

Comparison of the cytotoxic effects of single and divided treatment of 4-hydroxycyclophosphamide at the same total dosage amount in canine lymphoma cell lines

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Abstract: Cyclophosphamide is widely used in combination chemotherapy to treat dogs with lymphoma. The metabolite of cyclophosphamide, acrolein, can irritate the urinary bladder and cause sterile haemorrhagic cystitis. The divided administration of cyclophosphamide across multiple days may reduce the occurrence of the cystitis. However, the therapeutic effect of this modification has not been evaluated and compared to the traditional single maximum-tolerated dose. It is difficult to evaluate the cytotoxic effect by the single chemotherapeutic drug in dogs. In order to verify the effect of the single and divided treatment of cyclophosphamide in canine lymphoma, we used two canine lymphoma cell lines (CLBL-1, B-cell lymphoma and UL-1, T-cell lymphoma) to imitate the clinical conditions. The cell viability in the CLBL-1 and UL-1 cells treated by a single dosage of 4-hydroxycyclophosphamide after 48 h were 70.4% and 61.5%, respectively. The cell viability in the CLBL-1 and UL-1 cells treated by the divided dosage of 4-hydroxycyclophosphamide after 48 h were 109.4% and 50.8%. There were no significant differences between the two administration methods in the T-cell lymphoma cell line ($P = 0.215$). The single full dosage of 4-hydroxycyclophosphamide exhibited a significant cytotoxic effect rather than the divided dosage in B-cell lymphoma cell line ($P = 0.007$) did. The maximum-tolerated dose of cyclophosphamide is still recommended to be used in dogs with B-cell lymphoma.

Keywords: cyclophosphamide; cytotoxic effect; dog; lymphosarcoma

Lymphoma is one of the most common neoplasms in dogs. The CHOP-based chemotherapy protocol that includes the use of cyclophosphamide (CP), hydroxydaunorubicin, vincristine and prednisolone is most commonly used. A complete remission rate of 70–90% was reported with a median first remis-

sion duration of approximately nine months (Stone et al. 1991; Flory et al. 2008).

CP is an alkylating agent that is widely used in different combination chemotherapy protocols (Stanton and Legendre 1986). CP is oxidised by the cytochrome P450 system to form 4-hy-

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droxycyclophosphamide (4-HYCP). After entering the intracellular space, 4-HYCP is converted into phosphoramidate mustard and acrolein (Ren et al. 1998). Phosphoramidate mustard is mainly responsible for the antitumor effect, and acrolein is considered to cause cystitis (Dobson 2014). In addition, acrolein may cause oedema, haemorrhage, necrosis, and fibrosis of the bladder epithelium without a bacterial infection as a sterile haemorrhagic cystitis (SHC) (Crow et al. 1977). Some authors revealed that higher single doses, higher cumulative doses and the prolonged application of cyclophosphamide were the main risk factors for developing SHC (Crow et al. 1977; Gaeta et al. 2014; Laberke et al. 2014). Many studies have been conducted and practical suggestions have been provided to reduce the risk of SHC, some of which are as follows: CP should be administered in the morning, water intake and urination should be encouraged, CP should be administered with furosemide or glucocorticoids, and MeSNa (sodium methanethiolate) should be used to bind the acrolein in the urinary bladder (Dobson 2014; Laberke et al. 2014). Best and Fry (2013) reported that the divided maximum-tolerated dose of oral CP administered over three days in combination chemotherapy for canine lymphoma could reduce the incidence of SHC. However, the exact therapeutic effect of the divided CP dosage was not tested because the patients received a multidrug chemotherapy procedure. This modification was contrary to the traditional oncologic principle that a chemotherapeutic drug should be used at or near its maximum-tolerated dosage (Saba et al. 2009). The half-life of CP in the serum is variable, and its metabolites are excreted in the urine predominantly within the first 24 hours (Dobson 2014). The divided administration of CP over a few days may affect the maximum concentration of this cytotoxic drug in the serum.

In clinical practice, lymphoma is primarily treated with combination chemotherapy. Therefore, it is difficult to evaluate the cytotoxic effect of a single chemotherapeutic drug on dogs. To verify the effect of a single and divided treatment of CP on canine lymphoma, we used two canine lymphoma cell lines to imitate the clinical conditions. In this study, we evaluated the cytotoxic effects of a single and divided treatment of 4-HYCP at the same total dosage on canine lymphoma cell lines.

MATERIAL AND METHODS

Lymphoma cell lines

Two canine lymphoma cell lines (CLBL-1 and UL-1) were used in this study. CLBL-1 is a B-cell lymphoma cell line and was donated by Dr Rütgen's laboratory (University of Veterinary Medicine Vienna, Vienna, Austria) (Rutgen et al. 2010). UL-1 is a T-cell lymphoma cell line and was donated by Dr Tsujimoto's laboratory (The University of Tokyo, Tokyo, Japan) (Umeki et al. 2012). The lymphoma cells were cultured in RPMI 1640 (Simply, GeneDireX, Taipei, Taiwan) supplemented with 10% foetal bovine serum (FBS, Gibco, USA) and 1% antibiotics (Simply, GeneDireX, Taipei, Taiwan) at 37 °C in a humidified 5% CO₂ incubator.

Cell viability assay

The cytotoxic effect of 4-HYCP (Toronto Research Chemicals, Toronto, Canada) on the two lymphoma cell lines was examined using a cell viability assay. The lymphoma cells were seeded at 20 000 cells/well in 96-well plates in a medium containing different concentrations of 4-HYCP (10, 1, 0.1, 0.01, and 0.001 µg/ml) and incubated for 48 hours. At 48 hours after the drug treatment, the cells were collected and mixed with the cell proliferation reagent WST-1 (Roche, Mannheim, Germany) for three hours. The optical density was measured using a Multiskan GO (Thermo Fisher Scientific, Waltham, USA) at a wavelength of 450 nm for the cell viability test, and the LD-50 concentration was calculated by linear regression (Lieberman 1983). All the samples were tested in triplicate.

The two lymphoma cell lines were seeded at 20 000 cells/well in 96-well plates in the aforementioned culture medium. The control group cells were mixed with the LD-50 concentration of 4-HYCP and incubated for 48 hours. The experimental group was mixed with half of the LD-50 concentration of 4-HYCP for 24 h and further mixed with the second half of the LD-50 concentration of 4-HYCP for the next 24 hours. All the cells were collected at 48 h after the first time the drug was administered. The cells were then mixed with WST-1, and the optical density was measured for the cell viability test. The samples were tested in triplicate in three independent experiments.

Statistical analysis

The differences in the cytotoxic effects between the two treatment methods were evaluated using Student's *t* test. The *P* value of < 0.05 was considered significant for the analysis.

RESULTS

The mean LD-50 concentrations of 4-HYCP in the CLBL-1 and UL-1 cell lines were 39.5 ng/ml and 548.5 ng/ml, respectively. Doses of 40 and 500 ng/ml for the CLBL-1 and UL-1 cells, respectively, were administered in the subsequent experiments to evaluate the cytotoxic effect of the single and divided dosages of 4-HYCP. In the experimental group, the 4-HYCP concentrations of 20 and 250 ng/ml were added twice and mixed with the CLBL-1 and UL-1 cells in the beginning and 24 h later. The viability of the CLBL-1 and UL-1 cells treated with a divided dosage of 4-HYCP after 48 h was 109.4% and 50.8%, respectively. In the control group, the 4-HYCP concentrations of 40 and 500 ng/ml were added and mixed with the CLBL-1 and UL-1 cells only once in the beginning of the study. The viability of the CLBL-1 and UL-1 cells treated with a single dosage of 4-HYCP after 48 h was 70.4% and 61.5%, respectively. Compared with the divided dosage, the single full dosage of 4-HYCP exerted a significant cytotoxic effect on the B-cell

lymphoma cell line ($P = 0.007$) (Figure 1). No significant difference was observed between the effect of the two administration methods on the T-cell lymphoma cell line ($P = 0.215$) (Figure 2).

DISCUSSION

Studies in humans have reported an incidence of CP-induced haemorrhagic cystitis of 2–40% (Bennett 1974). Approximately 10% of canine lymphoma patients developed haemorrhagic cystitis after receiving CP (Gaeta et al. 2014). This adverse effect can be mild (self-limited) or severe (life-threatening). Two formulations of CP, namely parenteral and oral, are administered by veterinarians. One study indicated that intravenous and oral administrations can result in equal metabolites (Warry et al. 2011). The incidence of SHC following the two administration methods did not differ significantly (Laberke et al. 2014). In a clinical setting, oral medication is a fast and low-cost administration method. Oral medication can also reduce the damage of the peripheral veins and without producing any cytotoxic contaminants of the disposable medical equipment. Because of the advantage of the oral administration of the aforementioned CP, most veterinarians choose to use an oral CP instead of a parenteral CP. The main disadvantage is that the calculated dose must be rounded down to the nearest possible dose that is available. The owner

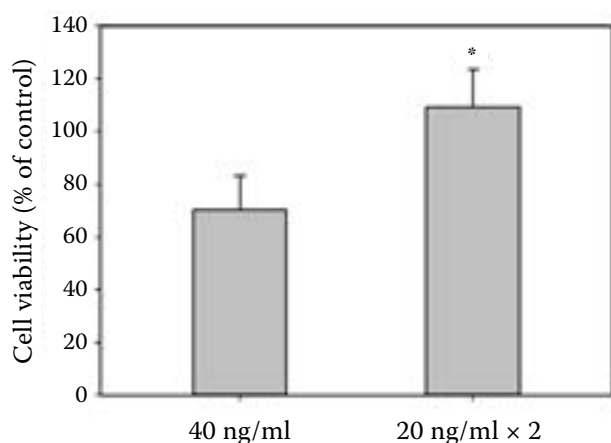


Figure 1. The cell viability of the CLBL-1 cells treated with a single or divided dosage of 4-hydroxycyclophosphamide after 48 hours. The single full dosage of 4-hydroxycyclophosphamide exerted a significant cytotoxic effect than did the divided dosage on the B-cell lymphoma cell line ($P = 0.007$). *Significant difference ($P < 0.05$)

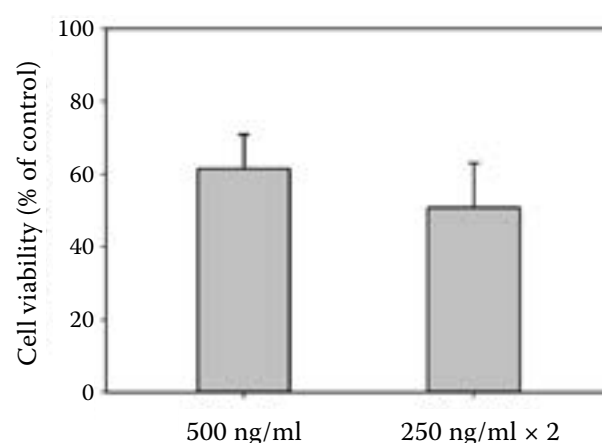


Figure 2. The cell viability of the UL-1 cells treated with a single or divided dosage of 4-hydroxycyclophosphamide after 48 hours. There are no significant differences between the single or divided dosage of 4-hydroxycyclophosphamide on the T-cell lymphoma cell line ($P = 0.215$)

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also must independently handle the cytotoxic medication at home (Warry et al. 2011; Best and Fry 2013; Laberke et al. 2014). Other possible disadvantages include a decreased cytotoxic effect. Best and Fry (2013) reported that no dogs developed CP-induced myelosuppression after the administration of a single maximum-tolerated dose of CP over three days. In contrast to the findings of other research, the incidence of CP-induced myelosuppression was 38% in their study when a dose of 250 mg/m² was administered as a single treatment (Morrison-Collister et al. 2003). Chemotherapy-induced neutropenia was associated with the prolonged remission and survival time in canine lymphoma (Wang et al. 2015). Dividing the maximum-tolerated dose of CP over three days may reduce the cytotoxic effect and affect the remission and survival time.

The incidence of SHC in dogs who received a divided dose of CP over three days was significantly lower than that in dogs who received a single dose of CP without diuretics (Best and Fry 2013). However, the cytotoxic effect of CP was not evaluated in their study. In this study, we used two lymphoma cell lines to determine the *in vitro* effect of the single and divided treatment of 4-HYCP, a metabolite of CP, at the same total dosage. Our results indicated that divided dosage of 4-HYCP significantly reduced the cytotoxic effect compared with a single dose of 4-HYCP in the B-cell lymphoma cells. Our data suggest, that a single administration of the maximal tolerated dose of CP is more effective than one divided over several days in B-cell lymphoma. However, prospective clinical studies are strongly warranted.

The same conclusion was not found for the T-cell lymphoma cells. The cytotoxic effect of the full or divided dosage of 4-HYCP on the T-cell lymphoma cells was not significantly different. This finding may be attributed to the different immunophenotypes and variance in the drug sensitivity. The LD-50 concentration of 4-HYCP differed considerably between the B-cell and T-cell lymphoma cells, ranging from 39.5 ng/ml to 548.5 ng/ml. The T-cell lymphoma (UL-1) required a 4-HYCP dosage that was approximately 13 times higher to induce the same cytotoxic effect compared with the B-cell lymphoma (CLBL-1) in our study. A high therapeutic dosage of this cytotoxic drug for T-cell lymphoma may inhibit its usage in clinical treatment, namely because a high dose may result in a high possibility of bone marrow and gastrointestinal toxicity.

CP is commonly used in combination with hydroxydaunorubicin, vincristine, and prednisolone to treat canine lymphoma. However, a clinical relapse occurs more frequently after the administration of the maximum-tolerated dosage of CP than after that of vincristine or doxorubicin, suggesting the low cytotoxic effect of the CHOP-based protocol (Wang et al. 2016). The divided dosage of CP may decrease the cytotoxic effect. According to the findings of our previous study and of this study, replacing CP with other cytotoxic drugs may improve the clinical outcomes. If CP is still required, then it should be administered in its full maximum-tolerated dosage rather than in its divided dosage.

We can use other methods to prevent SHC in clinical practice, such as administering CP in the morning to allow its excretion through urination during the day, encouraging water intake or combining diuretics or glucocorticoids to increase the urination frequency, and administering MeSNa to bind with the acrolein in the urine (Crow et al. 1977; Dobson 2014; Laberke et al. 2014). In the study conducted by Best and Fry (2013), the incidence of SHC in dogs who received a single dose of CP with diuretics was not significantly different from that of dogs who received a divided dosage of CP over the course of three days. This prophylactic method of combining CP with diuretics for reducing SHC is better than administering a divided dosage of CP in yielding significant treatment effects. The effects of the diuretics and glucocorticoids are also influenced by the degree to which the owner encourages and permits the dog's urination (Laberke et al. 2014). Some studies have reported that glucocorticoids may affect the biotransformation of CP through their effects on the hepatic microsomal enzymes (Toru et al. 1969). However, another study reported that the plasma or urine concentration of CP or its metabolites did not significantly differ between the group that received oral prednisolone and the control group (Hanasono and Fischer 1972). Additional studies are required to clarify the correlation between the glucocorticoids and CP.

We used 4-HYCP rather than CP in this study. The major reason is that CP is oxidised by the cytochrome P450 system to form 4-HYCP, which can enter the intracellular space and cause cytotoxicity (Ren et al. 1998; Huitema et al. 2000).

We usually divided the oral CP over two days in a canine lymphoma patient rather than three

days. The possible disadvantage of the three-day regimen included delayed myelosuppressive nadir and reduced efficacy. The metabolites of CP are excreted in the urine predominantly within the first 24 hours (Dobson 2014). Therefore, we used 48 h for the drug treatment in this study.

This study has some limitations. We used two lymphoma cell lines to demonstrate the cytotoxic effect of 4-HYCP, which may not represent the clinical outcomes of dogs with lymphoma. Second, the clinical maximum-tolerated dosage of CP was not assessed in this study. Therefore, we could not confirm whether the concentration of 4-HYCP we used was suitable to represent the bioavailability of CP.

In conclusion, the cytotoxic effect of the divided dosage of 4-HYCP was significantly decreased over the single full dose of 4-HYCP in the canine B-cell lymphoma cells. The maximum-tolerated dose of CP is still recommended to be used in dogs with B-cell lymphoma. The T-cell lymphoma cells need a higher dosage of 4-HYCP than the B-cell lymphoma cells to induce the death of the cancer cells. Further clinical research is needed to confirm the cytotoxic effect of the single or divided dosage of CP on dogs with lymphoma.

Conflict of interest

The authors declare no conflict of interest.

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