Original Article

Effects of Pinealectomy and Gonadectomy on Olfactory Bulb Dopaminergic Neurons in Rats

Yan Li[1], Jian Zhu[2], Ying Wang[3], Lei Guo[3], Lei Li[1], Dong Wang[1]

[1]Department of Neurology, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong 250013, China
[2]Department of Medical Imaging, Shandong Provincial Hospital, Jinan, Shandong 250014, China
[3]Department of Medical Imaging, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong 250013, China

Abstract

Background: Olfactory disorder is an early manifestation of Parkinson's disease (PD), likely to be associated with abnormalities of the dopaminergic neurons in the olfactory bulb (OB); however, the causes of olfactory disorder in PD are not entirely clear. Some studies showed that melatonin (MT) and androgens (mainly testosterone, T) might participate in the pathogenesis of PD. The research aimed to investigate effects of MT or T deficiency on OB dopaminergic neurons in rats.

Methods: One hundred and twenty normal male Wistar rats were randomly divided into the control, sham operation pinealectomy (PX), sham operation gonadectomy (GDX), PX, GDX, and PX + GDX groups. After 60 days, glial cell hyperplasia and neuronal apoptosis were examined with hematoxylin and eosin and the TUNEL method; the expression levels of tyrosine hydroxylase (TH), Bax, and Bcl-2 were measured using immunohistochemistry (IH) by the streptavidin peroxidase conjugated method. Comparison among multiple sets used analysis of variance and LSD method or Kruskal-Wallis test and Nemenyi method.

Results: There were no significant differences between the sham operation groups and the control group; thus, they were merged into Group A. There was no significant glial cell hyperplasia ($P > 0.05$) or change in shape in any of the groups after PX or GDX. The number of apoptotic cells in Groups A (1.41 ± 0.56), PX (12.31 ± 4.68), GDX (20.52 ± 5.13), and PX + GDX (30.23 ± 5.25) successively significantly increased ($P < 0.05$). The number of TH (+) cells in Groups A (42.62 ± 5.63), PX (37.31 ± 4.32), GDX (31.07 ± 4.21), and PX + GDX (25.22 ± 3.66) was successively significantly decreased ($P < 0.05$). The gray value of TH (+) cells and fibers in Groups A (98.51 ± 10.36), PX (108.96 ± 13.01), GDX (119.02 ± 12.98), and PX + GDX (128.99 ± 13.39) was successively significantly increased ($P < 0.05$). The results of Bax staining were as follows: Group A+, Group PX++, Group GDX++, and Group PX+ GDX+++, the results of Bcl-2 in all groups were +.

Conclusions: PX or GDX could lead to OB neurotoxicity in the following groups of rats in the following order: PX < GDX < PX + GDX. PX or GDX increased the ratio of Bax/Bcl-2. The effect of PX and GDX was equal, but both were less than that of PX + GDX. Neurotoxicity as a result of PX or GDX was not related to inflammation.

Key words: Apoptosis; Gonadectomy; Olfactory Bulb; Pinealectomy; Tyrosine Hydroxylase

Introduction

Parkinson's disease (PD) is a neurodegenerative disease caused by dopamine (DA) dysfunction in the brain. Dopamine is a very important neurotransmitter that exists in the substantia nigra striatum system, olfactory bulbs (OBs), marginal systems, and other areas.[1,2] Most (95.5%) patients with PD suffer olfactory disorder and 12% of them experience a decline in their olfactory function as the first symptom. Studies suggest that the olfactory disorder of PD may be associated with abnormalities in the OB DA neurons[3] and may involve apoptosis.[4]

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The cause of PD is not entirely clear, but people believe that it is multifactorial. Data suggest that the prevalence of PD in men is 1.5 times greater than that in women, a reminder that sex hormones may be involved in the pathogenesis of

Address for correspondence: Dr. Yan Li, Department of Neurology, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong 250013, China
E-Mail: liyanzhongxinyiyua@163.com

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PD.[5] Melatonin (MT) may also trigger the incidence of PD.[6] PD incidence increases with age; in contrast, MT and testosterone (T) levels in the body decrease with age.[5,6] Currently, there is insufficient research on the relationship between the levels of MT or T and PD. There is even less research on the effects of the levels of MT or T on the OB. There are no reports of multivariate studies.

MT and T are produced mainly in the pineal gland and testicles. After pinealectomy (PX) or gonadectomy (GDX), the levels of MT or T in the body drop significantly. A simultaneous deficiency of MT and T can simulate the hormone levels in elderly men better. Tyrosine hydroxylase (TH) is the limiting enzyme in the synthesis of DA and is often used as a marker of DA neurons. The aim of this experiment was to detect apoptosis, the variation of TH (+) cells and nerve fibers, glial cell hyperplasia, and expression of Bax and Bcl-2 in OB to elucidate the relationship between the PD olfactory disorder and inadequacy of MT and T.

Methods

Materials

Male Wistar rats (weight: 301–320 g, aged 12 months) were provided by the Experimental Animal Center of Shandong University. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Shandong University. Their environment was kept quiet and they had free access to food and water with exposure to 12 h (8:00–20:00) of light and 12 h (20:00–8:00) of darkness.

Animal model

One hundred and twenty male Wistar rats were completely randomly divided into the following six groups (20 rats in each group): control, sham operation PX, sham operation GDX, PX, GDX, and PX + GDX groups. The controls did not have their pineal gland or testicles removed. The other steps were the same. They were fed postoperatively for 60 days. The animals were sedated with an intraperitoneal injection of 10% chloral hydrate (4 ml/kg). The operation time was 17:00–21:00.

Main reagents

The following reagents were used: *In situ* Cell Apoptosis Kit produced by F. Hoffmann-La Roche AG; TH Primary Antibody by Santa Cruz Biotechnology in the United States; Bax, Bcl-2, PS, DAB Kit Primary Antibody by Beijing Zhong Shan Golden Bridge Biological Technology CO., LTD; Protease K by Sigma-Aldrich in the United States; SP Kit by Beijing Zhong Shan Golden Bridge Biological Technology Co., LTD. There was no any conflict of interest for all reagents.

Sample selection and pathological observation

Sixty days after the operation, the animals were sedated, infused with 4% paraformaldehyde for 2 h, beheaded, and then the OBs were removed. OBs with sizes between 4.6 mm to 1.6 mm were used as samples. The samples were fixed for 6 h before being dehydrated, cleared, soaked in wax, and sliced evenly into 25 slices. Five slices were taken from each group for the following procedures: (1) staining with hematoxylin and eosin (HE) to show the proliferation of glial cells; (2) detection of apoptosis with the Tuning method; (3) detection of the expression of Bax, Bcl-2, and TH using the streptavidin peroxidase method. When collecting the brains, we found that no pineal glands or testicles were incompletely removed. After the operation, there were a total of 6 rats in the control, sham operation PX, and sham operation GDX groups, 3 rats in the PX group, and 4 rats in the GDX and PX + GDX groups with symptoms such as forelimb or hind limb penalization, difficulty in walking, hair loss, lethargy, and difficulty eating before death. Sixteen animals from each group were used for analysis.

Statistical analysis

The number of OB TH (+) and apoptotic cells from the five slices were counted at 10 × 10 or 10 × 20 magnification, recorded, and the mean was calculated. The morphology of the cells was observed at 10 × 40 magnification. The number of glial cells in the three fields of each slice was randomly selected from 5 slices, recorded, and the mean was calculated. The TH (+) neurons and fiber gray values were measured in the OB by the Tangier digital medical image analyzer (Lambert-Beer law: the actual concentration of matter is inversely proportional to the gray value of matter). Slices for immunohistochemistry (IH) observation of Bax, Bcl-2 were semiquantitatively measured with the following system: negative (−), no expression; weakly positive (+), expressed in a number of cells, positive cells accounted for 0–25% of the entire field of vision, lightly stained; positive (++), positive cells accounted for 25–50% of the entire field of vision, medium stained; strong positive (+++). Positive cells accounted for more than 50% of the field of vision, deeply stained. The data are expressed as mean ± standard. Comparison among multiple sets was based on a completely random variance analysis, comparison between two groups was based on the LSD method. If the variance analysis was inconclusive, the Kruskal-Wallis test was adopted and a comparison between the two groups was based on the Nemenyi method. Statistical analyses were performed with the SPSS version19.0 (SPSS Inc., Chicago, IL, USA). The significance level was $P < 0.05$.

Results

There were no significant differences between the sham operation groups and the control group; therefore, they were merged to form Group A. The number of animals and the staining results were recorded and the means of the three groups were used for statistical analysis. There were no significant differences in the detection indexes between the two sides of the areas of the brain; therefore, only one side was taken for analysis.

Hematoxylin and eosin staining results

HE staining showed that the OB was a hierarchical structure, the layers from the outside to the inside were the olfactory nerve layer, glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer, and granular cell layer (GC1). The GL is composed

of many olfactory glomeruli, which are round, consisting of a layer of small neurons and glial cells. Under light microscopy, normal microglia (Mig) appeared long or triangular, the chromatin was evenly distributed, the nucleus had a coloring deep, and there were a few thick and short protrusions. Astrocytes (As) have a broadly rounded stellate shape with a light-colored nucleus and protrusions that appear dendritic. When compared to Group A, there was no significant proliferation of Mig and As ($P > 0.05$) and no significant change in the shapes in the other three groups. There was no significant difference in the number of glial cells among Groups A, PX, GDX, and PX + GDX ($P > 0.05$).

TUNEL method

Features of apoptosis include a pyknotic nucleus that dyes brown with uneven coloring and is typically ring shaped. Apoptosis was occasionally observed in group A with a varied position. Compared to Group A, apoptotic cells were significantly increased in groups PX, GDX, and PX + GDX. They were mainly distributed in the OB GL, with a small amount in the MCL and GC1. The number of apoptotic cells in Groups A, PX, GDX, and PX + GDX was significantly increased in succession (Hc = 274.130, $P < 0.001$; Table 1 and Figure 1a–1d).

Immunohistochemistry staining of tyrosine hydroxylase

The cytoplasm of TH (+) cells stained brown. The nuclei were large, round, and undyed. The nucleolus was small, in the center of nucleus, and cell spindle-or conical shaped with a number of protrusions. Group A TH (+) neurons were mainly distributed in the OB GL, periglomerular cells (PG) surrounded the olfactory glomerulus, and the protrusions were TH (+). TH (+) nerve fibers were long, thin, dense, continuous with prominent branches, and protrusions. There was a small amount of TH (+) cells and fibers in the EPL. Compared to group A, some of the protrusions of TH (+) cells and fiber were ruptured in groups PX, GDX, and PX + GDX. The number of TH (+) cells in groups A, PX, GDX, and PX + GDX were significantly decreased successively (Hc = 228.763, $P < 0.001$; Table 1 and Figure 2a–2d); the TH (+) fiber density in groups A, PX, GDX, PX + GDX was significantly decreased successively (since the gray value of TH (+) cells and fibers in Groups A, PX, GDX, and PX + GDX successively increased significantly) ($F = 88.028$, $P < 0.001$; Table 2 and Figure 2a–2d).

Bax and Bcl-2 immunohistochemical staining

Bax or Bcl-2 immunohistochemical-positive reaction was in the cytoplasm. Bax and Bcl-2 were mainly expressed in the GL with a small amount of staining in the MCL. The results for Bax in Group A were weak positive (+), positive in groups PX and GDX (++), and strong positive in group PX + GDX (+++); all groups Bcl2+.

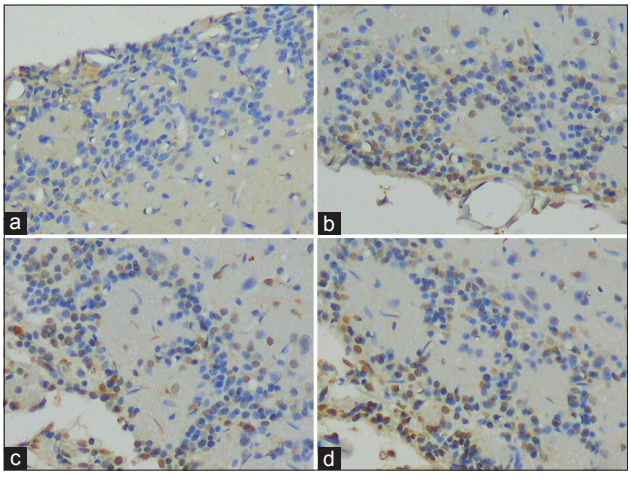

Figure 1: TUNEL method for neuronal apoptosis in OB (original magnification ×200). (a) Group A, (b) Group PX, (c) Group GDX, (d) Group PX + GDX. PX: Pinealectomy; GDX: Gonadectomy; OB: Olfactory bulb.

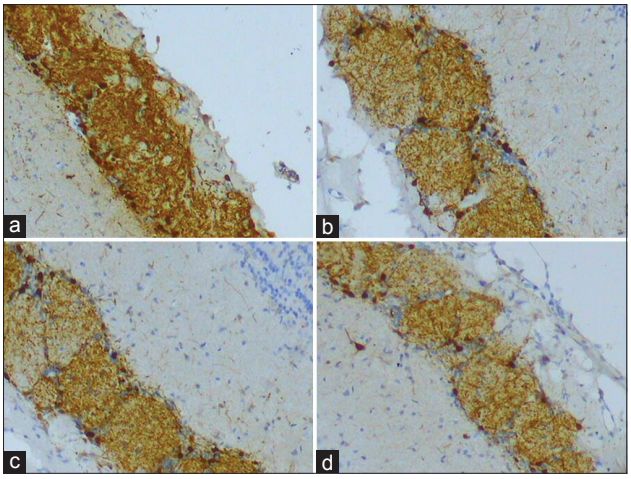

Figure 2: Immunohistochemistry for expression of TH (+) cells and fibers in OB (SP: Original magnification ×100). (a) Group A, (b) Group PX, (c) Group GDX, (d) Group PX + GDX. PX: Pinealectomy; GDX: Gonadectomy; OB: Olfactory bulb; TH: Tyrosine hydroxylase.

Table 1: Comparison of number of apoptosis and TH (+) cells in OB between all the groups (mean ± SD)

Group	Number of animals	TUNEL		Hc	*P*	TH (+) cell		Hc	*P*
		Count	Mean rank			Count	Mean rank		
A	16	1.41 ± 0.56*	40.50	–	–	42.62 ± 5.63†	258.26	–	–
PX	16	12.31 ± 4.68*	130.19	274.130	<0.001	37.31 ± 4.32†	206.06	228.763	<0.001
GDX	16	20.52 ± 5.13*	198.79	–	–	31.07 ± 4.21†	124.72	–	–
PX + GDX	16	30.23 ± 5.25*	272.51	–	–	25.22 ± 3.66†	52.96	–	–

*Comparison between two groups and †Comparison between two groups, all $P<0.05$. TH: Tyrosine hydroxylase; A: Sham operation groups and the control group; PX: Pinealectomy; GDX: Gonadectomy; OB: Olfactory bulb; SD: Standard deviation.

Table 2: Comparison of the gray value of TH (+) cells and fibers in OB between all the groups (mean ± SD)

Group	Number of animals	TH (+) cells and fibers	*F*	*P*
A	16	98.51 ± 10.36*	–	–
PX	16	108.96 ± 13.01*	88.028	<0.001
GDX	16	119.02 ± 12.98*	–	–
PX + GDX	16	128.99 ± 13.39*	–	–

*Comparison between two groups, all $P<0.05$. TH: Tyrosine hydroxylase; A: Sham operation groups and the control group; PX: Pinealectomy; GDX: Gonadectomy; OB: Olfactory bulb; SD: Standard deviation.

Discussion

The olfactory system consists of three parts: olfactory epithelium, OB, and olfactory center. The synaptic layer of the OB (also known as the GL) accepts the information transmitted by the sensory cells of the olfactory epithelium and pre-processes and transmits the information to the olfactory center. The GL consists of a variety of cells, of which the pericytes are a type of intermediate neurons. Approximately 11% of the cells release DA to express TH.[7] Olfactory disorder is the earliest biological marker of PD.[1,2] Its etiology and mechanism are not completely known and are widely believed to be caused by multiple factors. A study by Lazarini *et al*.[8] revealed that smell disorders are associated with pathological changes of the dopaminergic neurons in the OB.

MT secreted by the pineal gland is a type of indole neural endocrine hormone. Androgen is mainly produced by the Leydig cells and has steroid compounds, with T as the main ingredient. Levels of MT and T decrease with age and levels of MT and T in PD patients are even lower.[5,9] However, there are different conclusions regarding the relationship between the levels of MT and T with PD. This study showed that TH (+) cells and fibers are mainly distributed in the GL with a small amount in the MCL and GC1 of OB, which is consistent with previous studies. After PX or GDX, in the OB, TH (+) cells and fibers significantly decreased and apoptosis significantly increased. These findings indicate that a significant decrease in the levels of MT and T can lead to OB neurotoxicity in rats. The followings are possible mechanisms for the reduction of neurotoxicity by MT and T: (1) Anti-free-radical lipid peroxidation: free radicals are scavenged, thus inhibiting the expression of the gene which produces free radical-related enzymes and enhancing the active expression of the gene producing free radical metabolism related enzyme.[10,11] (2) Suppression of the inflammatory cascades activated by free radicals, alpha synuclein (α-SYN), etc.[12,13] (3) Direct and indirect inhibition of α-SYN expression.[14,15] (4) Neurotrophic effects: MT enhances the expression of neurotrophic factor (GDNF) mRNA and protein.[16] T increased the expression of nerve growth factor and expression of nerve regeneration.[17] (5) Regulation of the expression of the Bax/Bcl-2 gene and caspase-3 and inhibition of apoptosis.[18,19] MT and T are secreted mainly by the pineal gland or testicles. Levels of MT and T sharply decreased after PX or GDX. Their neuroprotective effects also decreased and the previously mentioned toxic factors in the brain increased. Brain tissues contain a large amount of polyunsaturated fatty acids and iron ions with catalytic activity. The level of antioxidant enzymes was lower than that of other tissues; therefore, it was susceptible to the toxic nerve factors, and PX or GDX led to OB neurotoxicity.

Studies have shown[20] that apoptosis is a fundamental course in the denaturation of PD dopamine neurons. In the above-mentioned experiment, no apoptosis or occasional apoptosis was observed in Group A, indicating that there is a physiological apoptosis process in the body of a normal rat. In Groups PX, GDX, and PX + GDX, cell apoptosis was observed and the number of apoptotic cells was significantly higher than that of Group A, indicating that PX or GDX could induce a significant increase of apoptosis in the OB, which means that apoptosis is involved in neurotoxicity induced by PX and GDX. This study shows that the apoptotic cells were mainly distributed in the GL of the OB, which is consistent with the distribution of TH (+) cells. The number of apoptotic cells significantly increased successively and the number of TH (+) cells and fibers significantly decreased successively in Groups A, PX, GDX, and PX + GDX, indicating that the increase in cell apoptosis and the decrease of TH (+) cell after PX or GDX are positively correlated. This proves that PX and GDX could cause neurotoxicity of the OB DA with the main pathological process being apoptosis. It also shows that PX and GDX have different causative effects on apoptosis in different groups of cells and OB DA neurons are more susceptible to damage. Studies have shown that the enzymatic degradation of DA neurons and their self-degradation process can produce a large number of free radicals. Levels of free iron are significantly higher than in other brain regions, resulting in an excessive production of hydroxyl radicals. GSH content in DA neurons is relatively low, resulting in poor antioxidant capacity and highly sensitive oxidative stress.[21] It is likely to be related to the OB DA neurons' susceptibility to damage.

Bax is a pro-apoptotic member of the Bcl-2 genes and Bcl-2 is the inhibitor of apoptosis. Bax in neurons is a necessary signal of the beginning of apoptosis. There is no unanimous conclusion about whether the expression of Bax or Bcl-2 in brain of PD patients was altered. Bax/Bcl-2 in group A was +/+, indicating that the apoptosis of cells in normal brain tissues is regulated to a balance through Bax, Bcl-2 regulation; while Bax/Bcl-2 in group PX,

GDX, PX + GDX was ++/+ or +++/+, indicating that PX and GDX could increase the expression of Bax, which is positively correlated with the increase in the number of apoptotic cells in the three groups. This suggests that PX and GDX can cause a Bax/Bcl-2 imbalance to accelerate cell apoptosis. In group PX and GDX, Bax/Bcl-2 are both ++, and GDX induced apoptosis more than PX, indicating that there are other ways to regulate cell apoptosis in OB. The synergistic effect is stronger than the individual effect, which is associated with the superposition of Bax upregulation. It has been found that inflammatory actions may be involved in the process of progressive degeneration of DA neurons in PD.[22] HE staining showed that there was no significant difference in the number of glial cells in Groups A, PX, GDX, and PX + GDX, indicating that PX or GDX did not lead to an obvious glial cell reaction. This suggests that the mechanism of OB neurotoxicity induced by PX and GDX had some differences from the pathogenesis of PD.

In groups A, PX, GDX, PX+GDX, the number of apoptotic cells of OB significantly increased successively and the number of TH (+) cells and fibres of OB significantly decreased successively. This indicates that in the OB, a lack of T had stronger neurotoxicity than the lack of MT, neurotoxicity by the lack of MT and T in the joint is stronger. It was unknown why GDX induced a stronger neurotoxicity than that of PX. Since the mechanism of neurotoxicity inhibition by MT and T involves many aspects, there are both similarities and differences, but no experiments have compared the ability to inhibit neurotoxicity between MT and T. Analysis of why PX+GDX caused the strongest neurotoxicity shows that it was related to weakened inhibition of neurotoxicity at the same time by MT and T.

We found that PX or GDX could lead to OB neurotoxicity and compared the effects of PX, GDX, and PX + GDX on OB neurotoxicity, the specific mechanism of which was also investigated, such as bcl-2, bax, and inflammatory reaction. However, other effects of this pathway have not been studied deeply, such as the influence on free radical oxidation.

In this study, we demonstrated the association of the alone or both decrease of MT and T with OB neurotoxicity. The results of our study supported that the inadequacy of MT or T after PX or GDX was associated with pathological changes in the OB, the effect of GDX was stronger than that of PX, but both were less than that of PX + GDX.

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Conflicts of interest

There are no conflicts of interest.

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