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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 G2M 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 reports1) 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) activity2,3,4). It is reasoned that inhibition of glutathione activity leads to impair the repair process from thermal cytotoxicity.

The purpose of this study is to clarify as to whether it is possible or not to enhance anti-proliferative 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 streptomycin sulfate and maintained at 37°C in humidified and thermostated bath containing 5% CO₂. 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, 0.1 µg) for one hour.

Experiment 3: Colony formation in contact with different concentrations (1 mM, 0.5 mM) 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 seventy-five per cent of 0.18 µg and 0.08 µg.

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 1 mM 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 0.08 µg as compared with the control. It was no influential concentration of BSO on colony formation that corresponded to 1 mM.

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 0.08 µg which corresponded to 75% of colony formation, and the concentration of BSO was 1 mM 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 (0.08 µg) for one hour  d) heating (40°C) in the presence of 1 mM BSO for one hour  e) heating 40°C in the presence of 0.08 µg cis DDP and 1 mM BSO for one hour.

RESULTS

Fig. 4 summarized the experimental results. The colony formation was reduced to 52% in
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 experimentally evaluated. Five x 10 of HeLa cells were collected in the dish and incubated for 24 hours in the thermostated incubator containing CO₂, 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, pipetted 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 G2M 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 G2M 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 G2M 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 G2M 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.
When raised to 43°C of heating, accumulaiton in G2M phase was obvious in both groups on the DNA histogram.

COMMENT

HeLa cells were accumulated in G2M phase at 43°C heating with 0.08μg cis DDP. On such a condition, the addition of BSO also enhanced accumulation in G2M 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 G2M phase. On the contrary, there was no tendency toward accumulaiton in G2M 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 Shiohara23). Five × 10 of HeLa cells were collected in the dish and incubated in the humidified and thermostated bath containing 5% CO₂ for 24 hours and left one, 12 and 24 hours at 41°C. The intracellular glutathion concentration 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

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

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<tr>
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<th>41°C 1h</th>
<th>41°C 12h</th>
<th>41°C 24h</th>
<th>41°C+BSO 1h</th>
<th>41°C+BSO 12h</th>
<th>41°C+BSO 24h</th>
<th>41°C+CDDP 1h</th>
<th>41°C+CDDP 12h</th>
<th>41°C+CDDP 24h</th>
<th>41°C+BSO+CDDP 1h</th>
<th>41°C+BSO+CDDP 12h</th>
<th>41°C+BSO+CDDP 24h</th>
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<tr>
<td></td>
<td>0.534</td>
<td>0.318</td>
<td>1.860</td>
<td>0.013</td>
<td>0.165</td>
<td>0.51</td>
<td>0.294</td>
<td>0.834</td>
<td>1.884</td>
<td>0.018</td>
<td>0.177</td>
<td>1.248</td>
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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.

**DISCUSSION**

Mitchell et al\(^{(1)}\) reported that combination of hyperthermia with BSO may play a key role in eliminating thermotolerance. Freeman et al\(^{(2)}\) also indicated that depletion of GSH with the help of BSO brings the enhancement of thermal sensitivity. Russo et al\(^{(3)}\) emphasized that depletion of GSH by the action of BSO suppresses the development of thermotolerance and reduces the production of heat shock protein in V-79 cells. On the other hand, Konings et al\(^{(4)}\) reported that there was no relationship in using DEA in LM cells and BSO in EAT cells between depletion of GSH and thermotolerance. 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\(^{(5)}\) confirmed that combination therapy by mitomycin C and cisplatin 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 clinical use as the thermochemotherapy and Maeda\(^{(6)}\) reported that the response 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\(^{(1)}\) 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 incompatible 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 the pattern of cell cycle, whereas high concentration of 1mg cis DDP provided accumulation in G2M 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 G2M phase after 24 hours.

Development of accumulation in G2M phase was inhibited in the presence of BSO and it had become manifest at 42°C. The result demonstrated that the presence of BSO in combination with hyperthermia with cis DDP inhibited development of accumulation in G2M phase.

It implies that repair process of cytotoxic cells is suppressed in G2M phase and also damaged cells by anticancer drugs are free of accumulation in G2M 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 G2M accumulation. It is assumed that it is mainly associated with the role of GSH in the repair process.

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REFERENCES


