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# Effects of Heating on Hydroxyl Radical-Generated Toxicity in Mouse Forebrain Tissue Culture

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***ABSTRACT*** *: This experiment was carried out to know the effects of heating and serum on hydroxyl radicals in embryonic mouse forebrain (cerebrum) culture. The heating to mouse embryonic cerebrum cells in culture was done in a water bath at 43°C for 60 min. After that, two supernatants were prepared at 20 hrs and 48 hrs respectively after heat treatment to the brain cells. To find out the heating effects on neuron cells, mouse cerebrum cells (13 embryonic day) were cultured in hydroxyl radical generation system composed of 20 mU/ml glucose oxidase (GO system), using condition of normal culture media (MEM, 5% serum, 5%* $CO_2$*) or supernatant prepared after heating at 43°C for 60 min in a water bath. Supernatant prepared at 20 hrs after heat treatment had a greater protective effects against hydroxyl radical than supernatant prepared at 48 hrs after heat treatment. Otherwise, the protective effect of serum against hydroxyl radicals in the cultured brain cells is higher than that in the heat treatment. These results indicated that serum in culture media reduced cytotoxicity of hydroxyl radicals in mouse forebrain culture, also that heat treatment showed the protective effects against hydroxyl radicals generated with 20 mU/ml GO system in mouse forebrain culture.*

***Key Words*** *: Mouse forebrain tissue culture, Hydroxyl radical, Heat treatment, MTT assay*

## I. INTRODUCTION

Effects of a mild heat shock on cells were firstly described several decades ago (Craig, 1985). Heat shock proteins (i.e. stress proteins or induction proteins) are essential for the survival of cells confronted with inducers such as abnormal temperature, stress, toxic materials and oxygen free radicals (Lee and Lim, 1996; Pelham, 1995; Schlesinger *et al.*, 1990).

Generally, we know that oxygen free radicals are produced by oxidation in metabolic processes. Although the amount produced by oxygen free radicals are in micromolar concentration, they damage to the lipid membrane of the neuron cells. Especially, the role of oxygen radicals is to do lipid peroxidation which is a causative agents of neuronal loss (Hall and Braughler, 1986; Saunders *et al.*, 1987) such as stroke, as well as degenerative neurological diseases, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Bracco *et al.*, 1991; Halliwell, 1992). Additionally, oxygen free radicals can act both as oxidant of lipid membrane of neuron cells and as stress protein inducers. Stress response is a universal phenomenon to survive any organisms in a biosphere laden with environmental hazards. In the relation between oxygen free radical and induction protein, addition of exogenous hydrogen peroxide to cultured hamster fibroblast (Spitz *et al.*, 1987) brought about increasing heat shock protein (HSP). Also superoxide radicals have a potential ability to produce the stress protein as inducers (Ropp *et al.*, 1983). Recently, many scientists postulate that hydrogen peroxide plays a role in signaling transducer inside cells (Candido, 1995).

In addition, the short-lived induction proteins by stress inducers were known to have several functions such as antigen, the RAD 6 gene (Vincent, 1989). Also, stress proteins produced by several stress inducers play a protective role against irreversible cell damage (Burdon *et al.*, 1990).

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Therefore, we assume that the heating to neuron cells can produce stress proteins which protect against oxygen free radical such as hydroxyl radicals. In these experiments, strongly we want to know whether effects of heating to neuron cells can protect against hydroxyl radical in GO system.

## II. MATERIALS AND METHODS

### 1. Cell culture

Mouse neuron cells in tissue culture were prepared from 13 day embryoic mouse (ICR) forebrain using the procedure previously described (Michikawa *et al.*, 1994). In brief, the brain tissues were cut out and then dissected into the small pieces of tissue. These small pieces were incubated in $Ca^{2+}$, $Mg^{2+}$-free Hank's balanced salt solution (HBSS) containing 0.25% trypsin and 20 μg/*ml* DNase for 60 min at 37°C, 5% $CO_2$. The mouse brain tissues were dissociated by trypsin into single cells observing with microscope. Then the cells were centrifuged at 1,000×g for 10 min, and then at 800×g for 10 min. Equal numbers of cells ($1.0\times 10^5$/well) were placed in each well of 96 well plate with minimum essential media (MEM feeding medium) containing 0.5% glucose, 20 μg/*ml* gentamycine and 5% horse serum. Total volume of media in each well was 100 μ*l*. The neuron cells were cultured at 37°C, 5% $CO_2$ for this experiment. The medium was renewed twice a week.

### 2. Preparation of supernatant

After 7~10 days of mouse brain cell culture in 96 multiwells, the culture plates were sealed and immersed at 43°C for 60 min in a water bath. Immediately after that, they were placed in a 5% $CO_2$ incubator at 37°C for 20 hrs or 48 hrs to allow the production of stress proteins. And then, the cells were lysed in multiwells by resuspending several times with a pasteur pipet after incubation for 20 hrs or 48 hrs. After lysis of cells in each well, 10 *ml* lysates were collected and centrifuged at 15,000×g for 10 min. The supernatants were stored respectively at -20°C for use as induction proteins in the following experiments.

### 3. Oxygen exposure

Mouse forebrain cells were exposed to enzymatically-generated radicals (i.e. hydroxyl radicals) for different incubation times. To oxygen exposure of the cultured cells, the media were changed with MEM feeding medium, containing 0.5% glucose, 20 mU/*ml* GO. And then supernatant prepared at 20 hrs or 48 hrs after heat treatment were added in 96 multiwell plates with from 4 μ*l* to 100 μ*l*/well. After 4 hrs of incubation with glucose oxidase (GO) (type X from *Aspergillus niger*, G8135), cells in multiwells were processed for MTT cytotoxicity assay.

### 4. MTT cytotoxicity assay

For the protective effects against hydroxyl radicals, MTT cytotoxicity assay was performed according to the method of Mosmann (1983). MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical] was dissolved in PBS at 5 mg/*ml* (stock solution) and filtered. After 4 hrs of incubation with GO system, the stock MTT solution (10 μ*l* per 100 μ*l* of total volume/well) was added to the each well and the plates were incubated for another 4 hrs at 37°C/5% $CO_2$. After that, 100 μ*l* of acidic propanol was added to the wells, and then shoken for a meanwhile. The plates were read on a Dynatech Microelisa reader at a wavelength of 570 nm.

### 5. Data analysis

All the values are expressed as a percentage of the control value. Control was not treated with GO to the cells. All the data were analyzed by single factor ANOVA analysis. Columns and bars represent means ±SEM for n=4.

## III. RESULTS

To evaluate the dose-response relationship of the GO-generated hydroxyl radical cytotoxicity on embryonic mouse forebrain cells, the cells in the 96 multiwells were exposed at concentrations of 1, 5, 10, 20, and 40 mU/*ml* GO for 4 hrs and then

processed by MTT assay. At 1 mU and 5 mU GO, more than 90.0% of the cells survived after 4 hrs of the treatment under incubation at 37°C, 5% $CO_2$. At 10 mU GO, approximately 76.4% of the cells survived, while at 20 mU and 40 mU GO concentrations, 44.6% and 33.1% respectively of the total cell population survived the hydroxyl radical-induced cytotoxicity (Fig. 1).

The time dependency of GO-induced cytotoxicity in embryonic mouse forebrain is shown in Fig. 2. At 20 mU/m*l* GO, cell viabilities were measured 91.0%, 81.0% and 65.0% after 0.5 hr, 1 hr and 2 hrs of GO treatment respectively. After 3 hrs and 4 hrs of GO treatment, viabilities of the neuron cells were 57.0% and 44.6% individually.

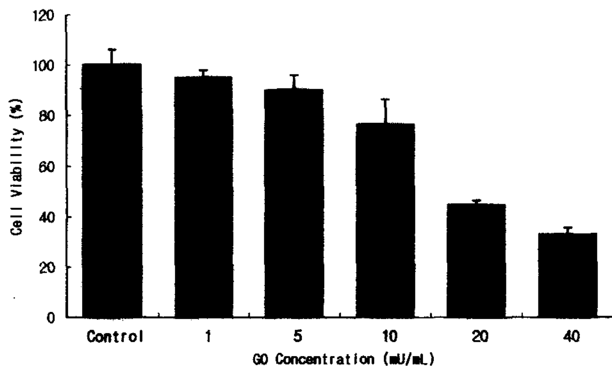


**Fig. 1.** Dose response relationship of glucose oxidase (GO) concentration. Cytotoxicity was measured by MTT assay in embryonic mouse forebrain culture. Cultures were exposed to 1, 5, 10, 20 and 40 mU/m*l* GO for 4 hrs. The values are expressed as a percentage to the control. The results are the means ±SED (n=4).

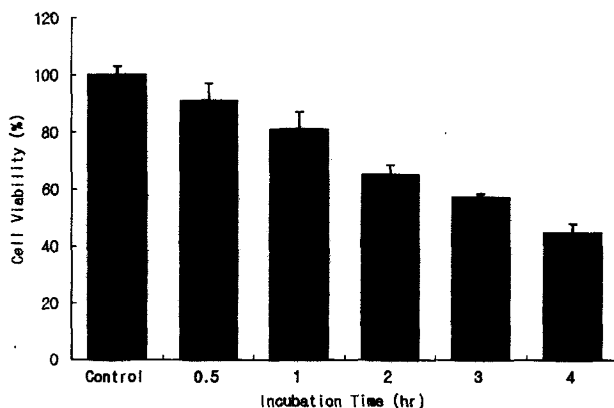


**Fig. 2.** The time dependency of GO-mediated hydroxyl radical cytotoxicity as measured by MTT assay in newborn mouse forebrain culture. The cerebrum cells were exposed to 20 mU/m*l* GO for 0.5, 1, 2, 3, and 4 hours. The values are expressed as a percentage to the control. The results are the means ±SED (n=4).

Therefore, a concentration of GO 20 mU/m*l*, and a incubation time of 4 hrs, with more than 50% cytotoxicity, were chosen for the hydroxyl radical-generating system (GO system) in subsquent studies.

### 1. Protective effects of supernatant prepared at different times after the heat treatment

Supernatants prepared at 20 hrs or 48 hrs after the heat treatment (43°C/60 min) were compared with the protective effects against hydroxyl radicals. The supernatants were added to culture media from 4 μ*l* to 100 μ*l*/well of final maximum volume respectively.

In the additional effects of 20 hrs supernatant, although small amounts (4 μ*l* addition) of supernatant prepared from 10 m*l* lysates were added to MEM (96 μ*l*) in 20 mU/m*l* GO system, there was the protective effects. Generally, the percentage of living cells in GO system was increased by rising of the volume of 20 hrs supernatant added. Such a tendency was also found similarly in the case of 48 hrs supernatant prepared after the heat treatment. In comparison to two different supernatants, the percentage of cell survival in the 20 hr supernatant was higher than that in the case of 48 hrs supernatant. In the 20 hrs supernatant, the protective effects were

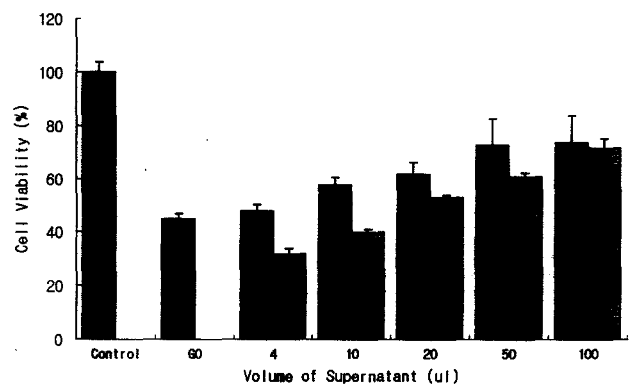


**Fig. 3.** The comparision of the protective effects between 20 hr supernatant and 48 hr supernatant prepared after the heat treatment at 43°C for 60 min. The neuron cells were exposed to GO 20 mU/m*l* and incubated for 4 hrs. ■; 20 hr supernatants, ▤; 48 hr supernatants, GO; 20 mU/m*l* glucose oxidase. The values are expressed as a percentage to the control. The results are the means± SED (n=4).

shown even at the lowest added volume of 4 μl (Fig. 3). In contrast, the protective effects in addition of 48 hrs supernatant were generally lower than in the 20 hrs supernatant under same culture conditions. In the 48 hrs supernatant, the protective effects to the hydroxyl radicals were shown from the 20 μl addition of supernatant in GO system (Fig. 3).

### 2. Protective effects of heat treatment for a long time

To know the time effects of heat treatment against hydroxyl radical, heating to the mouse forebrain cells was treated as previous procedures (materials and methods), at 43°C for 60 min. Therefore the cells were treated immediately with GO after heat without preparation of supernatant and without changing media. At 16 hrs, 20 hrs, 24 hrs, or 48 hrs after heat, the cells in two kind of media, with 5% horse serum or without serum, were exposed to GO 20 mU/ml for 4 hrs. After that, the exposed cells were done MTT assay and evaluated the number of living cell. The percentages of living cells among the different time after heat treatment were 26.1% at 16 hrs, 27.4% at 20 hrs, 27.8% at 24 hrs and 25.4% at 48 hrs respectively (Fig. 4).

### 3. Effects of serum

To get only real heating effects against hydroxyl radical, we tested the effects of serum, because

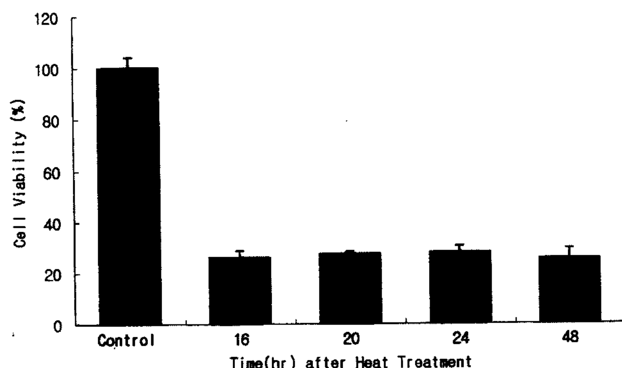


**Fig. 4.** Cytotoxicity of hydroxyl radicals generated by GO (20 mU/ml) at 16 hrs, 20 hrs, 24 hrs and 48 hrs after the heat treatment at 43°C for 60 min. The values are expressed as a percentage to the control. The results are the means ±SED (n=4).

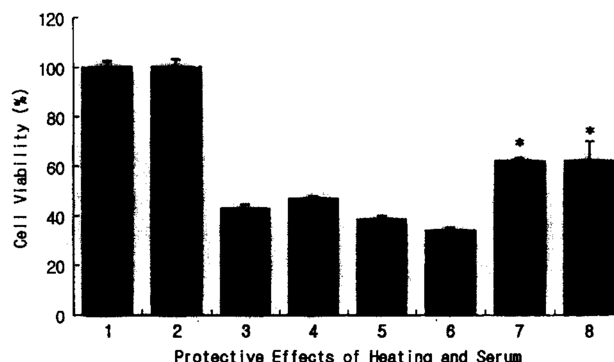


**Fig. 5.** The protective effects of serum on hydroxyl radicals generated by GO 20 mU/ml. The culture cells were treated by heating or non-heating and by serum or non-serum as previously described. 1; Control (non-heating), 2; Control (heating), 3; non-heating, new media +20 mU/ml GO, 4; heating, new media +20 mU/ml GO, 5; non-heating, old media +20 mU/ml GO, 6; heating, old media +20 mU/ml GO, 7; non-heating, with serum +20 mU/ml GO, 8; heating, with serum +20 mU/ml GO. The neuron cells were incubated for 4 hrs at 37°C, 5% $CO_2$. The values are expressed as a percentage to the control. The results are the means ±SED (n=4). *; $P<0.05$.

culture media contain 5% horse serum. Here, the neuron cells in trial were divided into several groups on the different treatments (with serum, without serum, with heat, without heat). After culture for 7~10 days at 37°C, 5% $CO_2$, the cells were treated with heat or non-heat at 43°C for 60 min and then exposed to 20 mU/ml GO in MEM containing 5% horse serum or non-serum at 37°C for 4 hrs. In the effects of heating, cell survival in media containing serum showed 62.1%, whereas it was 62.3% without heating. The percentage of living cells in the media without serum was 38.6% after heating at 43°C for 60 min, and 34% after non-heating respectively (Fig. 5). Generally, serum showed the protective effects against hydroxyl radicals in embryonic mouse forebrain culture. In the changing of the media (new media) after heat, the percentage of survived cells was 46.8%, whereas the percentage of the survived cells in new media was 42.7% after non-heating respectively.

## IV. DISCUSSION

Active oxygen radicals are very toxic to the spinal cord (Michikawa *et al.*, 1994) and oligodendroglial cells (Kim *et al.*, 1991). On the other hand, oxygen free radicals stimulate to induce stress

protein in cell culture system, because they act as stress inducer (Schlesinger *et al.*, 1990).

In this experiment, we investigated the cytotoxicity of hydroxyl radicals generated by glucose oxidase in embryonic mouse forebrain tissue culture. First, hydroxyl radicals produced by this GO system in embryonic mouse forebrain culture were very toxic and killed almost 50% of the neuron cells after 4 hrs of 20 mU/*ml* GO exposure (Figs. 1 and 2). These results indicate that hydrogen peroxide generated by GO system plays a role in cell killing through the formation of hydroxyl radicals, and that the cytotoxicity of hydroxyl radicals to neuron cells in this experiment showed similarity with Michikawa (1994).

Generally, the protective effects of 20 hr supernatant against hydroxyl radicals had greater than that of 48 hr supernatant, comparing with control which was not only non-heat but also normal culture in GO system (Fig. 3). Although, the supernatants prepared after heat had protective effects to the embryonic mouse neuron cells from hydroxyl radicals, the protective effects in the case of 48 hr supernatant were not better than that in 20 hr supernatant. This reason might be that the life time of induced protein is limited (Richard *et al.*, 1990).

To know heating effect, embryonic mouse brain cells were treated just by heat without preparing supernatants. At 16 hr, 20 hr, 24 hr, 48 hr after heat treatment cells were treated with GO and evaluated the number of living cells. At the different time after heat treatment, the results of protective effect against hydroxy radicals were not different (Fig. 4). The percentages of living cells were likely between 20 hr and 24 hr after heat treatment in 20 mU/*ml* GO system. This means that effects of heat to mouse embryo brain cells showed effectively just only between 20 hr and 24 hr after heat treatment, because supernatants prepared later than 24 hr after heating had no more different effects. Consequently, these protective effects to hydroxyl radical came together from heating and serum in culture media.

To know which one effect is stronger between heating and serum against hydroxyl radicals, these experiment carried out to test the effects of serum and of heating separately. In the serum case, the protective effects against hydroxyl radicals in embryonic mouse forebrain culture were obviously clear. As shown in Fig. 5, the number of living cells were definitely different in two media, either with serum or without serum in the culture media.

Interestingly, just only the heat treatment showed also the protective effects in our experimental system. Probably the heating to cells would be produced trace amount of the stress protein, because the cells survived until 48 hrs after heating treatment without changing of culture media. Normally the embryonic mouse forebrain cells would be entirely died, when the cells were treated with 20 mU/*ml* GO for 10 hrs. Even at 4 hrs after exposure of 20 mU/*ml* GO, the number of living cell was over 50% in the case of non-heat. In addition, the protective effects of serum against hydroxyl radicals were increased cell survival, by about 23.7% compared to cells in the absence of serum. This percentage, 23.7% live cells, was deduced from the subtraction between the effects of two treatments, with serum and without serum, as shown Fig. 5. In the real heating effects without serum, the number of living cells in heat treatment was by 4.6% higher than non-heating (Fig. 5). Although the effects of resistance to hydroxyl radicals generated by GO came mostly from horse serum, heating contribute also to protective effects. In condition of non-toxic level, active oxygen radicals as stress inducer have also capability to produce stress protein. (Burdon *et al.*, 1990). Also, hydrogen peroxide is necessary for the transcription of HSP genes (Pelham, 1985). Therefore, even though the protective effects of heating to hydroxyl radicals in the neuron cell culture were lower than serum effects, we believe that neuron cells probably produce stress peotein after heat treatment and then the induction proteins protect the cells from cytotoxigenic radicals such as hydroxyl radicals (Schlesinger 1990).

We concluded that hydroxyl radical generated by GO system was a toxic substance to embryonic mouse forebrain tissue, that heat treatment to the cells at 43°C for 60 min brought about results of the protection from hydroxyl radicals generated by GO in culture system, although trace amount of

stress protein might be produced by heat treatment.

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