·Original Article·

Iron contributes to the formation of catechol isoquinolines and oxidative toxicity induced by overdose dopamine in dopaminergic SH-SY5Y cells

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Abstract: Objective The selective loss of dopaminergic neurons in Parkinson's disease is suspected to correlate with the increase of cellular iron, which may be involved in the pathogenesis of PD by promotion of oxidative stress. This research investigated dopamine-induced oxidative stress toxicity contributed by iron and the production of dopamine-derived neurotoxins in dopaminergic SH-SY5Y cells. **Methods** After the SH-SY5Y cells were pre-incubated with dopamine and Fe^{2+} for 24 h, the cell viability, hydroxyl radical, melondialdehyde, cell apoptosis, and catechol isoquinolines were measured by lactate dehydrogenase assay, salicylic acid trapping method, thiobarbuteric acid assay, Hoechst 33258 staining and HPLC-electrochemical detection (HPLC-ECD), respectively. **Results** (1) Optimal dopamine (150 μmol/L) and Fe^{2+} (40 or 80 μmol/L) significantly increased the concentrations of hydroxy radicals and melondialdehyde in SH-SY5Y cells. (2) Induction with dopamine alone or dopamine and Fe^{2+} (dopamine/Fe^{2+}) caused cell apoptosis. (3) Compared with untreated cells, the catechol isoquinolines, salsolinol and *N*-methyl-salsolinol in dopamine/Fe^{2+}-induced cells were detected in increasing amounts. **Conclusion** Due to dopamine/Fe^{2+}-induced oxidative stress similar to the state in the parkinsonian substantia nigra neurons, dopamine and Fe^{2+} impaired SH-SY5Y cells could be used as the cell oxidative stress model of Parkinson's disease. The catechol isoquinolines detected in cells may be involved in the pathogenesis of Parkinson's disease as potential neurotoxins.

Keywords: Parkinson's disease; Fe^{2+}; dopamine; oxidative stress; salsolinol; *N*-methyl-salsolinol

1 Introduction

Iron is an essential element for life responsible for proper brain functions. It plays roles in a number of vital processes such as gene expression, neuronal development, enzymatic reactions, the tricarboxylic acid cycle, electron transportation, toxication, and detoxication reactions[1,2]. Numerous postmortem and intravitam studies have demonstrated that iron level was increased in the neuronal cells of parkinsonian substantia nigra pars compacta (SNpc)[3-5]. Although the mechanism of neuronal loss remains unclear, it is proposed that oxidative stress plays an important role in the pathogenesis of Parkinson's disease (PD)[6,7]. One of major contributors to the oxidative stress in PD might be changes of dopamine level, because the enzymatic and non-enzymatic degradation of dopamine can lead to H_2O_2 production. In addition, increased iron, especially ferrous iron (Fe^{2+}), can promote the conversion of H_2O_2 to ·OH via the Fenton reaction and favor a greater turnover in the Haber-Weiss cycle, resulting in an amplification of oxidative stress[8].

In this study, dopamine combined with iron (Fe^{2+}) was added into human SH-SY5Y neuroblastoma cells to induce the condition of oxidative stress, a similar state to that in the parkinsonian SNpc. Levels of cellular catechol isoquinolines, a kind of potential endogenous neurotoxin, were measured under the oxidative stress.

2 Materials and methods

2.1 Reagents The human dopaminergic neuroblastoma SH-SY5Y cell line was provided by Dr. Wei-Hong SONG

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(Department of Psychiatry, Brain Research Center, University of British Columbia, Canada). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Invitrogen Co., USA). Dopamine hydrochloride, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol), 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,5-dihydroxybenzoic acid (2,5-DHBA), sodium 1-heptanesulfonnate (SHS), and Hoechst 33258 were obtained from Sigma-Aldrich Chemical Company (USA). Lactate dehydrogenase (LDH) and melondialdehyde (MDA) kits were purchased from Promega (USA) and Jiancheng Biotechnological Research Center (Nanjing, China). Fe_2SO_4 and all other chemicals used in this study were analytical grade or better quality and obtained from Beijing Chemical Reagent Company (Beijing, China) or Fisher Scientific (USA). *N*-methyl-salsolinol (*N*MSal) was synthesized according to Teitel *et al*.[9]

2.2 Cell culture and treatment SH-SY5Y cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air. Cells were trypsinized and plated in 96-well plates at a density of 2×10^4 cells/well for LDH assay; cells in 6-well plates at a density of 1×10^5 cells/well were used for apoptosis measurement; cells in 75-cm^2 flasks at a density of 3×10^8 cells/flask were used for hydroxyl radical, MDA assays, and salsolinol and *N*MSal measurement. The plated cells were allowed to grow overnight prior to treatment. All 24 h dopamine/Fe^{2+} incubations were conducted in DMEM supplement with 10% fetal bovine serum at 37 °C in 5% CO_2 atmosphere.

2.3 Cellular viability assay SH-SY5Y cells were treated with different concentrations of dopamine (0, 50, 100, 150, 200, 250, 300, or 350 μmol/L) for 24 h. Cellular viability was measured by using an LDH kit to detect the release of LDH in the cultured medium, according to manufacturer's instructions (Promega, USA).

2.4 Hydroxyl radical assay The production of intracellular hydrogen radical in the SH-SY5Y cells were quantified by measuring the equivalent derivatives 2,3-DHBA or 2,5-DHBA, after a trapping reaction with salicylic acid as described previously[10]. After treated with Fe^{2+} alone, dopamine alone, or dopamine combined with Fe^{2+}, SH-SY5Y cells were washed with PBS (pH7.4) and harvested by centrifugation. The cell suspension was homogenized by ultrasonic homogenizer (Cole-Parmer Instrument Company) and treated with 0.1 mol/L perchloric acid containing 0.1 mmol/L disodium EDTA and sodium metabisulfite for deproteinization. After centrifugation (17 000 *g* for 20 min at 4 °C), the supernatant was filtered, and DHBAs were measured by HPLC-ECD (Esa. Bioscience Inc., USA). The HPLC column was Alltima C18 5 μm column (4.6 mm × 200 mm, Alltech Associates Inc., USA). The mobile phase was 35 mmol/L citric acid / 45 mmol/L disodium acetate, pH4.5, containing 130 μmol/L disodium EDTA (Na_2EDTA) and 100 μmol/L SHS, with 17% methanol (v/v) added at a flow rate of 0.8 mL/min. The voltage of a model 6210 analytical cell was set at -50, 50, 300, and 450 mV, respectively. The level of DHBAs in each sample was quantified by comparing the DHBA peak of sample areas with that of the DHBAs standard solutions.

2.5 Lipid peroxidation assay Lipid peroxidation was analyzed by measurement of MDA, a lipid peroxidation product. After treated with Fe^{2+} alone, dopamine alone, or dopamine combined with Fe^{2+} for 24 h, cells were sonicated, and the concentration of intracellular MDA was measured by thiobarbuteric acid (TBA) assay.

2.6 Hoechst 33258 staining After SH-SY5Y cells were treated with dopamine alone, or dopamine combined with Fe^{2+} for 24 h, apoptotic cell death was assessed by the Hoechst 33258 staining method[11]. Quantification of chromatin condensation was done by counting a minimum of 250 total cells as follows: Percentage of cells with chromatin condensation = (Total cells with chromatin condensation / Total number of cells counted) × 100%. The index assessment was repeated in three independent experiments.

2.7 DNA fragmentation analysis After SH-SY5Y cells were treated with dopamine or dopamine/Fe^{2+} for 24 h, DNA fragmentation was assessed according to Qing *et al*.[11].

2.8 Measurement of salsolinol and *N*MSal in SH-SY5Y cells Salsolinol and *N*MSal were also detected by HPLC-ECD. The supernatant from SH-SY5Y cells treated with dopamine alone or dopamine/Fe^{2+} for 24 h was obtained, following the methods described before, and the samples were injected into the HPLC system. The mobile phase was 40 mmol/L citric acid / 20 mmol/L disodium phosphate, pH 3.7, containing 350 μmol/L SHS and 300 μmol/L Na_2EDTA, and 3% methanol and 6% acetonitrile (v/v) were added. The flow rate was 1 mL/min and the voltage of analytical cells was set at -50, 50, 300, and 400 mV.

2.9 Statistical analysis Data are present as mean±SEM of at

least three independent experiments. The Student's *t*-test and one-way ANOVA were used for the comparisons between the control and experimental groups. The statistical significance was taken when $P < 0.05$.

3 Results

3.1 Effects of dopamine on cell viability. The death rate of SH-SY5Y cell is expressed as percentage of control, for the Triton-X-100-treated cells taken as 100%. The LDH activity indicated that dopamine decreased viability of SH-SY5Y cells in a dose-dependent manner. Treating the SH-SY5Y cells with gradually increased concentration of dopamine (50-350 μmol/L) significantly decreased the cell viability from 79.9% to 16.0% (Fig. 1).

The dosage of 150 μmol/L dopamine was used in the following experiments.

3.2 Effects of Fe^{2+} on dopamine-induced hydroxyl radical accumulation in SH-SY5Y cells Production of hydroxyl radicals in cells is expressed as the quantity of 2,5-DHBA because the chromatographic peak of 2,3-DHBA is impacted by the peak of foreign matter. The concentration of 2,3-DHBA had no significant difference between 80 μmol/L Fe^{2+} group and the untreated control group. Incubating SH-SY5Y cells with dopamine alone or dopamine (150 μmol/L) and Fe^{2+} (40-200 μmol/L) significantly increased the concentration of 2,5-DHBA, especially when dopamine was added with 40 or 80 μmol/L Fe^{2+} (Fig. 2). However, the further increase of Fe^{2+} (120-200 μmol/L) led to reduction of 2,5-DHBA formation, though which was still much higher than that in control group. This result indicated that Fe^{2+} (80 μmol/L) alone had no effect on hydroxyl radical formation in cells, while 40 or 80 μmol/L Fe^{2+} combined with dopamine could directly promote the accumulation of hydroxyl radicals.

3.3 Effects of Fe^{2+} on dopamine-induced MDA formation in SH-SY5Y cells To further explore the effect of Fe^{2+} on the production of dopamine-induced lipid peroxidation, we assessed the levels of MDA, which is a lipid peroxidation product. Compared with untreated control, the concentration of MDA has no statistically significant difference in the cells treated with Fe^{2+} alone, but it increased significantly in the cells treated with dopamine alone (Fig. 3). Moreover, Fe^{2+} (40 or 80 μmol/L) enhanced production of dopamine-induced MDA in SH-SY5Y cells. However, Fe^{2+} at the concentrations higher than 80 μmol/L resulted in a significant decline of MDA concentration. As a consequence, 40 μmol/L and 80 μmol/L were chosen as the optimal treatment concentrations of Fe^{2+} contributing to the dopamine-induced oxidative stress for further experiments.

3.4 Promotion effect of Fe^{2+} on dopamine-induced apoptosis in SH-SY5Y cells To examine the morphological features of cell death induced by dopamine alone and in combination with Fe^{2+} (dopamine/Fe^{2+}), Hoechst 33258 staining were performed (Fig. 4I). Untreated cells showed normal chromatin (Fig. 4I A), whereas the dopamine/Fe^{2+}-induced cells showed chromatin condensation and nuclear fragmention (Fig. 4I B, C and D). The quantitative data of chromatin condensation showed that dopamine/Fe^{2+} incubation significantly increased the index of chromatin condensation than that in control (Fig. 4II). The dopamine/Fe^{2+}-induced apoptosis was further substantiated by DNA fragmentation assay. As shown in Fig. 4III, DNA fragmentations were observed in the groups treated with dopamine alone, dopamine + 40 μmol/L Fe^{2+}, and

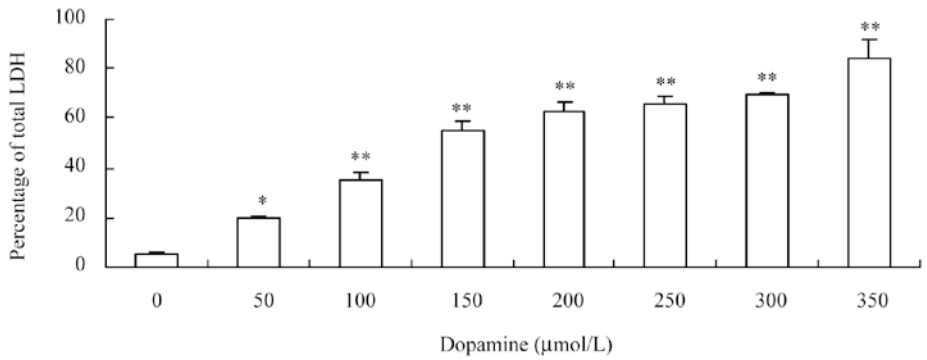

Fig. 1 Dopamine treatment significantly reduced the viability of SH-SY5Y cells. Cell viability was detected by lactate dehydrogenase (LDH) assay after incubation with different concentrations of dopamine (50-350 μmol/L) for 24 h. Values of 0% total LDH = 0% cell death, and 100% total LDH =100% cell death. *$P < 0.05$, **$P < 0.01$ *vs* 0 μmol/L dopamine group.

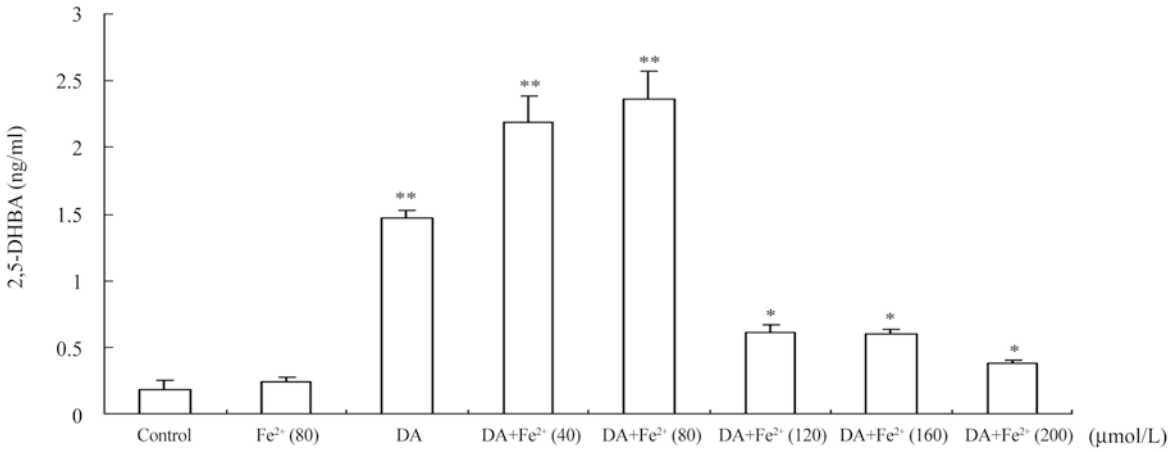

Fig. 2 Effects of Fe^{2+} on the concentration of dopamine-induced 2,5-DHBA in SH-SY5Y cells. Cells were treated with 150 μmol/L dopamine (DA) for 24 h in absence or presence of Fe^{2+} (40-200 μmol/L). Compare with untreated control, 2,5-DHBA formation was significantly increased by addition of 40 or 80 μmol/L Fe^{2+} to DA treated cells, other treatment groups can increased 2,5-DHBA formation as well. *$P < 0.05$, **$P < 0.01$ *vs* Control.

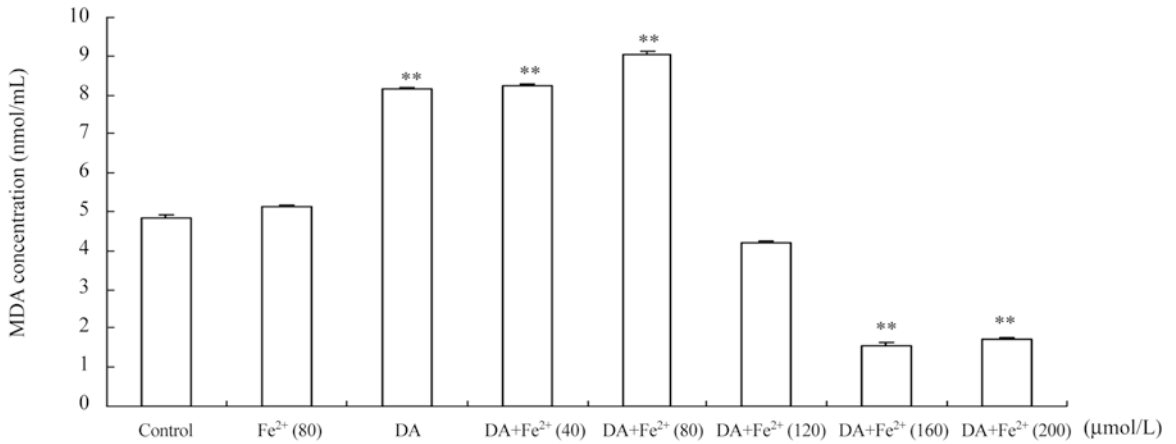

Fig. 3 Effects of Fe^{2+} on dopamine-induced MDA concentration in SH-SY5Y cells. Cells were treated with 150 μmol/L dopamine (DA) for 24 h in absence or presence of Fe^{2+} (40-200 μmol/L). Compared with untreated control, MDA concentration increased in DA (150), DA+Fe^{2+} (40) and DA+Fe^{2+} (80) groups, while reduced in DA+Fe^{2+} (160) and DA+Fe^{2+} (200) groups. There was no statistically difference between Fe^{2+} (80) or DA+Fe^{2+} (120) and control group. *$P < 0.05$, ** $P < 0.01$ *vs* Control.

dopamine + 80 μmol/L Fe^{2+}.

3.5 Effect of Fe^{2+} on dopamine-induced intracellular salsolinol and *N*MSal accumulation in SH-SY5Y cells The dopamine-derived alkaloids, salsolinol and *N*MSal have been confirmed to have selective neurotoxicity to dopamine neurons[12]. Table 1 shows the change of intracellular concentrations of dopamine, salsolinol, and *N*MSal in the SH-SY5Y cells which were incubated with dopamine alone or incubated combinedly with Fe^{2+} and dopamine for 24 h. dopamine/Fe^{2+} incubation led to a two-fold increase of dopamine concentration compared with that in control. Addition of Fe^{2+} significantly increased salsolinol concentration compared with that in control and dopamine-incubated cells; whereas at 80 μmol/L Fe^{2+}, a slight reduction in salsolinol concentration was observed. *N*MSal was not detected in control cells, but was detected in the cells exposed to dopamine/Fe^{2+}. Simultaneous treatment with dopamine and Fe^{2+} effectively increased *N*MSal concentration, and which increased further with the augment of Fe^{2+} concentration.

4 Discussion

PD is a progressive neurodegenerative disease charac-

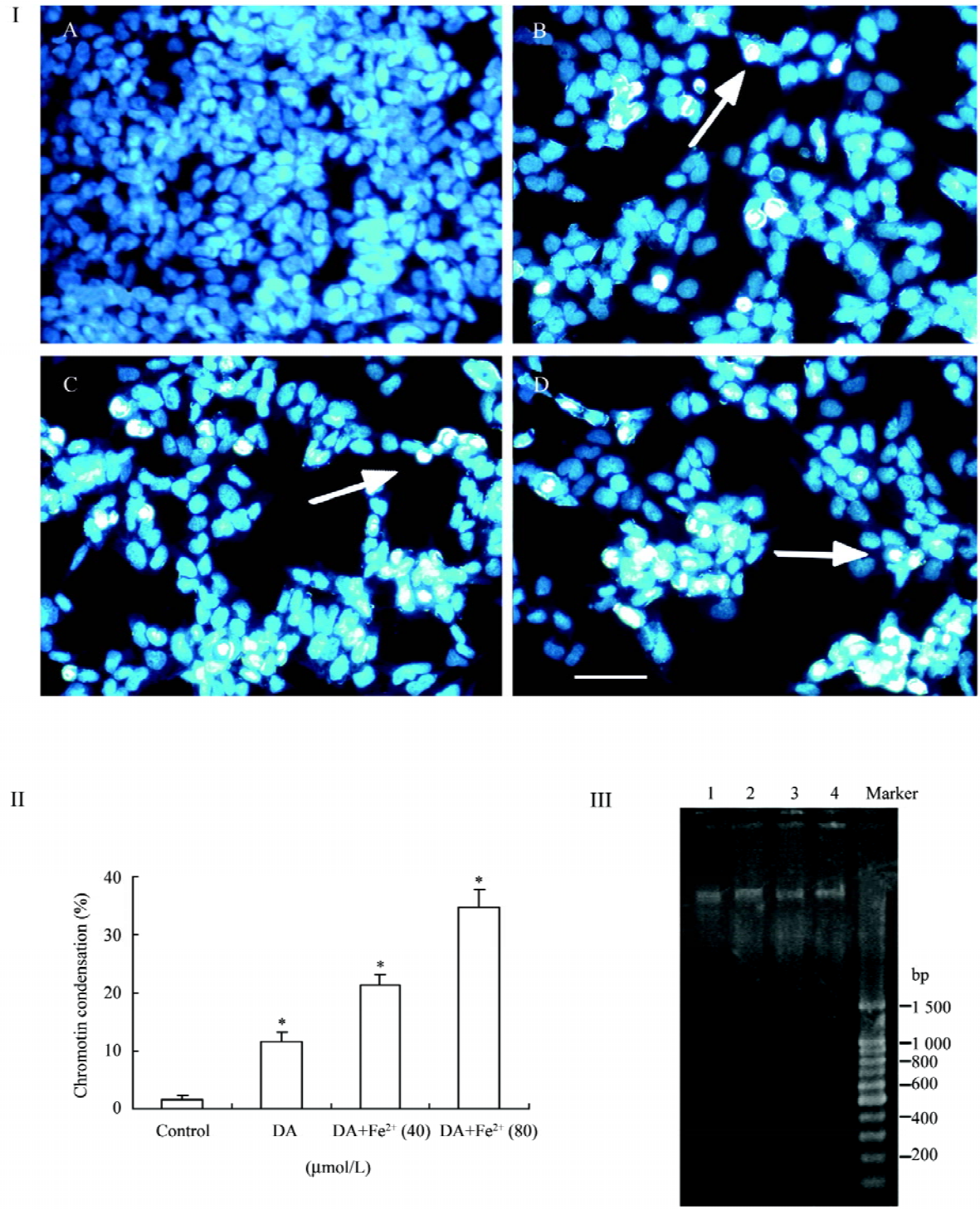

Fig. 4 Promotion effects of Fe^{2+} on dopamine-induced apoptosis. After 24 h exposure to 150 μmol/L dopamine alone or dopamine associated with 40 or 80 μmol/L Fe^{2+}, cells were stained by Hoechst 33258 and subjected to DNA fragmentation analysis. I: The Hoechst 33258 staining results. In the control group (A), the vast majority of the cells displayed normal nuclear size and blue fluorescence. However, in a culture treated with dopamine alone or DA and Fe^{2+} (B-D), many cells showed bright highly chromatin condensation and nuclear fragmentation (arrows). A: Control; B: 150 μmol/L DA; C: 150 μmol/L DA + 40 μmol/L Fe^{2+}; D: 150 μmol/L DA + 80 μmol/L Fe^{2+}. Scale bar: 10 μm. II: The percentage of cells with chromatin codensation. *$P < 0.05$, **$P < 0.01$ *vs* Control. III: DNA fragmentations induced by DA/Fe^{2+}. Lane 1, control; lane 2, DA alone; lane 2, DA with 40 μmol/L Fe^{2+}; lane 4, DA with 80 μmol/L Fe^{2+}. DNA fragmentations were observed in lane 2-4.

Tab. 1 Effects of Fe^{2+} on dopamine-induced intracellular dopamine, salsolinol and *N*MSal concentrations in SH-SY5Y cells

	Control	Dopamine	Dopamine + 40 μmol/L Fe^{2+}	Dopamine + 80 μmol/L Fe^{2+}
Dopamine	22.14±5.19	46.31±5.76	47.05±6.08	52.40±5.57*
Salsolinol	4.06±1.08	4.20±1.52	6.60±2.09*	5.68±1.72
*N*MSal	ND	1.95±0.47	2.69±1.14	6.92±2.56

ND: Not detected. *$P < 0.05$ *vs* Control.

terized by loss of dopaminergic neurons in the SNpc. Oxidative stress has been implicated to play a major role in the neuronal cell death associated with PD. Research evidence suggested that idiopathic PD may be primarily a disease resulting from oxidative stress[13]. The major facilitator of oxidative stress in PD is the unique biochemistry of the SNpc, which renders SNpc extremely vulnerable to oxidative insults and probably partially underlies the regional specificity of the neurodegeneration. One of the major features of this vulnerability is that the neurons in SNpc contain dopamine. Dopamine can be degraded both enzymatically and non-enzymatically. Both isoforms of monoamine oxidase (MAO-A and B) involved in the enzymatic degradation of dopamine are found at high levels within the SNpc. Hydrogen peroxide (H_2O_2) is produced as a byproduct of this reaction. Normally, H_2O_2 is relatively inert and non-toxic to cells, and is converted to H_2O by glutathione peroxidase using glutathione (GSH) as a reducing substrate. However, it has been demonstrated that the SNpc has a relatively low GSH content compared to other regions of the brain. The GSH levels are further reduced in the Parkinsonian SNpc, resulting in an inefficient clearance of H_2O_2 from this brain region[14,15]. In addition, the auto-oxidation of dopamine can produce 6-OHDA and other toxic end-products.

The selective loss of dopaminergic cells in PD is correlated with increased levels of cellular iron[16]. Both enzymatic and non-enzymatic catabolisms of dopamine are accelerated by the presence of redox active elements such as iron, zinc, or manganese[17]. The SNpc contains much higher concentration of iron than other brain regions, and it has been suggested that the increase in iron levels may catalyze the conversion of H_2O_2, which could be produced during the breakdown of dopamine, to highly reactive hydroxyl radicals, and thus resulted in increased oxidative damage in this region[18].

In this study, dopamine in combination with Fe^{2+} was used to induce SH-SY5Y cells in order to imitate the oxidative stress state in the parkinsonian SNpc. Our results showed that dopamine induction was toxic to the cells by causing oxidative stress, while a certain amount of Fe^{2+} did not induce oxidative stress. Moreover, addition of certain amount of Fe^{2+} could enhance dopamine-induced oxidative stress in the cells, which was demonstrated by the higher level of hydroxyl radicals, MDA, and increased apoptotic bodies. Previous research has found that addition of $FeSO_4$ could increase dopamine-induced toxicity, showing that $FeSO_4$ increased expression of the stress protein home oxygenase-1 (HO-1), enhanced nuclear condensation, and promoted caspase-3 activation[19].

Salsolinol was first detected in the urine of the PD patients administered with L-DOPA by Sandler M. in 1973[20]. This discovery stimulated the progress of researches on salsolinol derivatives in the brain. Recently, it has been confirmed that only the (*R*)enantiomers of salsolinol derivatives are selectively produced in the human brain. These are synthesized not by the nonenzymatic Pictet–Spengler reaction, but by enzymes *in situ*. A (*R*)salsolinol synthase catalyzes the enantio-specific synthesis of (*R*)salsolinol from dopamine and acetaldehyde or pyruvic acid, and then (*R*)salsolinol *N*-methyltransferase catalyzes (*R*)salsolinol into *N*-methyl(*R*) salsolinol[21]. Both *in vivo* and *in vitro* experiments demonstrated that salsolinol derivations affected the neurotransmitter levels in the brain by inhibiting the enzymes which participate in the metabolism of monoamines[22,23]. *N*MSal can cause selective apoptotic cell death in dopamine neurons[24]. The *N*MSal levels and the activities of enzymes related to the metabolism of *N*MSal are significantly higher in PD patients than that in the control group[25].

Our data showed that salsolinol and *N*MSal could be found in dopamine/Fe^{2+}-induced cells, whereas only salsolinol was detected in the control cells. Dopamine/Fe^{2+} treatment significantly increased the level of hydroxyl radical and the concentration of MDA in SH-SY5Y cells. These

results suggested that dopamine/Fe^{2+} treatment could induce oxidative stress in SH-SY5Y cells, and that the redundant free radicals may catalyze production of lipid peroxide (LPO). The condensation of dopamine, and a great amount of aldehyde analogue created by LPO, especially acetaldehyde, could offer the opportunity for the biosynthesis of salsolinol and *N*MSal. In addition, when there was no significant increase of intracellular dopamine in treatment groups, the presence of Fe^{2+} would increase intracellular concentrations of salsolinol and *N*MSal, suggesting that the formation of these catechol isoquinolines might relate directly to oxidative stress contributed by Fe^{2+}. In contrast, the normal cells only produce little *N*MSal due to lack of adequate oxidative stress. Furthermore, salsolinol could be synthesized by enzymatic synthesis or non-enzymatic Pictet-Spengler reaction, while enzyme(s) is necessary in the synthesis of *N*MSal. Thus our findings suggested that the enzymes related to the metabolism of *N*MSal might exist in the SH-SY5Y cells. *N*MSal-induced apoptosis in SH-SY5Y cells has been proven by Naoi *et al.*[26]. Therefore, salsolinol and *N*MSal, as endogenous neurotoxins, might be associated with the impairment of SH-SY5Y cells induced by dopamine/Fe^{2+}.

Though the pathogenesis of PD is still unknown, the observed biochemical change points to the involvement of oxidative stress associated with the catabolism of dopamine and iron. Increasing evidences indicate that endogenous neurotoxins, especially the catechol isoquinolines salsolinol and *N*MSal, are involved in the deterioration of nigrostriatal dopamine system in PD. In conclusion, our research indicates that dopamine/Fe^{2+} induction can lead to intracellular oxidative stress in SH-SY5Y cells. Salsolinol and *N*MSal could be an endogenous neurotoxin involved in the pathogenesis of PD. dopamine/Fe^{2+}-induced SH-SY5Y cells may be useful as an *in vitro* neurodegeneration model for investigating the potential role of endogenous dopamine-derived neurotoxin in PD.

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铁离子促进过量多巴胺引起 SH-SY5Y 细胞儿茶酚异喹啉物质生成和氧化损伤

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摘要：目的 帕金森氏病(Pakinson's disease, PD)中多巴胺能神经元选择性缺失与胞内铁水平升高有密切关系，提示铁可能通过参与氧化应激在PD发病机制中起重要作用。本研究使用一定浓度的Fe^{2+}和多巴胺诱导人多巴胺能成神经细胞瘤SH-SY5Y细胞产生氧化应激状态，并且检测胞内是否有多巴胺衍生类的神经内毒素物质产生。**方法** 多巴胺添加不同浓度的Fe^{2+}诱导SH-SY5Y细胞，24 h后用乳酸脱氢酶法、水杨酸捕获法、硫代巴比妥酸法、Hoechst 33258染色法和带有电化学检测器的高效液相色谱仪分别检测细胞存活率、羟自由基生成量、丙二醛含量、细胞凋亡和儿茶酚异喹啉物质的生成情况。**结果** (1) 150 μmol/L 多巴胺添加40或80 μmol/L Fe^{2+}后，胞内羟自由基和丙二醛含量较对照组显著增加；(2) 单独多巴胺以及多巴胺加40或80 μmol/L Fe^{2+}诱导后细胞发生凋亡；(3)在诱导后的胞内检测到Salsolinol和*N*-methylsalsolinol的含量高于对照组。**结论** 一定浓度的Fe^{2+}和多巴胺诱导SH-SY5Y细胞可模拟帕金森氏病人黑质区多巴胺能神经元所受到的氧化应激状态，胞内检测到的儿茶酚异喹啉物质，如去甲猪毛菜碱和*N*-methyl-salsolinol，可能作为一类潜在的神经毒性物质与帕金森氏病的发病有关。

关键词：帕金森氏病；Fe^{2+}；多巴胺；氧化应激；Salsolinol；*N*-methyl-salsolinol