THE TOXIC EFFECT OF ROTENONE ON THE MESENCEPHALIC ASTROCYTES AND DOPAMINERGIC NEURON MICROENVIRONMENT

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ABSTRACT

In the present study, we applied the mitochondrial complex I inhibitor rotenone to in vitro cultured astrocytes and examined the changes in lactate dehydrogenase (LDH) activity and the glutathione (GSH) level to determine whether glycolysis was elevated by the resulting dysfunction in oxidative phosphorylation, enhancement of oxygen free radical production and reduction in GSH in the astrocytes. Glial cell line-derived neurotrophic factor (GDNF) has a protective effect on dopaminergic neurons. Western blot was performed to examine whether GDNF expression was altered after rotenone treatment. After short-term exposure of astrocytes (11 h) to low dose of rotenone (20, 30, and 40 nM), LDH activity was increased in all rotenone treatment groups compared to the control group and there was significant difference in LDH activity between the rotenone treatment groups and the control group. Additionally, the 40 nM group displayed a decreased GSH level, whereas the other two groups did not significantly differ from the control group. GDNF expression was decreased in all rotenone treatment groups compared to the control group and there was significant difference in GDNF expression between the rotenone treatment groups and the control group and the group treated with 40nM rotenone showed more significant difference than the others.After the dopaminergic neurons were treated with 50μM 6-OHDA and the supernatant of astrocyte treated with different rotenone concentration for 24 hours, the cell viability of the groups treated with different concentration of rotenone has distinguished difference than control group. As the increase of the rotenone's concentration, the cell viability treated with rotenone decreased. The above results demonstrated that rotenone treatment damaged the astrocytes that constituted the midbrain microenvironment. As a result, the production of oxygen free radicals was increased, the GSH and GDNF level was reduced, thus damaging dopaminergic neurons.

***Keywords**: Parkinson's disease (PD), astrocyte, rotenone, GSH, LDH, GDNF.*

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Introduction

To date, the etiology of Parkinson's disease (PD) remains largely unknown. Recently, gene mutations have been shown to form the pathogenic basis of certain familial PDs. However, familial PD accounts for less than 10% of total PD cases. Moreover, genomic analysis cannot fully explain the disease pathogenesis of cases of sporadic PD with a relatively late onset and insignificant genetic background. An increasing amount of evidence has shown that environmental toxins may play a critical role in the pathogenesis of sporadic PD[(1)]. Rotenone is an analogue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Rotenone is extensively used in the natural world as a major component of herbicides. Rotenone causes typical symptoms similar to PD in experimental animals[(2)]. Researchers have also found that weed control workers who receive long-term exposure to herbi-

cides have a higher chance of developing PD than people in other occupations[3]. Therefore, the present study simulated the biochemical changes that occur in astrocytes during the pathological process of PD through treatment of astrocytes with rotenone and explored the significance of the biochemical changes of the midbrain micro environment in PD pathogenesis.

Materials and methods

Astrocyte and dopaminergic neurons culture

The midbrains were dissected from sacrificed newborn Wistar rats as previously described by McCarthy and de Vellis[4]. The dissociated cells were seeded and cultured in 75-cm2 flasks coated with poly-L-lysine in Dulbecco's modified Eagle's medium-high glucose (DMEM-HG) supplemented with 10% fetal calf serum and 100 U/ml of penicillin/streptomycin in a humidified 5% CO_2 and 95% air atmosphere at 37°C. When the cells grew to confluence, the flasks were shaken at 280 rpm for approximately 20 h at 37°C to get astrocyte. A Trypan blue exclusion test was performed to measure cell viability, and immunocytochemistry was performed to guarantee that the percentage of GFAP-positive cells was >95%.

For dopaminergic neurons culture, Neurons were cultured at 37°C with 5% CO_2 in Neurobasal medium supplemented with 1% B27, 1% fetal calf serum, and 500μm Glutamax. At 4, 7, and 10 d in vitro (DIV), half of the media was changed with media supplemented with 2% B27 and 500 μm Glutamax.

Establishment of a rotenone-induced cell injury model using in vitro cultured astrocytes

The astrocytes were divided into the experimental and control groups (n = 5). After changing the medium, rotenone was added to the experimental group to final concentrations of 20, 30 and 40 nM. Equal volumes of DMEM/Ham's nutrient mixture F-12 (F12) were added to the control group. Subsequently, the cells were cultured in a 5%CO_2 incubator for 11 h. Then, the cells were observed using an inverted phase-contrast microscope.

The samples (2 μl each) were diluted to 0.2 ml with double-distilled water. After the A640 values (absorbance at 640 nm) of the samples were determined by the Lowry method, the sample protein concentrations were calculated according to an established formula. If an obtained A640 value fell outside of the linear range of the standard curve, the concentration of the sample was increased or reduced by dilution until the value lay within the linear range of the standard curve. These measures were taken to avoid errors caused by protein concentrations either above the upper limit or below the lower limit of detection.

Western blot

After separation in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels (10 or 12%), the protein samples were transferred to PVDF membranes (Millipore). The membranes were blocked with 5% nonfat milk/TBST (0.1% Tween-20, TBS) for 1 h and then incubated with rabbit anti-GDNF (Santa Cruz sc-328) and rabbit anti-β-actin (Sigma) antibodies at 4°C overnight. β-actin was used as the immunoblotting loading control. After incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h, the immunoblot bands were detected using an advanced ECL detection kit (Millipore). The immunoblots were digitally acquired using the Bio-Rad System. The quantitative data analysis of the immunoblots was performed and is shown as the density ratio compared with the control groups.

The LDH release assay and GSH assay were performed using commercial kits.

Cell treatment Cell viability assay

Dopaminergic neurons were plated onto 96-well plates for viability tests incubated overnight as it grew into 80-90% confluency. The supernatantsof astrocytes treated with different concentration of rotenone were collected and centrifuged to remove cell fragments, then that treated dopaminergic neurons for 12 hours in a humidified 5% CO_2 and 95% air atmosphere at 37°C. Dopaminergic neurons were washed twice with PBS solution and incubated in fresh low-serum media with 6-OHDA at a final concentration of 50μM. Regular medium was replaced with low-serum medium (0.5% FBS/DMEM) immediately before treatment. Dopaminergic neurons were incubated for 12 h after exposure to 6-OHDA. After 12 hours, Cell viability was measured using the In Vitro Toxicology Assay Kit, MTT based(Sigma-Aldrich).

Statistical methods

The experimental data were subjected to statistical analysis using the SPSS 10.0 statistical software package. The data are expressed as the mean ± standard deviation. P values less than 0.05 were considered statistically significant.

Results

Changes in lactate dehydrogenase (LDH) activity in astrocytes after treatment with various rotenone concentrations

LDH activity was clearly increased in all groups treated with various rotenone concentration compared to the normal control group. There was significant difference in LDH activity between the groups treated with various rotenone concentrationand the normal control group (Figure 1).

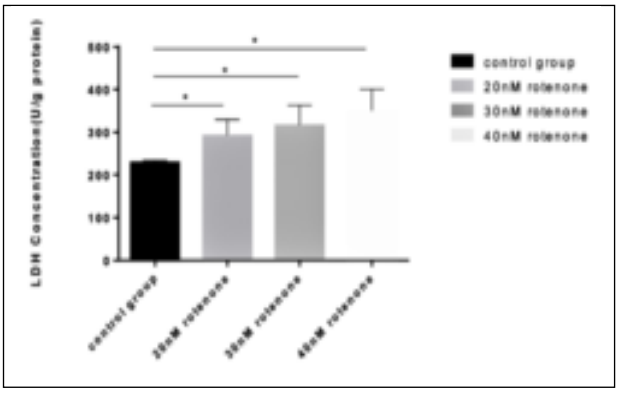

Figure 1: Changes in lactate dehydrogenase (LDH) activity in astrocytes after treatment with various rotenone concentrations.
**The difference was significant compared with the control group ($p < 0.05$).*

Changes in the glutathione (GSH) content in astrocytes after treatment with various rotenone concentrations

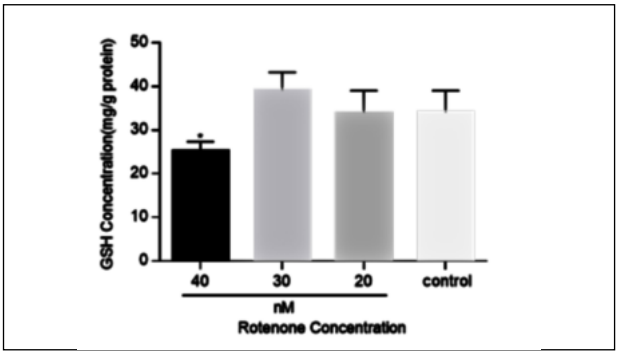

Figure 2: Changes in the glutathione (GSH) content in astrocytes after treatment with various rotenone concentrations.
**The difference was significant compared with the control group ($p < 0.05$).*

The GSH content was significantly decreased in the group treated with 40 nM rotenone compared to the normal control group. In contrast, the GSH levels in the groups treated with 20 nM and 30 nM rotenone were not significantly different from the level in the normal control group (Figure 2).

Changes in the expression of glial cell line-derived neurotrophic factor (GDNF) in astrocytes after treatment with various rotenone concentrations

The western blot results (Figure 3) showed that GDNF expression was decreased in all rotenone treatment groups compared to the control group and there was significant difference in GDNF expression between the rotenone treatment groups and the control group and the group treated with 40nM rotenone showed more significant difference than the others.

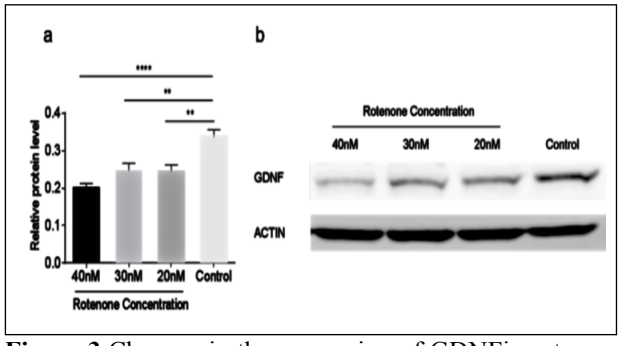

Figure 3:Changes in the expression of GDNFin astrocytes after treatment with various rotenone concentrations.
a*:Changes in the relative protein level expression of glial cell line-derived neurotrophic factor (GDNF) in astrocytes after treatment with various rotenone concentrations.* ***b****: Western blot of glial cell line-derived neurotrophic factor (GDNF) in astrocytes after treatment with various rotenone concentration. ** $p < 0.05$. **** $p < 0.01$.*

The cell viability of dopaminric neurons after exposed to 6-OH and incubated with the medium of astrocytes treated with different concentration of rotenone

After exposed to 6-OH, all treated group's cell viability were decreased compared to the control group. More over the cell viability in the group that incubated with the medium of astrocyte treated with 40nM was decreased significantly compared to the control group and the other treatment groups (Figure 4).

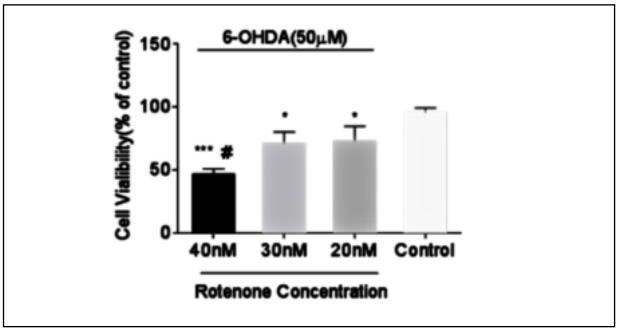

Figure 4: The cell viability of dopaminric neurons after exposed to 6-OH and incubated with the medium of astrocytes treated with different concentration of rotenone.

*Different concentration of rotenone group vs. control group. * $p < 0.05$,*** $p < 0.01$.*

 # $p < 0.05$ 40nM rotenone group vs. 20(30)nM rotenone.

Discussion

The results of our study showed that rotenone can block mitochondrial complex I in astrocytes, resulting in inhibition of oxidative phosphorylation, elevation of glycolysis and enhancement of the activity of a key enzyme in glycolysis (LDH) and the GSH level and GDNF expression level reducedas the dose of rotenone increased. The astrocytes consumed a large amount of GSH to defend themselves against the excess oxygen free radicals generated due to the suppression of the mitochondrial respiratory chain and the ability of producing GDNF decreased.The above biochemical changes in the mesencephalic microenvironment constituted by the astrocytes would certainly affect the dopaminergic neurons supported and nourished by the astrocytes. Our cell viability test showed when the dopaminergic neurons exposed 6-OH, they are more easy to die.

Scavenging of oxygen free radicals has a protective effect on dopaminergic neurons[5]. Because both 1-methyl-4-phenylpyridinium (MPP+) and rotenone cause Parkinson's syndrome, we speculated that energy metabolism disorder and inhibition of oxidative phosphorylation played important roles in PD pathogenesis.

Traditionally, astrocytes were thought to provide structural and nutritional support to neurons and to play a passive secondary role. However, deeper research has provided enhanced understanding of astrocyte structure and function. Researchers have found that astrocytes are not inert cells. Instead, these cells play critical roles in nervous system development, synaptic transmission, nervous tissue repair and regeneration, neuroimmunity and the pathological mechanisms of a variety of neurological diseases.Astrocytes transmit and exchange information with one another.

Moreover, astrocytes interact with neurons in a variety of ways and influence their biological activity. Specifically, the functions of astrocytes include:

• participating in synaptogenesis and ensuring the development of neurons;

• participating in the regulation of substance metabolism in neurons;

• regulating neural information[6,7].

Astrocytes and microglia normally play neuroprotective roles, but when chronically activated (i.e., under inflammatory/neurotoxic exposure or upon brain injury), glial cells produce a wide range of cytotoxic mediators, including reactive oxygen species, reactive nitrogen species, and neural proinflammatory cytokines and chemokines that may perpetuate/exacerbate glial activation, thereby increasing neuronal vulnerability and/or promoting dopaminergic cell death[8].

In a study conducted in 1999, McNaught and Jenner[9] constructed an in vitroco-culture system that consisted of dopaminergic neurons and astrocytes to simulate the mesencephalic microenvironment. Changes in glial cell function, such as an increase in oxygen free radicals, promoted dopaminergic neuronal death or increased the susceptibility of dopaminergic neurons to neurotoxins.The findings indicate that impaired mitochondrial function in glial cells or hypoxia induce oxidative stress. In turn, oxidative stress may promote dopaminergic neuronal death and cause PD.

Under normal physiological conditions, harmful free radicals are continuously produced during cellular metabolic processes. However, the body's defense system limits free radical levels and thus the damage of cells due to free radicals. A variety of substances are produced by the body to antagonize free radicals. A number of small molecules play important roles in cell defense against oxidative damage, including GSH, β-carotene, vitamin E and vitamin C.Under physiological conditions, a balance is reached between the production and elimination of free radicals.Therefore, defects in one or multiple antioxidants, regardless of the causeor the excessive production of free radicals may lead to insufficient scavenging of free radicals. The excessive levels of free radicals then induce oxidative stress.

The function of GSH is to eliminate oxygen radicals and convert H_2O_2 into water; GSH represents one of the most important mechanisms of antioxidative protection in the brain. Because the GSH concentration is fairly low in the perikaryon, GSH secretion by glial cells is required to antagonize oxidative stress.The present study demonstrated that the GSH level was reduced in glial cells under the action of rotenone. The results confirmed that the cells displayed elevated glycolysis, released an increased amount of oxygen free radicals and consumed large amounts of GSH upon suppression of the mitochondrial respiratory chain. We can infer from the results that the reduction in the GSH level in astrocytes weakens the ability of dopaminergic neurons to withstand oxygen radicals and promotes the production of hydroxyl radicals. Therefore, a reduced glial GSH content may represent one reason for the impairment of dopaminergic neurons. In 1999, Mytilineou et al.[10] applied an inhibitor of GSHsynthesisbuthioninesulfoximine (BSO) to reduce the GSH level. Although the reduction of the GSH level had no effect on neurons alone, reduction of the GSH level in a mixed culture of neurons and astrocytes resulted in neuronal death.The findings indicate that the reduction of the GSH level leads to increased neuronal susceptibility. Activation of glial cells increases the level of oxygen free radicals, resulting in neuronal impairment.

GDNF was initially isolated and purified in 1993 by Lin et al.[11] from the conditioned culture medium of the rat glioma cell line B49. GDNF has a specific nutritive effect on rat midbrain dopaminergic neurons by promoting the survival and growth of dopaminergic neurons and stimulating the uptake of dopamine (DA). A large number of in vivo and in vitro experiments have shown that GDNF exerts an apparent protective effect on dopaminergic neurons. Researchers have observed under various experimental conditions that the GDNF protein prevents and antagonizes the loss of dopaminergic neurons induced by 6-hydroxydopa and MPP+. Under the action of GDNF, the numbers of tyrosine hydroxylase-positive cells and neurites increase, the DA level is enhanced, and the symptoms of model animals with PD are improved[12-16].

In a study conducted in 2000 by McNaught and Jenner[17], administration of MPTP or deprivation of GSH in in vitro cultured astrocytes led to increased expression of tumor necrosis factor-alpha (TNF-α) and decreased expression of GDNF in the astrocytes. The results of the present study showed that GDNF expression was reduced in astrocytes after treatment with a certain dose of rotenone, which was consistent with the findings described above. Therefore, we speculate that changes in astrocyte functions lead to decreased GDNF expression and the subsequent apoptosis of dopaminergic neurons, which may explain the neuropathological degeneration of the substantia nigra in PD.

We can conclude from the above discussion that due to the intrinsic weaknesses of dopaminergic neurons (such as low cellular levels of GSH and mitochondrial complex I) and the toxic effects of DA metabolites, dopaminergic neurons rely on the neurotrophic factors and GSH provided by astrocytes to survive and defend against endogenous and exogenous damage. Astrocytic injury reduces the resistance of dopaminergic neurons. Furthermore, the harmful substances released by astrocytes aggravate dopaminergic neuronal injury.

Based on the findings of the present study, we can reasonably infer that functional changes in astrocytes have a certain relationship with the incidence of PD. This speculation prompted us to expand our perspective on the treatment strategies for PD by shifting our focus from rescuing dopaminergic neurons to improving the microenvironment essential for dopaminergic neuron survival, inhibiting the activation of astrocytes, and furthering our study on the pathogenesis and treatment of PD from a macroscopic perspective.

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