ORIGINAL RESEARCH

Protective effect of estrogen on apoptosis in a cell culture model of Parkinson's disease

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Abstract

Objectives: The protective effect of estrogen on the neurons in Parkinson's disease (PD) is unclear. The present study aimed to investigate the effect of estrogen on the apoptosis and dopaminergic function on a cultured cell model of PD.

Methods: The PD model was established by addition of 1-methyl-4-phenylpyridinium (MPP⁺) to PC12 cell culture. Estrogen was added to cell groups with MPP⁺ (Estrogen+MPP⁺), and without MPP⁺ (Estrogen only group). Cell viability, content of tyrosine hydroxylase (TH), apoptosis ratio, expression of apoptosis-suppression protein Bcl-x and apoptosis-acceleration protein IL-1 beta converting enzyme (ICE) were measured.

Results: Cell viability in the Estrogen+MPP⁺ group was similar to the control group but was higher than in the MPP⁺ group ($P<0.05$). The apoptosis ratios in the Estrogen+MPP⁺ group (33.6%), and the control group (31.3%), were also similar, but it was lower than in the MPP⁺ group (63.5%, $P<0.05$). Concentrations of Bcl-x were higher in the Estrogen+MPP⁺ group, whereas ICE concentrations were lower than in the MPP⁺ group ($P<0.05$).

Conclusions: Estrogen suppresses apoptosis and improves cell viability in MPP⁺ induced injuries in the PC12 cells.

The beneficial effects of estrogen on the PD model are due to the suppression of pro-apoptotic protein ICE, and stimulation of anti-apoptotic protein Bcl-x.

Parkinson's disease (PD) is characterized by progressive degeneration and loss of dopaminergic neurons in the substantia nigra. Recent experimental studies have suggested that estrogen has certain protective effects against neuronal cell injuries, and may be used to prevent or treat neuronal degenerative diseases such as PD.¹⁻⁴

PC12 cells, which are derived from rat pheochromocytoma, synthesize and store dopamine and norepinephrine. Because these cells share the main features with mid-brain dopamine neurons,^{5, 6} they have been widely used in neurotoxicological studies such as PD.^{2, 7-9} 1-methyl-4-phenylpyridinium (MPP⁺) is the active metabolite of 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine (MPTP), which is an inhibitor of complex I.¹⁰ MPP⁺ causes cell death by inducing mitochondria dysfunction and oxidative stress, depleting both ATP and GSH, and disrupting calcium homeostasis.¹¹ MPP⁺ is also directly toxic to neurons, and is able to induce a Parkinsonian-like state *in vitro*.¹² Using MPP⁺ induced cell injuries in PC12 cells as a PD model, the present study aimed to investigate whether estrogen is able to prevent MPP⁺ -induced cell death or apoptosis, and enhance the dopaminergic activities. The signaling pathways of estrogen-mediated PC12 cell protection were also evaluated.

Materials and Methods

Cell culture

The PC12 cells, both naïve and differentiated, were cultured at 37°C in RPMI 1640 media supplemented with 10% horse serum, 5% FCS, 2mM of L-glutamine, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin in a humidified atmosphere of 5% carbon dioxide in the air. The dispersed cells were plated onto collagen-coated 96-well plates at a density of 3×10^4 cells/well, and were cultured under various combinations of times and drug regimens.

The cultured cells were divided into four groups: control (vehicle), MPP⁺ (250µmol/L of MPP⁺), Estrogen + MPP⁺ (10 nM 17-beta-estradiol and 250µmol/L of MPP⁺), and Estrogen only group (10 nM 17-beta-estradiol). Using the method reported by Christis Chiniolos and Vera Adam-Vizi,¹³ the PC12 cells in the MPP⁺ group were treated with 50, 250, and 500 µmol/L of MPP⁺, inducing apoptosis similar to that seen in PD. In the Estrogen group, cells were cultured with 17-beta-estradiol at a concentration of 5 nM, 10 nM and 50 nM.

Thiazolyl blue tetrazolium bromide (MTT) assay of cell viability

After the PC12 cells were treated with MPP⁺ for 24 h, MTT solution (5mg/ml, Sigma-Aldrich), was added to each culture well. After incubation at 37°C for 4 h, the formazan crystals were dissolved in 100µl DMSO, and the MTT reduction was measured at 570 nm using a DG-3022A ELISA plate reader. Control values from addition of vehicle only to cells were taken as 100%, and experimental values were taken as percentage decrease in MTT reduction. The cell viability and metabolite state were evaluated by A570.

Immunocytochemistry of tyrosine hydroxylase (TH)

This was performed to assess the levels of catecholamine biosynthesis in the PC12 cells. Sections were incubated with 0.3% Triton X-100 in PBS for 1 h at room temperature, and then incubated with goat anti-rat TH monoclonal antibody. Bax polyclonal antibody (1:250 dilutions in 0.01 mmol/L phosphate buffer saline, PH 7.4), was added overnight at 4°C. Slides were then incubated with biotinylated rabbit anti-goat IgG and SABC-reagent for 30 min at 37°C. Subsequently, the cells were processed with 50µl of DAB solution (Boshide, Wuhan, China). Cells were stained with hematoxylin and dehydrated with ethanol. Under a microscope, cells with dark brown color are considered as positive expression, while light purple as the negative.

Detection of apoptotic cells by flow cytometry

The apoptosis rate of endothelial progenitor cells was measured by DNA flow cytometry and DNA electrophoresis. Annexin V binding was assessed using bivariate flow cytometry, and cell staining was evaluated with fluorescein isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclusion of propidium iodide (PI) (negative for red fluorescence).

In each group, the total cells and the surviving cells were also counted in five fields and the average values were derived.

Identification of Bcl-x and IL-1 beta converting enzyme (ICE) mRNA by Real-time PCR

Total RNA was isolated from PC12 cells by Trizol (GIBCO). First-strand cDNA was reverse transcribed from 1 µg RNA in a total volume of 25 µl with Rever-tAid™ M-MuLV. PCR primers were designed as described below: the Bcl-x: 5'-CGCCTCTCGGCGCTGCTGCATT-3' (sense primer); 5'-AGG CTGGCGATGATGTTGAA-3' (antisense primer); ICE: 5'-TGACGC CAAAGAGGAAGC-3' (sense primer); 5'-GTGATAAACATCTGG-3' (antisense primer); β -Actin: 5'-TATGAGAAGATTTGGCACC-3' (sense primer) 5'-GTCCAGACGCAGGATGGCAT-3' (antisense primer). PCR procedures were carried out in a total volume of 25 µl, 35 cycles of 5 min at 94°C (predenaturation), 30 s at 94°C (denaturation), 1 min at 50°C (annealing), and 1 min at 72°C (extension). About 5 µl of the product was taken for DNA fragment separation by electrophoresis through agarose gel.

Statistics

Data were expressed as means $\pm$ SD. Statistical analysis of the data for multiple comparisons was performed by ANOVA. For single comparison, the student *t* test was used. Categorical data were analyzed with Chi-square test. $P < 0.05$ was considered statistically significant.

Results

Cell viability

As shown in Table 1, the cell viability in the MPP⁺ group was lower than in the control ($P < 0.05$), Estrogen+MPP⁺ ($P < 0.01$), and three Estrogen groups ($P < 0.01$). The cell viability in the MPP⁺ 500 group was significantly lower than in the 250 and 50 groups

TABLE 1. Cell viability analysis.

Group	Cell viability (A570)
Control	0.49 $\pm$ 0.11
MPP ⁺	
50 µmol/L	0.37 $\pm$ 0.06
250 µmol/L	0.30 $\pm$ 0.07
500 µmol/L	0.21 $\pm$ 0.09
Estrogen+MPP ⁺	0.56 $\pm$ 0.16
Estrogen 5 nM	0.50 $\pm$ 0.02
Estrogen 10 nM	0.61 $\pm$ 0.17
Estrogen 50 nM	0.59 $\pm$ 0.14

(Table 1, $P < 0.01$). There was no significant difference in the cell viability between Estrogen +MPP⁺ and the control group ($P > 0.05$).

The cell viability in the 10 nM and 50 nM Estrogen groups were similar. Both were higher than in the 5 nM group ($P < 0.05$, Table 1).

Apoptosis and the average absorbency of TH positive PC12 cells

The TH positive cells were stained in brown color. The unstained nuclei in the TH positive cells were large and rounded, with an empty appearance (Fig 1). The cells treated with estrogen were larger than the cells in the control or MPP⁺ group, and most estrogen-treated cells had neurites (Fig 1). Some very small and rounded cells were detected in the MPP⁺ group (Fig 1).

Optical density in the TH positive cells in the Estrogen group was higher than in the control (0.46 $\pm$ 0.06 vs 0.22 $\pm$ 0.07, $P < 0.05$), the Estrogen+MPP⁺ (0.24 $\pm$ 0.04, $P < 0.05$), and the MPP⁺ only group (0.10 $\pm$ 0.03, $P < 0.05$). There was no significant difference in optical density in the TH positive cells between the control and Estrogen+MPP⁺ group ($P > 0.05$).

The apoptosis ratio in the Estrogen+MPP⁺ group and the control group was similar ($P > 0.05$, Table 2), which was lower than in the MPP⁺ group (Table 2, $P < 0.05$).

The lowest apoptosis ratio was found in the Estrogen only group (Table 2, $P < 0.05$).

FIGURE 1. The *tyrosine hydroxylase* positive PC12 cells were stained in brown color. Large P12 cells were seen in estrogen treated group (B), some with neuritis. There were very small and rounded cells in the MPP⁺ group (C). (A). Control (DAB×200), (B) Estrogen group (DAB×200), (C) MPP⁺ group (DAB×200), (D) Eestrogen+MPP⁺ group (DAB×200)

FIGURE 2. The expression in ICE and *Bcl-x*.

Lane 1: Marker. Lane 2: Control. Lane 3: Estrogen. Lane 4: Estrogen +MPP⁺. Lane 5: MPP⁺

Effects of estrogen on the expression of Bcl-x and ICE

The expression of *Bcl-x* and ICE in the control, MPP⁺, Estrogen+MPP⁺ and Estrogen groups is shown in Fig 2.

As shown in Table 3, the Estrogen group had the highest average level of *Bcl-x*, whereas the MPP⁺ group had the lowest ($P < 0.05$). There was no difference in *Bcl-x* levels between the control and Estrogen+MPP⁺ group ($P > 0.05$).

The average ICE levels were the highest in the MPP⁺ group, and the lowest in the Estrogen group ($P < 0.05$, Table 3). There was no significant difference in ICE concentrations between the control and Estrogen+MPP⁺ group ($P > 0.05$, Table 3).

Discussion

The major findings of the present study are: 1) Estrogen improves the survival rates of PC12 cells during

MPP⁺ induced cell injury; 2) Estrogen treatment leads to elevated phos-density in the TH positive cells and outgrowth of neurites. It also brings the phos-density in the MPP⁺ treated cells back to the control level; 3) Estrogen reduces the apoptosis ratio of PC12 cells by enhancing the expression of anti-apoptotic protein *Bcl-x*, and suppressing the expression of pro-apoptotic protein ICE. These results indicate that estrogen prevents cell injuries in this cultured cell model of PD, and enhances neuronal activities in the non-injured cells.

So far, there has been no convincing evidence to suggest that estrogen plays a critical role in the pathogenesis or treatment of PD. Some epidemiological studies reported an increased risk of PD in conditions causing an early reduction in endogenous estrogens, such as early menopause.¹⁴ A recent small clinical ob-

TABLE 2. The rate of apoptosis.

Group	Apoptotic cells (%)	Necrotic cells (%)	Living cells (%)
Control	31.3	3.6	65.1
MPP ⁺	63.5**	4.3	32.2
Estrogen	11.5*	0.8	87.7
Estrogen +MPP ⁺	33.6***	5.1	61.3

* $P < 0.05$ compared with Estrogen+MPP⁺ ** $P < 0.05$ compared with Control; *** $P < 0.05$ compared with MPP⁺

TABLE 3. Semi-quantitative RT-PCR ratio (objective product/ β -Actin)

	<i>Bcl-x</i>	<i>ICE</i>
Control	0.66 $\pm$ 0.37	0.31 $\pm$ 0.14
MPP ⁺	0.32 $\pm$ 0.18**	0.76 $\pm$ 0.25**
Estrogen+MPP ⁺	0.70 $\pm$ 0.26*	0.42 $\pm$ 0.13*
Estrogen	0.92 $\pm$ 0.19***	0.18 $\pm$ 0.05***

*** $P < 0.05$ compared with control, MPP⁺ group and Estrogen+MPP⁺ group.

** $P < 0.05$ compared with control and Estrogen+MPP⁺ group.

servation found that in early postmenopausal women with Parkinson disease, estrogen replacement therapy for 12 weeks significantly improves peak-dose dyskinesia without worsening motor disability, suggesting a possible benefit of estrogen in postmenopausal women with Parkinson disease.¹⁵ However, the reports on the effect of postmenopausal estrogen replacement therapy are inconsistent among other reports.¹⁴

In the present study, estrogen elevated the viability of PC12 cells, and increased survival cells from 65.1% in the control group to 87.7% in the Estrogen group. MPP⁺ diminished the cell viability and reduced the cell survival rate by more than 50%. However, in cells cultured with both estrogen and MPP⁺ the cell viability and survival rate was similar to the control group, which was almost 50% higher than in the MPP⁺ group. These results strongly suggest that estrogen has a considerable impact on the growth and development of neuronal cells in this PD model.

TH is a rate-limiting enzyme in the biosynthesis of catecholamines, and the levels of TH represent the enzymatic activity that determines the amount of catecholamine synthesis. Several studies have demonstrated that estrogen enhances the TH positive neurons in the mid-brain of animals, and stimulates the biosynthesis of catecholamines in a variety of neuronal cells, such as cultured bovine adrenal medullary cells.¹⁶⁻¹⁸ In the present study, estrogen increased the expression of TH in PC12 cells, and decreased the MPP⁺-induced reduction in TH activities in these cells. In addition, a greater neurite outgrowth was also present after the

treatment of estrogen, indicating improvement in differentiation and growth of dopaminergic neurons.

Several studies have tried to elucidate the mechanisms by which estrogen prevents neuronal cell death. A study in mice showed that estrogen directly acts on the nerve growth factor and enhances the number of nigral dopaminergic neurons.¹⁹ Estrogen is also found to bind to specific membrane receptors to regulate gene transcription, influencing the balance of the cell death suppression gene and cell-death-accelerating gene.²⁰⁻²²

Bcl-x is a member of the Bcl-2 family with apoptosis suppression being its major function. ICE is a proteinase whose main action is to mediate cell death. In the present study, MPP⁺ suppressed the expression of Bcl-x but increased the levels of ICE (Table 2). The addition of estrogen to the MPP⁺ group prevented the reduction in Bcl-x and diminished the levels in ICE, bringing Bcl-x and ICE to the control level. Furthermore, without the influence of MPP⁺, estrogen significantly improved Bcl-x expression and suppressed ICE production in the Estrogen group. These results indicate that both Bcl-x and ICE are involved in the cell protective effects of estrogen on the PC12 cells.

In summary, in the cultured PC12 cells, MPP⁺ induces cell injury which can be largely prevented by pretreatment with estrogen. Estrogen improves cell viability and survival rate, and reduces apoptosis. The enhancement in Bcl-x expression and reduction in ICE production appears to be associated with the cell protective effects of estrogen. It remains unclear as to whether these protective effects of estrogen on the cultured PC12 cells, can be translated into the same therapeutic effects in patients with PD. Future clinical trials on the effect of estrogen in patients with PD may be warranted.

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References

- Ramirez AD, Liu X, Menniti FS. Repeated Estradiol Treatment Prevents MPTP-Induced Dopamine Depletion in Male Mice. *Neuroendocrinology* 2003; 77:223-31.
- Gelinas S, Martinoli MG. Neuroprotective effect of estradiol and phytoestrogens on MPP⁺-induced cytotoxicity in neuronal PC12 cells. *J Neurosci Res* 2002; 70:90-6.
- Horstink MW, Strijks E, Dluzen DE. Estrogen and Parkinson's disease. *Adv Neurol* 2003;91:107-14.
- Sawada H, Ibi M, Kihara T, et al. Estradiol protects dopaminergic neurons in a MPP⁺ Parkinson's disease model. *Neuropharmacology* 2002;42:1056-64.
- Green L, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* 1976;73:2424-8.
- Timothy JS, William DA. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* 1991;12:473-92.
- Yamamoto N, Sawada H, Izumi Y, et al. Proteasome inhibition induces glutathione synthesis and protects cells from oxidative stress: relevance to Parkinson disease. *J Biological Chemistry* 2007;282:4364-72.
- Seegal RF, Brosch K, Bush B, Ritz M, Shain W. Effects of Aroclor 1254 on dopamine and norepinephrine concentrations in pheochromocytoma (PC-12) cells. *Neurotoxicology* 1989;10:757-64.
- Shafer TJ, Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* 1991;12:473-92.
- Nickals WJ, Heikkila RE. Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenylpyridine: a metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin. *Life Sci* 1985;36:2503-8.
- Tipton KF, Singer TP. Advances in our understanding of the mechanisms of the neurotoxicity of MPTP and related compounds. *J Neurochem* 1993;61:1191-206.
- Obata T. Role of hydroxyl radical formation in neurotoxicity as revealed by in vivo free radical trapping. *Toxicol Lett* 2002;132:83-93.
- Christis Chinopoulos, Vera Adam-Vizi. Mitochondria deficient in complex-I activity is depolarized by hydrogen peroxide in nerve terminals: relevance to Parkinson's disease. *J Neurochem* 2001;76:302-6.
- Ragonese P, D'Amelio M, Savettieri G. Implications for estrogens in Parkinson's disease: an epidemiological approach. *Ann New York Acad Sci* 2006;1089:373-82.
- Nicoletti A, Arabia G, Pugliese P, et al. Hormonal replacement therapy in women with Parkinson disease and levodopa-induced dyskinesia: a crossover trial. *Clin Neuropharmacol* 2007;30:276-80.
- Pau KY, Hess DL, Kohama S, Bao J, Pau CY, Spies HG. Oestrogen upregulates noradrenaline release in the mediobasal hypothalamus and tyrosine hydroxylase gene expression in the brainstem of ovariectomized rhesus macaques. *J Neuroendocrinol* 2000;12:899-909.
- Ivanova T, Beyer C. Estrogen regulates tyrosine hydroxylase expression in the neonate mouse midbrain. *J Neurobiol* 2003;54:638-47.
- Yanagihara N, Toyohira Y, Ueno S, et al. Stimulation of catecholamine synthesis by environmental estrogenic pollutants. *Endocrinology* 2005;146:265-72.
- Tripanichkul W, Jaroensuppaperch EO, Finkelstein DI. Estrogen enhances the number of nigral dopaminergic neurons of adult male mice without affecting nigral neuroglial number and morphology. *Neurosci Lett* 2008;25:435:210-4.
- Chan CR, Hsu JT, Chang IT, Young YC, Lin CM, Ying C. The effects of glutamate can be attenuated by estradiol via estrogen receptor dependent pathway in rat adrenal pheochromocytoma cells. *Endocrine* 2007;31:44-51.
- Merot Y, Ferriere F, Debroas E, Flouriot G, Duval D, Saligaut C. Estrogen receptor alpha mediates neuronal differentiation and neuroprotection in PC12 cells: critical role of the A/B domain of the receptor. *J Mol Endocrinol* 2005;35:257-67.
- Alexaki VI, Charalampopoulos I, Kampa M, et al. Estrogen exerts neuroprotective effects via membrane estrogen receptors and rapid Akt/NOS activation. *FASEB J* 2004;18:1594-6.

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