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Comparison with Some Antioxidants on Hydroxyl Radical in Mouse Whole Brain Culture

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ABSTRACT *: This experiment carried out to compare the protective effects of some antioxidants to hydroxyl radicals in embryonic mouse whole brain tissue culture. The ICR mouse whole brain (13 embryonic day) was cultured in hydroxyl radical system in which radicals were generated by 20 mU/ml glucose oxidase (GO). In this experiment, to make ferrous iron from ferric iron, iron as an accelerator, and ascorbic acid as a reductant were used. For comparison of the protective effects to hydroxyl radicals, antioxidants such as desferrioxamine (DFX), laccase, water or ethanol extracts from Rhus Verniciflua Stokes (RVS), and α-tocopherol were used, because they relate to metal ion. The results of this experiment showed that all antioxidants protected effectively the cytotoxicity from hydroxyl radicals in the brain cultures. More than 70% of cell viabilities among different antioxidants was at 1 mM DFX, 1.43 μM laccase, 12.5 μM water extract, 12.5 μM ethanol extract and 50 μM α-tocopherol individually, compared with 20 mU/ml GO alone. In comparison to the antioxidative activities of antioxidants, laccase and extracts from RVS showed strong antioxidative effects even at low concentration.*

Key Words *: Hydroxyl radical, Laccase, Mouse whole brain cells.*

I. INTRODUCTION

Oxidation is a common process in many biological metabolic systems. Free radicals are inevitable by-products of redox-reaction mechanisms such as other processes associated with normal aerobic metabolism as well as mitochondrial electron transport, lipid peroxidation, peroxisomes in the organism. Oxidants are produced in micromolar concentrations and mostly consist of two species, $O_2^{\cdot}$ and $^{\cdot}OH$. In the presence of certain metals, especially iron and copper, the radical reactions are catalysed and then damage the lipid membranes of living cells (Halliwell and Gutteridge, 1990).

Normally many defense enzymes, including catalase, superoxide dismutase (SOD), glutathione peroxidase, ascorbic acid and α-tocopherols, can protect the non-specific levels of reactive oxidants in the human body (Frei *et al.*, 1992). Therefore, most organisms have a tolerance against mild oxidative stress.

In condition of severe oxidative stress, however, reactive oxygen species can lead to lipid peroxidation which is a causative agent of neuronal loss after brain or spinal cord injury (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). Reactive oxygen radicals can also mutate protein and DNA structures or produce protein radicals, leading to cancer and cell death (Carney *et al.*, 1991).

Recently, many scientists have tried to obtain antioxidants from natural substances, because several artificial antioxidants, like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), were known as toxigenic and mutagens (Choe and Yang, 1982).

We probed that extracts from *Rhus Verniciflua* Stokes (RVS) were natural antioxidants. Especially, the ethanol or water extracts from RVS has specifically protective effects against hydroxyl radicals. The main component of extracts from RVS was laccase which has an antioxidative effects as bioactive substances in veterbrate animals (Lim and Sim, 1997; Lee and Lim, 1998).

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To compare antioxidative activity of laccase, the protective effects of some antioxidants were measured by MTT assay in embryonic mouse whole brain cultured cells.

II. MATERIALS AND METHODS

1. Chemicals

Laccase (Crude acetone powder from *Rhus vernificera*, L2157), glucose oxidase (GO) (type x from *Aspergillus niger*, G8135), ascorbic acid (sodium salt, A7631), ferric chloride (anhydrous, F7134), ferrous chloride (tetrahydrate, F2130), and MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, M2128] were obtained from Sigma Chemical Co. (St. Louis, MO). Desferrioxamine (DFX) was purchased from Aldrich Chemicals (Milwaukee, WI). All the other chemicals were of the highest purity commercially available.

2. Cell culture

The neuron cells in tissues were prepared from 13 day embryonic mouse whole brain (ICR) using the procedure previously described (Michikawa *et al.*, 1994). In brief, the mouse whole brain tissues were cut out, and then dissected into the smallest possible pieces of tissue. These were incubated for 60 min at 37°C, 5% CO_2 with 0.25% trypsin and 20 ug/m*l* DNase in phosphate buffered saline (PBS; pH 7.5). The enzyme-treated tissue was dissociated by resuspending it with a pasteur pipet into single cells. The single cells were centrifuged at 1,000×g for 10 min, and then at 800×g for 10 min. The fine single cells were divided into the same numbers of cells (1.0×10^5/m*l*) in 96 multiwells containing minimum essential media (MEM) with 0.5% glucose, 20 ug/m*l* gentamycin and 5% fetal bovine serum (FBS). The total volume of medium in each well was 100 μ*l*. The neuron cells were cultured with 5% FBS, at 37°C/5% CO_2 for this experiment. The medium was renewed twice a week.

3. Hydroxyl radical exposure

On days 7~14, the cultured cells were exposed to enzymatically-generated hydroxyl radicals for different incubation times. For radical exposure of the neuron cells, the media was changed with the MEM feeding medium, containing 0.5% glucose, 20 mU/m*l* glucose oxidase (GO). And then FBS was not adding, because serum was known as antioxidant. To know the role of iron to hydroxyl radical, different concentration of ferrous iron was added in culture media, while ferric iron was reduced with 30 μM ascorbic acid. After 4 hrs of incubation with the glucose oxidase (GO), hydroxyl radicals generating system, cells in multiwells were processed for MTT cytotoxicity assay.

4. MTT assay

To evaluate 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, M2128] was dissolved in PBS at 0.25 mg/m*l* (stock solution) and filtered to sterilize and remove insoluble residue. 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 each well and the plates were incubated again at 37°C for 4 hrs. After that, 70 μ*l* of acidic propan-2-ol (including 0.1% HCl) was added to the each well, and then shaken for a while. The plates were read on a Dynatech MicroELISA reader at a wavelength of 570 nm.

5. Statistical analysis

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

III. RESULTS

To estimate the protective effects of GO-generated hydroxyl radical cytotoxicity on embryonic mouse whole brain cells, the cells in the 96 multiwells were treated with concentrations of GO 20 mU/m*l* with ferric iron reducing by ascorbic acid for 4 hrs, with more than 70% cell death.

1. The role of iron against hydroxyl radicals

To study the role of iron to hydroxyl radicals, the two kinds of iron (ferrous and ferric iron), were used in this experiment. At 4 hrs after coincubation with different concentration of ferrous iron (Fe^{2+}) in 20 mU/ml GO, the cell survivals were determined to be 33.5% at 1 μM, 33.2% at 100 μM, 26.7% at 500 μM and 25.4% at 1 mM respectively (data not shown). In the case of 20 μM ferric iron (Fe^{3+}) with 30 μM ascorbic acid without GO, the number of living cells showed 94.8% at 4 hrs and 82.5% at 16 hrs after coincubation, however addition 20 mU/ml GO under the same condition showed to accelerate radical reaction.

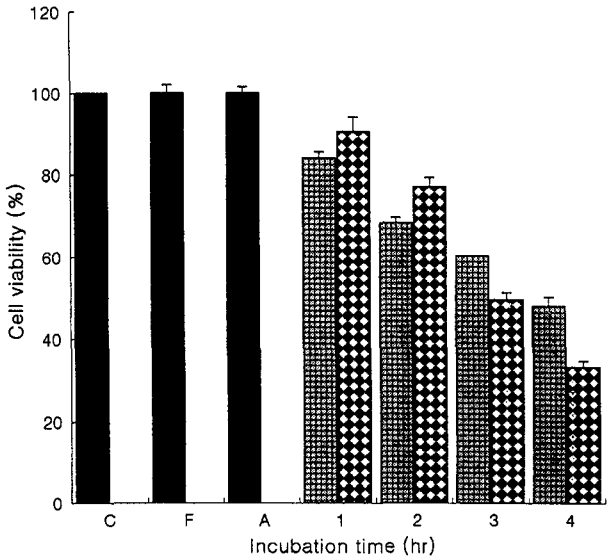

Fig. 1. The cytotoxic effects of 20 μM ferric iron (Fe^{3+}) reducing by 30 μM ascorbic acid in GO system. The cultured cells were exposed to 20 mU/ml glucose oxidase containing 20 μM Fe^{3+}, 30 μM ascorbic acid, at 37°C, 5% CO_2 for 1, 2, 3, and 4 hrs. Cell viabilities were evaluated by MTT assay. Antioxidative activities of antioxidants expressed to cell viability (%) compared with GO treatment alone. Values represent the mean±SEM (n=4). ▩; 20 mU/ml GO only, ▦; 20 μM ferric acid+30 μM ascorbic acid+20 mU/ml GO C; control, F; 20 μM ferric acid alone, A; 30 μM ascorbic acid alone.

Namely, in the mixture of 20 μM ferric iron and 30 μM ascorbic acid with 20 mU/ml GO, the percentage of the living cells were 90.5% in 1 hr, 70.2% in 2 hrs, 49.8% in 3 hrs and 33.2% in 4 hrs (Fig. 1). At 4 hrs, cytotoxicity of mixture of 20 μM ferric iron and 30 μM ascorbic acid in 20 mU/ml GO was shown to be more toxic, compared to that of 20 mU/ml GO alone.

2. Comparison with antioxidative effects between some antioxidants

To find the antioxidative activities of laccase against hydroxyl radical, activity of the laccase was compared with several natural antioxidants such as DFX, ethanol extracts or water extracts from RVS and α-tocopherol. The embryonic mouse whole brain cells were cultured with 5% FBS at 37°C/5% CO_2 and exposed to hydroxyl radical generated by 20 mU/ml GO with 20 μM ferric acid and 30 μM ascorbic acid. The antioxidative activities

Table 1. Comparison with antioxidative activities between some natural antioxidative substances

	control	Fe^{3+}	ascorbic acid	GO	mixture (20 μM Fe^{3+}+ ascorbic acid 30 μM+ GO 20 mU)	DFX	laccase		ethanol extract from RVS		water extract from RVS		α-tocopherol	
		20 μM	30 μM	20 mU		1 mM	1.43 μM	2.5 μM	12.5 μM	25 μM	12.5 μM	25 μM	50 μM	100 μM
GO 20 mU	×	×	×	○	○	○	○	○	○	○	○	○	○	○
Fe^{3+} 20 μM	×	○	×	×	○	○	○	○	○	○	○	○	○	○
ascorbic acid 30 μM	×	×	○	×	○	○	○	○	○	○	○	○	○	○
cell viability (%)	100.0	100.0 ±1.28	100.0 ±3.28	48.0 ±2.12	36.3 ±1.70	72.9 ±3.85	70.9 ±3.60	78.8 ±2.09	70.0 ±4.70	87.1 ±3.53	72.0 ±1.02	86.1 ±9.54	72.0 ±9.17	77.0 ±2.54

Mouse whole brain cells exposed to hydroxyl radical generated by 20 mU/ml glucose oxidase, reducing with 20 μM Fe^{3+} and 30 μM ascorbic acid, and then added to different antioxidants separately. After that, the cells were cultured at 37°C, 5% CO_2 for 4 hrs. Cell viabilities were evaluated by MTT assay. These data gathered just more than 70% of cell viability in neuron culture system.

of antioxidants expressed to cell viability, compared with GO treatment alone.

Table 1 assembled more than 70% of the antioxidative activity of several antioxidant in mouse brain culture. The protective effect of DFX showed obvious, namely 44.4% at 100 μM, and 72.9% at 1 mM DFX separatively. The laccase was added in the cultured neuron cells from 0.25 μM to 2.5 μM in 96 multiplates to find out the protective effects on hydroxyl radicals. After the addition of 0.25 μM, 0.5 μM and 1.0 μM laccase, cell viabilities were obtained 55.8%, 58.4% and 63.8% respectively (data not shown). 1.43 μM and 2.5 μM additions of the laccase were estimated 70.9% and 78.8% cell viabilities compared with control (Table 1).

In the case of water and ethanol extracts of RVS, cell viabilities were measured to be 86.1% for water extract, 87% for ethanol extract at 25 μM (10%/each well) addition of RVS extracts respectively. In addition of α-tocopherol, cell viabilities were evaluated to be 72% at 50 μM and 77% at 100 μM in the neuron cells. In table 1 shown, more than 70% cell survival of neuron cells showed at 1 mM DFX, at 1.43 μM laccase, at 12.5 μM extracts from RVS and at 50 μM α-tocopherol.

IV. DISCUSSION

The dissociated embryonic mouse whole brain tissue culture in this study provides a model system for oxidative stress which has been implicated in several neurological diseases. Oxidative damage to cells is the result of a series of cascade reactions by radicals. Hydroxyl radical produced from Fenton's reaction can act directly on cells and destroy the essential enzymes to cell survival or can degrade nucleic acids, cytoskeletal proteins and lipid membranes of cells, leading to cell death (halliwell and Gutteridge, 1990; Carney *et al.*, 1991). Therefore, we investigated whether laccase can play the role as an antioxidant or not in embryonic mouse whole brain tissue culture.

In this experiment, at 20 μM iron or at 30 μM ascorbic acid alone, cells were not damaged and the cell survival was >90% even at 16 hrs of incubation (data not shown). In the 30 μM ascorbic acid with 1 μM~1 mM Fe^{3+}, the number of living cells was over 90% even 16 hrs incubation, while the 1~100 μM ferrous iron in presence of ascorbic acid damaged entirely all neuron (Zhang *et al.*, 1993). Therefore, we used 20 μM ferrous iron and 30 ascorbic acid in this experiment. To make sure reduction of ferric iron into ferrous iron, we used excessiver amount of ascorbic acid than ferric iron. Here, 30 μM ascorbic acid which is excessive amount of stochiometric amount to reduce ferric iron has not any problem by itself.

In the case of both iron (Fe^{2+}, Fe^{3+}), Fe^{2+} was more toxic than Fe^{3+}, although they were not differed significantly. We assume that this based on Fenton's reaction in which the reaction of Fe^{2+} with H_2O_2 is very rapidly (Braughler *et al.*, 1986), resulting in the formation of hydroxyl radicals. In nature, ascorbic acid possesses two characteristics, reductant and antioxidant. One of the properties of ascorbic acid is the reduction of substances or metal, the other is its effect on antioxidants against oxygen free radicals. These properties can be changed by altering the several chemical conditions. As Fig. 1 shown, ascorbic acid played in 2 hrs after treatment as an antio- xidant, however it reacted from ferric iron to ferrous iron after 3 hrs coincubation as reductant.

In table 1, the number of living cells was shown 100.0% at 20 μM ferric iron alone, or at 30 μM ascorbic acid alone after 4 hrs of incubation respectively. In the case of 20 mU/m*l* GO alone, the cell viability was 48.0%, while with mixture (20 μM ferric iron, 30 μM ascorbic acid, 20 mU/m*l* GO), it was 36.3% after 4 hrs of incubation. This means that the certain concentration of iron or of ascorbic acid did not have any toxic effects by itself. However, the neurotoxicity of GO system is increased with the involvement of iron, while heavy metal chelators, desferrioxamine (DFX), decrease these cytotoxicity effects (Lim *et al.*, 1995). In this experiment, DFX had protective effects from 100 μM as iron chelator.

Otherwise, laccase is a copper-containing polyphenol oxidase and glycoprotein with carbohydrate content 11~25%. The molecular weight or structures of laccase were variable, depending on harvest season, part of tree and soil fertilization, but the properties of laccase were similar (Rodriguez *et al.*, 1997; Niku-Paavola *et al.*, 1990). Laccase is known glycoprotein which provides positive function to

cells, as a strong antioxidant.

When cell viabilities of 1.43 μM laccase showed approximately 70% antioxidative activities, to have similar character, RVS extracts had equivalent antioxidative activities at 12.5 μM in both RVS extracts (water, ethanol). These results suggest that the laccase can strongly act as an antioxidant. Although the laccase extracted from RVS by high polar solvent have 8.7 times low activity compared its commercial laccase, water or ethanol extracts from RVS act as a natural bioactive substance.

In the case of α-tocopherol, only it's high concentration had protective effects to hydroxyl radical, comparing with other antioxidants, such as laccase and RVS extracts. The reason on these results might be that α-tocopherol should be dissolved in DMSO, which is toxic solvent to cells, because the main role of DMSO in cells cause increasing membrane permeability (Mazur, 1977).

Conclusively, these results informed that the hydroxyl radicals generated by GO system are very toxic to neuron cells, that ferrous iron contributes to the increase in cytotoxicity of neuron in presence of hydroxyl radicals, that DFX as an iron chelator protects the neuron cells from the hydroxyl radicals, and that the laccase plays the apparent role as an antioxidant in GO system.

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