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Inhibition of Monoamine Oxidase B by Cigarette Smoke Constituents

Heung-Bin Lim, Hyung-Ok Sohn, Young-Gu Lee, Ja-Young Moon, Young-Kook Kang[1], Yong-Ha Kim, Un-Chul Lee and Dong-Wook Lee*

Korea Ginseng and Tobacco Research Institute, 302 Shinsung-dong, Yusung-ku, Taejon 305-345, [1]Department of Biology, Taejon University, Taejon 300-716, Korea

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ABSTRACT : Cigarette smoking is known to suppress both 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism and idiopathic Parkinson's disease (PD). However, the precise mechanism underlying its protective action against PD is not clearly elucidated yet. In order to find possible clue on the mechanism of protective action of smoking, we investigated the inhibitory effect of cigarette smoke components on rat brain mitochondrial monoamine oxidase B (MAO-B), responsible enzyme for the activation of MPTP to its toxic metabolites, and identified the components having an inhibitory potency on this enzyme from cigarette smoke. Total 31 eligible constituents including nicotine were selected from cigarette smoke condensates via solvents partitioning and silica gel chromatographic separation, and inhibitory potencies of 19 components on MAO-B were determined. Hydroquinone and methylcatechol, the phenolic components, showed the strongest inhibitory potencies on MAO-B activity in the components tested. 3,4-Dihydroxybenzylamine, myosmine and indole in basic fracton, eugenol in phenolic fraction, and farnesol in neutral fraction also inhibited the enzyme activity dose-dependently. Among tobacco alkaloids tested only myosmine was effective for the inhibition of this enzyme. These results suggest that the decrease in MAO-B activity by such components derived from cigarette smoke seems to be related to the suppression of MPTP-induced neurotoxicity and to the less incidence of Parkinson's disease in smokers than in nonsmokers.

Key words : **cigarette smoke, Parkinson's disease, MAO-B, MPTP, neurotoxicity**

Idiopathic Parkinson's disease (PD) is a typical neurodegenerative disease associated with a massive degeneration of dopaminergic nigrostriatal neurons. When approximately 60 to 80 percent of the dopamine producing neurons of substantia nigra are lost, the extrapyramidal system is no longer able to effectively promote movement and the symptoms of PD appear. This neuronal disease shows the clinical features of resting tremor, slowness of movement, rigidity, and postural instability. Unfortunately, etiology of the disease is not clearly elucidated and effective medicines for the treatment of patients with PD are limited yet. Interestingly, PD has been reported to occur more commonly in non-smokers than in cigarette smokers (Shahi *et al.*, 1991; Grandinetti *et al.*, 1994; Heller *et al.*, 1995). It has been observed that prevalence of PD in the smokers is in an inverse proportion to not only smoking duration but also the number of cigarette smoked daily (Grandinetti *et al.*, 1994). This negative association between smoking and PD has long been interested and led many investigators to suggest that some facet of cigarette smoking exerts a neuroprotective influence. Eluci-

* Corresponding author : *Korea Ginseng and Tobacco Research Institute. 302 Shinsung-Dong. Yusung-Ku, Taejon 305-345, Korea*

dation of the possible association of smoking with apparent protection on development of PD may contribute to understanding of the pathogenetic mechanism of this disease.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin, causes a syndrome of dopamine depletion in animal and human. Because this syndrome is very similar to that of idiopathic PD, it has been proposed that some environmental neurotoxins may be responsible for the incidence of PD (Langston, 1987). MPTP itself is not neurotoxic, but is converted to one or more toxic metabolites by mitochondrial monoamine oxidase B (MAO-B). Since MAO-B inhibitors such as deprenyl are capable of antagonizing the degenerative process induced by neurotoxic mechanism, it has been suggested that administration of these drugs during an early phase of parkinsonian symptom may slow down the progression of motor deficits and block the neurotoxic effect of MPTP (Poli *et al.*, 1990; The Parkinson Study Group, 1990). MAO-B is, therefore, considered to play at least as a part in the pathophysiology of PD.

Previous studies have shown that monoamine oxidase activity was found to be reduced by a solution of cigarette smoke and relatively lowered in platelets from smokers (Yu and Boulton, 1987; Boulton *et al.*, 1988). Recently, Fowler and his colleagues (Fowler *et al.*, 1996) demonstrated that MAO-B activity is also significantly low in the brains of smokers than in nonsmokers. In previous study, we demonstrated that cigarette smoke exposure suppresses MPTP-induced neurotoxicity and inhibits the activity of MAO-B in mouse brain (Lim *et al.*, 1996; Moon *et al.*, 1997). All these results from the current studies suggest that decrease in the activity of this enzyme may be related to the protective action of cigarette smoking on PD.

We hypothesized that the protective effect of smoking against PD development would probably be involved in the inhibition of MAO-B activity and that some components derived from cigarette smoke may function or act as inhibitors. Cigarette smoke contains numerous components more than 3,800 chemical components, including nicotine, carbon monoxide, and others. Except for nicotine, however, the biological or neuropharmacological implications of these agents were not well described so far. Therefore, in this study, we investigated the inhibitory potency of 4 fractions of cigarette smoke condensates fractionated by pH control and by solvent partitioning methods on MAO-B activity, identified smoke components having a potential for the inhibition of the enzyme from the four fractions, and evaluated their inhibitory potencies using authentic compounds.

MATERIALS AND METHODS

Chemicals. Chemicals such as nicotine, cotinine, epinephrine, norepinephrine and thiocyanate were obtained from Sigma Chemicals Co (St Louis, MO, USA). Hydroquinone, indol, farnesol, and vanillin were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Other chemicals used in this experiment were of reagent grade quality.

MAO-B assay. Brain mitochondrial MAO-B of rat was used for the screening of inhibitory potency of the components identified from cigarette smoke condensates (CSC). The enzyme activity was assayed by the method of Kalaria *et al.*(1987). A suitable amount of smoke components ranging from 25 to 100 ug per ml was preincubated with the reaction mixture for 5 min at 37 °C prior to start the reaction. Data were expressed as mean of three repeated assays.

Total MAO assay. Total activity of monoamine oxidase (MAO-T) was fluorometrically assayed in mitochondrial fractions of rat brain based on the direct measurement (Morinan and Garratt, 1985) of the intensity of 4-hydroxyquinoline produced using kynuramine as a substrate with some modifications (Lim *et al.* 1996). A suitable amount of smoke components ranging from 25 to 100 ug per ml was preincubated with the reaction mixture for 5 min at 37°C prior to start the reaction. Data were expressed as mean of three repeated assays.

Isolation of cigarette smoke components. To identify components which have an inhibitory potency against MAO-B activity, we selected candidate components from CSC using the following procedures. Smokes from 1,500 filter cigarettes were

collected on cambridge filter pad using a smoking machine (Heiner Borgwaldt, Germany). CSC was prepared by extracting the filter pads with methanol and was further fractionated to acidic, basic, neutral, and phenolic fractions by pH control of the extract and by solvent partitioning (Murase *et al.*, 1994). The neutral fraction (3.76 g) was applied onto a silica gel column (4.5 x 25 cm) and eluted by solvents with a polarity gradient described in figure legends. Fractions containing a potential for MAO-B inhibition were pooled after the screening of their inhibitory potency and components in the active fractions were further fractionated via the 2nd silica gel chromatography (1.5 x 11 cm) with different mobile phases. Then the components in the active fractions were identified by GC-MS spectroscopy.

Components in acidic, basic, and phenolic fractions were also further fractionated using a similar manner to that conducted for the neutral fraction with different eluents and candidate components were also identified by GC-MS. Inhibitory potencies of all the components identified on MAO-B were determined using their synthetic compounds obtained from commercial sources.

RESULTS

CSC is a complex mixture containing numerous chemical components. Therefore, it was fractionated to four bulk groups including acidic, basic, neutral, and phenolic fractions (Fig. 1). Table 1 shows the fractionation yield of CSC and their inhibitory potencies on MAO-B activity. Neutral fraction was the highest in yield, but basic and phenolic fractons were relatively low, in the four fractions. All the four fractions had the inhibitory potencies on MAO-B activity *in vitro*, that is, when preincubated in the presence of CSC fractions, the activity of rat brain MAO-B was remarkably reduced. The inhibitory potency was the strongest in basic fractions and the following order was neutral>acidic>

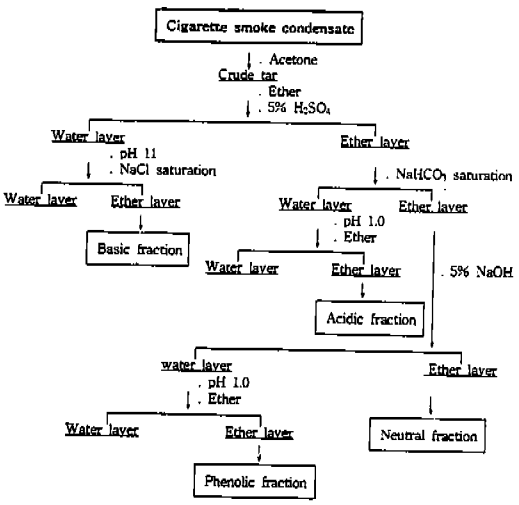

Fig. 1. The flow chart for the fractionation of CSC

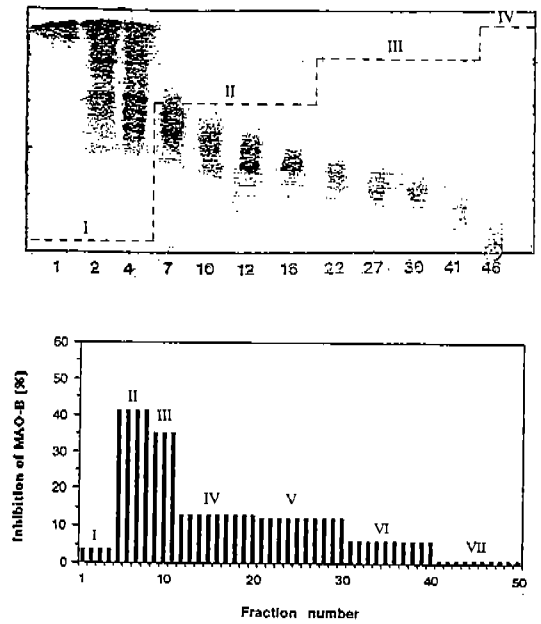

Fig. 2. Thin Layer Chromatography of the neutral fraction of CSC separated from silica gel column chromatography. Sample fraction was adsorbed to the silica gel in the presence of ethylacetate/hexane (1:5) and was dried before loading to the column. Sample-adsorbed silica gel was loaded on the column (4.5x25cm) prepacked with silica gel in the presence of ethylacetate/hexane (1:5). Four kinds of solvent systems, I. ethylacetate/hexane (1:5), II. ethylacetate/hexane (1:2), III. ethylacetate/hexane (1:1), and IV. ethylacetate, were eluted by discontinuous polarity gradient. TLC spots of fractions (upper) and their inhibitory potency on MAO-B (bottom) were expressed.

Table 1. Inhibitory potency of smoke components in each fraction of CSC on MAO-B

Fractions	Yield(g)	Inbibitory potency(%)
Acidic	1.17	29.8
Basic	0.63	77.3
Neutral	3.76	42.6
Phenolic	0.66	23.5

Amount of each fraction was received from smoke generated from 1,500 filter cigarettes by automatic smoking machine. The smoke components(25ug/ml) were preincubated with the reaction mixture for 5 min at 37℃.

phenolic fraction.

We further fractionated smoke components from each of the four fractions of CSC through silica gel chromatography with various mobile phases. In this paper, only a fractionation profile for the neutral fraction was described in detail (Figs. 2 and 3). Fig. 2 shows thin layer chromatography (TLC) pattern of fractions obtained from the 1st chromatography of the neutral fraction and their inhibitory potencies on MAO-B activity. It shows that, when compared with others, components corresponding to peak II (fraction numbers 5-8) and peak III (fraction numbers 9-11) which were eluted in ethylacetate/hexane (1:2) system had relatively high

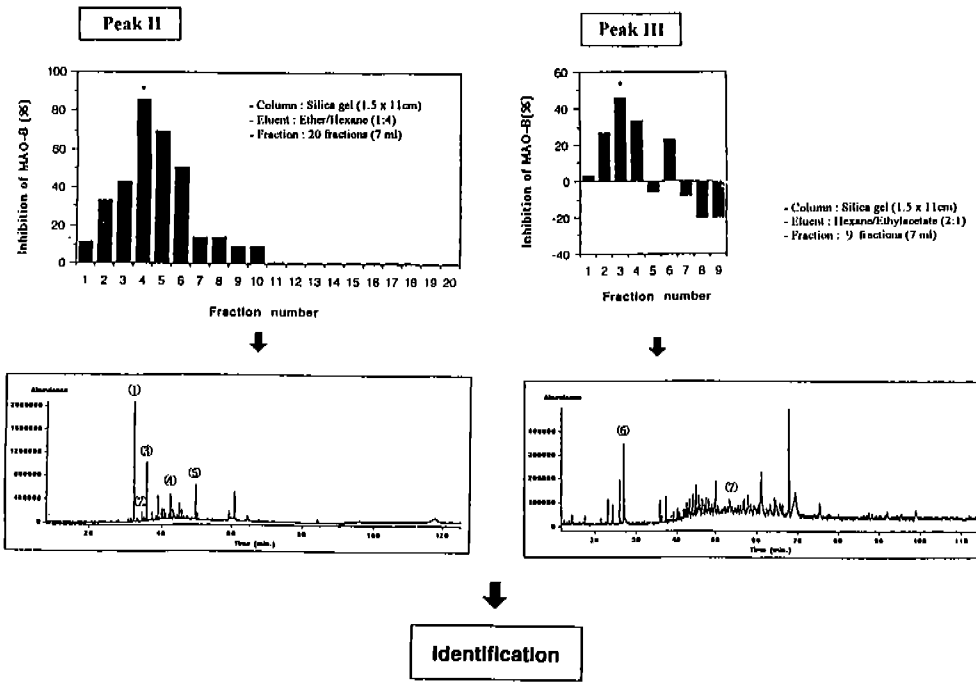

Fig. 3. The inhibitory potencies on MAO-B activity of the new fractions separated from peaks II and III using the 2nd silica gel column chromatography and total ion current (TIC) profiles of components in the peak fractions (asterisked) having the strongest inhibitory potency. Peaks II and III were obtained from the 1st silica gel column chromatography of neutral fraction of CSC. Candidate components were identified from these profiles by library of the chemicals. Peaks ①; Solanone, ②; β-Damascenone, ③; Geranyl acetone II, ④; 6,10,14-Trimethyl-2-pentadecanone, ⑤; Farnesol, ⑥; 2,3-Butanediol, ⑦; Caryophyllene.

inhibitory potentials against MAO-B activity. Therefore, each of the fractions corresponding to peaks II and III was applied to the 2nd silica gel column chromatography to further fractionate more specific components using more polar solvent systems.

Fig. 3 shows the inhibitory potencies on MAO-B activity of the new fractions separated from peaks II and III (Fig. 2) using the 2nd silica gel column chromatography and total ion current (TIC) profiles of components in the peak fractions (asterisked) having the strongest inhibitory potency. We finally identified 6 components including 2,3-butanediol, farnesol, and solanone as candidate components for MAO-B inhibitor from GC/MS analysis. Based on GC-MS analysis of other CSC fractions, we also selected 3 components from acidic, 9 from basic, and 13 from phenolic fraction, respectively (Tables 2 and 3).

Table 2 shows the inhibitory potency of the components isolated from neutral and acidic fractions of CSC on MAO-B activity. In the neutral fraction, only farnesol among the components tested greatly suppressed MAO-B activity. 2,3-Butanediol, β-damascenone, and solanone were not shown any inhibitory potencies on the enzyme activity at all. In acidic fraction, dibutylphthalate and diethylphthalate had the weak inhibitory potencies on MAO-B activity. Unfortunately, 1,2-benzenedicarboxylic acid was not tested for the inhibitory potency on this enzyme.

In basic fraction, 3,4-dihydroxybenzylamine was the strongest inhibitor of the MAO-B activity and indole and myosmine were also effective for the enzyme inhibition, whereas cotinine and nicotine known as representative alkaloids in cigarette smoke were not shown any inhibitory potencies at all (Table 3). In phenolic fraction, three components, eugenol, hydroquinone, and methylcatechol, had strong inhibitory effects on MAO-B activity. Especially both hydroquinone and methylcatechol inhibited MAO-B activity completely at the concentrations of 25, 50, and 100 ug/ml. On the contrary, acetovanillon, phenol, and vanillin had weak or no inhibitory effect on the MAO-B activity. As shown in Tables 2 and 3, the order of the CSC components tested in inhibitory potency on MAO-B activity at the concentration of 25 ug/ml was hydroquinone>methylcatechol>3,4-dihydroxy-benzylamine>farnesol>eugenol>indole>myosmine.

To verify the inhibitory effects of these compo-

Table 2. Inhibitory potency of smoke components selected from neutral and acidic fractions of CSC on MAO-B activity in rat brain

Components identified from CSC	Inhibition of MAO-B activity (%)		
	25	50	100*
Neutral fraction			
2,3-Butanediol	0	0	0
Caryophyllene	-	-	-
β-Damascenone	0	0	0
Farnesol	85	93	95
Geranyl acetone II	-	-	-
Solanone	0	0	0
6,10,14-Trimethyl-2-pentadecanone	-	-	-
Acidic fraction			
1,2-Benzenedicarboxylic acid	-	-	-
Dibutylphthalate	0	16	17
Diethyphthalate	7	9	16

* Applied amounts (ug/ml), - ; Not tested

Table 3. Inhibitory potency of smoke components selected from basic and phenolic fractions of CSC on MAO-Bactivity in rat brain.

Components identified from CSC	Inhibition of MAO-B activity (%)		
	25	50	100*
Basic fraction			
Cotinine	0	0	0
3,4-Dihydroxybenzylamine	94	97	98
2,3-Dipyridyl	0	0	0
Indole	49	70	84
4-Methylpentanamide	-	-	-
Nicotine	0	0	0
Myosmine	36	80	94
Guaiacycloacetone	-	-	-
Triacetine	0	3	6
Phenolic fraction			
Acetovanillon	0	0	0
Cyclotene	-	-	-
Dimethylphenol	-	-	-
Eugenol	62	81	98
Hydroquinone	100	100	100
Methylcatechol	98	98	98
5-Methyl-2-(1-methylethyl) phenol	-	-	-
Phenol	6	17	35
Phenylphenol	-	-	-
Pyrocatechol	-	-	-
Resorcinol	-	-	-
Scopoletin	-	-	-
Vanillin	11	16	21

* Applied amounts (ug/ml), - ; Not tested

Table 4. Inhibitory potency of smoke components selected from CSC on total MAO activity in rat brain

Smoke components	Inhibition of Total MAO activity (%)		
	25	50	100*
3,4-Dihydroxybenzylamine	14	25	41
Eugenol	19	31	47
Farnesol	64	72	78
Hydroquinone	27	42	58
Indole	60	71	82
Methylcatechol	17	30	63
Myosmine	16	18	27

* Applied amounts (ug/ml), - ; Not tested

nents on MAO-B activity, we tested the effects of these components on total activity of monoamine oxidase using kynuramine as a substrate (Table 4). Even though showing relatively lower inhibitory potencies on total MAO activity than those on MAO-B activity, these components also revealed to have similar inhibitory effects on MAO-T activity (Table 4). Among the components having the strong inhibitory potencies on MAO-B activity, farnesol and indole also showed relatively strong inhibitory potencies on the MAO-T activity at the three concentrations tested, and others showed relatively weak inhibition. The order of these components in inhibitory potency on MAO-T activity shows being different from those in MAO-B inhibition. These results indicate that hydroquinone, methylcatechol, 3,4-dihydroxybenzy-lamine, and eugenol are specific inhibitors for MAO-B enzyme. These results also suggest that farnesol and indole are possible inhibitors for both MAO-A and MAO-B enzymes.

DISCUSSION

The repeated finding of apparent protective effects of cigarette smoking on the risk of PD is one of the few consistent results in the epidemiology of that disorder (Shahi *et al.*, 1991; Grandinetti *et al.*, 1994; Heller *et al.*, 1995). There is a consistent opinion that environmental factors rather than genetic ones are the more important determinants of risk of PD (Calne and Langston, 1983). In contrast to these possible causes, could cigarette smoking be an environmental factor that protects against PD? We demonstrated that it could be possible in this study.

Several attempts have been made to find possible mechanism underlying such a protective effect of cigarette smoking on PD (Yu and Boulton, 1987; Yong and Perry, 1986; Carr and Rowell, 1990; Lim *et al.*, 1996). Nicotine is a representative component in tobacco alkaloids, increases the firing of dopaminergic neurons and causes the increase in striatal dopamine release (Clarke *et al.*, 1985). Thus, nicotine and other tobacco alkaloids have been assumed preferentially as effective components on MAO-B activity. However, as shown in our experiment, tobacco alkaloids tested except myosmine did not give any effect against this enzyme activity, which was in accord with that of previous report (Sershen *et al.*, 1988). These results indicate that nicotine is not the responsible component of cigarette smoke for MAO-B inhibition.

As second possibility for MAO-B inhibition by cigarette smoking, components known to be increased in the smokers body after smoking were considered. Both smoking itself and nicotine administration are known to increase concentrations of thiocyanate, norepinephrine and epinephrine in blood and tissues (Benowitz, 1995). So we tested the inhibitory potential of these components against MAO-B activity in rat brain. Even though thiocyanate did not show any inhibitory potency on MAO-B activity, epinephrine and norepinephrine strongly inhibited the enzyme activity *in vitro* (Data not shown). It is not surprising phenomenon because such catecholamines can react with the enzyme competitively during the assay as a substrate. However, further study is necessary whether the inhibition of MAO-B activity is caused by the increased level of catecholamines in the brain of smokers from the repeated smoking.

One interesting finding regarding to MAO-B inhibition by cigarette smoking has been made by Yong and Perry (1986) who suggested that some metabolites of hydrazine present in cigarette smoke may inhibit the activity of platelets MAO. Because cigarette smoking could enhance the metabolism and elimination of xenobiotics, metabolites of some smoke components by the enzymes responsible for their bioactivation may inhibit the MAO-B activity (Gresham *et al.*, 1993). In this study, we have tested only cotinine out of nicotine metabolites, but it was not effective on MAO-B activity. All of the results obtained by current studies indicate that the major smoke components or the components increased *in vivo* after smoking are not responsible for MAO-B inhibition.

The significance of the present study suggests the possibility that some unknown minor components of cigarette smoke may block the metabolic processes of neurotoxins such as MPTP. Among the candidate components selected from CSC, hydroquinone, methylcatechol, 3,4-dihydroxybenzy-lamine, farnesol, eugenol, and indole showed high potentials for MAO-B inhibition. Another interesting

finding is that these are not major components of cigarette smoke and contained hydroxyl groups in their molecular structures. Results of our experiment imply that some phenolic compounds in cigarette smoke have a high potential for MAO-B inhibition, even though they have different chemical structures from MAO-B inhibitors well known such as deprenyl. In order to reduce MPTP neurotoxicity in rodents, MAO-B activity should be inhibited more than 40% of the control activity (Jossan *et al.*, 1987). Therefore, the inhibition of MAO-B activity by such multiple components might have an important role for the reduction of endogenous or environmental neurotoxins.

Since phenolic compounds are known to have antioxidant activity, in general, such compounds might be contributed to scavenging free radicals as well as MAO-B inhibition. In regarding to this hypothesis, Calne and Langston (1983) have proposed that carbon monoxide could create an environment that protects the substantia nigra from oxidative damage. It is, however, far from the inhibition of MAO-B activity although it might be a possible explanation for low incidence of PD in smokers. Because the formation of free radicals from MPTP occurs after its activation to toxic by MAO-B.

Our results suggest that the inhibition of MAO-B by such smoke components seems to be related to the suppression of MPTP-induced parkinsonism and less incidence of PD in smokers than in non-smokers. We also suppose that other smoke compounds unidentified yet may have more potential to inhibit the activity of brain MAO-B. Therefore, the identification of other smoke components for MAO-B inhibition and whether MAO-B inhibitable components identified already in cigarette smoke can penetrate into the blood brain barrier remain for further study.

Finally, data discussed here, however, do not suggest that cigarette smoking could be used to prevent against or to treat parkinsonism. These data may be helpful, in elucidating the etiology and pathophysiology of PD and in suggesting the effective measures for the prevention and therapeutics of the patients with PD because effective drugs for the treatment of PD did not yet develop.

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