Isradipine Can Alleviate Iron-Induced Toxicity caused by Elevated Intracellular Calcium in MES23.5 Cell

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Abstract: Previous research demonstrated that the progressive accumulation of iron in the substantia nigra pars compacta (SNpc) may contribute to dopaminergic (DA) neurons selective degeneration in Parkinson's disease (PD). However, the etiology and mechanism underlying iron-induced neurotoxicity processes are as yet unresolved. It has been reported that L-type calcium channels (LTCCs) may mediate iron influx into neuronal cells and can compete with calcium for common routes to enter primary neurons. The present study, we found that isradipine can alleviate iron-induced toxicity caused by raising intracellular calcium in MES23.5 cells. Analysis of experimental results revealed that an increase in extracellular free CaCl2 (500 μmol/L) is sufficient to promote FeSO4 (100 μmol/L) entry by activating L-type Ca2+ channels (LTCCs) significantly. The enhancement of calcium and/or iron influx was accompanied by a corresponding decrease of cell viability and higher susceptibility of toxicity, such as decrease of mitochondrial membrane potential (ΔΨm) and increase of nucleus pyknosis and cleaved caspase-3 protein expression in MPP7 (5 μmol/L) treatment MES23.5 cells. Pre-treatment with isradipine (10 μmol/L), a LTCCs blocker, for 15 min, can antagonize calcium and/or iron-induced neurotoxicity. These results suggest that application of isradipine may be a potential method for the treatment of the neurodegenerative disease induced by calcium and/or iron dysmetabolism.

Keywords: Iron, Mitochondria, Neurotoxicity, L-Type Ca2+ Channel

Introduction
Parkinson's disease (PD), also known as quiver paralytic, is a neuronal degenerative disease characterized by selective death of DA neurons in SN and in part by the presence of lewy bodies constituted of a-synuclein (a-syn) rich intracellular inclusions in cytoplasm of residual neurons1. Subsequent studies found the identification of a series of central PD hallmarks in SN, including oxidative stress, mitochondrial dysfunction, abnormal iron accumulation, abnormal protein aggregation, apoptosis, inflammation and so on. Currently, there are still no effective drugs or surgical treatments to delay or cure the progression of PD. However, studies have shown that progressive accumulation of iron in abnormal aging brain or pathologic alterations of iron homeostasis can be a contributory cause in the neurodegeneration processes observed in many neurologic disorders2.

As an indispensable part of the body's trace metal elements, the life activity system relies on iron availability for many essential functions such as the synthesis of hemoglobin in erythrocytes, the transport of oxygen and nutrients in the blood and the composition of many enzymes and oxidoreductase activators. In addition, it is also involved in the formation of myelin and the synthesis and metabolism of neurotransmitters in the brain. Iron plays a significant role in maintaining normal brain function, so deficiency, excess or metabolic disorder of iron can give rise to a series of brain diseases3. Actually, some study found that with the increase of iron levels, especially under the influence of aging factors, may result in various diseases such as heart disease, cardiovascular disease and even cancer. There is growing evidence that overload of iron and increase of hydroxyl radical formation resulted from steady-state imbalances of iron in the brain are considered to be the pathogenic cause of Parkinson's disease4.

Previous research has demonstrated that the concentration of iron in the brain interstitial fluids exceeds the binding capacity of transferrin (TF), which suggests the presence of non-transferrin-bound iron (NTBI) in the brain5. Results obtained in cardiomyocytes and neuronal cell lines suggest NTBI uptake was mediated by LTCCs activity. Research report L-type calcium channels were identified as the target of clinically used calcium channel blockers,

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2. Introduction Parkinson’s disease (PD), also known as quiver paralytic, is a neuronal degenerative disease characterized by selective death of DA neurons in SN and in part by the presence of lewy bodies constituted of a-synuclein (a-syn) rich intracellular inclusions in cytoplasm of residual neurons1. Subsequent studies found the identification of a series of central PD hallmarks in SN, including oxidative stress, mitochondrial dysfunction, abnormal iron accumulation, abnormal protein aggregation, apoptosis, inflammation and so on. Currently, there are still no effective drugs or surgical treatments to delay or cure the progression of PD. However, studies have shown that progressive accumulation of iron in abnormal aging brain or pathologic alterations of iron homeostasis can be a contributory cause in the neurodegeneration processes observed in many neurologic disorders2.

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containing many high-affinity drug binding sites of dihydropyridines (DHPs) and other organic chemistry. Isradipine, a DHP Ca\(^{2+}\) channel antagonist, is readily available to the brain with minor side effects and the same affinity for Cav1.2 and Cav1.3-type calcium channels. Studies have shown that isradipine has neuroprotective effects in the animal and cell line models of PD. Reduction of the calcium and/or iron influx mediated by isradipine markedly reduces the neurotoxin sensitivity of SNc DA neurons. In the study, our target is to clarify the protective effect of neurotoxicity induced by calcium and/or iron dysmetabolism.

### Materials and methods

#### Chemicals

1-methyl-4-phenylpyridinium (MPP\(^+\)), iron (II) sulfate heptahydrate (FeSO\(_4\)*7H2O), 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and Rhodamine123 (Rh123) were purchased from Sigma Chemical Co (Sigma, St. Louis, MO, USA). Isradipine was supplied by Shanghai Yangfan Pharmaceutical Technology Co., Ltd. (Shanghai, China). Cleaved caspase-3 primary antibody was purchased from Cell signal technology (CST, Beverly, MA USA).

#### Cell culture

MES23.5 cell line was purchased from Dr Weidong Le (Baylor College of Medicine, TX, USA) and maintained in DMEM 12 medium supplemented with 10% fetal bovine serum (10% FBS). MES23.5 cells were seeded in a 96-well plate with a density of 1 x 10\(^5\) cells/well, then grown in a humid incubator (37°C, 5% CO\(_2\)) before use.

#### MTT assay

MES23.5 cells were seeded in a 96-well plate (8 x 10\(^4\) cells/well) to approximately 70% confluency. Cells were treated with MPP\(^+\) (5 \(\mu\)mol/L) for 24 h, and some groups were pre-treated with/without isradipine (10 \(\mu\)mol/L) for 15 min, which was followed by treatment with CaCl\(_2\) and/or FeSO\(_4\) for 3 h, then washing three times with HBS. Subsequently, 20 \(\mu\)L MTT made up in sterile PBS (5 \(\mu\)g/ml) was added to 96-well plate for being incubated 4 h at humid incubator (37°C, 5% CO\(_2\)). After 4 h later, the MTT was removed and 100 \(\mu\)L DMSO was added to each well to dissolve the precipitate. The absorbance of MTT was measured using a Microplate Reader at 490 nm.

#### Cell morphological assessment

MES23.5 cells were grown in a 24-well plate with glass coverslips (4 x 10\(^5\) cells/well) overnight, then cells were treated with MPP\(^+\) (5 \(\mu\)mol/L) for 24 h. After pre-treated with/without isradipine (10 \(\mu\)mol/L) for 15 min, some groups treated with CaCl\(_2\) (500 \(\mu\)mol/L) and/or FeSO\(_4\) (100 \(\mu\)mol/L) for 3 h. After the end of different drugs treatment, cells were washed in PBS (0.01 mol/L) and fixed in 4% paraformaldehyde solution for 10 min. Cells were stained with Hoechst (500 \(\mu\)l of 10 \(\mu\)g/L/well) for 15 min at room temperature and washed with PBS for three times to remove the excessive dye. Images were observed and recorded with an inverted phase-contrast microscope equipped with a digital camera (Olympus, Japan) after incubation at 352 nm excitation and 461 nm emission wavelengths.

#### Detection of mitochondrial transmembrane potential (ΔΨm)

The presence of cell ΔΨm allows some lipophilic cationic fluorescent dyes (Rh123), to bind to the mitochondrial matrix. MES23.5 cells (1 x 10\(^5\) cells/well) were seeded on Poly-L-lysine-coated 6-well plate overnight. In brief, cells were treated as described previously. Subsequently, the cells were incubated with 1ml Rh 123 (5 \(\mu\)g/mL) for 30 min at 37°C and then washed three times with HBS. Fluorescence intensity was recorded by flow cytometry (Beckton Dickinson, USA) at 488 nm excitation and 525 nm emission wavelengths.

#### Western blot analysis

Cells (1 x 10\(^5\) cells/well) were seeded in 6-well plate overnight and treated as described before. Western blotting was used to detect the expression of caspase3 protein in different treatment groups. Each experiment was repeated at least six times, with P < 0.05 being considered to be statistically significant.

### Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as means ± S.E.M. One-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was used to compare difference between means in more than two groups. Each experiment was repeated at least six times, with P < 0.05 being considered to be statistically significant.

### Results

#### 1. MTT assay in MES23.5 cells

MTT assay can measures the effect of a drug on the cell number and the cellular enzyme activity. As shown in figure 1, the result showed that there was no significant difference in MPP\(^+\) and MPP\(^+/\)CaCl\(_2\) groups compared with the control group, while the cell
viability obviously decreased in MPP+/FeSO₄ and MPP+/CaCl₂/FeSO₄ groups (P < 0.001, compared with MPP⁺ group). While pre-incubation with isradipine for 15 min, the cell viability was increased respectively, and the difference was statistically significant (P < 0.001, compared with MPP⁺/FeSO₄ group) and (P < 0.05, compared with MPP⁺/CaCl₂/FeSO₄ group).

![Figure 1](image)

**Figure 1**

2. **Cell morphological assessment.**

   We stained cells with Hoechst 33258 and observed changes in nuclear morphology in different treatment groups. As shown in figure 2, the results showed that the nucleus were round, large and uniform dispersion of low-density fluorescence in MPP⁺ and MPP⁺/CaCl₂ groups compared with the control, while there were different degrees of obvious morphological changes such as nucleus pyknosis, fragmentation and bright fluorescence in MPP⁺/FeSO₄ groups (P < 0.001, compared with the MPP⁺ group) and MPP⁺/FeSO₄/CaCl₂ (P < 0.001, compared with the MPP⁺ group). There was an obvious phenomenon that the cell damage in MPP⁺/CaCl₂/FeSO₄ group was much greater than in MPP⁺/FeSO₄ group and the results were statistically significant (P < 0.01). Pre-incubation with isradipine can improve cell damage caused by CaCl₂ and/or FeSO₄, and the difference is statistically significant (P < 0.01, compared with MPP⁺/FeSO₄ group) and (P < 0.01, compared with MPP⁺/CaCl₂/FeSO₄ group).
3. **Isradipine prevents FeSO₄–induced ΔΨm reduction in MES23.5 cells.**

We observed ΔΨm changes with calcium and/or iron treatment in MES23.5 cells by flow cytometry. As shown in figure 3, most of the cells appeared in the high Rh123 fluorescence field in the control group, which is indicative of complete, viable cells with a high ΔΨm. There were slightly lower in MPP⁺ group and MPP⁺/CaCl₂ group than control group, however about 25% reduction in MPP⁺/FeSO₄ group and 37% reduction in MPP⁺/FeSO₄/CaCl₂ group and the difference was statistically significant ($P < 0.001$, compared to MPP⁺ group). However, ΔΨm decreased 12% in MPP⁺/CaCl₂/FeSO₄ group ($P < 0.05$, compared to MPP⁺/FeSO₄ group), indicating mitochondrial dysfunction more serious than iron alone. Pretreatment with isradipine partially alleviated the ΔΨm reduction and the ΔΨm rose 13% ($P < 0.05$, compared with MPP⁺/FeSO₄ group) and 18% ($P < 0.001$, compared with MPP⁺/CaCl₂/FeSO₄ group), respectively.
4. **Isradipine inhibits FeSO$_4$-induced cleaved caspase-3 expression in MES23.5 cells.**

In order to confirm this hypothesis and elucidate the mechanism of iron-induced cell apoptosis and protective effect of isradipine, we performed expression of cleaved caspase-3, a signaling protein in the process of apoptosis by Western blot assay. As shown in figure 4, the results showed the expression of cleaved caspase-3 protein at different treatment groups.

The expressions of cleaved caspase-3 in MPP$^+$/FeSO$_4$ group increased ($P < 0.05$, compared to MPP$^+$ group) and more importantly expressions of cleaved caspase-3 in the MPP$^+$/FeSO$_4$/CaCl$_2$ group and even was 1.3 times than the MPP$^+$/FeSO$_4$ group ($P < 0.05$), but significantly inhibited after pretreatment with isradipine ($P < 0.05$, compared with MPP$^+$/FeSO$_4$ group) and ($P < 0.001$, compared with MPP$^+$/CaCl$_2$/FeSO$_4$ group), respectively.
Discussion

As previously mentioned, the pathological hallmarks of PD are represented by loss of DA neurons selectivity in SN and presence of α-syn-containing inclusions called Lewy bodies in residual neurons. Recently, several researches are in favor of the possibility iron enters cardiomyocytes via the LTCC. Also, as a divalent metal ion, extraneous iron may also enter other types of cells via LTCC. We speculate that LTCC, ubiquitously expressed, may be an important route of excessive entry for both calcium and iron, contributing to cell toxicity or death. Research shows that LTCCs such as Cav 1.2 and Cav 1.3 Ca\(^{2+}\) channel, contain pore-forming (α1-subunit isoforms) in complex with an accessory α2, β and γ subunits. Two subtypes LTCCs are also expressed in DA neurons in SN and pharmacologically blocked by dihydropyridines (DHPs) channel inhibitors. There is evidence that isradipine has neuroprotective effects in animal models and cell models of PD, possibly by reducing calcium load and mitochondrial metabolic stress of DA neurons in SN.

In order to prove the protective effect of isradipine on MES23.5 cells, we evaluated calcium and/or iron-induced neurotoxicity from the following aspects: cell viability, nuclear morphology, mitochondrial membrane potential, and cleaved caspase-3 expression. Above all, we observed that the cell survival rate in the pre-treatment with isradipine group was higher than in MPP\(^+/\)FeSO\(_4\) group and in MPP\(^+\)/CaCl\(_2\)/FeSO\(_4\) group, indicating that calcium and/or iron may enter the cell through a common pathway, namely LTCC. Next, we performed Hoechst staining on cells in different treatment groups. The results showed that the nuclear damage in MPP\(^+\)/CaCl\(_2\)/FeSO\(_4\) co-treatment group was more serious than in MPP\(^+\)/FeSO\(_4\) group and isradipine could indeed improve the nucleus pyknosis and fragmentation caused by calcium and/or iron. This further confirms that the use of isradipine can protect cells from neurotoxicity. Furthermore, we detected ΔΨm changes in different treatment groups by flow cytometry. In addition, the results also showed that ΔΨm in MPP\(^+\)/CaCl\(_2\)/FeSO\(_4\) group co-treatment group was much lower than MPP\(^+\)/FeSO\(_4\) group, indicating that the addition of calcium aggravated the cell damage caused by MPP\(^+\)/FeSO\(_4\), while isradipine significantly improved the ΔΨm reduction caused by MPP\(^+\)/CaCl\(_2\)/FeSO\(_4\) co-treatment. This suggests that isradipine may reduce the influx of calcium and/or iron by blocking calcium channels to protect mitochondrial function. Finally, we examined the expression of cleaved caspase-3 protein in different treatment groups by western blot. Consistent with the above results, caspase-3 was significantly activated in the calcium and/or iron treatment group. While the expression of cleaved caspase-3 in isradipine pre-incubation group showed a corresponding decrease, suggesting that isradipine can partially block cell damage caused by calcium and/or iron.

In summary, we investigated the protective effect of isradipine on iron-induced cytotoxicity. The most straightforward conclusion from our findings is that the decrease of cell viability by calcium and/or iron-induced can be partly improved by isradipine pre-incubation. Additionally, our results demonstrated that calcium can aggravate neuronal injury induced partially by increasing intracellular iron concentrations, inducing oxidative stress and leading to a neurodegenerative disorder. However, isradipine can
inhibited cell damage by decreasing nucleus pyknosis ratio increasing ΔΨm and down-regulating cleaved caspase-3 proteins. These findings suggested that isradipine might be a novel therapy for prevention of calcium and/or iron-induced-induced neurodegenerative diseases such as PD.

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