The Effects of 17β-estradiol on Neuronal PC12 Cells Injuried by OGD-R and NO/iNOS System Mechanism

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Abstract

Estrogen also plays an important role in normal development or differentiation of the brain. It's found recently that estrogen can not only affect reproductive activity through the regulation of serving Gn-RH inferior colliculus neurons, but also affect the other neurons of the brain in electrophysiology, neurological nutrition and metabolism. And, what concern most is the protection to central neuron of it. Through establishing the model of PC12 cells injuried by OGD-R, investigating the effect of Estrogen receptor antagonist ICI182780 Intervention on 17β-estradiol of PC12 cells injuried by OGD-R and ON/iNOS system. [Life Science Journal. 2010; 7(1): 34– 40] (ISSN: 1097 – 8135).

Key words: ICI182780; estradiol; PC12 cell; neuron; oxygen-glucose deprivation; iNOS

1. Objectives

Through establishing the model of PC12 cells injuried by OGD-R, investigating the effect of Estrogen receptor antagonist ICI182780 Intervention on 17β-estradiol of PC12 cells injuried by OGD-R and ON/iNOS system. For more, explore further the neuro-protective mechanism of 17β-estradiol on PC12 cells injuried by OGD-R, so as to provide the theoretical basis for clinical therapeutic application of 17β-estradiol in acute brain injuries induced by ischemia or reperfusion injury.

2. Methods

PC12 cells were cultured in DMEM medium, and then were induced to differentiate in DMEM medium containing 50ng/ml NGF for 5 days. These cells were named neuronal PC12 cells after exhibiting characteristic neuronal morphology. These neuronal PC12 cells which grew well and were well induced, and were dissected into suspension and then were inoculated on well plates with the cell concentration according to the necessity of different experiments. After induced to differentiate with NGF for 48 hours, they attached the wall and completely expanded. These cells were randomly divided into five groups（Table 1）: the normal control group, the OGD-R group, the 17β-estradiol group, the ICI182780 Intervention group and the ICI182780 control group. PC12 cells in the normal group did not receive OGD-R and were cultured in DMEM medium recovered glucose as usual during oxygen-glucose deprivation and during reperfusion. While PC12 cells in OGD-R group were cultured in medium without glucose. Cells in 17β-estradiol group containing 10ng/ml 17β-estradiol and medium without glucose during OGD-R. The ICI182780 intervention group containing 10ng/ml 17β-estradiol and medium without glucose during OGD-R. adding 100ng/ml ICI182780. The ICI182780 control group was cultured in 100ng/ml ICI182780 DMEM without glucose. Then were put into the chamber in which oxygen was driven off, after receiving oxygen-glucose deprivation for 30 minutes, PC12 cells in the groups were taken out and were changed into medium with glucose, 10ng/ml 17β-estradiol and medium with glucose, 10ng/ml 17β-estradiol and 100ng/ml ICI182780 DMEM with glucose and 100ng/ml ICI182780 DMEM with glucose respectively. They were put back to cells culture incubator and cultured successively during reperfusion. Cellular morphological changes were observed after oxygen-glucose deprivation.

The viability of cells and the concentration of NO in the culture medium were measured at each time point of 2h, 4h, 8h, 16h, and 24h after reperfusion. Cell apoptosis and cell death were detected with flow cytometry 24 hours after oxygen-glucose deprivation. The expression level of iNOS and protein were examined by Western Blotting 24 hours after oxygen-glucose deprivation. The expression level of iNOS mRNA and protein were examined by RT-PCR analysis 24 hours after oxygen-glucose deprivation.

Table 1: group experiments and the experimental process

groups	period of oxygen-glucose deprivation (30min)	period of reperfusion
normal control group	were cultured in DMEM medium and did not receive OGD-R in the chamber	were cultured in DMEM medium
OGD-R group	were cultured in medium without glucose and receive OGD-R in the chamber	were cultured in DMEM medium
17β-estradiol group	containing 10ng/ml 17β-estradiol and medium without glucose during OGD-R in the chamber	10ng/ml 17β-estradiol and medium with glucose
ICI182780 Intervention group	containing 10ng/ml 17β-estradiol and medium without glucose during OGD-R, adding 100ng/ml ICI182780	10ng/ml 17β-estradiol and 100ng/ml ICI182780 DMEM with glucose
ICI182780 control group	were cultured in 100ng/ml ICI182780 DMEM without glucose	100ng/ml ICI182780 DMEM with glucose and receive OGD-R in the chamber

3. Results

(1) The morphological observation of PC12 cells after oxygen- glucose deprivation for 30 minutes

The PC12 cells in normal control group grew well as usual (Figure 1). After oxygen-glucose deprivation for 30 minutes, the PC12 cells in OGD-R group (Figure 2) and the ICI182780 control group (Figure 5) became round because of serious edema and their prominence completely disappeared.

The PC12 cells in 17β-E_2 group (Figure 3) reserved the neuron-like characteristics, but the cell body of them had slighter edema and their prominence became thinner and shorter. the PC12 cells in the ICI182780 Intervention group (Figure 4) seem similar with that in 17β-E_2 group. The PC12 cells in five different groups had a very different appearance.

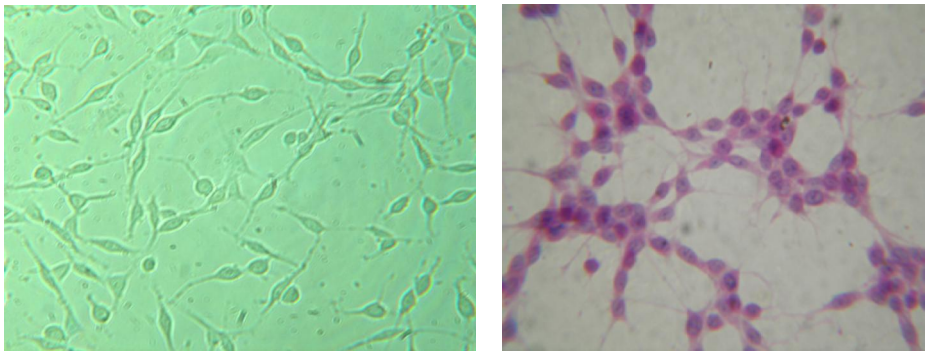


Figure 1: The characteristics of PC12 cells in normal control group are very similar to these of neurons. The right figure is HE staining. (Observed under the microscope 10×40)

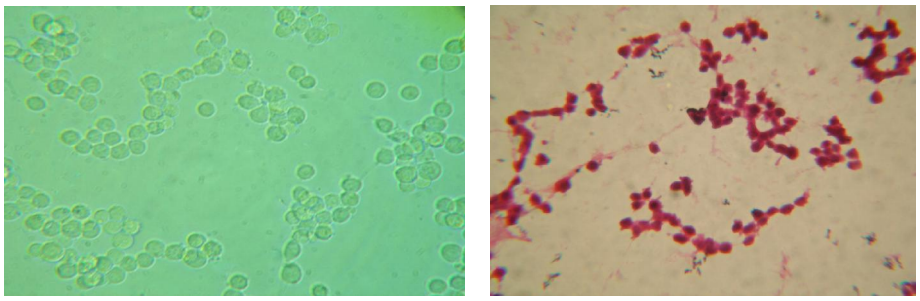


Figure 2: After oxygen-glucose deprivation for 30 minutes, the PC12 cells in OGD-R group became round because of serious edema and their prominence completely disappeared. They group gathered. The right figure is HE staining. (Observed under the microscope 10×40)

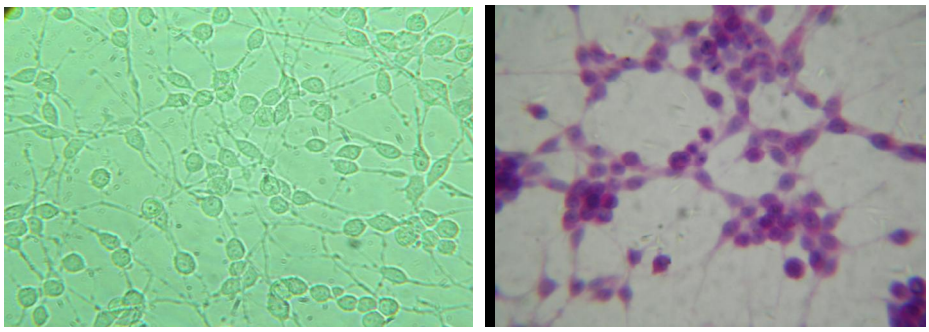


Figure 3: After oxygen-glucose deprivation for 30 minutes, most PC12 cells in 17β-E_2 group retained the spindle-shaped cells, but the cell body of them had slighter edema and their prominence became thinner and shorter. Small number of cells became round and their prominence disappeared; the group gathered is not obvious. The right figure is HE staining. (Observed under the microscope 10×40)

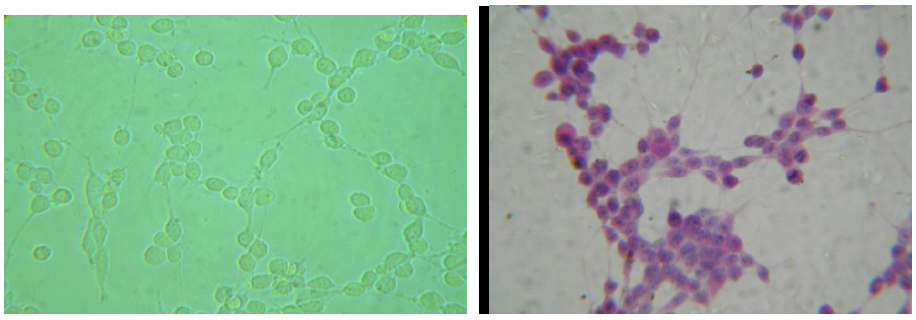


Figure 4: After oxygen-glucose deprivation for 30 minutes, most PC12 cells in the ICI182780 Intervention group retained the spindle-shaped cells, but the cell body of them had slighter edema and their prominence became thinner and shorter. Small number of cells became round and their prominence disappeared, the group gathered is not obvious. A small number of cells floated. The right figure is HE staining. (Observed under the microscope 10×40)

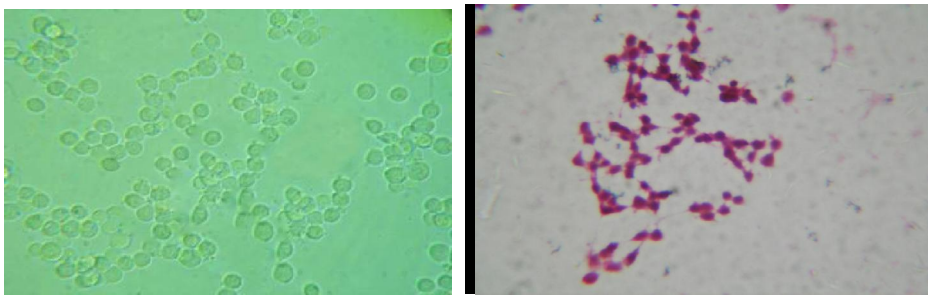


Figure 5: After oxygen-glucose deprivation for 30 minutes, the PC12 cells in the ICI182780 control group became round because of serious edema and their prominence completely disappeared. They group gathered. Their characteristics are very similar to these of OGD-R group. The right figure is HE staining. (Observed under the microscope 10×40)

(2) The determination of NO in cell medium

The concentration of NO in cell medium was measured respectively at each time point of 2h, 4h, 8h, 16h, and 24h after reperfusion. At each time point, the concentration of NO was highest in OGD-R group or Fulvestrant control group, and lowest in normal control group, and the concentration of NO in 17β-E_2 group and ICI182780 Intervention group was between the two. The differences among five groups were obvious (P<0.01). Along with the reperfusion time, the differences of the concentration of NO among five groups changed more significantly. (Table 2)

Table 2: The concentration of NO in cell medium of every group in each time ($\bar{x} \pm s$)

groups	*n*	*The concentration of NO (μM)*				
		2h	**4h**	**8h**	**16h**	**24h**
the normal control group	6	0.566±0.212	0.605±0.178	0.606±0.178	0.645±0.122	0.685±0.130
OGD-R group	6	1.830±0.415$^{\triangle}$	2.027±0.277$^{\triangle}$	2.146±0.193$^{\triangle}$	2.303±0.245$^{\triangle}$	2.422±0.178$^{\triangle}$
17β-E_2 group	6	1.080±0.178$^{\triangle\star\#}$	1.159±0.130$^{\triangle\star\#}$	1.238±0.178$^{\triangle\star\#}$	1.356±0.245$^{\triangle\star\#}$	1.435±0.193$^{\triangle\star\#}$
the ICI182780 Intervention group	6	1.372±0.156$^{\triangle\star\#}$	1.405±0186$^{\triangle\star\#}$	1.532±0.179$^{\triangle\star\#}$	1.631±0.185$^{\triangle\star\#}$	1.746±0.253$^{\triangle\star\#}$
the ICI182780 control group	6	1.790±0.451$^{\triangle}$	1.998±0.321$^{\triangle}$	2.120±0.213$^{\triangle}$	2.259±0.241$^{\triangle}$	2.389±0.117$^{\triangle}$

Annotate: Compare with the normal control group, $^{\triangle}P$<0.01 ; compare with OGD-R group $^{\star}P$<0.01 ; compare with the ICI182780 control group, $^{\#}P$<0.01.

(3) Detecting the expression of iNOS protein in neuronal PC12 cells after 24 hours of reperfusion

The iNOS protein in the cytoplasm of PC12 cells after 24 hours of reperfusion was showed by Western Blotting method, and then was measured by computer picture analysis software. The expression of iNOS protein was lowest in normal control group and highest in OGD-R group and ICI182780 control group, but the expression of iNOS protein in 17β-E_2 group and ICI182780 Intervention group was between the two (Figure6.7). The differences of the expression of iNOS protein among five groups were obvious (P<0.05) (Table 3).

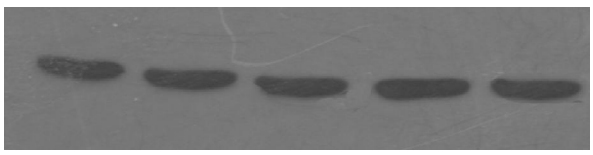


Figure 6

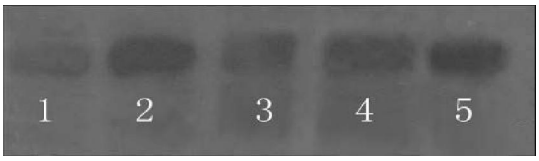


Figure 7

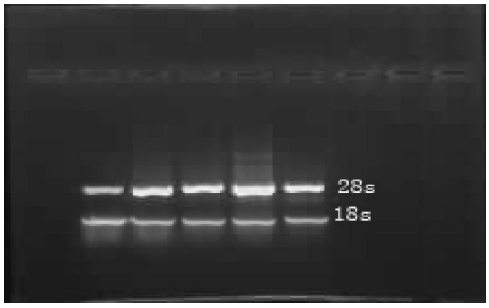
Figure 6: **A**fter oxygen-glucose deprivation for 30 minutes and after 24 hours of reperfusion, the expression of protein. (β-action). Figure 7: **A**fter oxygen- glucose deprivation for 30 minutes and after 24 hours of reperfusion, the expression of iNOS protein in five groups. In the figure, 1 is for the normal control group, 2 is for the OGD-R group, 3 is for the 17β-estradiol group, 4 is for the ICI182780 Intervention group, 5 is for the ICI182780 control group.

Table 3: Comparison of iNOS protein gray level ($\bar{x} \pm s$)

groups	*n*	*iNOS*
normal control group	3	0.31±0.01
OGD-R group	3	$0.62\pm0.01^{\triangle}$
17β-E_2 group	3	$0.45\pm0.02^{\triangle\star\#}$
the ICI182780 Intervention group	3	$0.39\pm0.01^{\triangle\star\#}$
the ICI182780 control group	3	$0.61\pm0.02^{\triangle}$

Annotate: Compare with the normal control group, $^{\triangle}P<0.05$; compare with OGD-R group $^{\star}P<0.01$; compare with the ICI182780 control group, $\#P<0.01$.

(4) The expression of iNOS mRNA examined by RT-PCR after 24 hours of reperfusion. (Figure 8, 9) The level of iNOS mRNA was expressed as ratio expression rate of iNOS gene/β-actin according to RT-PCR. The level of iNOS gene expression was 0.640±0.028 in normal control group, 1.322±0.094 in OGD-R group, 1.361±0.065 in ICI182780 control group, 0.772±0.032 in 17β-E_2 group and 0.764±0.0453 in ICI182780 Intervention group. The expression of iNOS mRNA was different among five groups ($P<0.05$). The difference between OGD-R group and normal control group was more significant ($P<0.01$) (Table 4).



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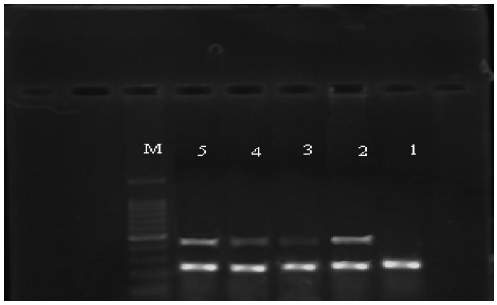

Figure 9

Figure 8: The expression of iNOS mRNA examined by RT-PCR after 24 hours of reperfusion. Figure 9: **A**fter oxygen-glucose deprivation for 30 minutes and after 24 hours of reperfusion, the expression of iNOS mRNA in five groups. In the figure, M is for mark, 1 is for the normal control group, 2 is for the OGD-R group, 3 is for the 17β-estradiol group, 4 is for the ICI182780 Intervention group, 5 is for the ICI182780 control group.

Table 4: Comparison of iNOS mRNA gray level ($\bar{x} \pm s$)

groups	*n*	*iNOS mRNA*
the normal control group	3	0.630±0.028
the OGD-R group	3	$1.342\pm0.094^{\triangle}$
the 17β-estradiol group	3	$0.772\pm0.032^{\triangle\star\#}$
the ICI182780 Intervention group	3	$0.869\pm0.029^{\triangle\star\#}$
the ICI182780 control group	3	$1.361\pm0.065^{\triangle}$

Annotate: Compare with the normal control group, $^{\triangle}P<0.05$; compare with the OGD-R group, $^{\star}P<0.01$; compare with the ICI182780 control group, $\#P<0.01$.

4. Discussions and Conclusions

In the experiment, oxygen glucose deprivation and reperfusion injury of PC12 cells model is more realistic than a simple lack of oxygen, or lack of sugar, and even oxygen-glucose deprivation period of application of sugar-free Earle's solution in the simulation of cerebral ischemia-reperfusion in which nerve cells ischemic perfusion environment [1]. After numerous pre-tests we found that the model repeatability and stability. It can meet the needs of this experiment [2]. Through inhibiting NO / iNOS system, 17β-estradiol can reduce the injury of PC12 cells in oxygen-glucose deprivation and reperfusion, also, it reduce their early apoptosis as a neuroprotective role [3]. However, the exact mechanism of action and role of the target is not clear [4]. In order to further understand the mechanism of estrogen action, we introduced estrogen receptor (ER) of the specific antagonist ICI182780 (Faslodex) [5] to interfere in the 17β-estradiol treatment, with its observation of the object as a control study was to investigate estrogen receptor ER [6, 7], in the cerebral protective effect of estrogen in the meaning. In this experiment, five groups, in the oxygen-glucose deprivation and reperfusion 2h, 4h, 8h, 16h, 24h of each time point. We found NO concentration in the supernatant of the cells in the experimental group; the cell activity was reduced reverse. We can see that high concentrations of NO have obvious cytotoxicity[8]. So NO / iNOS system activation may lead to oxygen-glucose deprivation-reperfusion injury in PC12 nerve cell injury and early apoptosis [9, 10]. It's one of the important reasons [11, 12]. Detection of RNA and protein expression levels of the test results also prompted that iNOS gene and protein expression have a consistency in all experimental groups[3], and directly related to the measured NO concentrations; compared with the normal group, the iNOS gene and protein expression of PC12 cells in the 24h oxygen-glucose deprivation-reperfusion model group is the highest. The cell activity in microscopic observation is the worst. ICI182780 followed by the group below. While the iNOS gene and protein expression of estrogen-treated PC12 cells is the lowest, activity of microscopic cells is the closest to the normal group. The ICI182780 Intervention group is between model group and treatment group. So we can see through inhibiting NO / iNOS system, 17β-estradiol can reduce the injury of PC12 cells in oxygen-glucose deprivation and reperfusion, and reduce their early apoptosis as a neuroprotective role [13]. In this experiment, the iNOS gene, protein expression, NO concentration and cell activity observations of PC12 cells have shown that the ICI182780 intervention groups still have a certain protection to PC12 cells on oxygen-glucose deprivation and reperfusion [14]. Using enough high-purity of estrogen receptor antagonist can not make an effective rivalry to the protection of estrogen. So, we speculate that the neuroprotective effect of estrogen is multipath. We point out it may play a neuroprotective effect through means other than its receptor [15].

This experiment was successfully prepared by oxygen-glucose deprivation-reperfusion injury model PC12 cells, the application interfere with estrogen receptor antagonist[16.17], estrogen treatment, compared to observe the protective effect of estrogen, and further explored 17β-estradiol on the oxygen-glucose deprivation and then reperfusion injury in PC12 cells[18.19], and NO / iNOS system, the impact mechanism, draw the following conclusions:

(1) High concentrations of NO has a strong cytotoxic, NO / iNOS system activation is the important cause of PC12 cells injuried by OGD-R.

(2) 17β-E2 can effectivly inhibit NO/iNOS system from activating and performing in PC12 cells of OGD-R. It can relieve cell edema, preserve cell morphous and enhance cell activity.

(3) Estrogen receptor antagonist ICI182780 could not effectively block the 17β-estradiol inhibited NO / iNOS system in neuroprotection.

(4) The protective effects of estrogen are probably many ways, and 17β-E_2 can exert neuroprotective effects and relieve injuries on neuronal PC12 cells injuried by OGD-R through other means other than receptor.

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