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A Study on Enhancement of Hyperthermochemotherapy —— in the Presence of Buthionine Sulfoximine (BSO) ——

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ABSTRACT : The aim of this study was enhancement of the effect of hyperthermia which is a part of the multidisciplinary treatments for carcinomas. Many physicians suffered from practical difficulty in keeping the uniformity of 43°C in the deep organs.

Buthionine sulfoximine (BSO) is a specific inhibitor of glutathione (GSH) synthesis, and suppress GSH activity which is related to the repair process of cell damage. In this study, colony formation was tested using HeLa cells on the condition of one hour heating in the presence of 1mM BSO which is suitable for clinical application.

The concentration of 0.08ug cis DDP was used, corresponding to LD25. Colony formation was reduced to 45% and 19% at 40°C and 41°C in combination cis DDP in the presence of BSO. From the analysis of flowcytometry inhibition of accumulation in G_2M phase was observed at 12 and 24 hours exposing to 41°C and 42°C heating in the presence of BSO. The cells were increased in number for 24 hours at 41°C heating on the basis of analysis of cell growth curve.

A total of GSH were reduced to 2.4% in the presence of BSO as compared with 41°C heating alone. It is concluded that BSO helps escape repair process from thermal cytotoxicity and BSO plays an important role in promoting the effect of chemohyperthermia for the treatment of carcinomas.

INTRODUCTION

Many reports[1] have substantiated that hyperthermia plays a key role in a part of multidisciplinary treatment for malignant tumors. In fact, it is difficult to keep the intratumorous temperature uniformly kept at 43°C. It is well known that the addition of buthionine sulfoximine (BSO) inhibits glutathione (GSH) activity[2,3,4]. It is reasoned that inhibition of glutathione activity leads to impair the repair process from thermal cytotoxicity.

The purpose of this stydy is to clarify as to whether it is possible or not to enhance antiproliferative effect of hyperthermia in combination with the use of anticancer drugs in the presence of inhibitor of GSH, which is characterized by impairment of the repair process from cell damage.

MATERIAL AND METHOD

HeLa cells were incubated in RPMI with 10% fetal calf serum with penicillin G potassium and streptmycin sulfate and maintained at 37°C in humidified and thermostated bath containing 5% CO_2. HeLa cells in the semilogarithm proliferation stage were transferred to 6 well plates and each well contained 500 cells. After 24 hours, they were warmed and added with cis DDP, and then treated with BSO for one hour.

After 8 days these cells were washed twice, fixed for 30 to 60 minutes by using methyl alcohol, washed and stained by Giemsa' method and counted as a cluster of a colony over 50 cells.

Experiment 1: Colony formation at exposure of heating for one hour at 37°C, 49°C, 41°C, 41.5°C, 42°C, 42.5°C and 43°C, respectively.

Experiment 2: Colony formation in contact with cis DDP at exposure to different concentrations (6.4 3.2 1.6 0.8 0.4 0.2 0.1μg) for one hour.

Experiment 3: Colony formation in contact with different concentrations (1mM, 0.5mM) of BSO for 2 hours.

RESULTS

Experiment 1: **Fig. 1** shows colony formation on the condition of heating alone for one hour. It shows that colony formation was reduced to 48% at 41.5°C heating as compared with the control. When heating exceeded 42°C, colony formation was significantly inhibited.

Experiment 2: **Fig. 2** shows colony formation in contact with cis DDP for one hour. Fifty and

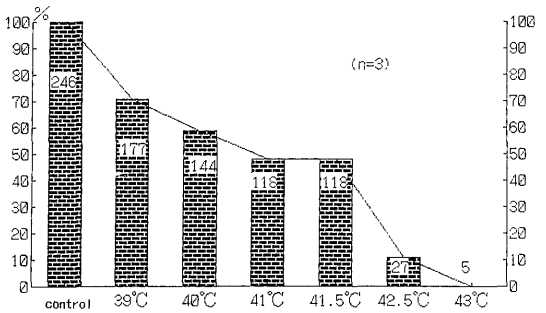

Fig. 1. Colony formation according to various tempertures of heating

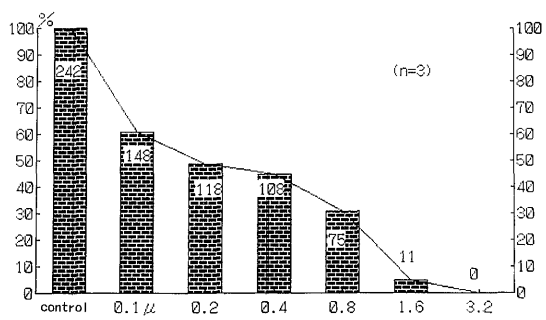

Fig. 2. Colony formation of various concentrations of cisDDP

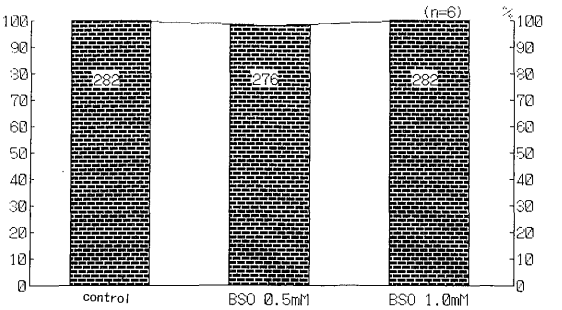

Fig. 3. Colony formation in contact with different concentrations of BSO

seventy-five per cent of 0.18μg and 0.08μg.

Experiment 3: **Fig. 3** shows colony formation in contact with BSO at various concentrations for 2 hours. No favorable effect on colony formation was provided by 1mM BSO.

COMMENT

From the standpoint of heating alone, hyperthermia over 42°C provoked a reduction of colony formation.

Seventy-five per cent colony formation was produced by contact with cis DDP at the concentration of 0.08μg as compared with the control. It was no influential concentration of BSO on colony formation that corresponded to 1mM.

From the above results of preliminary study, colony formation was evaluated by application of cis DDP and BSO in combination with heating. In this study, heating of 40°C or 41°C was commonly used for clinical feasibility, and the concentration of cis DDP was determined as 0.08μg which corresponded to 75% of colony formation, and the concentration of BSO was 1mM that did not alter the effect on colony formation. In experiment 1, the experimental plan was made as follows.

a) control (37°C) b) heating (40°C) for one hour c) heating (40°C) in the presence of cis DDP (0.08μg) for one hour d) heating (40°C) in the presence of 1mM BSO for one hour e) heating 40°C in the presence of 0.08μg cis DDP and 1mM BSO for one hour.

RESULTS

Fig. 4 summarized the experimental results. The colony formation was reduced to 52% in

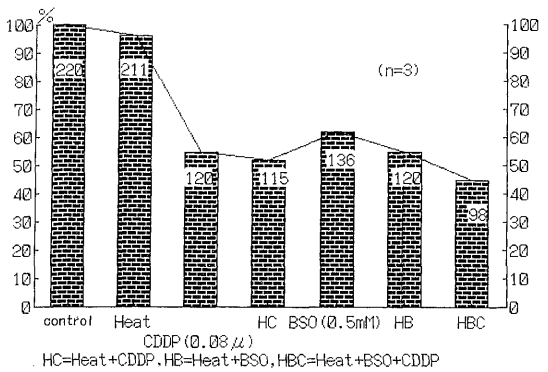

Fig. 4. Colony formation at 40°C for one hour in contact with drugs

combination with heating and cis DDP and to 55% in combination with heating and BSO as compared with heating alone. Meanwhile, when heating was combined with cis DDP and BSO, colony formation had shown a reduction of 45%. In experiment 2, the grade of heating was changed from 40°C to 41°C for one hour. The experiment was planned as follows.

a) control (37°C) b) heating (40°C) for one four c) heating (41°C) in combination with cis DDP (0.08μg) for one hour d) heating 41°C for one hour in combination, with BSO (1mM) e) heating (41°C) for one hour in combination, with cis DDP (0.08μg) and BSO 1mM.

RESULTS

Fig. 5 shows the result of heating (41°C) alone and in combination with anticancer drug. The colony, fell down to 61% in the presence of cis DDP and to 62% in the presence of BSO as

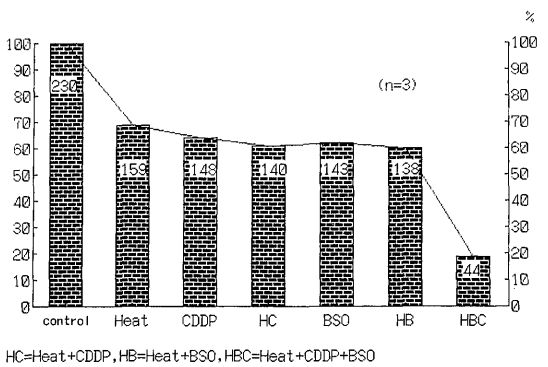

Fig. 5. Colony formation at 41°C heating in combination with 0.08 Cis DDP and 1mM BSO

compared to controls. On the other hand, heating (41°C) in combination with cis DDP and BSO enabled colony formation to reduce to 19% of the control.

COMMENT

Heating (41°C) in combination with cis DDP (0.08μg) and BSO (1mM) was of great benefit to reduce the rate of colony formation of HeLa cells effectively. It was certified that the combination with BSO and low grade heating, which was clinically applicable, allows inhibition of cell proliferation.

The effect of chemothermotherapy in the presence of BSO was experimentlly evaluated. Five × 10 of HeLa cells were collected in the dish and incubated for 24 hours in the thermostated incubator containing CO_2, thereafter heated at 37°C, 40°C, 41°C, 42°C and 43°C for one hour. It was carefully observed as to whether changes in cell cycle were introduced or not at 12 and 24 hours on the different conditions of heating for one hour, in the presence of cis DDP (0.08μg), heating for one hour in the presence of BSO (1mM) and heating for one hour in combination with cis DDP (0.08μg) and BSO (0mM).

HeLa cells on the above conditions at one hour were washed twice with PBS solution, pippeted to the glass tubes with 0.02% EDTA and 0.05% trypsin solutions, centrifuged at 600 to 800 rpm for 5min and supernatant was discarded, mixed and added 3 to 5 ml of 70% ethanol, and then left at −4°C for 30min. Then, 5ml of propidium iodide (PI) was added and stained for 30min. Flow cytometry (FACS IV) was used for the analysis of nuclear DNA content in accordance with Dean' method.

RESULTS

At 37°C, the patterns of cell cycle was not affected at 12 hours and 24 hours in heating alone and the presence of BSO (1mM) for 1 hour as shown in **Fig. 6** and there was no definitive difference between each other.

Meanwhile, the DNA histogram showed a remarkable accumulation in G_2M phase at 24 hours after one-hour exposure of the concentration of 1mg cis DDP and also it was the same as that in the presence of 1mM BSO as shown in **Fig. 7.**

At the time of 40°C heating and cis DDP and BSO in combination at 12 and 24 hours, the pattern of DNA histogram was almost similar as shown in **Fig. 8.**

At 41°C heating and cis DDP and BSO in combination, the DNA histogram indicated in analysis of Dean's method that G_2M phase displayed 7.7% at 12 hours and 12.3% at 24 hours in the group in combination with cis DDP. On the other hand, it was 4.69% at 12 hours, and 4.61% at 24 hours in the group in combination with BSO, indicating the inhibition of accumulation in G_2M phase with the help of BSO as shown in **Fig. 9.**

When heated at 42°C in combination with cis DDP and BSO, the DNA histogram showed accumulation in G_2M phase that was 14.9% at 12 hour and 21.1% at 24 hour. However, while BSO was added, it had become 7.6% at 12 hour and 8.1% at 24 hour as shown in **Fig. 10.**

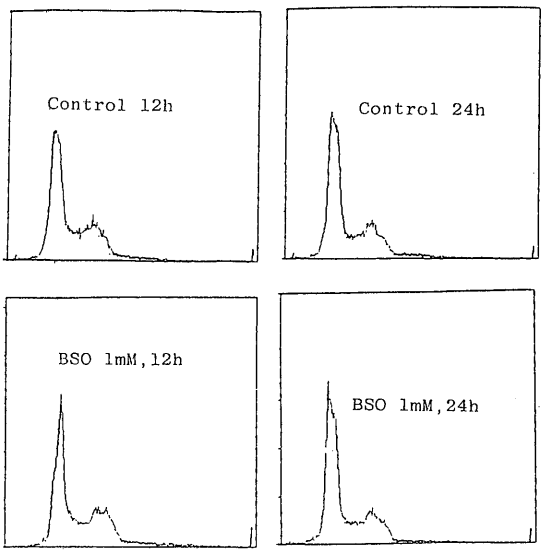

Fig. 6. DNA histograms of HeLa cells (37°C)

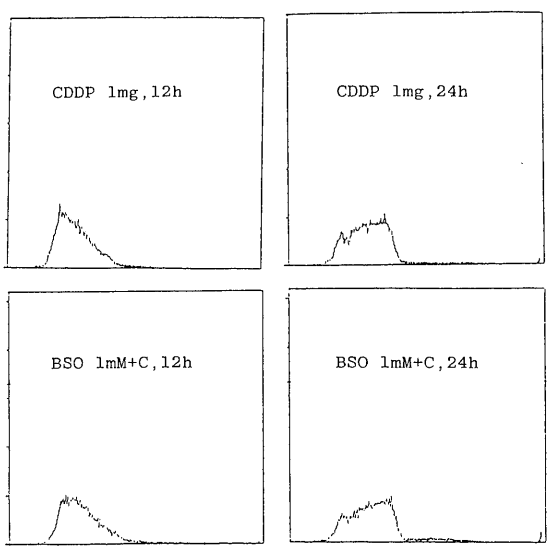

Fig. 7. DNA histograms of HeLa cells (37°C)

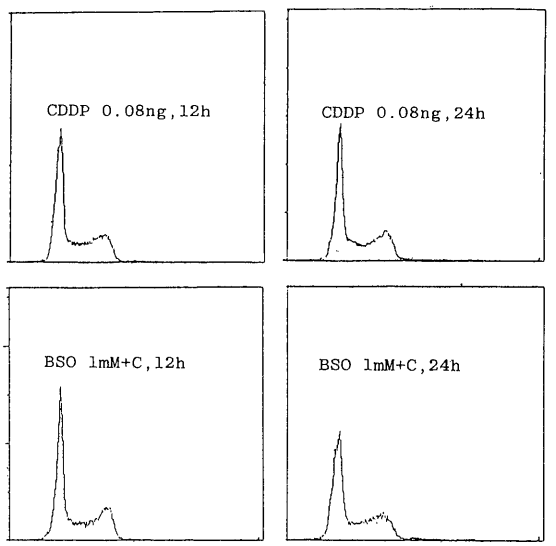

Fig. 8. DNA histograms of HeLa cells (40°C)

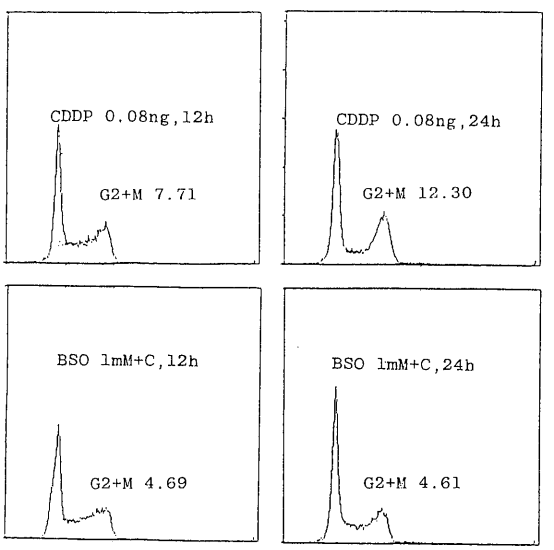

Fig. 9. DNA histograms of HeLa cells (41°C)

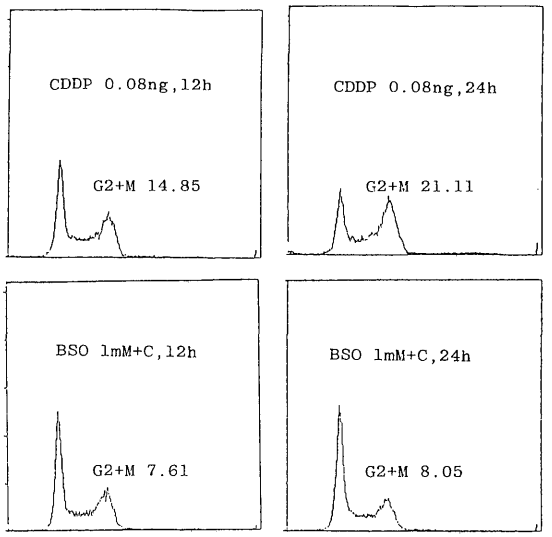

Fig. 10. DNA histograms of HeLa cells (42°C)

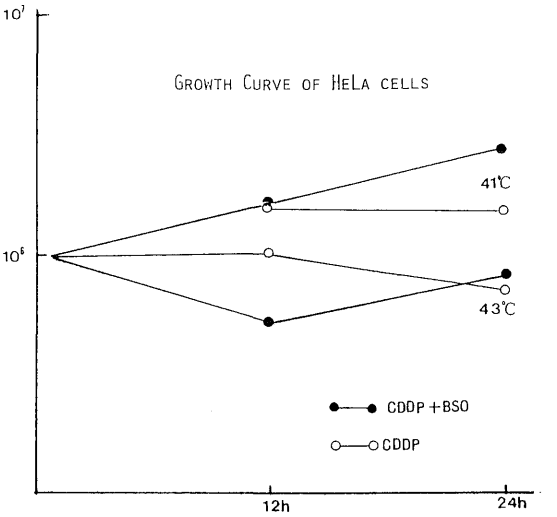

Fig. 11. Cell growth curves at 41°C and 43°C heating in the presence of CDDP alone and in combination with BSO

When raised to 43°C of heating, accumulaiton in G_2M phase was obvious in both groups on the DNA histogram.

COMMENT

HeLa cells were accumulated in G_2M phase at 43°C heating with 0.08μg cis DDP. On such a condition, the addition of BSO also enhanced accumulation in G_2M phase did not provide a change in any of cell cycle.

On the other hand, 41°C and 42°C heating in combination with 0.08μg/ml of cis DDP produced accumulation of G_2M phase. On the contrary, there was no tendency toward accumulaiton in G_2M phase by the addiiton of BSO under this circumstances. The cell growth curve was compared between 41°C to 43°C heating with cis DDP for 12 and 24 hours and in addition of BSO. As shown in **Fig. 11,** the cells increased at 41°C to 43°C heating with the addition of BSO. It is indicated that BSO, which acts as a reducer of intracellular glutathion level, impairs the repair process for cell damage at heating of 41°C and 42°C. The following experiment was made for the measurement of intracellular glutathion by using GSH reductase-DTNB method according to modified Owen's method by Shiohara[23]. Five × 10 of HeLa cells were collected in the dish and incubated in the humidified and thermostated bath containing 5% CO_2 for 24 hours and left one, 12 and 24 hours at 41°C. The intracellular glutathion concertration in each time was measured. And total glutathion volume was also measured when incubated HeLa cells were in one-hour contact with 0.08μg cis DDP and 1mM BSO.

RESULTS

Intracellular glutathion volume increased at 41°C heating alone, in combination with cis DDP and/or BSO at 24 hour. On the contrary the

Table 1. Changes in total glutathion level at 41°C heating according to heating time and drugs in combination

41°C 1h	0.534
41°C 12h	0.318
41°C 24h	1.860
41°C+BSO 1h	0.013
41°C+BSO 12h	0.165
41°C+BSO 24h	0.51
41°C+CDDP 1h	0.294
41°C+CDDP 12h	0.834
41°C+CDDP 24h	1.884
41°C+BSO+CDDP 1h	0.018
41°C+BSO+CDDP 12h	0.177
41°C+BSO+CDDP 24h	1.248

addition of BSO reduced glutathion in volume. In particular, it was decreased to 2.4% immediately after the addition of BSO as compared with that of heating alone as shown in **Table. 1.**

DISUCUSSION

Mitchell *et al*[5] reported that combination of hyperthermia with BSO may play a key role in eliminating thermotolerance. Freerman *et al*[6] also indicatd that depletion of GSH with the help of BSO brings the enhancement of thermal sensitivity. Russo *et al*[7] emphasized that depletion of GSH by the action of BSO suppress the development of thermotolerance and reduces the production of heat shock protein in V-79 cells. On the other hand, Konings *et al*[8] reported that there was no relationship in using DEA in LM cells and BSO in EAT cells between depletion of GSH and thermotolarance. It is assumed that this discrepancy is based on volume difference of GSH in the cells used for the study, it is well accepted that combined therapy of anticancer drugs with hyperthermia provides synergistic influence on anticancer effect. Barlogie *et al*[9] confirmed that combination therapy by mitomycin C and cisplation with low grade heating below 42°C by the total body hyperthermia method is of great clinical benefit in either exponential or stationary phase. In fact, cisplatin with hyperthermia is mostly prevalent in clincal use as the thermochemotherpy and Maeda[10] reported that the respones rate was 27.6%.

In this series, combination therapy of BSO with cis DDP was evaluated on the condition of 40°C and 41°C heating, it is concluded that there was no definite difference in colony formation of HeLa cells between BSO + cis DDP and in combination with BSO or cis DDP. On the other hand, the inhibition of colony formation in combination with BSO and cis DDP was more manifest, indicating 68.1% of BSO combination group and 68.6 of cis DDP combination one. In this series, changes in intracellular GSH volume were analyzed. The results showed that intracellular GSH volume at 41°C for one hour heating in combination with BSO was reduced much more than that at 41°C alone. This tendency was similar to that in combination with BSO and cis DDP. However, GSH 24 hours after exposing to BSO at 41°C and BSO + cis DDP at 41°C increased in volume and colony formation was significantly depressed and much accelerated in combination with BSO and cis DDP at 41°C.

As Lee *et al*[11] stressed, it may be in association with cell volume. The fact of increasing GSH volume and increasing cell counts at 41°C in combination with BSO and cis DDP is imcompatible with the result of colony formation. For the purpose of elucidating the contradictory result, cell cycle study was performed. The addition of one mM BSO did not affect th pattern of cell cycle, whereas high concerntration of 1mg cis DDP provided accumulation in G_2M phase at 24 hours and low concentration of 0.08μg cis DDP did not alter the pattern of cell cycle. On the other hand, at 41°C 0.08μg cis DDP produced accumulation in G_2M phase after 24 hours.

Development of accumulation in G_2M phase was inhibited in the presence of BSO and it had become manifest at 42°C.

The result demontrated that the presence of BSO in combination with hyperthermia with cis DDP inhibited development of accumulation in G_2M phase.

It implies that repair process of cytotoxic cells is suppressed in G_2M phase and also damaged cells by anticancer drugs are free of accumulaton in G_2M phase and transfers to the process of cell division, finally becomes cell death.

In conclusion, thermochemotherapy of cis DDP with low grade heating at 41°C in the presence of BSO in HeLa cells causes significant inhibition of colony formation, due partly to impairment of repair process by inhibition of G_2M accumulation. It is assumed that it is mainly associated with the role of GSH in the repair process.

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A main point of this paper announced with

the 47th gyrus Japanese cancer academy.

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