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Effect of Cigarette Smoke Exposure on MPTP-Induced Neurotoxicity in Mice

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흡연이 MPTP에 의해 유발되는 신경독성에 미치는 영향

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ABSTRACT : Effect of cigarette smoke exposure on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity was investigated in C57BL6 mice. Cigarette smoke exposure of mice to the mainstream smoke generated from 15 cigarettes for 10 mins per day, 5 days per week, for 6 weeks, effectively attenuated the decline both in the level of striatal dopamine and the number of tyrosine hydroxylase-positive cells in the brain caused by MPTP treatment. Exposure to cigarette smoke significantly decreased monoamine oxidase B activity in the cerebral cortex and cerebellum. The activity of brain antioxidant enzymes such as catalase, glutathione peroxidase, and Cu, Zn-superoxide dismutase, was not changed by cigarette smoke exposure or MPTP treatment. Sulfhydryl compounds content in all brain regions except for the striatum was uniquely increased by MPTP treatment, however, such an effect of MPTP was not observed in mice exposed to cigarette smoke. These results suggest that cigarette smoke exposure inhibits MPTP-induced neurotoxicity without influencing free radical metabolism in the brain of mice. This protective effect of cigarette smoke seems to be closely related with the decreased activity of brain monoamine oxidase B.

Key words : cigarette smoke exposure, dopamine, monoamine oxidase B, antioxidant enzymes, MPTP.

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Parkinson's disease (PD) is a typical neuronal disease rarely detected before the age of 40. The major pathological and neurochemical features of PD are destruction of dopamine producing nerve cells, however, the cause for these deficits is unknown. Recently, glial cell-derived neurotrophic factor has been proposed as a potential treatment for parkinsonism based on its effects on dopaminergic neurons of rodents and monkeys (Tomac *et al.*, 1995; Gash *et al.*, 1996), but little is known about its action in humans. Interestingly, epidemiologic studies have repeatedly shown an inverse association between cigarette smoking and idiopathic PD (Grandinetti *et al.*, 1994; Heller *et al.*, 1995). However, the possible mechanism underlying the positive aspect of cigarette smoking on PD remains unclear.

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin, has been known to destroy the striatal dopaminergic cells selectively and to induce a very similar symptom of PD clinically and pathologically in animals and humans (Snyder and D'Amato 1986; Maret *et al.*, 1990). The discovery that MPTP is a specific toxin to the nigrostriatal neurons has provided a conventional experimental model for the PD research.

Several investigators have attempted to evaluate smoking effect on MPTP-induced neurotoxicity. However, the effect of cigarette smoke exposure on the neurotoxicity in rodents has been controversal. Perry and his coworkers (1987) reported that exposure of mice to cigarette smoke for 12 days had no effect on MPTP-induced neurotoxicity, while Carr and Rowell (1990) claimed that it retarded the neurotoxicity. Chronic nicotine treatment also showed inconsistent results (Sershen *et al.*, 1988; Fung *et al.*, 1991; Bahmand and Harik, 1992). The reasons of these disagreement may be related to differences in the experimental conditions conducted because MPTP is a protoxin metabolically activated by monoamine oxidase B (MAO-B) before it excerts a neurotoxic action.

Differences *in vivo* antioxidant status of animals used also can be considered as another possible reason for the disagreement of smoking effect on MPTP neurotoxicity. Because toxic metabolite formed from MPTP by MAO-B blocks complex I of the respiratory chain within mitochondria of dopaminergic cells, which leads to the generation of free radicals and disruption of energy metabolism in the brain (Chiba *et al.*, 1984). These facts imply that neurotoxic potency of MPTP is clearly dependent on the ability of host for the activation of MPTP and their antioxidant capacity. Cigarette smoke exposure may affect these biochemical parameters.

Therefore, in this study, we investigated the effect of long-term exposure of cigarette smoke on MPTP-induced neurotoxicity in mouse brain and its possible protective mechanism by evaluating the changes in the activity of MAO-B and antioxidant enzymes.

MATERIALS AND METHODS

Animals and their treatment

Male C57Bl6 mice weighing 20-22g were used in this study. The mice were divided into four groups; control, MPTP-treated (MPTP), cigarette smoke exposed (CS), and MPTP-treated and cigarette smoke exposed mice (CS+MPTP). Twelve mice were housed in each cage in a temperature (20 ± 2 ℃) and light-controlled facility room (12/12 hrs, light/dark cycle) with free access to food and water. Mice of CS and CS+MPTP groups were exposed to mainstream of cigarette smoke generated from 15 filter cigarettes (tar and nicotine content, 11 mg and 1.1 mg/cigarette) for 10 mins a day, 5 days per week, for 6 weeks using an exposure chamber (D

37 cm x H 13 cm). Smoke exposure was continued until sacrifice. Mice of MPTP and CS+MPTP groups were injected subcutaneously with MPTP (10 mg/kg body weight) once a day for 6 consecutive days from the 4th week to induce neurotoxicity. Ten days after the final treatment, 8 animals were sacrificed by decapitation. Brains were removed quickly and dissected to cerebral cortex, striatum, hippocampus, hypothalamus, and cerebellum on an ice bath. Tissues from each brain region were frozen immediately in liquid nitrogen. The remaining mice were used for the immunohistochemical analysis of tyrosine hydroxylase-positive cells.

Immunohistochemistry for tyrosine hydroxylase-positive cells

Tyrosine hydroxylase (TH) positive cells in the substantia nigra of brain were counted by microscopic observation after immunohistochemical staining (Specht *et al.*, 1981). Briefly, animals sacrificed were perfused transcardially with a syringe containing 1% paraformaldehyde (60 ml/100g body weight) followed by 4% paraformaldehde (70 ml /100g body weight). Brains were removed, stored in 4% paraformaldehyde overnight and then cut as 40 ㎛ free-floating horizontal sections. Prior to overnight incubation with primary antibody (rabbit antisera to TH : Boehringer Mannheim ; 1:500), the sections were prewashed in 0.2% Triton X-100 for 15 mins followed by 4% goat serum. After 24 hrs incubation with primary antiserum, the sections were incubated with the secondary biotinylated antiserum (1 : 600 dilution) for 1 hr. Sections were washed 3 times with PBS (pH 7.4) between each incubation step. 3,3-Diaminobenzidine was used as a chromogen. Cell population in the region of substantia nigra was counted in 3 or 4 slices per animal.

Determination of striatal levels of dopamine and homovanillic acid

The striata were homogenized in 20 times volume of 0.1N perchloric acid containing 0.1 mM sodium metabisulfite at 4℃. The resulting supernatant from the striatal homogenates after centrifugation at 22,000 x g for 15 min was analyzed for dopamine (DA) and its metabolites, homovanillic acid (HVA) by reverse-phase HPLC (Lichrospher 100 RP-18.5 ㎛, 250 x 4 mm) using an electrochemical detector (ESA, Coulochem II). The mobile phase was 0.15 M sodium phosphate (pH 3.4) containing 0.1 mM EDTA and 0.5 mM sodium octane sulfonic acid and the flow rate was 1.0 ml per min.

Determination of monoamine oxidase activity

Total activity of mitochondrial monoamine oxidase (MAO-T) was measured in each brain region by fluorometric assay (Morinan and Garratt, 1985). Namely, mitochondrial fractions (100 ㎍ protein) were incubated for 5 mins with 870 ㎕ of 10 mM potassium phosphate buffer (pH 7.2). The reaction was initiated by the addition of 30 ㎕ of 3.0 mM kynuramine to the reaction mixture, which was shaken for 15 mins before it was terminated by the addition of 300 ㎕ of 0.4 M perchloric acid. The tubes were then capped, and centrifuged at 11,600 g for 15 secs to remove precipitated proteins. An 1.0 ml aliquot of the supernatant was transferred to a test tube containing 2.0 ml of 1.0 M sodium hydroxide. After mixing, the 4-hydroxyquinoline fluorescence was measured at an excitation wavelength of 315 nm and an emission wavelength of 380 nm in a Perkin-Elmer fluorospectrophotometer. MAO-B activity was measured by colorimetric determination of hydrogen peroxide using benzylamine as a substrate (Kalaria *et al.*, 1987). The reaction mixture (0.7 ml) contained 100 mM sodium phosphate buffer (pH 7.4), 3 mM

sodium azide, 100 μg of mitochondrial proteins, and 1 mM benzylamine. The reaction mixture was incubated at 37 ℃ for 30 min, and the reaction was stopped by the addition of 0.5 ml of the hydrogen peroxide-measuring solution which contained 0.5 mM phosphate/citrate buffer (pH 4.0), 1.8 mM 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), and 5 units of horseradish peroxidase. After 15 secs, 0.25 ml of 0.75 M hydrochloric acid containing 5% sodium dodesylsulfate was added, and the colored product was measured at 414 nm.

Assay of antioxidant enzymes and thiol compounds

Levels of antioxidant enzymes and thiol compounds were determined in the cytosol obtained from the homogenate of each dissected brain region. The activity of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) was measured by the method of McCord *et al.* (1972) which monitors the inhibition of cytochrome c oxidation at 550 nm by using xanthine oxidase to generate superoxide. Catalase activity was assayed based on the direct measurement of decomposition of hydrogen peroxide at 240 nm spectrophotometically (Abei, 1983). The activitiy of glutathione peroxidase and the content of thiol compounds were determined according to the method of Flohe and Gunzler (1984), and Sedlak and Lindsay (1968), respectively.

Statistics

Statistical analysis of results was performed using Students t test.

RESULTS

Effects of MPTP treatment and cigarette smoke exposure on dopaminergic neurons

During the experimental period, mice did not show any abnormal sign in food intake and body weight gain by the exposure to cigarette smoke or MPTP treatment. Under the smoking conditions, blood carboxyhemoglobin content of the mice immediately after exposure was 15 ± 1.2% when assayed by the method of Watson *et al.* (1987).

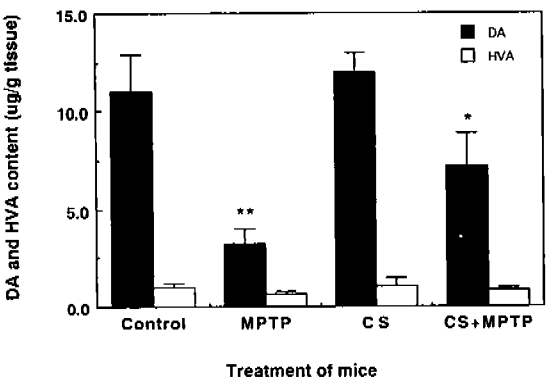

Fig. 1. Effect of cigarette smoke exposure on changes of striatal dopamine (DA) and homovanillic acid (HVA) caused by MPTP treatment in mouse brain. Data are expressed as mean±SD of eight animals per group. *Significantly different from the MPTP group ($P<0.05$). **Significantly different from the control ($P<0.01$).

Fig. 1 shows the effect of MPTP treatment and/or cigarette smoke exposure on the striatal DA level in mouse brains. The striatal DA level decreased significantly from 12.9 ± 2.1 to 3.9 ± 2.2 μg per g tissue after administration of MPTP ($P<0.01$). However, DA decrease by MPTP treatment was effectively attenuated by cigarette smoke exposure as shown in Fig. 1 ($P<0.05$). HVA levels were also slightly decreased by treatment with MPTP and it was retarded by exposure to tobacco smoke. However, there was no significant difference in HVA level between tobacco smoke exposed groups and non-exposed ones.

To determine whether exposure of cigarette smoke affected the activity of DA synthesis, the change in the number of TH-positive cells in the substantia nigra of brain, a rate limiting enzyme for the synthesis of DA, was investigated. As shown in Fig. 2, the number of TH-positive cells decreased significantly after MPTP administration ($P<0.05$). The decrease in the cell number was attenuated by cigarette smoke exposure although tobacco smoke exposure alone did not affect on the cell population.

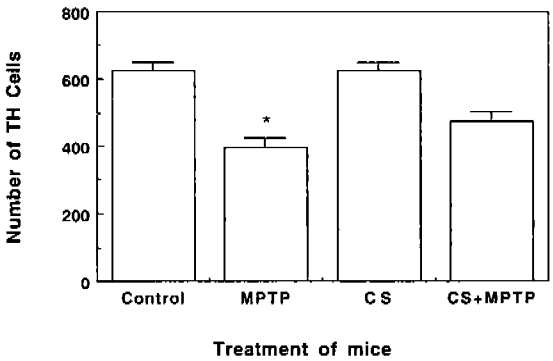

Fig. 2. Change in the number of tyrosine hydroxylase (TH) positive cells in the brain of mice by MPTP treatment and/or cigarette smoke exposure. TH-positive cells were counted by microscopic observation after immunohistochemical staining. Data are expressed as mean ±SD of 3-4 animals per group. *Signifcantly different from control ($P<0.05$)

Fig. 3 shows the change in the activity of MAO-T and MAO-B in various regions of the brain after MPTP treatment and/or exposure to cigarette smoke. The activity of the both enzymes was the highest in the cerebral cortex and the lowest in the hypothalamus. Two major regions, the cerebral cortex and cerebellum, contained about 75% of total brain MAO activity.

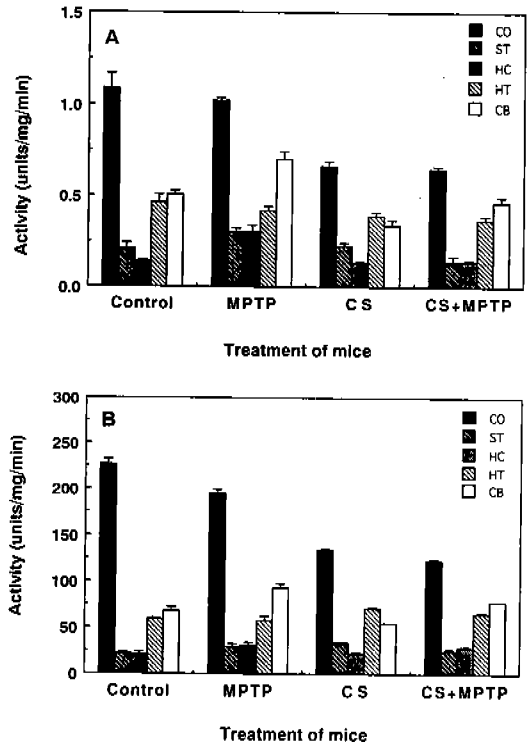

Fig. 3. The activity of monoamine oxidases B (A) and total activity of monoamine oxidase (B) in various regions of the brain from control, cigarette smoke exposed (CS), MPTP treated (MPTP) and MPTP treated with cigarette smoke exposure (CS+MPTP) groups. The data in nmoles hydrogen peroxide produced per mg protein per min for MAO-B and arbitrary units of fluorescence intensity for total MAO, are means±SD of three repeated experiments using brain fractions pooled from 7 mice. (CO; cerebral cortex, ST; striatum, HC; hippocampus, HT; Hypothalamus, CB; cerebellum).

Cigarette smoke exposure caused a decrease in the activity of the both enzymes, especially, in the cortex and cerebellum. Interestingly, MAO activity in the striatum was not changed by exposure to

tobacco smoke. However, it was remarkably decreased in all brain regions when mice were exposed to tobacco smoke together with MPTP treatment.

Effects of MPTP treatment and cigarette smoke exposure on the brain antioxidant enzymes

Table 1 shows the effect of cigarette smoke exposure and MPTP treatment on the activity of catalase in various regions of the brain. Catalase activity was relatively high in the cerebral cortex and cerebellum, and low in the striatum, compared to other brain regions. This enzyme activity was not significantly changed by exposure to tobacco smoke or MPTP treatment. SOD and glutathione peroxidase activity was also not significantly changed either by MPTP treatment and/or exposure to cigarette smoke (Table 2 and 3). Sulfhydryl compounds were increased remarkably in all brain regions except striatum after MPTP administration in the absence of exposure to tobacco smoke. It was, however, decreased especially in the striatum and hippocampus

Table 1. Effect of cigarette smoke exposure and MPTP treatment on catalase activity in various regions of brains

	Control	CS	MPTP	CS+MPTP
Cortex	0.73 ± 0.03	0.64 ± 0.05	0.71 ± 0.02	0.62 ± 0.04
Striatum	0.09 ± 0.01	0.15 ± 0.02	0.11 ± 0.02	0.16 ± 0.01
Hippocampus	0.13 ± 0.01	0.14 ± 0.04	0.11 ± 0.02	0.15 ± 0.04
Hypothalamus	0.28 ± 0.01	0.27 ± 0.01	0.27 ± 0.06	0.29 ± 0.01
Cerebellum	0.42 ± 0.02	0.37 ± 0.01	0.44 ± 0.04	0.39 ± 0.02

The enzyme activity was measured in cytosolic fractions obtained from the homogenate of each brain region. The data are expressed as mean ± SD of three repeated experiment using samples pooled from 8 mice. A unit of the enzyme was defined as umoles of hydrogen peroxide degradation per mg protein per min. CS; smoke exposure, MPTP; MPTP treatment, CS+MPTP; smoke exposure with MPTP.

Table 2. Effect of cigarette smoke exposure and MPTP treatment on Cu, Zn-SOD activity in various regions of brains

	Control	CS	MPTP	CS+MPTP
Cortex	6.0±0.6	4.7±0.2	5.0±0.2	5.0±0.2
Striatum	5.1±0.5	5.6±0.4	4.0±0.4	4.6±0.9
Hippocampus	3.3±0.3	3.8±0.6	3.9±0.3	3.9±0.5
Hypothalamus	3.0±0.3	4.3±0.4	4.9±0.6	5.4±0.5
Cerebellum	4.0±0.4	4.2±0.5	4.2±0.3	5.9±0.7

Enzyme activity was determined by monitoring the inhibition of cytochrome c oxidation at 550 nm by using xanthine oxidase to generate superoxide. Data are expressed as mean ± SD of three repeated experiments. Abbreviations are the same as in Table 1.

Table 3. Effect of cigarette smoke exposure and MPTP treatment on glutathione peroxidase activity in various regions of brains

	Control	CS	MPTP	CS+MPTP
Cortex	51± 4	48± 6	52± 3	41±7
Striatum	56± 6	58± 4	44± 4	63±8
Hippocampus	62± 6	63± 6	57±10	45±3
Hypothalamus	60± 2	57±10	55± 7	53±5
Cerebellum	83±10	61± 2	62± 1	79±9

The data are expressed as mean ± SD of three repeated experiments using cytosolic fraction obtained from the homogenate of each brain region. The activity was measured with coupled-enzymes system using cumene hydroperoxide as a substrate. A unit of the enzyme was defined as nmoles per mg protein per min. Abbreviations are the same as in Table 1.

by exposure to cigarette smoke regardless of MPTP treatment as shown in Fig. 4.

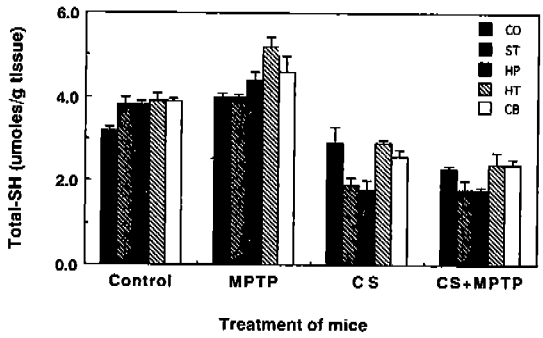

Fig. 4. The effect of cigarette smoke exposure and MPTP treatment on sulfhydryl compounds in various regions of the brains. The data are expressed as means±SD of three repeated experiments using brain homogenates pooled from 8 mice. Abbreviations are the same as Fig. 3.

DISCUSSION

Data obtained in the present study demonstrate that cigarette smoke exposure attenuates the decrease of striatal DA caused by MPTP in mice. Our data is not consistent with studies previously reported by other investigators (Carr and Rowell 1990; Perry *et al.*, 1987), but accorded with that of Carr and Rowell (1990) who exposed mice to cigarette smoke for one month for the change of DA level. These aspects may suggest that, in the case of mice, the duration of cigarette smoke exposure should be at least 4 weeks prior to MPTP treatment in order to suppress MPTP-induced neurotoxicity. The time of sacrifice after MPTP treatment also could be an important determinant for studying the protective influence of cigarette smoke exposure.

In our study, long-term cigarette smoke exposure to mice treated with MPTP suppressed the loss of striatal TH positive cells responsible for DA synthesis. The cell number was proportional to DA content. This result suggests that the suppressive effect of cigarette smoke on DA depletion is not due to the stimulation of DA release by a simple action of nicotine, but due to unknown factor(s) which protect the cells from being destroyed. This fact implies that some components in cigarette smoke may inhibit the metabolic activation of MPTP.

Based on these data, the effect of cigarette smoke exposure on the activity of brain MAO-B converting MPTP to its active metabolite was investigated. Monoamine oxidases (MAO) exist as two types, MAO-A and MAO-B, which are distinguished according to their specificities for substrates and inhibitors. MAO-B is responsible for approximately 80% of the total MAO activity in human brain (Youdim and Finberg 1991). In mice, the activity pattern of MAO-T and MAO-B in various brain regions was similar each other as shown in Fig. 3. Interestingly, cigarette smoke exposure did not affect the striatal MAO-B activity although DA levels were decreased significantly in this region ($P<0.01$). Contrary to this, MAO-B activity was greatly decreased in the cortex and cerebellum by exposure to cigarette smoke. These results indicate that MAO-B existing in the cerebral cortex or cerebellum regions plays a pivotal role for the activation of MPTP in the brain although the striatal region was severely affected by this neurotoxin. Carr and Rowell (1990) and Shahi *et al.* (1991) have demonstrated that MAO-B activity of the mouse brain is decreased by cigarette smoke exposure. However, they measured the enzyme activity in homogenates of the whole brain. The decreased activity of brain MAO-B by cigarette smoke exposure might contribute to the suppression of

MPTP activation.

The involvement of abnormal metabolism of free radicals in the brain has been suggested as another risk factor possibly responsible for the onset of PD (reviewed by Olanow, 1992). Therefore we investigated whether treatment of mice with MPTP or exposure to cigarette smoke could influence the antioxidant defense status of brain since MPTP generates reactive oxygen species (ROS) during its metabolism (Zang and Misra 1992) and cigarette smoke contains various free radicals (Church and Pryor 1985). Our results, however, showed that the activity of brain antioxidant enzymes responsible for the scavenging of ROS was not significantly changed by MPTP treatment or exposure to cigarette smoke. These data suggest that ROS be not a primary factor in MPTP-induced neurotoxicity.

The content of sulfhydryl compounds was unusually increased by MPTP treatment although it was slightly decreased by cigarette smoke exposure. This is in agreement with a previous report (Desole *et al.*, 1995). However, it is not clear why only sulfhydryl compounds are enhanced by MPTP administration in the absence of significant alterations in the activity of other major antioxidant enzymes.

In general, long-term exposure to cigarette smoke is known to increase the activity of antioxidant enzymes and to decrease the level of antioxidants such as sulfhydryl compounds and ascorbic acid in both rat lung and human serum (Sohn *et al.*, 1993; Dickinson *et al.*, 1994). Such changes were modulated by the treatment with antioxidants (Lee et al., 1993; Sohn et al., 1993). These facts indicate that cigarette smoke causes the increase of the oxidative stress *in vivo*. However, the data obtained in the present study indicate that cigarette smoke exposure does not severely affect the antioxidant system in the brain and these suggest that the protective action of cigarette smoke against MPTP neurotoxicity seems to result from the suppression of MPTP activation, but not from the regulation in the metabolism of toxic free radicals in the mouse brain.

In conclusion, we demonstrated clearly that long-term exposure to cigarette smoke attenuates MPTP neurotoxicity in mice. Such an effect of cigarette smoke exposure seems to be due to the inhibition of MAO-B by an unknown compound(s) in cigarette smoke or its metabolite(s). These biochemical changes may be a probable reason for the decreased incidence of PD in smokers.

요 약

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP)의 신경독성에 대한 흡연의 영향을 생쥐에서 조사하였다. 생쥐를 15개피의 담배를 연소하여 얻은 연기에 일일 10분, 주 5회, 6주 동안 전신폭로법으로 노출시키고, 신경독성을 유발하기 위해 흡연 1개월 후 부터 MPTP (10mg/kg)를 매일 1회씩 6일간 연속 주사하였다. 마지막 주사 후 10일째에 생쥐를 희생시키고 몇가지 신경생화학적 지표성분을 조사하였다. 그 결과 담배연기에 노출된 생쥐는 MPTP에 의해 유발되던 뇌 선조체의 dopamine 함량의 감소와 tyrosine hydroxylase-positive 세포수의 감소현상이 유의하게 억제되었다. 반면, MPTP를 활성화 시키는 monoamine oxidase B (MAO-B)의 활성도는 흡연에 의해 특히 대뇌와 소뇌에서 억제되었다. 그러나 뇌의 catalase, glutathione peroxidas, Cu, Zn-superoxide dismutase 등의 항산화효소들의 활성도는 흡연이나 MPTP의 투여로 인해 크게 변화되지 않았다. 흥미롭게도, 뇌 조직의 sulfhydryl 화합물의 함량은 MPTP의 투여에 의해 증가되었다. 그러나 이와같은 MPTP의 영향은 흡연한 생쥐에서는 관찰되지 않았다. 이 결과들은 장기적인 흡연이 뇌의 free radical대사에는 영향을 주지 않으면서 MPTP에 의해 유발되는 신경독성을 억제한

다는 것을 암시해 주며, 이와같은 흡연의 긍적적인 효과는 MPTP를 활성화시키는 MAO-B의 활성도가 억제된 것과 깊은 관련이 있는 것으로 사료된다.

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