggested that consecutive low-dose CDDP had the same inhibitory effect on tumor growth and the number of lung metastases as single high-bolus-dose CDDP. In addition, prolongation of survival time was noted when a consecutive low dose of CDDP or a single high bolus dose of CDDP was administered. This suggests that the consecutive low-dose CDDP has clinically the same anti-tumor effect as the single high-bolus-dose CDDP in malignant bone and soft tissue tumors.

In this study, no significant adverse drug reactions were observed in the HD-CDDP, LD-CDDP, or caffeine-assisted groups, even when the total dose of CDDP was 6 mg/kg. Restriction factors of CDDP administration in the clinical setting are renal insufficiency, nausea and vomiting. It is obvious that dose-limiting toxicities such as renal insufficiency and peripheral neuropathy can be reduced by consecutive low-dose CDDP (25-38). Low-dose CDDP may

provide reduced adverse drug reactions, with the same therapeutic effect as high-bolus-dose CDDP.

Significant inhibition of tumor growth was observed in the LD-CDDP + caffeine and HD-CDDP + caffeine groups compared to the control group when the total amount of CDDP was 3.5 mg/kg. However, the difference in inhibition of tumor growth between the caffeine-assisted groups (HD-CDDP + caffeine and LD-CDDP + caffeine groups) and the CDDP groups (HD-CDDP and LD-CDDP groups) was not significant. In short, no additional effect of caffeine could be observed in this study. The maximum concentration of caffeine was about 60  $\mu\text{l/ml}$ , which was high but below the acute toxic level (29-31). Clinically, since caffeine is administered by continuous intravenous drip infusion, its concentration is maintained at high levels. In this study, the concentration of caffeine could not be maintained, which might be one of the reasons that no additional effects could be observed. Clinically, radiographic evaluations are performed following 3 cycles of caffeine-assisted chemotherapy. Even if the effect of chemotherapy is not observed by radiographic evaluations after only one cycle of chemotherapy, an effect may be observed following 2 and 3 cycles of chemotherapy (32). The evaluation of the effect of caffeine-assisted chemotherapy after only one cycle may have no validity even in experimental studies. The add-on effect of caffeine can be reinforced for tumor cells that are only sensitive to CDDP (29-30). Therefore, the effect of caffeine-assisted CDDP chemotherapy may depend mainly on the sensitivity of tumor cells to CDDP.

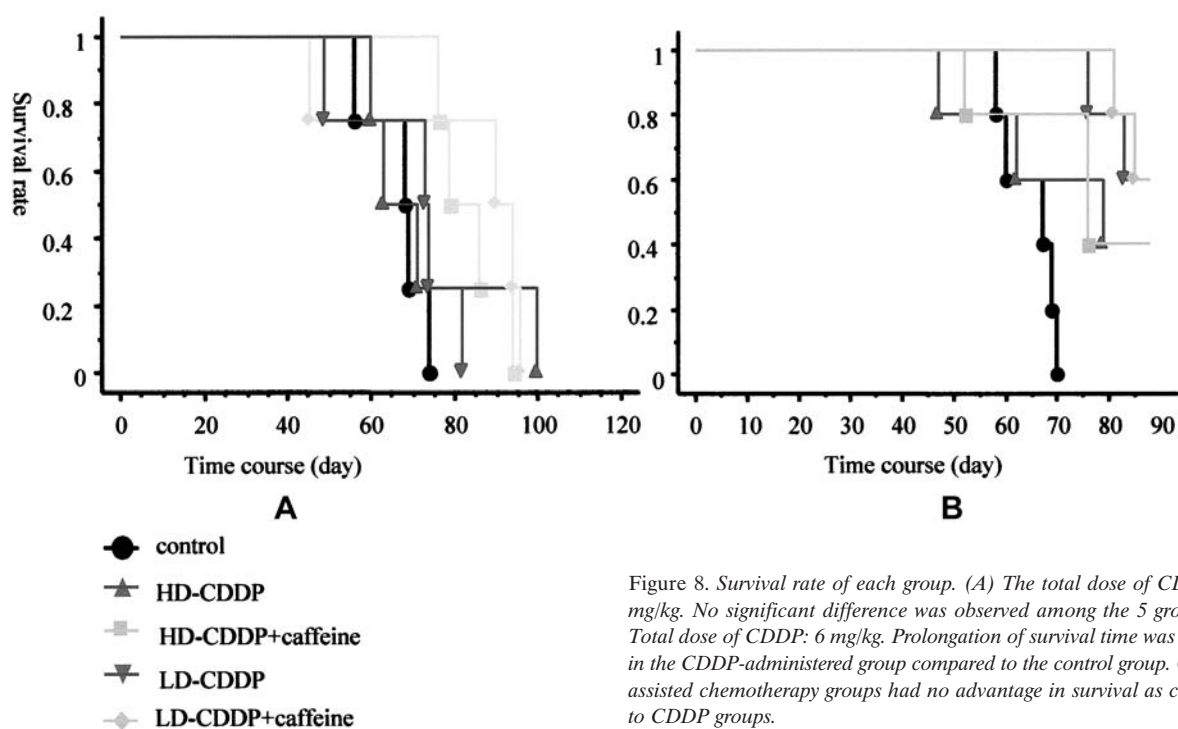


Figure 8. Survival rate of each group. (A) The total dose of CDDP: 3.5 mg/kg. No significant difference was observed among the 5 groups. (B) Total dose of CDDP: 6 mg/kg. Prolongation of survival time was observed in the CDDP-administered group compared to the control group. Caffeine-assisted chemotherapy groups had no advantage in survival as compared to CDDP groups.

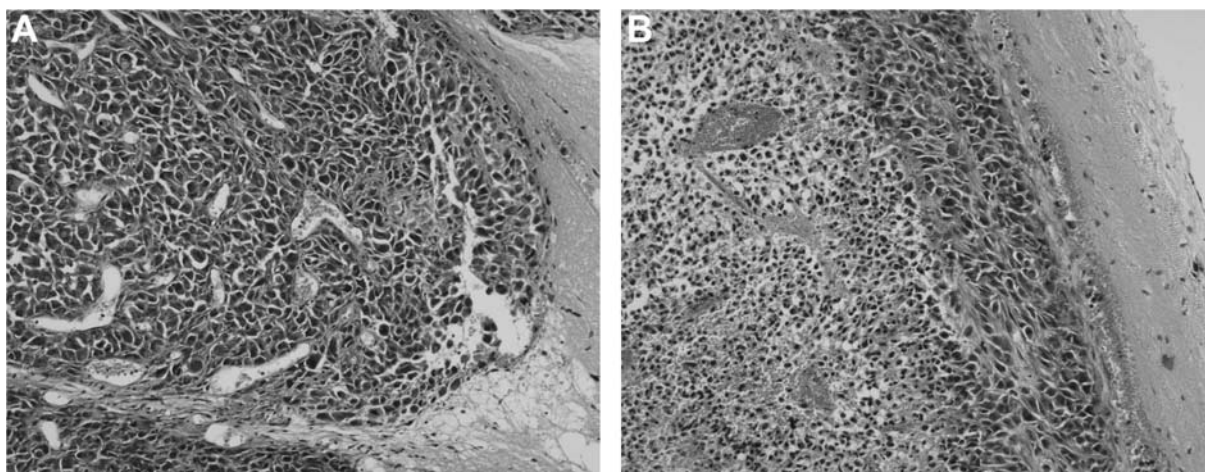


Figure 9. Histological response to chemotherapy. (A) Control group. Little effect of chemotherapy was observed. (B) LD-CDDP group. Some areas of histologically viable tumor cells, as well as areas of acellular tumor, were observed.

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