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Research Paper

Effect and mechanism of homocysteine on Parkinson's disease induced by 6-OHDA ☆

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Abstract

Objective: To study the effects and mechanism of homocysteine(Hcy) on Parkinson's disease(PD) induced by 6-hydroxydopamine (6-OHDA) in vivo. **Methods**:Forty rats were divided into 4 groups. 6-OHDA or the solvent of 6-OHDA was focally administrated to induce PD, 2 h later Hcy or 0.9% sodium chloride was administrated in the ipsolateral substantial nigra(SN). We used behavioral testing, Immolunohistochemical techniques, biochemistry techniques to detect the injury of SN. **Results**:The rotary turns of PD rats induced by 6-OHDA showed significant increase after treatment with Hcy compared with the controls($P < 0.05$). Also the numbers of tyrosine hydroxylase(TH)-stained neurons were decreased, and dendrites were fragmented and truncated. Free radicals were increased and antioxidant enzymes decreased. **Conclusion**:Focal infusion of Hcy into the SN increased the vulnerability of the dopaminergic neurons to 6-OHDA-induced degeneration, it seems that the endangering effect of Hcy is due to exacerbating oxidative stress.

Key words: Parkinson's disease; 6-hydroxydopamine; homocysteine; tyrosine hydroxylase

INTRODUCTION

Parkinson's disease(PD) is a progressive neurodegenerative disorder characterized mainly by tremor, akinesia, bradykinesia, rigidity, and postural instability. PD is associated with selective loss of the dopaminergic neurons in substantial nigra pars compacta(SN_{pc}). The cause of it is unknown, but growing evidence suggests that it may be due to a combination of environmental and genetic factors. 6-OHDA-induced rat rotation PD models by stereotactic injection into SN_{pc} have been extensively used in PD studies[1-2]. Hcy is the precursor of methionine. Normally Hcy levels are maintained low by enzymes(remethylation to methionine) that require folate or cobalamin(vitamin B_{12}), and by catabolism to cysteine by the pyridoxine(vitamin B_6)-dependent enzyme cystathionine β-synthase. It has been known for many years that the elevated plasma Hcy concentration is the independent risk factor of atherosclerosis, epidemiological data and clinic studies suggest that part of hyperhomocysteine patients have PD or Alzheimer's disease(AD) symptoms[3]. The relation between Hcy and PD is not clear, and for Hcy's influence on the development of PD, no report is as yet available. In our study, we used model of PD induced by 6-OHDA in vivo to study the effect and mechanism of Hcy on dopaminergic neurons, expecting to expend the study of PD's etiology.

MATERIALS AND METHODS

Materials

Experimental animals

Forty healthy female Sprague-Dawley(SD) rats, weighing 180-200 g, were purchased from the Experimental Animal Center of Tongji Medical College.

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Reagents

6-OHDA, Apomorphine, Hcy and anitibody of mouse against TH were provided by Sigma Co,USA. Kit of SP immolunohistochemistry(rat against mouse IgG antibody) was products of Beijing Zhongshan Bio-engineering Co Ltd Kits of superoxide dismutase(SOD), malondialdehyde(MDA) and catalase(CAT) were products of Nanjing Jiancheng Co Ltda. The Ⅱ-C brain stereotactic instrument was purchased from Shanghai Jiangwang apparatus Factory.

Grouping

The animals were randomly divided into four groups with each group having ten rats: control group(Solvent of 6-OHDA and 0.9%Sodium Chloride), 6-OHDA-treated group(6-OHDA and 0.9%Sodium Chloride), Hcy-treated group(Solvent of 6-OHDA and Hcy), 6-OHDA and Hcy-treated group(6-OHDA and Hcy). Rats of each group were treated as following.

Methods

Animal model

According to the procedures reported by Thomas[4], unilateral 6-OHDA-lesioned rats model of PD was made. 5 μl 6-OHDA solution(0.2%6-OHDA, dissolved in saline containing 0.27% ascorbic acid) or solvent of 6-OHDA(just 0.27% ascorbic acid) was respectively injected at the locus of SN_{pc} and mid-brain ventral tegmental area(VTA) according the grouping. 2 h later, each rats was administrated at SN_{pc} with 0.9% Sodium Chloride or 43 ng/μl Hcy 1 μl.

Behavioral testing

One day before the 6-OHDA(or solvent of 6-OHDA) injecting and two weeks after the Hcy(or 0.9% Sodium Chloride) injecting, rats were injected intraperitoneally with apomophine(APO) 0.5 mg/kg(Concentration 0.1mg/ml) to evoke rotational behavior. Eliminating the auto-rotating rats, we quantified and recorded the rotating rats number of each group, the generator time, the average rings every 30 min and the continuance. The qualified PD models were those with turning behaviors more than 210 rings 30 min.

Immunohistochemistry staining of TH in Nigra and analysis

Slices were fixed, then embedded in wax and cut into sections. Labbed StreptAvidin Biotin Enzyme method (SP method) was used to detect TH, according to the instruction of the kits of Beijing Zhongshan Bio-engineering Co Ltd. Three adjacent slides in every group were selected, cell bodies and dendrites in the similar area of nigra were observed, the numbers of neurons positive for TH and the percentage of positive cells with intact dendrites in right nigra were counted under a light microscope. MDA content, SOD activity and CAT content assay.

Plasma MDA content was measured using the TBA method. Plasma SOD activity was measured according to the xanthine-xanthinoxidase method. Plasma CAT content was measured using the Claiborne method. Rat right nigra was rapidly removed 0.2 g in ice bath, then added 1.8 ml distilled water to become 10% brain tissue homogenate, according to the instruction of the kits. The results were indicated as concentration or activity at per mg wet brain tissue.

Analysis of image

Each section for Immunohistochemistry was analyzed by media mix color pathological diagram analysis system, using the same amplifying power(× 40) and the same intensity(4.0). The three neighboring sections in each case were subjected to Immunohistochemistry to calculate the numbers of neurons positive for TH and the percentage of positive cells with intact dendrites in right nigra.

Statistical analysis

SPSS11.0 software package was used for the statistical analysis. All the data were expressed as $\bar{x} \pm s$. Hypothesis testing methods included Chi-square(χ^2 test), paired samples t test and analysis of variance (ANOVA). AP% value less than 0.05 was considered statistically significant.

RESULTS

Common Observation

One of the forty rats died from infection at the tenth day after the operation, the others were healthy. Apomophine can evoke rotational behavior. The classical rotational behaviors of PD rats were described as follows: heads connecting tails, PD rats rotated toward un-affected side in a clockwise direction. Hind limbs were taken as fulcrum. Parts of rats showed masticatory movement, hindlimb scratching, tearing and agitation, et al.

Rotational Behaviors of Rats

There were significant differences in the number of rats which showed rotational behaviors (>210 rings 30 min) between 6-OHDA group and control group, so did between 6-OHDA+Hcy group and Hcy group($P < 0.05$), from which we can say that our PD models were successful. 6-OHDA+Hcy group can increase the number of rotational rats, and there was a significant difference from 6-OHDA group($P < 0.05$)(shown in Tab 1, χ^2 test). But there were no significant difference in the generator time, the average rings every 30 min and the continuance between the two groups(Tab 2, t text). There were no significant difference in the frequency of rotational

behaviors between Hcy group and control group, but many rats in the Hcy group showed smelling, tail rigor, and the left hindlimb staying long time in air, et al. In the Hcy group, also, there were some rats constantly rotated toward left(four, <210 rings 30 min)(Tab 1).

Tab 1 The rotational behaviors of rats after operating 2 weeks (%, $\bar{x} \pm s$)

group	n	constant rotation toward 30 min left more than 210 rings	constant rotation toward 30 min left less than 210 rings	constant rotation toward left	No rotation
Control	10				10
6-OHDA-treated	10	3*		1	6*
Hcy-treated	10		4		6
6-OHDA+Hcy-treated	9	7*	1	0	1*▲

6-OHDA-treat group compared with control group, 6-OHDA+Hcy-treat group compared with Hcy-treat group, *$P < 0.01$; 6-OHDA+Hcy-treat group compared with 6-OHDA-treat, ▲$P < 0.01$.

Tab 2 Constant rotation toward left more than 210 rings 30 min observation ($\bar{x} \pm s$)

group	average generator time(min)	average rings every 30 min(r/min)	Continuance(min)
6-OHDA-treated	3.50 ± 0.707	306 ± 71.4	71 ± 12.73
6-OHDA+Hcy-treated	5.75 ± 4.349	255 ± 42.0	60 ± 8.54

There were no difference between these two groups($P > 0.05$).

Immunohistochemistry Staining of TH in Nigra

Shown in Fig 1 and Tab 3; ANOVA. Immunohistochemical labeling of dopaminergic neurons resulted in dense brown black staining of both the cell bodies and the dendrites. In sections of 6-OHDA group, the number of TH-positive neurons in the SN and the percentage of positive cells with intact dendrites had significant difference($P < 0.05$) compared with control group. But Hcy alone could not cause changes as stated above, there were no significant difference($P > 0.05$) compared with control group. In the 6-OHDA and Hcy-treated group, the number of TH-positive neurons and the percentage of positive cells with intact dendrites were significantly decreased when compared with every other group($P < 0.01$).

MDA Content, SOD Activity and CAT Content

There were significant difference between 6-OHDA group and control group, between 6-OHDA+Hcy group and Hcy group(*$P < 0.01$, **$P < 0.05$). In the 6-OHDA and Hcy-treated group, MDA content increased, and SOD activity and CAT content decreased, all these had significant difference($P < 0.01$) compared with 6-OHDA-treated group. There was no significant difference between Hcy group and control group(Tab 4; ANOVA).

DISCUSSION

Epidemiological data and clinic studies suggest that part of hyperhomocysteine patients have PD or AD symptoms. The relation between Hcy and PD being is not clear, and the pathological mechanism is still unclear.

Tab 3 The affection of 6-OHDA and Hcy to dopaminergic neurons in right SNpc ($\bar{x} \pm s$)

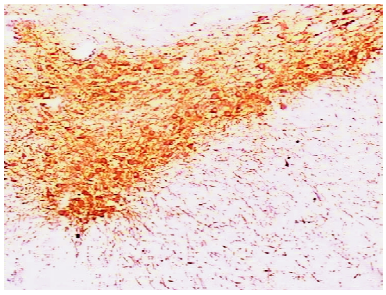
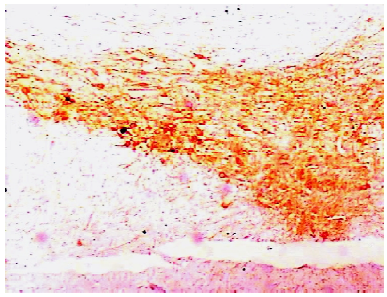
group	The section number of every group(pieces)	The number of TH-positive neurons	the percentage of positive cells with intact dendrites in all TH-positive neurons(%)
control	12	122.1 ± 4.2	0.771 ± 0.042
6-OHDA-treated	12	62.6 ± 4.6*	0.525 ± 0.066*
Hcy-treated	12	118.8 ± 4.5	0.730 ± 0.050
6-OHDA+Hcy-treated	12	40.9 ± 3.5*	0.256 ± 0.036*▲

6-OHDA-treat group compared with control group, 6-OHDA+Hcy-treat group compared with Hcy-treat group, *$P < 0.01$; 6-OHDA+Hcy-treat group compared with 6-OHDA-treat, ▲$P < 0.01$.

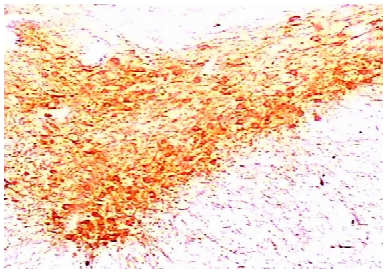
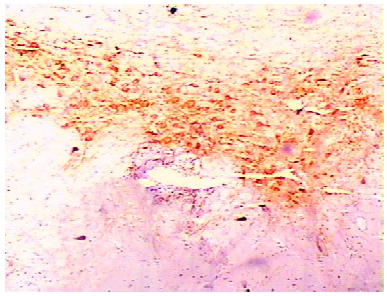
Tab 4 MDA content, CAT content, SOD activity ($\bar{x} \pm s$)

group	MDA [nmol/(mg • protein)]	CAT [(U/(mg • protein)]	SOD [nU/(mg • protein)]
control	3.099 ± 0.071	0.784 ± 0.026	2.894 ± 0.432
6-OHDA-treated	4.459 ± 0.153*	0.577 ± 0.072**	1.989 ± 0.458**
Hcy-treated	3.410 ± 0.720	0.731 ± 0.017	2.844 ± 0.273
6-OHDA+Hcy-treated	5.433 ± 0.326*▲	0.455 ± 0.037*▲	1.058 ± 0.261*▲

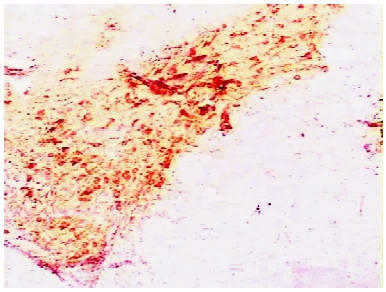
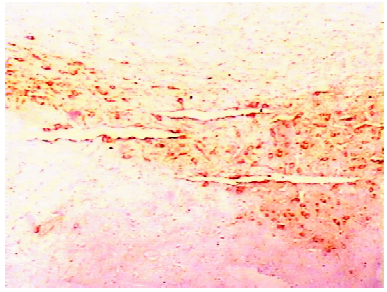
6-OHDA-treat group compared with control group, 6-OHDA+Hcy-treat group compared with Hcy-treat group, *$P < 0.01$, **$P < 0.05$; 6-OHDA+Hcy-treat group compared with 6-OHDA-treat, ▲$P < 0.01$.

left  right 

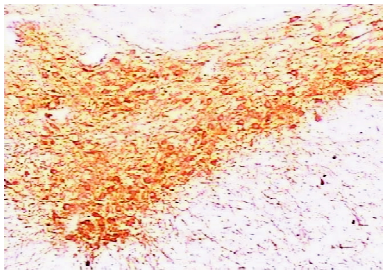
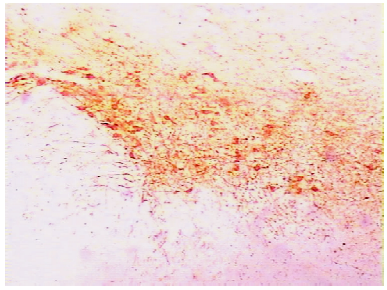
Control group:There were no significant difference on the number of TH-positive neurons and the percentage of positive cells with intact dendrites between the right and the left.

left  right 

6-OHDA-treated group: The number of TH-positive neurons and the percentage of positive cells with intact dendrites on the right were less than those on the left.

left  right 

Hcy-treated group: The number of TH-positive neurons and the percentage of positive cells with intact dendrites on the right were approximately equal with those on the left.

left  right 

6-OHDA+Hcy-treated group: The number of TH-positive neurons and the percentage of positive cells with intact dendrites on the right were remarkably less than those on the left.

Fig 1 Immunohistochemistry staining result of TH (Nigra, × 40)

In our study, There were significant differences in the number of rats which showed rotational behaviors (>210 rings 30 min) between 6-OHDA group and control group, so did between 6-OHDA+Hcy group and Hcy group($P < 0.05$), from which we can say that our PD models were successful. 6-OHDA+Hcy group can increase the number of rotational rats, and there were significant difference from 6-OHDA group($P < 0.05$). But there were no significant difference in the generator time, the average rings every 30 min and the continuance between the two groups. The reason of this phenomenon cannot be explained by documents. And our results were not all in accordance with Fei Cao's[5].

6-OHDA is a homolog of noradrenaline, which has selective damaging reaction to cate cholamine neuron and its ending. It could destroy the synthesis of dopamine in SN as well as its transportation pathway to corpus striatum, which would lead to imbalance between cat-

echolamine neurotransmitters and acetylcholine neurotransmitters, and hence a series of characteristic pathological changes and symptoms similar to human PD, such as rotation, would be produced[6-8]. If 6-OHDA directly injected into SN_{pc}, the mechanism of its selective damaging reaction might be that it would lead to oxidized stress through excessive producing free radicals in vivo and therefore damaging dopamine.

In our study, 6-OHDA alone can decrease the number of TH-positive neurons in the SN_{pc} and the percentage of positive cells with intact dendrites. It also can increase free radicals and decrease antioxidant enzyme as compared with control group, and our results were in accordance with documents. By only injected Hcy into SN_{pc} in midbrain, rats had no significant difference compared with those in control group on rotational behaviors, the number and shape of TH-positive neurons, free radicals and antioxidant enzyme. But when we combined 6-OHDA with Hcy, there were more rotational rats, less TH-positive neurons and percentage of positive cells with intact dendrites, and increasing MDA, decreasing CAT, SOD, compared with 6-OHDA-treated group ($P < 0.05$). Our study shows that the rats' symptoms of PD caused by 6-OHDA were significantly exacerbated in rats which Hcy was infused into SN_{pc}, but Hcy have not direct adverse effect on dopaminergic neurons. The underlying mechanism of Hcy enhanced PD induced by 6-OHDA is not clear now. We analyzed the results of our studies combining with documents, and thought it may mainly be: ①In the 6-OHDA and Hcy-treated group, MDA content increased and CAT content, SOD activity decreased. Hcy is believed to exert its effects through mechanisms involving free radical generation and as a result, oxidative damage[9-10]. Hcy is oxidized principally as a consequence of auto-oxidation resulting in the generation of ROS[11-12]. H_2O_2, hydroxyl radicals and superoxide anion radicals are generated during this reaction, and especially the hydroxyl radical initiates lipid peroxidation. Finally Hcy destroy biomembrane[13-14]. ② One of the explanations regarding how Hcy initiates lipid peroxidation is via the inhibition of GSH-Px activity. Upchurch et al. showed that Hcy not only inhibits GSH-Px activity but also causes a dramatic reduction in its mRNA levels. GSH-Px is the main antioxidant enzyme of the brain and is known as a neuroprotective enzyme[15-16]. ③ An early and pivotal event in the adverse effects of Hcy on neurons is increased DNA damage. The DNA damage results from a combination of impaired DNA repair and increased oxidative stress, as indicated by increased uracil misincorporation and increased oxidative modification of DNA bases[18-19]. DNA damage, in turn, triggers a cell death pathway involving poly(ADP-ribose) polymerase and the tumor suppressor protein p53, leading to mitochondrial dysfunction and activation of death proteases called caspases[20-21].

In our study, 6-OHDA could cause MDA content increase and CAT, SOD decrease, having significant difference compared with control, which illustrates that there is markedly peroxide reaction in the SN_{pc}. And this reaction became more obvious after the rats were infused with Hcy 2 h later. So, we can state that the endangering effect of Hcy is due to exacerbating oxidative stress.

Our findings provide the first direct evidence that Hcy can sensitize dopaminergic neurons to dysfunction and death in models of PD. There are a number of approaches that can be taken to lower Hcy levels and that would, therefore, be expected to reduce risk of PD. The major approach already in use is dietary supplementation with folate(typically 400 mg per day). Dietary supplementation with vitamins B_{12} and B_6, as well as preventing hereditary factor and renal diease, can also lower Hcy levels, and may enhance the effect of folate.

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