Suppressive Effect of Tetrahydrobiopterin on L-β-(3,4-Dihydroxyphenyl)alanine-Induced Cytotoxicity in PC-12 Cells

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Summary

We examined the inhibitory effect of tetrahydrobiopterin (BPH₄) on L-β-(3,4-dihydroxyphenyl)alanine (DOPA)-induced cytotoxicity in PC-12 cells. First *in vitro* inhibitory efficacy of BPH₄ on lipid peroxidation induced by Fe³⁺-DOPA complex was confirmed by using PC-liposomes. Fe³⁺-DOPA complex at 50 μM caused drastic lipid peroxidation, which was significantly inhibited by BPH₄ in a dose-dependent manner. Next, we confirmed the *in vitro* potency of BPH₄ in cultured PC-12 cells. Fe³⁺-DOPA complex exerted time- and dose-dependent cytotoxicity, which was also dose-dependently inhibited by BPH₄ possessed almost the same potency as deferoxamine and α-tocopherol. We also examined the cytotoxicity of DOPA itself. A higher dose of DOPA (250 μM) was necessary to afford the same cytotoxic potency towards PC-12 cells as that of Fe³⁺-DOPA complex (50 μM). The toxicity was essentially abolished by SOD and BPH₄, and ameliorated by catalase. However, deferoxamine did not modify the toxicity. In conclusion, BPH₄ protects both PC-liposomes and PC-12 cells from damage induced by DOPA with and without ferric ion, and may inhibit the degeneration of dopamine neurons through its antioxidative activity.

Key words : Tetrahydrobiopterin, DOPA, Cytotoxicity, PC-12 cells

Introduction

5,6,7,8-Tetrahydrobiopterin (BPH₄), which is biosynthesized from guanosine triphosphate (GTP) (1, 2), acts as a cofactor for phenylalanine, tyrosine and tryptophan hydroxylases (3-5). It plays an important role in regulating the *in vivo* activities of tyrosine and tryptophan hydroxylases, which are the rate-limiting enzymes in the biosynthesis of the catecholamines and serotonin, respectively. BPH₄ also has a regulatory role in cytokine-induced NO biosynthesis in various cells (6-9).

Parkinsonism is pathologically manifested as a premature degeneration of dopamine neurons, leading to depletion of dopamine, and it has been suggested that the possible elevation of tyrosine hydroxylase activity in surviving dopamine neurons by BPH₄ administration may provide a therapeutic means of increasing striatal synaptic dopamine (10-12). It has been speculated that the degeneration of dopamine neurons in Parkinson's disease is induced, at least in part, by active oxygen radicals (13, 14).

We have recently observed strong antioxidative activity of BPH₄, and suggested a possible new function of this compound as an endogenous an-

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tioxidant (15, 16). Based on the hypothesis that degeneration of neurons is caused by active oxygen radicals, BPH₄ has been suggested as a potential therapeutic agent for Parkinson's disease on the basis of not only its potency to increase the supply of dopamine as a cofactor for hydrolases, but also its antioxidative efficacy.

In this study, suppression of DOPA-induced cytotoxicity by BPH₄ was investigated in the PC-12 cell line (derived from rat adrenal medulla), which has dopamine-synthesizing, -storage, and -releasing potentials similar to those of neurons.

Materials and Methods

Materials

RPMI 1640 was purchased from Nissui Co., Ltd., Tokyo, Japan. Fetal bovine serum (FBS) was from BIO Whittaker Co., Maryland, U.S.A.

Horse serum (HS) was from Gibco Lab., Germany. β -NADH was obtained from Oriental Yeast Co. Ltd., Tokyo Japan. Superoxide dismutase (SOD, 3900 units/mg), catalase (7500 units/mg), α -tocopherol acetate (α -TP), sodium pyruvate, phosphatidylcholine (PC) from egg yolk, L- β -(3,4-dihydroxyphenyl)alanine (L-DOPA), ferrous chloride, and adenosine 5'-diphosphate monosodium salt (ADP) were obtained from Wako Chemicals Co. Ltd., Tokyo, Japan. Kanamycin sulfate was from Meiji Seika Co., Ltd., Tokyo, Japan. Deferoxamine (DF) was from Nippon Ciba Geigy Co. Ltd., Tokyo, Japan. α -Phenyl-N-t-butyl nitron (PBN) was obtained from Aldrich, Tokyo, Japan. 5,6,7,8-Tetrahydrobiopterin (BPH₄) was a gift from Suntory Co. Ltd., Tokyo, Japan.

Inhibitory effect of BPH₄ on autoxidation of PC-liposomes

Phosphatidylcholine (PC) from turkey egg yolk (100 mg) was dissolved in chloroform: methanol (2:1) solution, and evaporated under nitrogen gas to form a thin layer on the wall of a glass vessel. Phosphate-buffered saline (PBS) was then added and agitated vigorously for 10 min to obtain a liposome suspension. One ml of the solution was incubated with Fe³⁺-DOPA complex in the presence or absence of BPH₄ or another scavenger at 37°C. An aliquot of the liposomal preparation (0.2 ml) was removed at intervals and boiled for 40 min in 1 ml of 15% trichloroacetic acid and 0.25% thiobarbituric acid. It was cooled, then 4 ml of butanol was added. The mixture was vortexed vig-

orously and centrifuged at 3,000 rpm for 30 min. The absorbance of the butanol layer was measured at 532 nm to estimate the content of malondialdehyde (MDA). Fe³⁺-DOPA complex was prepared according to the method of Sugioka and Nakano (17) with a slight modification.

Tissue culture

PC-12 cell line was obtained from Riken Cell Bank, Tsukuba, Japan. The cells were routinely cultured in RPMI 1640 medium supplemented with 10% HS and 5% FBS at 37°C in an atmosphere of 95% O₂ and 5% CO₂. The cells suspended in the medium were seeded into 24-well plates at a concentration of 1×10^6 cells per dish and were cultured for 24 hr. Fe³⁺-DOPA, DOPA, BPH₄ and other scavengers including SOD (1000 U/ml), catalase (200 U/ml), PBN (100 μ M), DF (100 μ M), mannitol (MAN, 10 μ M) and α -TP (100 μ M), dissolved in the medium, were then added as appropriate and incubation was continued in a CO₂ incubator.

Assay of lactate dehydrogenase (LDH)

LDH released into the medium from PC-12 cells was assayed as a marker of cell damage. An aliquot of the supernatant of the cultured medium was collected at each time interval and subjected to LDH assay with LDH CII-Test Wako (Wako Chemicals Co., Ltd., Tokyo, Japan). The activity was expressed as Wroblewski units/ml medium.

Assay of lipid peroxides

After incubation, lipid peroxides in the cells were measured by a thiobarbituric acid procedure according to the method of Gavino *et al.* (18). The concentration of lipid peroxides was expressed as nmol of malondialdehyde (MDA) per dish.

Statistical analysis

Statistical significance was determined by Student's *t* test for the comparison between two groups or two way repeated measures analysis of variance (ANOVA) and Dunnett's tests for multiple comparison where appropriate. The criterion of significance was taken as $P < 0.05$.

Results

Oxidation of PC-liposomes and inhibition by BPH₄

As shown in Figure 1, significant increases of

Figure 1. Time courses of the effect of Fe³⁺-DOPA complex on the lipid peroxidation of PC-liposomes. PC-liposomes suspended in phosphate-buffered saline were incubated with 10, 50, 100, and 200 μM Fe³⁺-DOPA complex for 24 hr. Aliquots of sample were collected at intervals and subjected to lipid peroxide assay. Lipid peroxides are represented as the content of malondialdehyde (MDA). Each value is the mean ± S.E.M. of 3 independent assays. Significantly different from the normal control (0 μM): *P<0.05, ***P<0.001.

Figure 2. Effects of BPH₄ on the lipid peroxidation of PC-liposomes induced by Fe³⁺-DOPA complex. PC-liposomes suspended in phosphate-buffered saline were incubated with 50 μM Fe³⁺-DOPA complex in the presence of BPH₄ for 3 hr, and lipid peroxide was assayed. Lipid peroxides are represented as the content of malondialdehyde (MDA). Each value is the mean ± S.E.M. of 3 independent assays. "Nor" indicates the control without Fe³⁺-DOPA. Significantly different from Fe³⁺-DOPA complex-treated group: **P<0.01.

MDA content were observed at concentrations of more than 50 μM Fe³⁺-DOPA complex, without

Table 1. Inhibitory potency of BPH₄ and other scavengers against lipid peroxidation of PC-liposomes induced by Fe³⁺-DOPA

Scavenger	50%-inhibitory conc. (IC ₅₀ , μM)
BPH ₄	650
DF	240
α-TP	120
SOD	-
catalase	-
PBN	-
MAN	-

BPH₄, 5,6,7,8-tetrahydrobiopterin; DF, deferoxamine; α-TP, α-tocopherol; SOD, superoxide dismutase; PBN, α-phenyl-N-t-butyl nitone; MAN, mannitol. -, no potency.

Figure 3. Time courses of the effect of Fe³⁺-DOPA complex on LDH release by PC-12 cells as a mark of cell damage. The cells were incubated with 10, 50, 100, and 200 μM Fe³⁺-DOPA complex for 48 hr. Aliquots of the medium were collected at intervals and subjected to LDH assay. LDH activity is represented as Wroblewski units/ml medium. Each value is the mean ± S.E.M. of 4 independent assays. Significantly different from the normal control (0 μM): *P<0.05, ***P<0.01.

apparent dose-dependency. The content increased in a time-dependent manner, reaching a plateau at about 3 hr. We then examined the effect of BPH₄ on the elevation of lipid peroxides in PC-liposomes treated with 50 μM Fe³⁺-DOPA for 3 hr. The elevation was suppressed by BPH₄ in a dose-dependent manner at concentrations ranging from 50 to 1000 μM (Figure 2). The efficacy of BPH₄ was compared with those of other scavengers, i.e., DF and α-TP. As shown in Table 1, the 50%-inhibitory concentrations (IC₅₀) for BPH₄, DF and α-TP were 650 μM, 240 μM and 120 μM, respectively. SOD (1000 U/ml), catalase (200 U/ml), PBN (100 μM), and MAN (10 mM) were ineffective.

Figure 4. Effects of BPH₄ on the elevation of LDH activity induced by Fe³⁺-DOPA complex in cultured PC-12 cells. The cells were incubated with 50 μM Fe³⁺-DOPA complex in the presence of BPH₄ for 48 hr, and LDH activity was assayed. LDH activity is represented as Wroblewski units/ml medium. Each value is the mean ± S.E.M. of 4 independent assays. "Nor" indicates the control without Fe³⁺-DOPA or BPH₄. Significantly different from Fe³⁺-DOPA complex-treated group: **P<0.01, ***P<0.001.

Elevation of LDH activity induced by Fe³⁺-DOPA in PC-12 cells

Release of LDH, a marker enzyme for cytotoxicity, from PC-12 cells was assessed at different concentrations of Fe³⁺-DOPA complex. Namely, PC-12 cells were incubated with 10, 50, 100 and 200 μM Fe³⁺-DOPA complex and the release of LDH from the cells was assayed as a function of time for 48 hr. As shown in Figure 3, no significant increase of LDH activity was observed 24 hr after the start of treatment with Fe³⁺-DOPA complex at any dose examined. However, significant increases of LDH activity were observed at 48 hr, though the LDH activity of control cells was also increased at 48 hr-incubation. Based on these data, we fixed the concentration of Fe³⁺-DOPA complex at 50 μM for further experiments.

Effect of BPH₄ on Fe³⁺-DOPA-induced cytotoxicity in PC-12 cells

The effect of BPH₄ on the elevation of LDH activity at 48 hr after treatment of PC-12 cells with Fe³⁺-DOPA (50 μM) was examined. As can be seen in Figure 4, the Fe³⁺-DOPA complex-induced elevation of LDH activity was inhibited by BPH₄ in a dose-dependent manner at concentrations ranging from 50 to 400 μM. BPH₄ itself

Figure 5. Effect of BPH₄ and other scavengers on the elevation of LDH activity and lipid peroxides induced by Fe³⁺-DOPA complex in cultured PC-12 cells. A fixed concentration (100 μM) of BPH₄ or another scavenger was added to the medium simultaneously with 50 μM Fe³⁺-DOPA complex, and the mixture was incubated for 48 hr. LDH activity in the medium and lipid peroxides in the cells were assayed. LDH activity and lipid peroxides content are expressed in Wroblewski units/ml and as malondialdehyde content per mg protein (MDA/mg protein), respectively. BPH₄, 5,6,7,8-tetrahydrobiopterin; DF, deferoxamine; α-TP, α-tocopherol. Each value indicates the mean ± S.E.M. of 4 determinations. "Nor" indicates the control without Fe³⁺-DOPA or scavenger. Significantly different from Fe³⁺-DOPA complex-treated group: *P<0.05, **P<0.01.

did not increase the release of LDH in the cultures. The potency of BPH₄ was compared with those of other scavengers in terms of LDH activity and MDA content at a constant concentration of 100 μM. All of these scavengers significantly suppressed the elevations of both chemical markers induced by Fe³⁺-DOPA complex (Figure 5).

Effect of BPH₄ on DOPA-induced cytotoxicity in PC-12 cells

We next investigated the effect of BPH₄ on cytotoxicity induced by DOPA without Fe³⁺. The dose-dependence of the effect of DOPA on LDH release from PC-12 cells was preliminarily investigated (data not shown). The cells were incubated with 50, 100, 250 and 500 μM DOPA and the release of LDH in the culture medium was assayed as a function of time for 48 hr. No significant increase of LDH was observed at concentrations of less than 100 μM, but LDH was elevated at 250 and 500 μM. The activity at 24 hr after treatment with 250 μM DOPA was elevated to almost the same degree as with 50 μM Fe³⁺-DOPA complex. Thus, we examined the effect of BPH₄ on the cytotoxicity induced by 250 μM

Figure 6. Effect of BPH₄ and other scavengers on the elevation of LDH activity induced by DOPA in cultured PC-12 cells. An appropriate concentration of BPH₄ or another scavenger was added to the medium simultaneously with 250 μ M DOPA, and the mixture was incubated for 24 hr. LDH activity was assayed. LDH activity was expressed as Wroblewski units/ml. Each value indicates the mean $\pm$ S.E.M. of 4 determinations. "Nor" indicates the control without DOPA or scavenger. Significantly different from DOPA-treated group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. BPH₄, 5,6,7,8-tetrahydrobiopterin (100 μ M); SOD, superoxide dismutase (1000 U/ml); CAT, catalase (200 U/ml); MAN, mannitol (10 mM); PBN, α -phenyl-N-t-butyl-nitron (100 μ M); DF, deferoxamine (100 μ M).

DOPA. As can be seen in Figure 6, 100 μ M BPH₄ significantly suppressed the elevation of LDH activity induced by 250 μ M DOPA, as it did in the case of that induced by 50 μ M Fe³⁺-DOPA complex. SOD (1000 U/ml), a scavenger of O₂⁻, markedly reduced the elevation of LDH and catalase (200 U/ml), a scavenger of H₂O₂, showed partial inhibition, while MAN (10 mM) and PBN (100 μ M), scavengers of $\cdot$ OH and ¹O₂, respectively, were ineffective. DF, a specific chelator of iron, had no effect under these conditions.

Discussion

The degeneration of nigro-striatal dopaminergic neurons is considered to be a predominant pathogenetic factor in Parkinson's disease (19), and may involve oxygen free radicals such as O₂⁻, $\cdot$ OH, H₂O₂ and ¹O₂ (13, 14). This view is supported by the acceleration of lipid content (20) and increased iron level in the substantia nigra (21, 22) decreased peroxidase and catalase levels (23) and so on (24, 25). Generation of oxygen free radicals through the autoxidation of DOPA

has been well documented (26-30). Further, Sotomatsu *et al.* (31) demonstrated that ferric ions significantly promoted peroxidative cleavage of unsaturated phospholipids in liposomes *in vitro* after coordinating with DOPA and dopamine, and that α -TP and DF completely abolished the Fe³⁺-DOPA-induced lipid peroxidation, while SOD, catalase and sodium benzoate did not. Ceruloplasmin, a ferroxidase, also significantly inhibited the lipid peroxidation. Based on these results, they proposed that not oxygen free radicals, but rather oxene complexes such as Fe(V)=O and/or Fe(IV)=O generated after further peroxidation would abstract hydrogen atoms from unsaturated phospholipids to initiate lipid peroxidation as a possible mechanism of Fe³⁺-DOPA-induced lipid peroxidation.

Interaction of iron with melanin could be another important trigger for neuronal cell death, because melanin avidly binds Fe³⁺ and reduces it to Fe²⁺, resulting in O₂⁻($\cdot$ OH) generation (32).

BPH₄ is the cofactor for monoamine oxygenase, which is involved in the biosynthesis of biogenic amines such as catecholamines, and is also a potent antioxidant (15, 16). In the present study, we examined the inhibitory effect of BPH₄ on DOPA-induced cytotoxicity in PC-12 cells. First, the *in vitro* inhibitory potency of BPH₄ on lipid peroxidation induced by Fe³⁺-DOPA complex was assessed by using PC-liposomes. The complex at 50 μ M or more caused drastic lipid peroxidation (Figure 1), though there was no significant difference among the 50, 100 and 200 μ M treated groups. These findings are consistent with an earlier report (31). The lipid peroxidation induced by 50 μ M Fe³⁺-DOPA was significantly inhibited by BPH₄ in dose-dependent manner (Figure 2). Other scavengers, DF and α -TP, also suppressed the oxidation (Table 1). Since tetrahydropterin was shown to stabilize iron (III) by weak complex formation (33), BPH₄ may have a similar action, in addition to its antioxidant potency.

Next, we confirmed the *in vitro* potency of BPH₄ in cultured PC-12 cells. Fe³⁺-DOPA complex increased LDH activity time- and dose-dependently (Figure 3). The elevation of this activity was also dose-dependently suppressed by BPH₄ (Figure 4), which possessed almost the same potency as DF and α -TP (Figure 5). BPH₄ also inhibited DOPA-induced cytotoxicity, though as shown in Figure 6, much higher doses of DOPA were necessary to cause the same cytotoxic effect in PC-12 cells as compared with Fe³⁺-DOPA complex. The toxicity was decreased markedly by SOD, and

partially by catalase, while other scavengers were ineffective. Although the above results indicate that O₂⁻ and H₂O₂ mostly contribute to DOPA-induced cytotoxicity, other radicals may be involved. Basma *et al* (34) proposed that O₂⁻ and H₂O₂ are intermediates in the formation of the more toxic radical, ·OH, via trace metal catalyzed Fenton- and/or Haber/Weiss-type reaction. Another somewhat different hypothesis has also been proposed (35). In any event, it appears that the cytotoxicity induced by Fe³⁺-DOPA and DOPA is due, for the most part, to the action of oxygen free radicals formed via autoxidation. Thus, the protective effect of BPH₄ on both PC-liposomes and PC-12 cells against damage induced by DOPA with and without iron ion can be ascribed to the potent radical scavenging ability of BPH₄. We consider that BPH₄ is a potential therapeutic agent for Parkinson's disease through not only its potency to increase the supply of dopamine as a cofactor for hydrolases, but also its antioxidative potency.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent inducer of an animal model of Parkinson's disease. We have preliminarily examined the effect of BPH₄ on MPTP-induced PC-12 cell damage, and have already found that 1-methyl-4-phenylpyridinium (MPP⁺), which is considered as an ultimate active metabolite of MPTP, markedly induces PC-12 cell damage and BPH₄ significantly protects the cells from this damage (data not shown). Further investigations are under way.

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References

1. Blau, N. and Niederwieser, A. GTP cyclohydrolases: A review. *J Clin. Chem. Clin. Biochem.* 1985; 23: 169-176.
2. Nichol, C.A., Smith, G.K. and Duch, D.S.: Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Ann. Rev. Biochem.* 1985; 54: 729-764.
3. Kaufman, S.: The structure of phenylalanine hydroxylation co-factor. *Proc. Natl. Acad. Sci. U.S.A.* 1963; 50: 1085-1093.
4. Shiman, R., Akino, M., and Kaufman, S., Solubilization and partial purification of tyrosine hydroxylase from bovine adrenal medulla. *J. Biol. Chem.* 1971; 246: 1330-1340.
5. Friedman, P.A., Kappelman, H., and Kaufman, S., Partial purification and characterization of tryptophan hydroxylase from rabbit hind brain. *J. Biol. Chem.* 1972; 247: 4165-4173.
6. Kwon, N.S., Nathan, C.F., and Stuehr, D.J., Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. *J. Biol. Chem.* 1989; 264: 20496-20501.
7. Werner-Felmayer, G., Werner, E.R., Hausen, A., Reibnegger, G., and Wachter, H., Tetrahydrobiopterin-dependent formation of nitrite and nitrate in murine fibroblasts. *J. Exp. Med.* 1990; 172:1599-1607.
8. Schmidt, K., Werner, E.R., Mayer, B., Wachter, H. and Kukovetz, W.R., Tetrahydrobiopterin-dependent formation of endothelium derived relaxing factor (nitric oxide) in aortic endothelial cells. *Biochem. J.* 1992; 281: 297-300.
9. Kim, Y.M., and Lancaster, JR-Jr. Tetrahydrobiopterin-dependent nitrite oxidation to nitrate in isolated rat hepatocytes. *FEBS Lett.* 1993; 332: 255-259.
10. Keller, R., Bartholini, G., and Plescher, A., *In vivo* enhancement of tyrosine hydroxylation in rat striatum by tetrahydrobiopterin. *Nature* 1974; 249: 476-478.
11. Curtius, H.C.H., Niederwieser, A., Levine, R., and Muldner, H., Therapeutic efficacy of tetrahydrobiopterin in Parkinson's disease. *Adv. Neurol.* 1984; 40: 463-466.
12. Koshimura, K., Miwa, S., Lee K., Fujiwara, M., and Watanabe, Y., Enhancement of dopamine release in vivo from the rat striatum. *J. Neurochem.* 1990; 54: 1391-1397.
13. Cohen, G., Oxygen radicals and Parkinson's disease. In: *Oxygen Radicals and Tissue Injury*, Halliwell B, eds. Bethesda FASEB 1988; 130-135.
14. Adams, J.D., and Odunze, I.N., Oxygen free radicals and Parkinson's disease. *Free Radical Biol-Med.* 1991; 10: 161-169.
15. Kojima, S., Ona, S., Iizuka, H., Arai, T., Mori, H., and Kubota, K., Antioxidative activity of 5,6,7,8-tetrahydrobiopterin and its inhibitory effect on paraquat-induced cell toxicity in cultured rat hepatocytes. *Free Rad. Res.* 1995; 23: 419-430.
16. Kojima, S. Neo Neopterin as an endogenous antioxidant. *Pteridines* 1995; 6: 181-184.
17. Sugioka, K., and Nakano, M., Mechanism of phospholipid peroxidation induced by ferric ion-ADP-adrinamycin coordination complex. *Biochim. Biophys. Acta.* 1982; 713: 333-343.
18. Gavino, V.C., Miller, J.S., Ikharebha, S.O., Milo, G. E., and Cornwell, D.G., Effect of polyunsaturated fatty acids and antioxidants on lipid peroxidation in tissue cultures. *J. Lip. Res.* 1981; 22: 763-769.
19. Forno, L.S., Pathology of Parkinson's disease. In: *Movement Disorders*, Marssden CD and Fahn S, eds. Butterworth Scientific, London: (1982).
20. Dexter, D.T., Carter, C., Agid, F., *et al.* Lipid peroxidation as a cause of nigral cell death in Parkinson's disease. *Lancet* 1986; 2: 639-640.
21. Sofic, E., Paulus, W., Jellinger, K., Riederer, P., and Youdim, M.B.H., Selective increase of iron in sub-

- stantia nigra zona compacta of parkinsonian brains. *J Neurochem.* 1991; 56: 978-982.
22. Dexter, D.T., Carayon, A., Agid, F., *et al.* Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neuro-degenerative diseases affecting the basal ganglia. *Brain* 1991; 114: 1953-1975 (1991).
 23. Ambani, L.M., Van Woet, M.H., and Murphy, S., Brain peroxidase and catalase in Parkinson's disease. *Arch. Neurol.* 1975; 32: 114-118.
 24. Perry, T.L., and Yong, V.W., Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione and metabolism in the substantia nigra of patients. *Neurosci. Lett.* 1986; 67: 269-274.
 25. Riederer, P., Sofic, E., Rausch, W.D. *et al.* Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brain. *J. Neurochem.* 1989; 52: 515-520.
 26. Rosenberg, P.A., Catecholamine toxicity in cerebral cortex in dissociated cell culture. *J. Neurosci.* 1988; 8: 2887-2894.
 27. Michel, P.P., and Hefti, F., Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. *J. Neurosci. Res.* 1990; 26: 428-435.
 28. Ogawa, N., Edamatsu, R., Mizukawa, K., Asanuma, M., Kohno, M., and Mori, A., Degeneration of dopaminergic neurons and free radicals: possible participation of levodopa. *Adv. Neurol.* 1993; 60: 242-250.
 29. Ben-Shachar, D., and Youdim, M.B.H., Intranigral iron injection induces behavioral and biochemical "parkinsonism" in rats. *J. Neurochem.* 1991; 57: 2133-2135.
 30. Jellinger, K., Kienzl, E., Rumpelmair, G. *et al.* Iron-melanin complex in substantia nigra of Parkinsonian brain: An X-ray microanalysis. *J. Neurochem.* 1992; 59: 1168-1171.
 31. Sotomatsu, A., Nakano, M., and Hirai, S., Phospholipid peroxidation induced by the catechol-Fe³⁺ (Cu²⁺) complex: a possible mechanism of nigrostriatal cell damage. *Arch. Biochem. Biophys.* 1990; 283: 334-341.
 32. Hintz, P., and Kalyanaraman, B., Metal ion-induced activation of molecular oxygen in pigmented polymers. *Biochim. Biophys. Acta.* 1986; 883: 41-45.
 33. Schäfer, A., Fischer, B., Bosshard, R., Hesse, M. and Viscontini M: Formation of FeIII or FeII complexes with acetylacetonate and 5,6,7,8-tetrahydropterin as ligands and their activation of oxygen. In *Chemistry and Biology of Pteridines and Folates*, Ayling JE, Nair MG and Baugh CM, eds. New York; Plenum Press, 1993; 29-32.
 34. Basma, A.N., Morris, E.J., Nicklas, W.J., and Geller, H.M., L-DOPA cytotoxicity to PC 12 cells in culture is via its autoxidation. *J. Neurochem.* 1995; 64: 825-862.
 35. Olney, J.W., Zorumski, C.F., Stewart, G.R., Price, M. T., Wang, G., and Labruyere, J., Excitotoxicity of L-DOPA and 6-OHDOPA: implications for Parkinson's and Huntington's diseases. *Exp. Neurol.* 1990; 108: 269-272.