## Original Article
# Protective effect of IL-6 pre-treatment against oxidative stress injury in primary cultured neural cells and related mechanisms

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**Abstract:** Oxidative stress-induced mitochondrial dysfunction has been recognized as the major reason causing Parkinson's disease (PD). This study explored the *in vitro* protective effect of interleukin-6 (IL-6) pretreatment against $H_2O_2$-induced oxidative stress injury on neural cells, in an attempt to further illustrate PD related pathogenesis mechanism. Primary cultured neural cells were pre-treated with IL-6 (1 ng/mL or 5 ng/mL) and were challenged with $H_2O_2$. MTT assay was used to detect the viability of neural cells, while apoptosis was quantified by flow cytometry using Annexin-V and 7-AAD double labelling. Markers for oxidative stress including superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH) were tested by enzyme-linked immunosorbent assay (ELISA). The expression of signal transducer and activator of transcription 3 (STAT3) and p-STAT3 was studied by immunohistochemical (IHC) staining and Western blotting. Viability of IL-6 pretreated cells after oxidative stress was significantly elevated compared to model cells ($P<0.01$), with more significant effect in 5 ng/mL group. The pretreatment of IL-6 also endowed significantly depressed cell apoptosis ($P<0.01$). SOD and GSH levels were also elevated after IL-6, whilst MDA level was decreased ($P<0.01$). STAT3 expression level was also elevated in IL-6 group ($P<0.01$). IL-6 pretreatment may exert a protective function on neural cells against $H_2O_2$-induced oxidative stress injury.

**Keywords:** Interleukin-6, neural cell, oxidative stress, cell apoptosis, cell viability

## Introduction

Parkinson's disease (PD) is one common neurodegenerative disease mainly occurred in aged populations, with averaged onset age around 60 years. The incidence of PD in Chinese people over 65 years old was as high as 2%, occupying more than 40% of worldwide PD patients. The exact mechanism of PD has not known yet, although the loss of dopaminergic neurons in substantia nigra compacta has been recognized as the major pathological feature of PD [1-4]. The oxidative stress-induced mitochondrial dysfunction is known as the major reason causing PD [5]. In fact, oxidative stress has been proved to be involved in the occurrence and progression of multiple neurodegenerative diseases including PD, Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) [6].

As one inflammatory cytokine, interleukin-6 (IL-6) participates in the pathological process of acute inflammatory injury of cells [7, 8]. Recent studies have revealed the protective role of IL-6 pretreatment on myocardial cells against $H_2O_2$-induced oxidative stress [9]. Moreover, the pretreatment of PC12 cells with IL-6 can up-regulate intracellular concentration of glutathione (GSH), thus alleviating HNE-induced oxidative stress cell injury [10]. Based on this previous knowledge, we proposed that IL-6 pretreatment might also exert certain protective functions on cultured neural cells against $H_2O_2$-induced oxidative stress. This study thus observed the effect of IL-6 pretreatment on $H_2O_2$-induced oxidative stress in a neural cell model of oxidative stress, in addition to the illustration of related mechanisms.

## Materials and methods

### Animals

Adult ICR mice were purchased from Laboratory Animal Institute of Huazhong University of Science and Technology (Certificate No. SCXK2001-0031). Male and female mice were housed in the same cage. Pregnancy was checked on every morning. Fetal mice at 14~18 days were used for isolating neural cells. experimentation protocols were submitted to and approved by the ethics committee of Huazhong University of Science and Technology.

### Isolation and culture of neural cells

Female mice at pregnant day 14~18 were sacrificed by cervical dislocation. The uterus was immediately removed and immersed in Hank's balanced salt solution (HBSS), followed by the extraction of fetal mice. Dura and major vessels of the brain were dissected to expose the brain cortex, which was cut into small pieces (~1 mm$^3$). Brain tissues were digested in EDTA containing 0.05% trypsin. The digestion was terminated by adding DMEM medium containing 15% fetal bovine serum (FBS). After 10,000 g centrifugation for 3 min, the supernatant was discarded and cell pellets were re-suspended in 1 mL DMEM medium. Using trypan blue for cell quantification, cell concentration was adjusted to $1 \times 10^6$/mL. Cells were then seeded into 6-well plate which was pre-coated with 10 μg/mL PDL, followed by overnight incubation. Culture medium was changed every other day until day 6.

### IL-6 pretreatment

Cells were treated with 1 ng/mL IL-6 (group C) or 5 ng/mL IL-6 (group D) according to previous study [9] along with blank control (group A) and oxidative stress model (group B). Oxidative stress model was prepared as previously reported [9]. In brief, cells were treated with DMEM medium containing 0.6 mM $H_2O_2$ for 4 hours.

### Cell viability assay

20 μL MTT solution was added to all cultured cells in 96-well plate. After 4 hours, the supernatant was removed, followed by the addition of 150 μL DMSO. After 10-min *incubation, optical density (OD) values at 490 nm were measured.*

### Cell apoptosis assay

Annexin-V and 7-AAD double labelling method was used to detect cell apoptosis using test kit following the manual instruction. In brief, cells were firstly rinsed in PBA containing 1% bovine serum albumin (BSA), and were re-suspended in Annexin-V binding buffer at $1 \times 10^7$/mL concentration. 0.1 mL cell suspension was mixed with 5 μL Annexin-V and 7-AAD dye. Cells were developed in dark for 15 min at room temperature, followed by the addition of 0.4 mL Annexin-V binding solution. Samples were then loaded for flow cytometry quantification of apoptosis.

### SOD, MDA and GSH assay

Superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH) were tested by enzyme-linked immunosorbent assay (ELISA) using test kits (Beyotime, China; R&D Inc., US) following the manual instruction. In brief, serially diluted standards and test samples were added into 96-well plate (0.1 mL each). After 37°C incubation for 90 min, 0.25 mL washing buffer was added into each well for rinsing (4 changes). Biotin-labelled antibody working solution was then added into each well (0.1 mL per well), followed by 37°C incubation for 60 min. After a gentle washing, enzyme-linked working solution (0.1 mL each) was applied for 37°C incubation for 30 min. The color was developed by 0.1 mL developing reagent and dark incubation for 10~20 min. The reaction was quenched by 0.1 mL stopping buffer, followed by the measurement of OD values at 450 nm immediately.

### Immunocytochemical (ICC) staining

All cells were tested for the expression and localization of signal transducer and activator of transcription 3 (STAT3) by ICC method, using rabbit anti-mouse STAT3 (Cell signaling, US) and goat anti-rabbit IgG conjugated with FITC antibodies.

### Western blotting

Total proteins were extracted from all cultured neural cells. After separation by 8% SDS-PAGE, proteins were transferred to nitrocellulose membrane, which was blocked by TBST containing 5% BSA for 2 hours at room temperature. Rabbit anti-mouse STAT3 antibody was applied for overnight incubation. After rinsing in
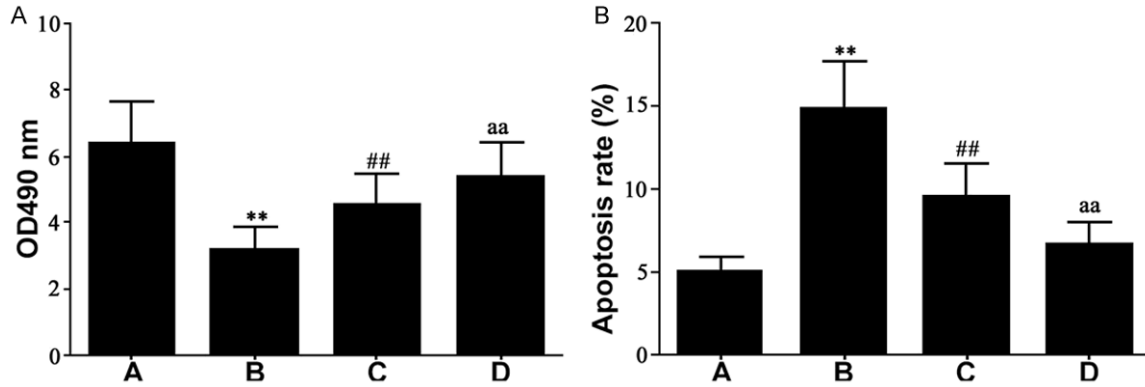
**Figure 1.** Viability and apoptosis of cultured neural cells. A: Cell viability as shown by OD values at 490 nm by MTT assay. B: Apoptotic cell percentage (in %) by flow cytometry. A, blank control; B, model cell. C: 1 ng/mL IL-6 pretreatment. D: 5 ng/mL IL-6 pretreatment. **P<0.01 compared to group A; ##P<0.01 compared to group B; aaP<0.01 compared to group C.
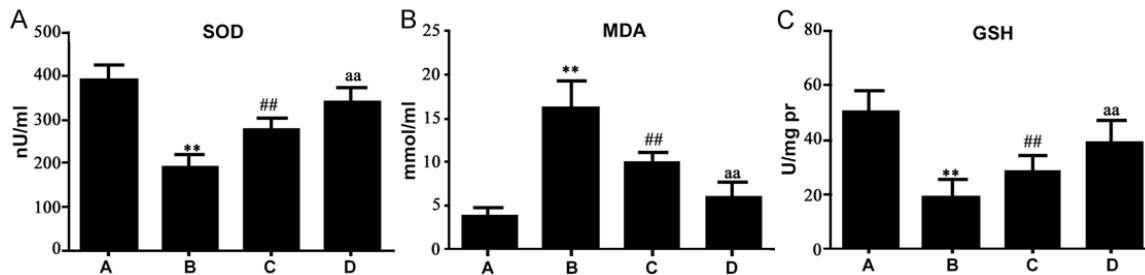


**Figure 2.** SOD, MDA and GSH expression in neural cells. A: Blank control; B: Model cell; C: 1 ng/mL IL-6 pretreatment; D: 5 ng/mL IL-6 pretreatment. **P<0.01 compared to group A; ##P<0.01 compared to group B; aaP<0.01 compared to group C.

0.1% TBST for three times, goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) was added for 1-hour incubation at room temperature. Chromogenic substrates (Supersignal West Femot/Pico HRP) were used to develop the membrane using actin as the internal reference.

*Statistical analysis*

All experiments were performed in at least triplicates. Data was represented as Mean ± SD. SPSS 16.0 software was used to compare all means by two-way analysis of variance (ANOVA). A statistical significance was defined when *P*<0.05.

**Results**

*Neural cell morphology*

12 hours after seeding, most cells adhered to the wall with round or oval shape. Few cells dis-

played 1~2 processes. 24 hour later, more cells had processes growth. 6 days later, neural cells had spindle shape with processes forming a network.

*Cell viability and apoptosis*

As shown in **Figure 1A**, model cells had significantly decreased cell viability compared to blank control cells (3.14 ± 0.73 vs. 6.34 ± 1.31, *P*<0.01). The pre-treatment of IL-6, however, significantly elevated cell viability (1 ng/mL IL-6: 4.52 ± 0.94; 5 ng/mL IL-6: 5.36 ± 1.05) with more significant improvement in 5 ng/mL IL-6 treated group.

When examining the percentage of apoptotic cells, it is found that oxidative stress increased cell apoptosis (14.84 ± 2.93 vs. 4.94 ± 1.04), while IL-6 pretreatment significantly decreased apoptotic cell percentage (1 ng/mL: 9.53 ± 2.01; 5 ng/mL: 6.63 ± 1.52).
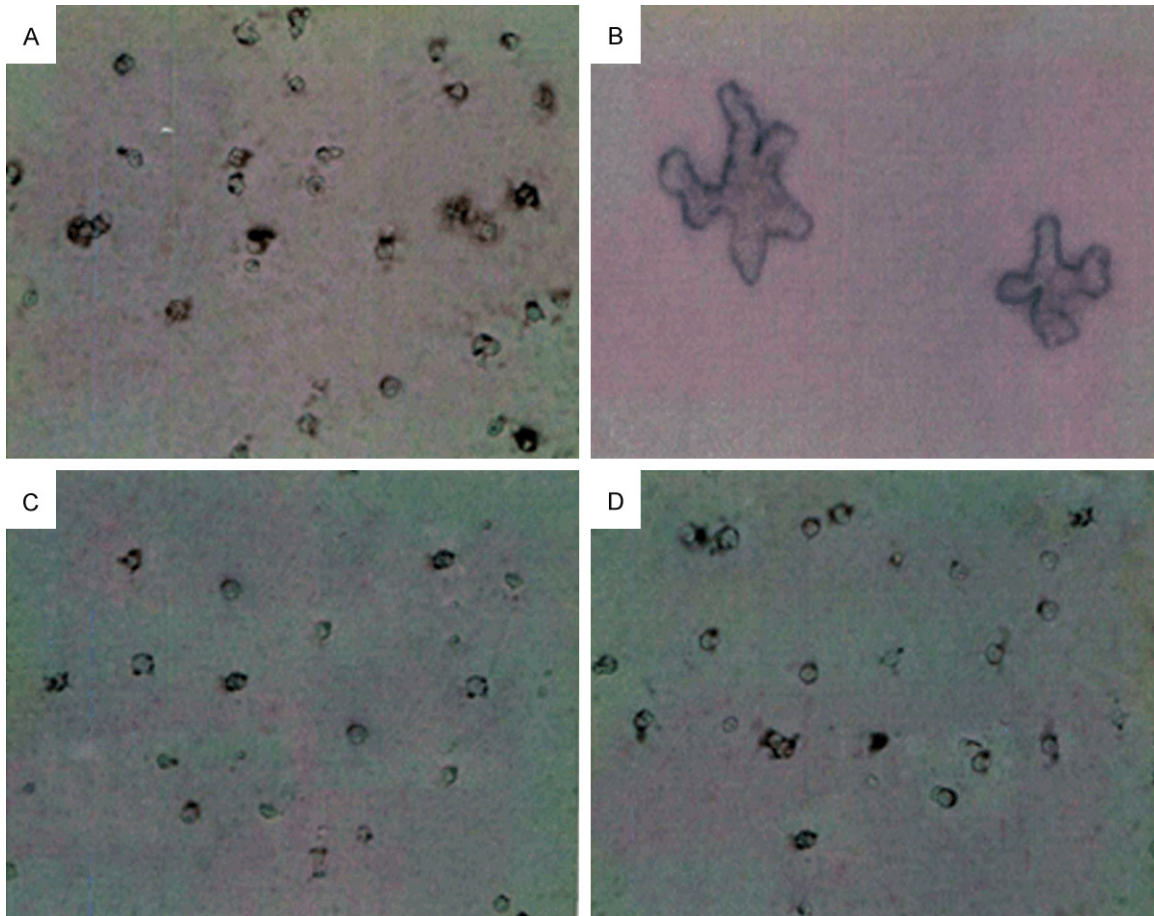
**Figure 3.** STAT3 expression in neural cells. A: Blank control; B: Model cell; C: 1 ng/mL IL-6 pretreatment; D: 5 ng/mL IL-6 pretreatment.
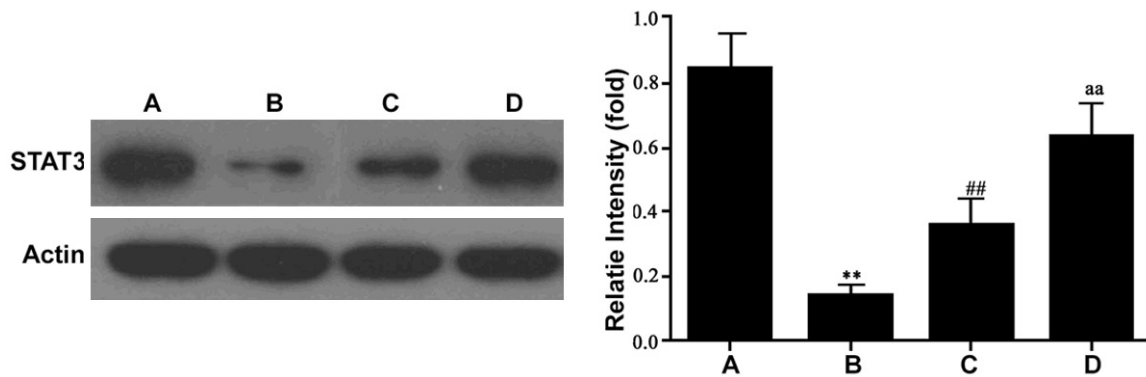


**Figure 4.** STAT3 and p-STAT3 expression levels. A: Blank control; B: Model cell; C: 1 ng/mL IL-6 pretreatment; D: 5 ng/mL IL-6 pretreatment. **$P<0.01$ compared to group A; ##$P<0.01$ compared to group B; aa$P<0.01$ compared to group C.

*SOD, MDA and GSH expression level*

SOD activity in IL-6 treated cells was significantly higher than model cells (**Figure 2A**, 1 ng/mL IL-6: 274.74 ± 27.84 nU/mL; 5 ng/mL IL-6: 337.54 ± 31.94 nU/mL; model cell: 189.56 ± 22.83 nU/mL). MDA showed opposite patterns as it decreased after IL-6 pretreatment (**Figure 2B**, 1 ng/mL IL-6: 9.64 ± 1.43 mM; 5 ng/mL IL-6: 5.73 ± 1.88 mM; model cell: 15.93 ± 3.36

mM). GSH level showed similar patterns as that in SOD (**Figure 2C**, 1 ng/mL IL-6: 27.93 ± 6.94 U/mg Pr; 5 ng/mL IL-6: 38.93 ± 8.42 U/mg Pr; model cell: 19.47 ± 5.45 U/mg Pr). In summary, high concentration of IL-6 pretreatment (5 ng/mL) exerted more significant alleviation on oxidative stress-induced cellular injury.

*STAT3 expression in neural cells*

STAT3 is widely distributed in the cytoplasm of normal neural cells (**Figure 3A**). After induction of oxidative stress, most cells showed fusion without STAT3 expression (**Figure 3B**). The pretreatment by IL-6 significantly elevated STAT3 expression (**Figure 3C**, **3D**).

*Western blotting*

As consistent with the cell staining results, high levels of STAT3 was found in normal neural cells, while oxidative stress significantly depressed STAT3 expression. The pretreatment of IL-6 can rescue cells by elevating STAT3 levels (P<0.01). High concentration of IL-6 (5 ng/mL) led to higher STAT3 levels (**Figure 4**).

## Discussion

As one progressively neurodegenerative disease, PD is mainly manifested as slow movement, static tremor and rigidity [11-15]. Although the exact pathogenesis mechanism of PD has not been fully illustrated, it is known that oxidative stress injury participate in the occurrence of PD [5]. This study tested the oxidative stress injury model on $H_2O_2$-induced primary cultured neural cells. Results showed significantly suppressed cell viability and elevated apoptosis in model cells. Meanwhile, $H_2O_2$ could depress intracellular SOD and GSH level whilst increase MDA expression. It has been known that SOD is one key enzyme in body's defense system against oxidative stress as it is the major enzyme for degrading intracellular oxygen radical species [16]. GSH is one sulfhydryl-containing tripeptide synthesized from glutamate, cysteine and glycine, and has anti-oxidation and detoxification functions [17]. The activity of SOD and GSH can reflect the body's ability to clear free radicals. As one important byproducts of membrane lipid peroxidation, MDA level can reflect the peroxidation level of membrane lipids [18] and internal clearance of free radicals. Therefore, our results including cell viability, apoptosis, SOD, GSH and MDA levels all suggested the successful generation of oxidative stress cell injury model.

IL-6 is one lymphocyte-derived cytokines produced from activated T cells and fibroblasts, and has been known to exert a protective role against $H_2O_2$-induced oxidative stress injury on myocardial cells [9]. Recent studies also showed the alleviation of HNE-induced oxidative stress injury on PC12 cells by IL-6 pretreatment [10]. This study thus investigated the protective function of IL-6 pretreatment against $H_2O_2$-induced oxidative stress injury on neural cells. Our results showed significantly elevated cell viability and depressed apoptosis in pretreated cells compared to mode cells. Such pretreatment by IL-6 can also elevate intracellular SOD and GSH levels while decrease MDA expression. All these results suggested the protective function on neural cells against oxidative stress injury, as consistent with previous studies in other cell types.

STAT is a family of transcriptional factors activated by peptide ligands such as cytokines and growth factors [19]. As one important member of STATs family, STAT3 exert crucial role in regulating cell proliferation, differentiation and apoptosis [19]. It has been known that STAT3 can be activated by IL-6 signal for downstream regulating intracellular gene expression [20]. Therefore we proposed that IL-6 might regulate STAT3 expression and phosphorylated activation status, thus exerting its protective functions against $H_2O_2$-induced oxidative stress injury on neural cells. Our studies showed significantly suppressed STAT3 expression level in model cells, and partially reversal of such down-regulation by IL-6 pretreatment. These results supported the role of IL-6 in mediating STAT3 expression in neural cells for further protection against $H_2O_2$-induced oxidative stress injury.

In summary, this study demonstrated that IL-6 pretreatment on neural cells can protect against $H_2O_2$-induced oxidative stress injury, possibly via regulating intracellular STAT3 expression.

## Disclosure of conflict of interest

None.

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