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## Effects of citalopram on serum deprivation induced PC12 cell apoptosis and BDNF expression

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**Objective** To investigate the protective effects of citalopram on neural cells and its possible mechanism. **Methods** Rat PC12 cells were induced with 100 mg/L nerve growth factor for 7 d before they were randomly divided into following groups: serum-free group, citalopram treatment groups (20, 40, 60, 80 and 100  $\mu\text{mol/L}$ ) and serum group ( $n=8$ ). Cell viability was determined by MTT assay. Cell apoptosis rate and cell cycles were examined by flow cytometry. Hoechst 33342 staining was employed for cell morphology observation. Expression of BDNF was checked by Western blot and RT-PCR. **Results** Cells treated with 60  $\mu\text{mol/L}$  citalopram showed significantly higher viability than the serum-free group ( $P<0.01$ ). Apoptosis rate of the citalopram treatment group remarkably decreased according to flow cytometry ( $P<0.01$ ) and fewer cells were arrested in G1 phase ( $P<0.01$ ). Hoechst 33258 staining further demonstrated the improved survival of cells ( $P<0.01$ ) and chromosome condensation after citalopram treatment. Western blotting and RT-PCR results both indicated that citalopram treatment could significantly ( $P<0.01$ ) increase BDNF expression. **Conclusion** Citalopram has an anti-apoptotic effect on PC12 cells. Up-regulation of BDNF might be one of its nerve protection mechanisms.

### 1. Introduction

Nowadays serotonin-specific reuptake inhibitors (SSRIs) are the best anti-depressants in the treatment of depression. Although their effectiveness is generally acknowledged, the sophisticated working mechanism is yet to be unveiled.

Current theory believes that depression occurrence is related to the brain neurotransmitters and their receptors, and is a direct result of nutritional limitation and nervous system plasticity in specific regions of the brain. Neurotrophic factors could participate in the anti-depression process. Brain-derived neurotrophic factor (BDNF) is an important member of the nutritional factor family. It promotes the growth, development, survival, differentiation and functional expression of multiple neurons (Mattson 2008). BDNF also maintains the function and synaptic plasticity of mature neurons (Mattson and Wan 2008).

PC12 cells were derived from rat pheochromocytoma. When induced with nerve growth factor (NGF), axons grow to form synapse, synthesize and release dopamine, noradrenaline and other neurotransmitters. These neurotransmitters which have both physiological and biochemical properties of sympathetic neurons have been widely used in relative studies on neurons. This study aims to investigate the effect of citalopram through observation of serum deprivation induced PC12 cell apoptosis and expression of BDNF, and to further understand the anti-depressant mechanism of SSRIs.

### 2. Investigations and results

#### 2.1. Cell culture observation

PC12 cells cultured in regular medium containing serum appeared round-shaped either dispersed or in clusters before they

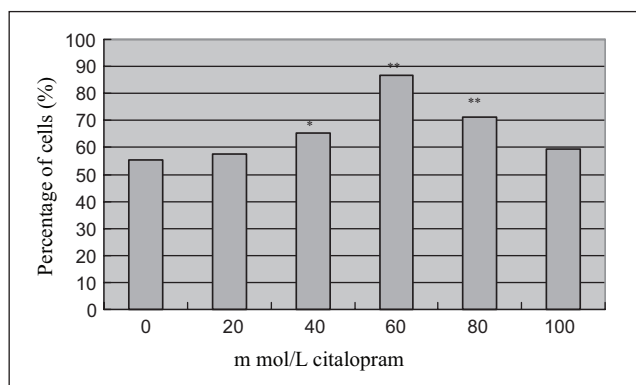
were differentiated. With increasing culturing time, cell density continued to increase but there was no obvious differentiation. The NGF treated cells, however, started to grow dendrites after 12 h of treatment. Cells were spindle-, triangle- or irregular-shaped and showed apparent nerve-fiber-like structures. The  $\beta$ 2 tubulin III staining result of the treated cells was positive. Dendrites were also stained, indicating that PC12 cells have neural cell properties after NGF induction.

#### 2.2. Effect of citalopram dosage on PC12 cell viability in serum free medium

Cell viability was examined by MTT assay after PC12 cells had been cultured for 48 h. Results showed that viability of the serum-free group was  $55.3 \pm 4.1\%$ , while it was  $57.6 \pm 3.4\%$  for the 20  $\mu\text{mol/L}$  citalopram treatment group,  $71.1 \pm 3.9\%$  for the 40  $\mu\text{mol/L}$ ,  $86.8 \pm 3.2\%$  for the 60  $\mu\text{mol/L}$ ,  $62.3 \pm 5.4\%$  for the 80  $\mu\text{mol/L}$ , and  $59.4 \pm 2.1\%$  for the 100  $\mu\text{mol/L}$  treatment groups. As shown in Fig. 1, the 40, 60 and 80  $\mu\text{mol/L}$  citalopram treatment groups showed much higher cell viabilities than the serum-free group ( $P<0.05$  or  $P<0.01$ ). As the citalopram concentration increased, cell viability gradually dropped. Differences between the 20, 100  $\mu\text{mol/L}$  citalopram treatment groups and the serum-free group were not statistically significant ( $P>0.05$ ). Based on these results, 60  $\mu\text{mol/L}$  citalopram will be used for cell treatment in subsequent studies.

#### 2.3. Detection of apoptosis and cell cycle change with flow cytometry

Apoptosis rate of the citalopram (60  $\mu\text{mol/L}$ ) treatment group was much lower than in the serum-free group ( $P<0.01$ ); more



\*  $p < 0.05$ ; \*\*  $p < 0.01$

Fig. 1: Effect of citalopram dosage on PC12 cell viability in serum-free medium

**Table: Flow cytometry for cell apoptosis rate and cell cycle analysis**

Group	Well	Apoptosis rate	Cell cycle phase (%)		
			G1	S	G2
Serum (+)		14.5 ± 2.1**	50.2 ± 5.3	42.2 ± 4.7**	1.4 ± 0.3**
Serum (-)		49.1 ± 3.3	82.5 ± 3.2	13.6 ± 2.4	4.3 ± 0.5
Citalopram		24.6 ± 2.7**	61.3 ± 3.7	24.7 ± 3.3*	2.2 ± 0.3**

v serum(-): \*  $P < 0.05$ , \*\*  $P < 0.01$

cells were found in G1 and G2 phases in the serum-free group than in the other two groups ( $P < 0.01$ ), while less were found in the S phase ( $P < 0.01$ ) (Table).

### 2.4. Hoechststaining

Nuclei in cells of the serum group were stained blue evenly, while majority of that in the citalopram treatment group stained and 2-3 chromosome condensations were found in each view field with occasional scenes of nucleus decomposition. Chromosome condensation was common for cells in the serum-free group and nuclei of cells were decomposed to pieces (Fig. 2).

### 2.5. Expression of BDNF as detected by Western blotting and RT-PCR

As shown in Figs. 3, 4 (Western Analysis) and Figs. 5, 6 (RT-PCR analysis), compared to the  $\beta$ -Actin levels of PC12

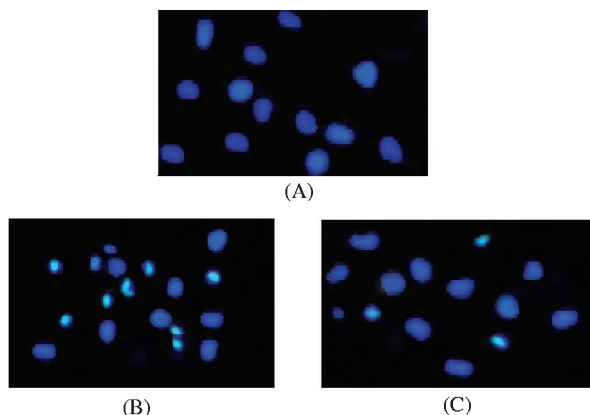


Fig. 2: Comparison of PC12 cell morphological changes. A, serum (+); B, serum (-); C, 60  $\mu$ mol/L citalopram and serum (-). Cells were stained with Hoechst 33258 and observed under a light microscope ( $\times 200$ )

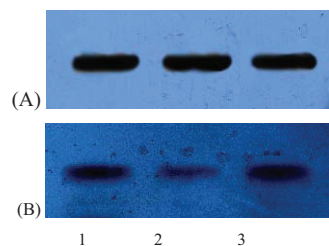
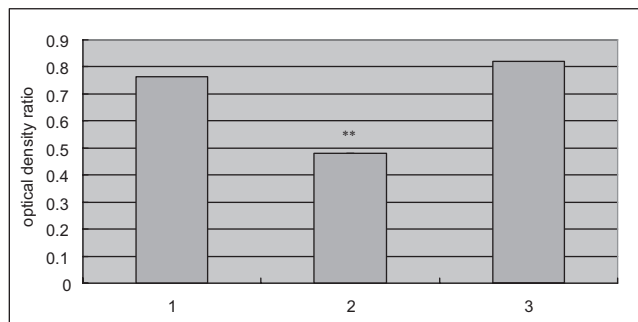


Fig. 3: Western blot analysis of BDNF and  $\beta$ -Actin expression levels. A, BDNF; B,  $\beta$ -Actin; 1, serum (+); 2, serum (-); 3, citalopram (60  $\mu$ mol/L) and serum (-)



\*\*  $p < 0.01$

Fig. 4: Comparison of BDNF protein expression among the three groups. Data were normalized to  $\beta$ -Actin levels. 1, serum (+); 2, serum (-); 3, citalopram (60  $\mu$ mol/L) and serum (-)

cells, the BDNF expression in the serum group significantly decreased ( $P < 0.01$ ) while that in the citalopram treatment group increased.

### 3. Discussion

Using PC12 cell line as the model, this study investigated the protective role of SSRIs compounds on NGF induced differentiation of neural cells and its possible mechanism.

Data showed that different doses of citalopram could improve survival of PC12 cells after serum-deprivation to different extents. Citalopram concentrations of 40  $\mu$ mol/L, 60  $\mu$ mol/L and 80  $\mu$ mol/L could significantly improve cell viability ( $P < 0.05$  or  $P < 0.01$ ). However, when the concentration of citalopram was increased further, cell viability gradually decreased, suggesting that the protective effect of citalopram on PC12 cells was dose-dependent.

Flow cytometry analysis showed that apoptosis rate in the serum-free group was much higher than that in the serum group and the citalopram treatment group. Meanwhile, more cells were arrested in the G1 phase indicating that the transition to the S phase was blocked in cells deprived of serum, which caused the

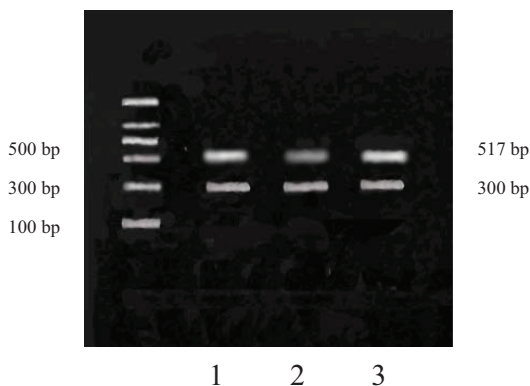


Fig. 5: RT-PCR analysis of BDNF (517 bp) and  $\beta$ -Actin (257 bp) expression levels. 1, serum (+); 2, serum (-); 3, citalopram (60  $\mu$ mol/L) and serum (-)

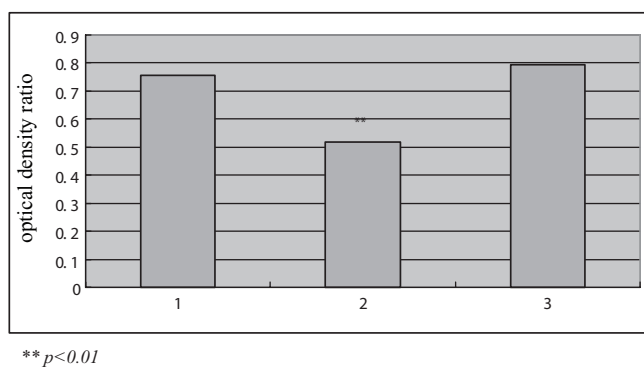


Fig. 6: Comparison of BDNF mRNA expression among the three groups. Data were normalized to  $\beta$ -Actin levels. 1, serum (+); 2, serum (-); 3, citalopram (60  $\mu$ mol/L) and serum (-)

accumulation of G1 phase cells, prevented the DNA synthesis and replication, and ultimately made cell replication difficult. Apoptosis was also found in the citalopram treatment group. However, the apoptosis rate was much lower. Cells in G1 phase were also significantly less than those found in the serum-free group. In conjunction with the Hoechst 33342 staining results, data suggested that even though certain amounts of cells in the citalopram treatment group went through apoptosis, the majority of them were rested in an early stage of apoptosis. Therefore, at a certain concentration, citalopram has an anti-apoptotic effect. Until now, the initiation mechanism of depression has not been fully elucidated. Most hypotheses on depression origin were built on the pharmacology basis of anti-depression drugs. However, the pathogenesis for depression is in fact far more complicated than merely the concentration change of monoamine transmitters between synapses. With the development of nerve imaging, more researchers have recognized the relationship of depression with brain structure change, including decreased neurogenesis, reduced hippocampus volume and neural cell atrophy (e.g., the hippocampal CA3 cone cells) (Frodil et al. 2002; McKittrick et al. 2000). Numerous studies have demonstrated decreased size of hippocampus in depression patients as compared to healthy individuals (Sheline et al. 2003, 1999, 1996). It has been proposed that anti-depressant drugs could increase neurogenesis in hippocampus of depressed rats. Therefore, hippocampal cell neurogenesis has become another important path for studying the mechanism of anti-depressants and the etiology of depression. Results in this study showed the protection role of citalopram on nerve cells *in vitro*. Since *in vitro* neural cell model has better homogeneity and less influencing factors, it can effectively reflect the drug effects on neurons (Zhong et al. 2008). Qiu et al. (2007) studied the interference of SSRIs reagent fluoxetine on rat depression models, and reported that fluoxetine could reduce neural cell apoptosis through inhibition of Bax and promotion of Bcl-2 mRNA expressions in hippocampal tissues also reported the protective role of paroxetine on glucocorticoid damaged neural cells in rat hippocampus.

In addition, this study showed that citalopram increased the expression of BDNF in serum-free medium cultured PC12 cells. BDNF is an important member of the nutrition factor family. It promotes the growth, development, survival, differentiation and function of multiple neurons, maintains proper function of mature neurons and synaptic plasticity, protects neurons from injury and plays pivotal roles in neuron regeneration (Allaman 2008; Aan 2009). Its critical role in injury protection and regeneration of neurons has also been recognized. The brain of depressed human who committed suicide has relatively less mRNA expression of hippocampal tyrosine kinase receptor B and BDNF than their healthy counterpart (Dwivedi 2009).

Patients treated with anti-depressant had more hippocampal BDNF expression than those who did not receive the treatment before death (Kim et al. 2008). Anti-depressants were also shown to up-regulate BDNF expression in rat hippocampus and endothelium at both protein and mRNA levels (Deltheil et al. 2008). Furthermore, Garcia (Garcia 2002) suggested that the working mechanism of different types of anti-depressants could all be rooted to BDNF, which might have increased the connection of hippocampal synapses (the nerve plasticity effect after positive synapse).

In summary, data in this study provided evidence that at a certain concentration of citalopram has anti-apoptotic effects on PC12 cells cultured in the serum-free medium. Results also preliminarily showed the protection role of SSRIs in clinical treatment of nerve cells and its possible contribution in increasing expression of BDNF.

## 4. Experimental

### 4.1. Drugs and reagents

The rat pheochromocytoma (JCRB0733) derived PC12 cell line was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Nerve growth factor (NGF), methylthiazolylidiphenyl-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma Inc (USA). Fetal bovine serum (FBS) and DMEM medium (pH7.14, high glucose) were obtained from Gibco Inc. (USA). Primers for BDNF (Forward: 5'-CTGGAGAAAGTCCCGGTAT-3'; Reverse: 5'-GGTAG1TRCGGCA1TRCGGAGT-3') and  $\beta$ -Actin (Forward: 5'-CATCTCTTGCTCGAAGTCCA-3'; Reverse: 5'-ATCATGTGTTGAGAC-CTTCAACA-3') were synthesized by Shanghai Yingjun Biological Technology Corp. Ltd. (China).

### 4.2. PC12 cell culture

PC12 cells were cultured in DMEM medium containing 100 mg/L NGF, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 1% v/v FCS. Cells were incubated in a 24-well plate at 37 °C, 5% CO<sub>2</sub>. New medium was replaced every other day. When cells reached confluence, they were collected with 0.125% Trypsine-EDTA. Cells were passaged once every 2-3 d and exponential phase cells were used for experiments.

### 4.3. Examination of drug effects on PC12 cell viability by MTT

Exponential phase cells were trypsinized and resuspended to a density of  $5 \times 10^4$  cells/ml in DMEM medium containing 10% fetal bovine serum. Cells were transferred to a 96-well plate with 200  $\mu$ l volume in each well. After 24 h of culturing, cells were washed and replaced with a serum free medium. Various doses of citalopram were then added to each well (0, 20, 40, 60, 80, 100  $\mu$ mol/L) except for the no treatment control group wells, which only received the replacement medium containing serum. Each treatment group had 8 replicates and was assayed by MTT 24 h after drug treatment. For MTT assay, briefly cells were washed with phosphate buffered saline (PBS), replaced with the same medium and added 20  $\mu$ l of MTT solution to reach a final concentration of 500  $\mu$ g/ml. Cells were then incubated in a 37 °C, 5% CO<sub>2</sub> incubator for 4 h before the supernatant in each well was carefully discarded with a pipette. Optical density (570 nm with reference wavelength at 630 nm) of the wells was then examined with a Dynatech MR4000 ELISA reader (Turku, Finland). Cell viability (%) was calculated by dividing the optical density reading of each well to the no treatment control wells. Independent experiment was repeated 3 times.

### 4.4. Drug effects on PC12 cell apoptosis and cell cycles

Induced cells were divided into 3 groups: serum free group, serum group and citalopram (60  $\mu$ mol/L) treatment group. After 72 h of incubation, cells were stained with either the Annexin V2F ITC kit (Beckman Coulter, USA) or the DNA2Prep Reagent kit (Beckman Coulter). Stained cells were respectively submitted for apoptosis and cell cycle analyses with a flow cytometer (Model Elite ESP).

### 4.5. Morphological observation of PC12 cells

PC12 cells were cultured with the following three conditions: the serum-free medium containing 60  $\mu$ mol/L citalopram, the serum-free medium only and the regular medium containing serum. After incubation for 72 h, cells were stained with 10 mg/L Hoechst 33342 dye (see below) at 37 °C for 30 min,

fixed with 4% (w/w) paraformaldehyde for 10 min, sealed and observed under a BX60 fluorescent microscope (Olympus, Japan).

#### 4.6. Hoechst 33258 staining

PC12 cells were inoculated in a 24-well plate with each well containing a preplaced poly-L-lysine coated cover slip. For observation of morphological changes in apoptotic nucleus, cells were washed twice with PBS after drug treatment, fixed with 4% paraformaldehyde for 30 min and washed 3 times with PBS before the Hoechst 33258 staining solution was added and let react in the dark at room temperature for 10 min. Subsequently, cells were washed twice with PBS and observed under the fluorescent microscope. Four view fields were randomly selected from each cover slip and each experimental group contained 4 cover slips. Images of these views were taken and apoptotic cells were counted. Apoptosis rate was expressed as percentage of apoptotic cells over all cells.

#### 4.7. Western blot analysis for BDNF protein expression

After SDS-PAGE electrophoresis, proteins on the gel were transferred to a nitrocellulose membrane in the buffer filled electrophoresis tank for 2 h at 4 °C. The membrane was then briefly washed for 1 min in TBS and placed into a sealed container containing 5% fat-free milk in TBST buffer for blocking. After 1 h of blocking at room temperature with shaking, the membrane was washed 3–4 times with TBS for 15–20 min each. Later, the membrane was transferred to the anti-goat primary antibody solution (1:1000) and incubated on an orbital shaker for 1 h before it was put to 4 °C overnight. The next morning, the membrane was again washed with TBS for 3–4 times for 20–30 min each before it was incubated on the orbital shaker for 1 h with the goat anti-rabbit IgG secondary antibody (1:1000). Finally, the membrane was washed 3–4 times with TBS and bands were exposed to film in the dark with the CL detection solution.

#### 4.8. Analysis of BDNF mRNA expression through RT-PCR

RT-PCR was performed according to instructions of Qiagen ONEStep RT-PCR Kit. The mRNA of both BDNF and internal control (a 257 bp long  $\beta$ -Actin product) was amplified with the following PCR condition: reverse transcription at 50 °C for 30 min, denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s and extension at 72 °C for 1 min. At the final step, a 72 °C extension was performed for 10 min. RT-PCR amplification products were analyzed on a polyacrylamide gel and signals were detected with the gel document system (Gel Doc 2000™ CleMiDoc™) and statistically quantified with the Bio-Rad software as mentioned below.

#### 4.9. Statistical analysis

Data were expressed as  $\bar{x} \pm s$  and analyzed with SPSS 11.0 software (SPSS Inc., USA) for one-way ANOVA. Differences between groups were tested with Least Significant Different Test *t* (LSD2*t*) method. Optical densities of bands from electrophoresis were quantified with the BIO-RAD Quantity One quantitation software package (Bio-Rad, USA) and analyzed for one-way ANOVA with the PEMS statistics software package (PEMS Software Inc., USA).

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