SPANDIDOS PUBLICATIONS

Combining anti-tumor drugs with mild hyperthermia increases the cytotoxic effects of drugs on human leukemia cells *in vitro*

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Abstract. Although the benefits of using a combination of hyperthermia and chemotherapy or radiotherapy in the treatment of cancer have been theoretically established, the use of such combination therapy is not widespread at the clinical level, as the application of hyperthermia is complex and maintaining a tumor temperature of 43˚C or higher is exceedingly difficult. Consequently, in the present study, the effects of chemotherapy combined with mild hyperthermia at 41˚C (which is easier to apply than standard hyperthermia) were examined in the NALM-6 leukemia cell line. The results were as follows: i) NALM-6 leukemia cells, like most cells, survived mild hyperthermia at 41˚C, but were killed at temperatures over 43˚C. ii) Low concentrations of adriamycin (0.1 μg/ml) or mild hyperthermia applied separately did not have a visible effect on the survival rate of NALM-6 cells, whereas combined treatment with these therapies decreased the survival rate of NALM-6 cells in a time-dependent manner. The anti-tumor effect after 5 h of the combination of 0.1 μg/ml adriamycin and mild hyperthermia was the same as that observed with a 10-fold higher concentration (1 μg/ml) of adriamycin alone. iii) Another anti-tumor drug, vincristine, exhibited the same behavior as adriamycin. The anti-tumor effect after 1 h of the combination of $5x10^{-11}$ M vincristine and mild hyperthermia was the same as that observed with a 10-fold higher concentration ($5x10^{-10}$ M) of vincristine alone. The results indicate that it may be possible to reduce the required concentrations of anti-tumor drugs by using them in combination with mild hyperthermia. In this way, the side effects of chemotherapy may be reduced in clinical settings. Mild hyperthermia is a useful and practical heating method, and could result in the increasing clinical application of hyperthermia in the treatment of cancer.

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Introduction

There are four therapeutic modalities used in the treatment of human cancer: surgery, radiotherapy, chemotherapy and hyperthermia. The efficacy of hyperthermia in combination with chemotherapy or radiotherapy, or of the combination of all three agents, has been established experimentally and clinically (1,2). However, although chemotherapy is used at virtually all hospitals and medical institutions, very few institutions employ it in combination with hyperthermia.

At present, the use of hyperthermia is not widespread due to the difficulty of heating and maintaining tissues at a temperature of 43˚C or more, and because the devices used for applying clinical hyperthermia along with the associated personnel costs are high. Therefore, in the present study, the effects of mild hyperthermic heating were examined at a lower temperature of 41˚C, as opposed to hyperthermic temperatures of 43˚C or more. The temperature used for mild hyperthermia, 41˚C, is not sufficient for tumor cell apoptosis. However, it is relatively easy to heat and maintain cells and tissue at this temperature. In addition, mild hyperthermia is a less severe form of therapy for cancer patients than standard hyperthermia.

Hyperthermia as a form of cancer therapy is generally applied to local solid tumors, and there have been few reports concerning its use in patients with leukemia. However, there have been reports examining its effects on leukemia cells *in vitro* (3,4). Since standard hyperthermia is not applicable in a systemic cancer such as leukemia, a safe and mild form of hyperthermia suitable for whole-body use would be beneficial.

The treatment of leukemia has been improved by advances in chemotherapy, radiotherapy and blood-forming stem cell transplants (5-7). However, these therapies are not necessarily effective, as leukemia patients face problems involving drug tolerance, toxicity, treatment side effects and advanced age.

In this study, the effects of a combination of mild hyperthermia and chemotherapy on leukemia cells were examined *in vitro*.

Materials and methods

Cell line. NALM-6 is an acute lymphoblastic leukemia cell line. NALM-6 cells were cultured in RPMI-1640 culture

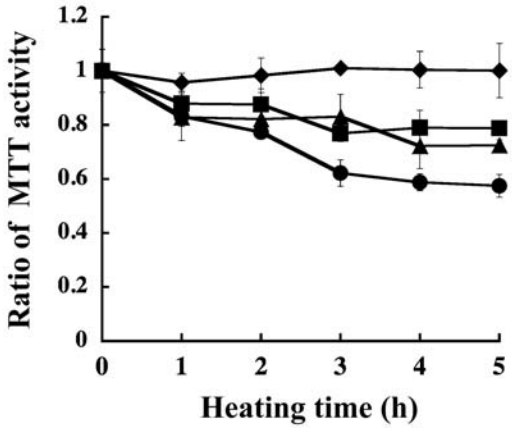

Figure 1. Thermal sensitivity of the human leukemia cell line NALM-6. NALM-6 cells were incubated at 41 (♦), 43 (■), 45 (▲) or 47˚C (●) for 0, 1, 2, 3, 4 and 5 h. Cell viability of the NALM-6 cells was measured using the MTT method after incubation. A lower MTT activity ratio indicates lower cell numbers.

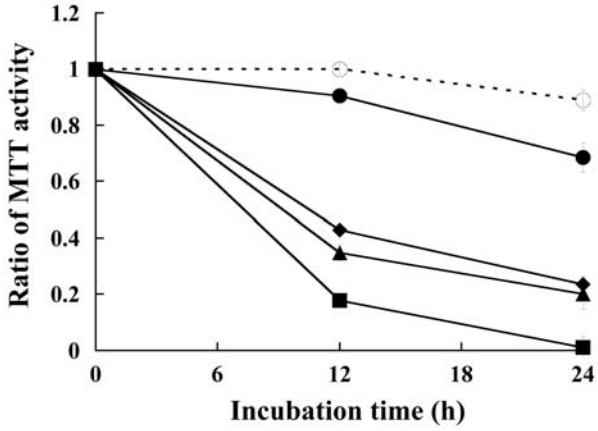

Figure 2. Changes in survival fraction for the human leukemia cell line NALM-6 after exposure to the anti-tumor drug adriamycin. NALM-6 cells were incubated with adriamycin at concentrations of 0 (○), 0.1 (●), 1 (♦), 10 (▲) and 100 μg/ml (■) at 37˚C for 0, 12 and 24 h. Cell viability was measured using the MTT method after the indicated incubation periods.

medium containing 10% FCS, and were used in experiments during the log phase of growth. The cells were grown in 48-well plates with $4x10^5$ cells in 0.4 ml of culture medium per well, and plates were used in experiments one day after being prepared and incubated in a standard humidified CO_2 incubator at 37˚C.

Cell survival rate. The rate of cell survival was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method (Cell Proliferation Kit; Roche Diagnostics, Mannheim, Germany) (8). After cells were treated, MTT reagent was added to an aliquot of cell medium (1/10 volume). This was incubated at 37˚C for 2 h, then an equal volume of 10% SDS was added to the reaction. Absorbance was measured at 550 nm and was proportional to the number of cells.

Thermal sensitivity of NALM-6 leukemia cells. NALM-6 cells were incubated in 48-well plates for 0-5 h at 41, 43, 45 and 47˚C, respectively, with a device capable of maintaining very precise temperatures (temperature accuracies of ± 0.01˚C; Nihon Kouseikagaku Res. Co., Osaka, Japan). The survival rate for the heated cells was measured using the MTT method.

Response of NALM-6 leukemia cells to anti-tumor drugs. Adriamycin (ADR) and vincristine (VCR), anti-carcinogenic drugs typically applied in the treatment of leukemia, were used in the experiments. NALM-6 cells were treated with ADR at final concentrations of 0.1, 1, 10 and 100 μg/ml in medium, and with VCR at final concentrations of $5x10^{-11}$, $5x10^{-10}$, $5x10^{-9}$ and $5x10^{-8}$ M.

In experiments measuring cell sensitivity to anti-tumor drugs, NALM-6 cells were treated with four concentrations of ADR or VCR for 12 and 24 h, respectively. Cell viability was then measured with an MTT assay.

Combination of chemotherapy and mild hyperthermia. In experiments designed to combine drug exposure and mild hyperthermia, NALM-6 leukemia cells were exposed to final ADR concentrations of 0.1 and 1 μg/ml or to VCR concentrations of $5x10^{-11}$ and $5x10^{-10}$ M at 41˚C for 0-5 h.

The total incubation time for all samples was 12 h, at both 41 and 37˚C. For example, if samples were treated at 41˚C for 3 h, they were then incubated at 37˚C in a CO_2 incubator for an additional 9 h, for a total of 12 h. Subsequently, MTT reagent was added to samples of incubation medium from the cells, and the cell survival rate was determined.

Results

Thermal sensitivity of leukemia cells. As shown in Fig. 1, ~100% of the NALM-6 leukemia cells survived a 5-h incubation at 41˚C. After exposure to 43˚C temperatures, the survival rate of NALM-6 cells was ~90% after 2 h incubation and ~80% after 3-5 h. At 45˚C, the survival rate of NALM-6 cells was ~80% after 1-3-h and ~70% after 4-5 h. At 47˚C, the surviving fraction of NALM-6 cells decreased almost linearly over a 3-h incubation, and then more slowly to 60% after 4-5 h.

The surviving fraction of NALM-6 leukemia cells was not affected by exposure to temperatures of 41˚C, and cell death after exposure to 43˚C was typical of cells in culture.

Sensitivity of leukemia cells to anti-tumor drugs. Fig. 2 shows the surviving fractions of NALM-6 leukemia cells after exposure to ADR concentrations of 0.1-100 μg/ml. The surviving fraction of NALM-6 cells after exposure to 0.1 μg/ml ADR was not strongly affected, even after 24 h of incubation. At this concentration, the tumoricidal effect in NALM-6 cells was barely apparent after 12 h, and the survival rate decreased slightly at 24 h. The surviving fraction of NALM-6 cells decreased drastically with increasing concentrations of ADR (1-100 μg/ml) after 12 h of incubation. The surviving fractions of NALM-6 cells after VCR concentrations of $5x10^{-11}$ to $5x10^{-8}$ M are shown in Fig. 3. Survival decreased linearly with increasing incubation time for each concentration of VCR.

Combination of anti-tumor drugs and mild hyperthermia. The surviving fraction of NALM-6 leukemia cells after exposure to

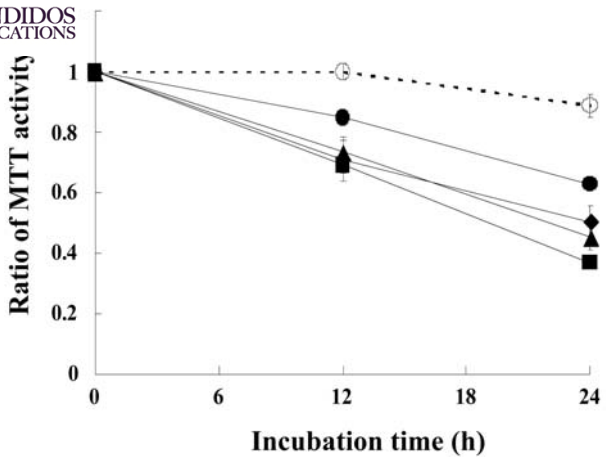

Figure 3. Changes in the surviving fraction of human leukemia NALM-6 cells after exposure to the anti-tumor drug vincristine (VCR). NALM-6 cells were incubated with vincristine at concentrations of 0 (○), $5x10^{-11}$ (●), $5x10^{-10}$ (♦), $5x10^{-9}$ (▲) and $5x10^{-8}$ M (■) at 37˚C for 0, 12 and 24 h. Cell viability was measured using the MTT method after the indicated incubation periods.

anti-tumor drugs and mild hyperthermia is shown in Fig. 4. As in Fig. 1, the survival of NALM-6 cells was not altered by treatment with mild hyperthermia at 41˚C for 5 h, nor was it strongly affected by low concentrations of anti-tumor drugs, such as 0.1 μg/ml ADR for 12 h (Fig. 2). However, though mild hyperthermia or low concentrations of the anti-tumor drugs ADR and VCR alone had no influence on the survival of NALM-6 cells, survival rates decreased when low concentration drug exposure (0.1 μg/ml ADR or $5x10^{-11}$ M VCR) was combined with mild hyperthermia at 41˚C. The viability of NALM-6 cells was observed to decrease after 1 h in the presence of a combination of mild hyperthermia and 0.1 μg/ml ADR (from 0.8 to 0.67), and decreased survival (0.53) was noted after 3 and 5 h of exposure.

The surviving fraction of NALM-6 cells was 0.47 after exposure to 1 μg/ml ADR without mild hyperthermia. Exposure to 0.1 μg/ml ADR with mild hyperthermia at 41˚C for 5 h had the same killing effect on the cell viability of NALM-6 cells as a 10-fold higher concentration of 1 μg/ml ADR alone.

The surviving fraction of NALM-6 cells after exposure to 1 μg/ml ADR decreased from 0.47 to 0.3 when cells were exposed to the drug in combination with mild hyperthermia at 41˚C for 1 h, and to 0.23 after 5 h of exposure. Therefore, the addition of mild hyperthermia at a 5-h exposure doubled the killing effect of 1 μg/ml ADR.

In a manner similar to that observed with ADR, the cell viability of NALM-6 cells after exposure to $5x10^{-11}$ M VCR decreased from 0.95 to 0.73, 0.67 and 0.66 in combination with mild hyperthermia at 41˚C for 0, 1, 3 and 5 h, respectively. Cell viability after exposure to a $5x10^{-10}$ M concentration of VCR was 0.76. Cell viability in the presence of $5x10^{-11}$ M VCR combined with mild hyperthermia after 1 h of exposure was identical to the effect of a 10-fold higher concentration of VCR alone ($5x10^{-10}$ M).

The viability of NALM-6 cells in the presence of $5x10^{-10}$ M VCR decreased from 0.76 to 0.6% when combined with mild hyperthermia at 41˚C for 1 h, and to 0.54 after 5 h. The addition of mild hyperthermia at 41˚C for 5 h served to increase the toxic effect of $5x10^{-10}$ M VCR by a factor of 1.5.

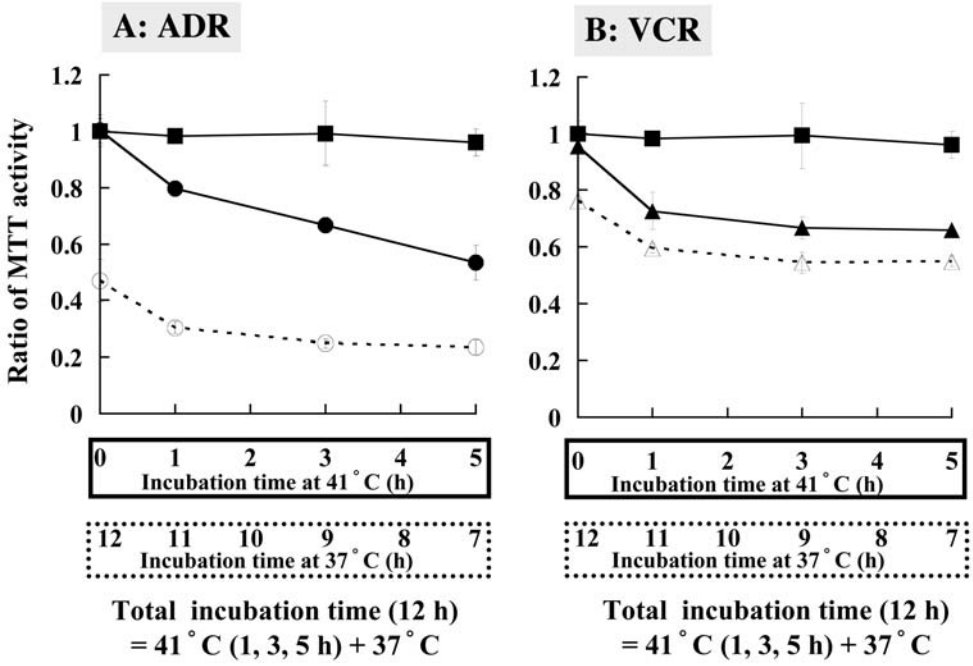

Figure 4. Effects of the anti-tumor drugs adriamycin (A; ADR) or vincristine (B; VCR) in combination with mild hyperthermia at 41˚C on the NALM-6 human leukemia cell line. Cells were incubated under mild hyperthermiac conditions at 41˚C (■) for 0, 1, 3 and 5 h, and then incubated at 37˚C (A and B). The total incubation time for these cells (at 41˚C plus 37˚C) was 12 h. Cells were incubated with adriamycin at concentrations of 0.1 (●) or 1 μg/ml (○) (A) or with vincristine at $5x10^{-11}$ (▲) or $5x10^{-10}$ M (△) (B) at 41 and 37˚C, respectively. The total incubation time at 41 and 37˚C for the cells was 12 h. After the 12-h incubation period, the viability of the NALM-6 cells was measured using the MTT method.

Discussion

When hyperthermia is used for cancer therapy, heating to 43˚C or more is required in order to kill tumor cells. However, for technical reasons, some organs and tissues are difficult to heat and to maintain at this temperature. Mild hyperthermia is relatively easy to apply, safe and cost-effective, and can be performed at almost any medical facility. The present *in vitro* experiments were conducted in order to determine whether mild hyperthermia at 41˚C would produce a synergistic effect with drug therapy; whether it would be effective in combination with chemotherapy, providing an alterative to hyperthermia at 43˚C.

Most reports concerning hyperthermia have examined the effects of local hyperthermia on solid tumors. There have been very few reports on the effects of whole-body mild hyperthermia on a systemic blood cancer like leukemia. Therefore, in these experiments, the thermal sensitivity and anti-tumor effects of drug therapy combined with mild hyperthermia were investigated in the NALM-6 leukemia cell line. The thermal sensitivity of NALM-6 leukemia cells was the same as that of normal control cells in culture. The cell survival rate of the NALM-6 cells was not affected by mild hyperthermia at 41˚C, but decreased after heating at temperatures over 43˚C.

ADR and VCR are established anti-tumor drugs used in chemotherapy for leukemia. Applied independently, ADR at a low concentration of 0.1 μg/ml or mild hyperthermia at 41˚C had no effect on the survival rate of NALM-6 leukemia cells. However, this survival rate decreased after exposure to a combination of the same low ADR concentration of 0.1 μg/ml and mild hyperthermia at 41˚C, and varied with incubation time. The anti-tumor effect of a low ADR concentration (0.1 μg/ml) with simultaneous exposure to a temperature of 41˚C for 5 h resulted in the same survival rate as would be expected from a 10-fold increased concentration of ADR of 1 μg/ml. VCR, another anti-tumor drug, induced a similar response. Used at a concentration of $5x10^{-11}$ M with hyperthermia at 41˚C for 1 h, it resulted in the same survival rate as a 10-fold increased VCR concentration of $5x10^{-10}$ M at 37˚C.

These results indicate that low concentrations of anti-tumor drugs, which under normal conditions have little or no effect on tumor cell killing, may have a 10-fold increased anti-tumor effect (equivalent to a 10-fold increase in drug concentration) when used in combination with mild hyperthermia at 41˚C. In addition, using a higher concentration of 1 μg/ml ADR in combination with mild hyperthermia at 41˚C for 5 h had a 2-fold increased anti-tumor effect (as measured by cell apoptosis). Similarly, the effect of $5x10^{-10}$ M VCR was increased 1.5-fold when combined with mild hyperthermia. This suggests that the tumor cell killing-inducing activity of anti-tumor drugs is increased when the drug treatment is combined with mild hyperthermia.

The anti-tumor mechanism of ADR functions by inhibiting DNA and RNA synthesis. ADR first forms a complex with DNA in tumor cells, then inhibits both DNA and RNA polymerase reactions (9,10). In addition, ADR is known to inhibit topoisomerase II (11,12). VCR is reported to act by binding to tubulin in microtubules, forming nucleospindles and inducing cell cycle arrest during the metaphase of cell division (13-15).

Hyperthermia has been found to inhibit DNA damage repair in lesions produced by anti-tumor drugs and radiation, resulting in increased chemotherapy and radiotherapy anti-tumor activity (16). Shioura *et al* (17) and Kano *et al* (18) reported an increase in the cytotoxic effects of combined treatment using low level hyperthermia at 40˚C and bleomycin *in vitro*. Ono *et al* reported that mild hyperthermia increased blood flow and enhanced the uptake of anti-tumor drugs into tumor tissue (19). In addition, mild hyperthermia has been reported to be more effective than therapy at high temperatures in promoting the expression of the heat-mediated suicide gene (HSP 70) (20). Mild temperatures below 41˚C showed significantly smaller energies in Arrhenius plots for some anti-tumor drugs than those observed with temperatures above 41˚C (21). Takahashi *et al* found no breakpoint in the D_0-temperature plot for pirarubicin (THP-adriamycin) with heat, indicating that pirarubicin cytotoxicity is more effectively enhanced by mild temperatures (40-42˚C) than by temperatures over 43˚C (22). Recently, Ahmed *et al* reported the mild hyperthermia-induced and hyperthermia-induced enhancement of drug cytotoxicity in apoptosis (23). Based on these reports, it can be surmised that mild hyperthermia enhances apoptosis and the anti-tumor effects of chemotherapy through an increase in the uptake of carcinostatic agents into tumor cells, and inhibits the repair of tumor cell lesions produced by anti-tumor drugs.

These experimental results suggest that the dose required for anti-tumor drug efficacy can be reduced by using the drugs in combination with mild hyperthermia. In this way, a higher efficiency of drug action than could normally be expected may be obtained, and the side effects of the drugs in the clinical setting may be reduced.

In conclusion, the cell killing activity of the anti-tumor drugs adriamycin and vincristine in leukemia cells was found to increase when these drugs were used in combination with mild hyperthermia. A high effective leukemia cell killing rate can therefore be expected with decreased drug concentrations during chemotherapy when these drugs are used in combination with mild hyperthermia. Mild hyperthermia is easier to apply than hyperthermia, and should be clinically useful in combination with chemotherapy.

References

1. Mizuno S: Biological and medical grounds of combination with hyperthermia and radiotherapy. In: Hyperthermia Manual. Matsuda T (ed). Magupuros Publication Co., pp1-5, 1991.
2. Mitsuhashi N: Positioning in combination therapy and future subjects. In: Hyperthermia - Guide Book of Hyperthermia for Cancer Therapy Japanese Society for Thermal Medicine (ed). Mainichi-Kenkousaron Publication Co., pp6-7, 2008.
3. Toffoli G, Bevilacqua C, Franceschin A and Boiocchi M: Effect of hyperthermia on intracellular drug accumulation and chemosensitivity in drug-sensitive and drug-resistant P388 leukaemia cell lines. Int J Hyperthermia 5: 163-172, 1898.
4. Shen J, Zhang W, Wu J and Zhu Y: The synergistic reversal effect of multidrug resistance by quercetin and hyperthermia in doxorubicin-resistant human myelogenous leukemia cells. Int J Hyperthermia 24: 151-159, 2008.
5. Hemant P, Surendra C, Suresh A and Manik C: Single and combination treatment with Vitamin K3 and adriamycin; *In vitro* effects on cell survival and DNA damage in human chronic myeloid leukemia cells. Select Cancer Therapeutics 7: 127-135, 1991.

lech J, Georget MT, Gihana E, Colombat P, Bremond J-L, Saigne M, Lamagnere J-P and Binet C: Evaluation of doxorubicin in combination with mafosfamide for *in vitro* elimination of myeloid and lymphoid tumor cells from human bone marrow. Bone Marrow Transplant 9: 101-106, 1992.
7. Sugimoto K: Chemotherapy of hematopoietic cancer. Juntendouigaku 52: 528-535, 2006.
8. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.
9. Di M: Adriamycin (NSC-123127): mode and mechanism of action. Cancer Chemother Rep 6: 91-106, 1975.
10. Nemoto T and Takahira H: Uptake of adriamycin into the cells and the interaction with DNA. Yakugakuzasshi 93: 1498-1508, 1973.
11. De Graff WG, Myer LS, Michell JB and Hahn SM: Protection against adriamycin cytotoxicity and inhibition of DNA topoisomerase II activity by 3,4-dihydroxybenzoic acid. Int J Oncol 23: 159-163, 2003.
12. Shuhendler AJ, O'Brien PJ, Rauth AM and Wu XY: On the synergistic effect of doxorubicin and mitomycin C against breast cancer cells. Drug Metabol Drug Interact 22: 201-233, 2007.
13. Owellen RJ, Owens AH Jr and Donigllan DW: The binding of vincristine, vinblastine and colchichine to tubulin. Biochem Biophys Res Commun 47: 685-691, 1972.
14. Schrek R: Cytotoxicity of vincristine to normal and leukemia cells. Am J Clin Pathol 62: 1-7, 1974.
15. Don VJ Jr and Richard AB: Cytotoxic thresholds of vincristine in a murine and human leukemia cell line *in vitro*. Cancer Res 39: 4346-4349, 1979.
16. Hall EJ and Roizin T: Biological effects of heat. Cancer Res 44: 4708s-4713s, 1984.
17. Shioura H, Hayashi S, Matsumoto H, Kitai R, Ohtsubo T, Nishida T, Zhang SW, Yoshida M, Ishii Y and Kano E: The effects of combined treatments with low hyperthermia and bleomycin on survival of murine L cells. Clin Cancer Res 16: 147-152, 1997.
18. Kano E, Furukawa-Furuya M and Nitta K: Sensitivity of bleomycin-resistant variant cells enhanced by 40˚C hyperthermia in vitro. Int J Hyperthermia 4: 5547-5553, 1988.
19. Ono H, Ando S, Suzuki T, Monzen H, Amano M, Terai K, Takahashi T and Hasegawa T: The drug uptake in the tumor when the mild-hyperthermia treatment in combination with the chemotherapy *in vivo*. Jpn J Hyperthermic Oncol 22: 23-33, 2006.
20. Huang Q, Hu JK, Lohr F, Zhang L, Braun R, Lanzen J, Little JB, Dewhirst MW and Li CY: Heat-induced gene expression as a novel targeted cancer gene therapy strategy. Cancer Res 60: 3435-3439, 2000.
21. Urano M, Kuroda M and Nishimura Y: For the clinical application of thermal chemotherapy given at mild temperatures. Int J Hyperthermia 15: 79-107, 1999.
22. Takahashi T, Mitsuhashi N, Sakurai H, Murata O, Kitamoto Y, Matsumoto H, Higuchi K and Niibe H: Thermal enhancement of pirarubicin (THR-adriamycin) by mild hyperthermia in vitro. Int J Hyperthermia 13: 317-324, 1997.
23. Ahmed K, Hori T, Yu DA, Wet ZL, Zhao QL, Nakashima M, Hassan MA and Kondo T: Hyperthermia chemo-sensitization, chemical thermo-sensitization and apoptosis. Thermal Med 24: 1-12, 2008.