의학박사 학위논문

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지도교수 강 병 철

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ABSTRACT

Establishment of a Parkinson's disease model and evaluation of therapeutic effects of dopaminergic precursor cells in a MPTP-treated common marmoset

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Parkinson's disease (PD) is one of the most important neuro-degenerative diseases. Studies investigating cell transplantation as an alternative to *L*-3,4-dihydroxyphenylalanine administration or deep brain stimulation surgery are being actively conducted. Many PD animal models are used for PD treatment or prevention. However, most of them are rodent models, and the most representative is the model established with 1-meth-

yl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Compared to other models, nonhuman primate (NHP) MPTP-treated models show clinical symptoms similar to human patients and facilitate behavioral evaluation, suggesting the use of various MPTP injection models according to experimental needs. Most NHP MPTP-treated models are optimized for short-term studies within three months and are not suitable for long-term studies such as cell transplantation. Since fetal mesenchymal cell transplantation in early studies, studies using mesenchymal stem cells or embryonic stem cells (ESCs) have been conducted. Studies have also been conducted using induced pluripotent stem cells, which can resolve ethical concerns and immune rejection. Despite advances in efficacy evaluation and safety of cell transplantation, studies on differentiation and discovery of homogeneous classification marker have yet to be investigated systematically since the degree of differentiation and homogeneity of cells after differentiation are directly related to clinical recovery and reduction of side effects. Accordingly, a Parkinson's disease model was established by subcutaneous administering "2-2-1-1-1" mg/kg of MPTP to common marmosets (*Callithrix jacchus*) to induce a long-term and stable clinical manifestations. Daily observation showed stable and persistent clinical symptoms. The results of tower test also reduced the motor function compared with pre-treatment with MPTP. In striatal positron emission tomography (PET) images, radioactivity was significantly reduced compared with prior MPTP administration. Immunohistrochemical analysis showed loss

of tyrosine hydroxylase (TH)-positive cells and fibers in substantia nigra. In addition, 2.0×10^6 cells were implanted intracranially into the stratum of marmoset PD model to evaluate the therapeutic effect of dopaminergic (DAergic) precursor cells from human ESCs differentiating into DAergic neurons associated with PD symptoms using trophoblast glycoprotein, a new differentiation marker. The results of daily observation showed that the clinical symptoms recovered significantly from the third week after the cell transplant compared with the group exposed to MPTP. The tower test result confirmed that significant increase in the number of levels the marmosets climbed from the seventh week after the cell transplant. In the striatal PET image, the specific uptake ratio value was significantly increased from the fourteenth week after the cell transplant compared to the MPTP treatment group. The histopathological analysis revealed no excessive inflammatory reactions or tumor-like neoplasms, and TH-positive cells developed from implanted DAergic precursor cells in the cell transplant site. Based on the above results, it is purposed that the marmoset model produced by the new MPTP treatment method is suitable for long-term studies such as cell transplantation, and it is suggested that DAergic precursor cells represent potential as PD treatments for human patients.

Keywords: Parkinson's disease; nonhuman primates; common marmoset; cell therapeutics; animal model; stem cell; cell transplantation

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PD	Parkinson's disease
DAergic	Dopaminergic
SN	Substantia nigra
L-DOPA	*L*-3,4-dihydroxyphenylalanine; Levodopa
DBS	Deep brain stimulation
6-OHDA	6-hydroxydopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ESC	Embryonic stem cell
NHP	Nonhuman primate
PET	Positron emission tomography
TH	Tyrosine hydroxylase
TPBG	Trophoblast glycoprotein

LITERATURE REVIEW

Parkinson's Disease (PD)

Parkinson's disease (PD) is one of the most important neuro-degenerative disorder triggered by dopaminergic (DAergic) cell death in the substantia nigra (SN) and dopamine depletion substantially, leading to manifestations such as tremor, rigidity and slow movement. PD was described by Dr. James Parkinson in 1817 in the essay, "Shaking Pasly", and was named in 1862 by Jean-Martin Charcot in honor of Dr. Parkinson (1). PD is one of the top three geriatric diseases, along with Alzheimer's disease (AD) and stroke (2), and is the second-largest degenerative brain disease with a prevalence of 200 per 100,000 people worldwide (3, 4). The number of patients in their 60s and over increases rapidly (5).

Classification and Etiology of PD

According to Jean-Martin Charcot, PD is characterized by tremor and rigid/akinesia depending on the clinical motor symptoms; however, PD patients do not necessarily need to manifest tremor (1). PD is classified into different types depending specific features; idiopathic, inherited, and other atypical PD (6). Idiopathic type of PD is the most common although underlying etiology is unknown, unfortunately. Some studies have reported that PD is caused by factors such as smoking, coffee and tea consumption,

and exposure to pesticides, traumatic brain damages, organic solvents, uric acid, daily products, nonsteroidal anti inflammatory drugs, statin and calcium channel blockers (7). Other studies have reported that diabetes and vitamin D deficiencies are also associated with PD (8). However, it is attributed to a combination of genetic and environmental conditions, and not to a specific etiological factor alone.

Five to 10% of PD patients present with the inherited type, and specific genes are known to be associated with PD such as *Parkin*, *DJ-1*, *PINK1* and others (3, 9, 10). *PARK* genes 1 to 18 are currently linked to familial PD, since the publication of the first mutant genetic map associated with possible PD in 1996 (11). *PARK8* and *PARK17* were associated with general PD occurring in older age groups, with corresponding mutant genes *LRRK2* and *VPS35*, respectively. Early-onset PD is associated with *PARK2* (*Parkin*), *PARK6* (*PINK1*), *PARK7* (*DJ-1*), *PARK9* (*ATP13A2*), *PARK14* (*PLA2G6*), and *PARK15* (*FBXO7*) excluding *PARK1*, whose gene name is *SNCA*. Among them, *SNCA*, *LRRK2*, *Parkin*, *PINK1*, *DJ-1*, and *ATP13A2*, are linked to PD, and the identity of the rest is still being investigated for possible linkage to PD (12).

Motor and non-motor symptoms of PD

Several neuropathological and neurochemical studies have shown that major clinical symptoms of PD, including motor symptoms are associated with dopamine. Subsequent studies have reported that motor symptoms do not appear until dopamine levels in the striatum are reduced significantly along with extensive loss of DAergic neuron in the SN (13). In the "subclinical state" characterized by symptoms of motor abnormalities, the dopamine level in the striatum decreases to 80%, and nearly 60% of DAergic cells in the SN appear to be lost, with eventual dopamine reduction and loss of DAergic neurons. However, it is known that motor symptoms do not occur due to various compensatory mechanisms until the disease is at an advanced stage (14). Thus, major motor symptoms such as tremors appear at late stage of disease, and it is very difficult to treat or prevent progressive disease because of the loss of a large number of DAergic neurons. Therefore, treatment initiation before the onset of "subclinical state" with motor symptoms can most effectively prevent disease progression (15, 16). However, it is very difficult to identify specific early symptoms, especially non-motor symptoms such as sleep disorder, anxiety and depression, because those symptoms vary among patients and it is not easy to realize specific non-motor symptoms (17, 18). Nonmotor symptoms can be largely classified into neuropsychological abnormalities, sleep disorders, autonomic neurological abnormalities, sensory ab-

normalities, and pain, and according to Witjas et al., anxiety, severe sweating, delayed information processing, fatigue, hypersensitivity, and hallucinations appear to be approximately 50% of those in PD patient (19). Non-motor symptoms are important not only as an indicator for early diagnosis in PD patients, but also in terms of quality of life in both patients and caregivers as well as motor symptoms (20).

Animal models of PD

In vitro and *in vivo* models are used to study PD mechanisms and treatments. *In vitro* models facilitate rapid pathological investigations inexpensively, and without the ethical concerns associated with animal models. In addition, genetic manipulation is easier and the reproducibility is high as large-scale experiments can be performed in a short time. However, since actual PD occurs via interaction with various neurons and other cells or tissues in addition to dopamine neurons, it can be studied only in living animal models. For this reason, results obtained from the *in vitro* model for this reason should be verified in studies using animal models (21). A search of SCOPUS (Elsevier) with the keywords “Parkinson ’s disease” and “Animal models” returned about 13,000 articles. In particular, in the last 5 years, the number of studies using animal models has increased, with about 1,000 articles in each year (Figure 1). The rat model for the PD study was first developed in 1970 by Ungerstedt and Arbuthnott

by administering a neurotoxic substance called 6-hydroxydopamine (6-OHDA) into the brain (22). In 1971, Hockman established a cat model by including thermal damage in the brain tissue surgically (23). Subsequently, studies using dog and rat models have been conducted, and primate models have been reported in Japan in 1979 (24). The PD animal models include acute models developed via neurotoxin administration and chronic model involving genetically modified animals. The animal model used for most PD studies is established via a neurotoxin administration (25) (Table 1, Figure 2).

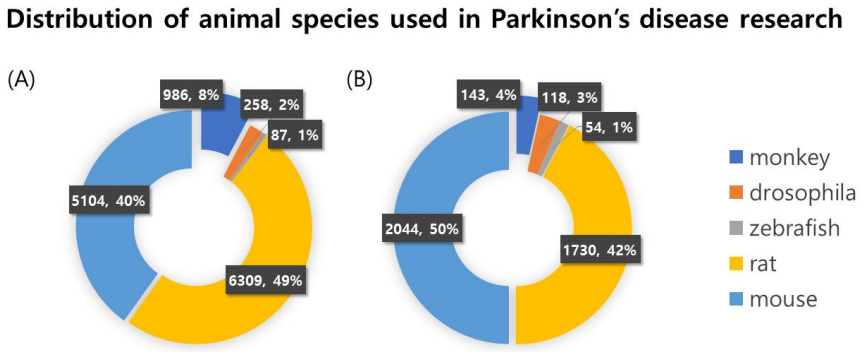


Figure 1. Species distribution in animal models for PD. As a result of literature search in SCOPUS with "Parkinson's disease" and "animal model", rodent models such as mice and rats were mostly used in PD studies, and primate models were used within 10%. (A) Total period (1974-2019). (B) Last 5 years (2015-2019).

Table 1. Characteristics of animal models used in PD

Type	Methods	Characters	
		Advantage	Disadvantage
Acute (Neurotoxin based)	6-OHDA	· Simultaneous acquisition of normal and pathological consequences in an individual (ethical aspect)	· Stereotaxic surgery required · Not all clinical and pathological symptoms are expressed
	MPTP	· Various routes of administration · All clinical symptoms can be observed	· Danger to health when exposed · Difference in Lewy body (LB) formation among histopathological characteristics
	Rotenone	· Results of epidemiological investigation · LB analog formation among histopathological properties	· Only some animal species can establish a model
Chronic	Aging	· Results of epidemiological investigation	· Cannot use uniform and controlled models
	Genetic modification	· Expression of specific proteins derived from humans	· Special operation skills required · A long time to establish a model strain

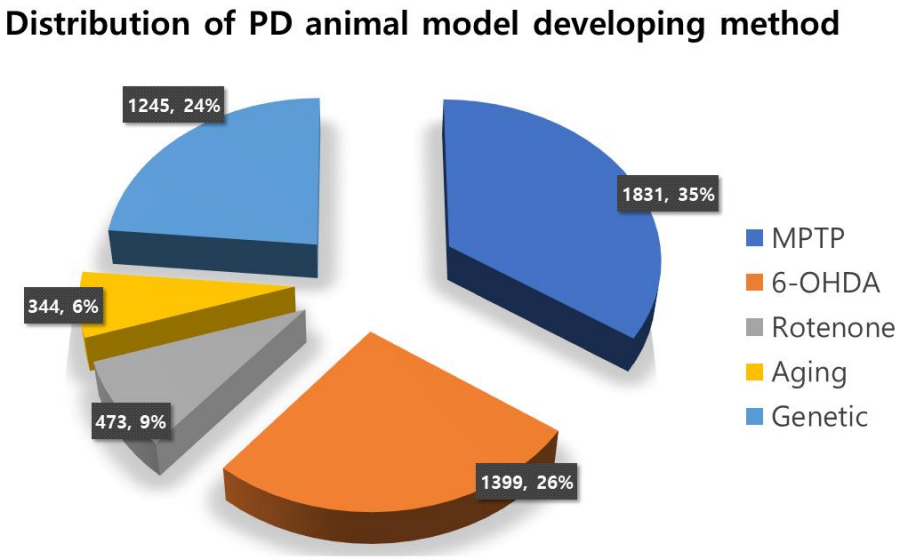


Figure 2. Methods used in animal models for PD from 1974 to 2019. As a result of literature search in SCOPUS with "Parkinson's disease" and "animal model", most studies were conducted using the MPTP treatment (35%), followed by 6-OHDA treatment (26%), and the genetic manipulation (24%).

6-OHDA models

The 6-OHDA model is established via topically application of a chemical neurotoxin, and various models including mice, cats, dogs, and monkeys as well as rats have been developed (26). Although studies using 6-OHDA models were conducted until 1985, administering 6-OHDA directly to brain tissue was technically difficult because 6-OHDA cannot cross the blood-brain barrier. Particularly, a bilateral lesion induced via intraventricular or intracranial administration results in death, due to motor symptoms and the inability to feed or drink water (27). Therefore, the 6-OHDA model is most commonly used to administer 6-OHDA directly to the substantia nigra, the nigrostriatal tract, or the striatum. In addition to the model retention rate due to reduced mortality, a single animal can be used in the experimental and animal groups simultaneously (28).

MPTP models

The MPTP model was constructed in 1982 by Langston et al., who found symptoms similar to PD in drug addicts who injected MPTP-contaminated heroin (29). In particular, most of the histopathological findings along with the characteristic clinical symptoms in PD patients were observed in the nonhuman primate (NHP) model, and in the mouse model, DAergic neurons degeneration was observed, although no representative clinical symptoms were observed. However, the rat model was inadequate be-

cause it was resistant to MPTP due to the species characteristics (30). In addition, in the case of the mouse model, no inclusion body similar to the Lewy body (LB), one of hallmarks of PD, was found, although the model was established with acute, subchronic, and chronic MPTP treatment via injection or using an osmotic pump (31, 32). However, acute or subacute MPTP treatment leads to necrosis of DAergic neurons in the SN, but is limited by a spontaneous recovery due to the reversible response.

Other neurotoxin models

As studies investigated the role of environmental factors in human PD, a model was developed by chemical treatment the assumption that exposure to herbicides or pesticides may be a cause. Among the herbicides, paraquat (N, N′-dimethyl-4-4′-bipiridinium) was structurally similar to MPP^+ (1-methyl-4-phenylpyridinium), a metabolite of MPTP, and therefore used in mouse studies. Decreased DAergic nerve cell fibers in the striatum and neurons of the SN were reported in patients with decreased motor abilities (33). In contrast, only a small number of necrotic nigrostriatal DAergic neurons were observed in other studies (34) without behavioral abnormalities or neural circuit destruction (35). However, based on several models developed using the method reported by Betarbet et al., (36) only about half of the rats treated with rotenone showed necrosis of nigrostriatal neural cells, and the model was not established in mice or monkeys except

rats (37).

In recent years, inflammation has been reported as an important factor contributing to PD. A rat model in which the endotoxin lipopolysaccharide (LPS) was directly injected to the nigrostriatal pathway has been developed based on inflammation in the nigrostriatal pathway triggered by neurotoxins administered (38). LPS administered topically to the SN or striatum is not directly toxic to DAergic neurons, but cytotoxins released by microglia activation disrupted the dopamine neural circuit. Hunter et al. (39) confirmed a decrease in dopamine concentration and accumulation of α-synuclein (α-syn) in the striatum along with SN cell necrosis in a mouse model administered LPS in the striatum.

Genetic models

After studies using mouse and rat transgenic animal models exposed to oxidative stress, which believed to cause PD, cell transplantation therapy was developed using genetically modified animals (40, 41). In the late 1990s, various genetically modified mouse models were used to investigate pathological mechanisms: MAO-B transformation or knockout (KO) mouse model (42, 43), neuronal nitric oxide synthase or inducible NOS KO mouse model (44, 45), and dopamine transfer factor (DAT) or dopamine receptor KO mice (46, 47). However, this strategy is difficult to accept as practical transgenic PD models. In the early 2000s, mice with knockout of *α-syn* (48, 49), *DJ-1* (50), *Parkin* (51), *PINK1*

(52), and other genes were investigated. However, a non-mammalian transgenic animal model was developed as an alternative to compensate for the poor nigrostriatal neuronal necrosis in transgenic mouse models. The representative non-mammalian transgenic animal models, fruit flies (*Drosophila*) (53, 54), *Caenorhabditis elegans* (55), and zebrafish (56) models are economical in terms of model development and maintenance compared with rodent or NHP models, and a large number of such models can be tested simultaneously (57). As a result, an optimal model for evaluating the effect of α-syn deletion or neuroprotection was developed while maintaining the nigrostriatal pathway, which increases the concentration of dopamine and dopamine metabolites in the striatum and increases the concentration of α-syn in the striatum (58).

Limitation of rodent models of PD

In general, rodents have been utilized as animal models compared with NHP based on accessibility, easy handling and manipulation, ease of husbandry, and economics of management. As an animal model for PD studies, the 6-OHDA-treated rat model presents behavioral disorders such as limb movement abnormalities, ataxia, and sensory-motor disorders; however, the standard 6-OHDA model carries a hemisphere lesion, which is different from the patient's symptoms (59). The MPTP-treated mouse model exhibits motor symptoms such as tremor and gait abnormalities,

but unlike the primate model, it does not show dyskinesia, because the nerve pathways associated with MPTP damages are presumed to differ from those of the mouse and NHP, and several therapies may explain the difference in interpretation of results in mouse models and human patients (60). The transgenic mouse model is easy to develop and maintain compared to a neurotoxin-treated model, except that genetic engineering techniques are essential. In addition, in a mouse model, the overexpression or deletion a gene underlying hereditary PD represents an optimal condition to identify the causative role of a specific gene. However, the common intrinsic genetic factor underlying dopamine neuronal necrosis is not observed in the characteristic mouse species (61).

NHP models of PD

The ideal animal model of PD should show motor or non-motor symptoms in PD patients and responses to therapies used clinically, and similar histopathologic lesions in the brain (62-64). However, no animal models meeting the above conditions completely are available. Therefore, NHP models have an advantage over other species models because NHP models are the closest genetically with human, exhibit similar anatomy of central nervous system (CNS) including the brain and other organs, and operate arms or legs like human (29, 65, 66).

Neurotoxin models - 6-OHDA, MPTP

Neurotoxin-treated primate models were mainly developed by MPTP and 6-OHDA, especially 6-OHDA models of baboons (*Papio papio*) (67) and rhesus monkeys (*Macaca mulata*) (68), but mainly common marmosets (*Callithrix jacchus*). The characteristics of 6-OHDA NHP model vary according to the location and frequency of administration of 6-OHDA (69-76). In general, the striatum, SN, and medial forebrain bundle, which is the nigrostriatal pathway, are the treatment sites. New models have been developed to control dose frequency due to the nature of recovery after about 10 weeks after treatment with 6-OHDA similar to MPTP (77). In order to develop the 6-OHDA model, although a very small needle is used in the brain tissue, it is asscociated with the risk of physical damage following multiple injections, and appropriate stereotaxic surgery is required to inject it in the correct position. In addition, administering 6-OHDA to induce symptoms in only one hemisphere, the various clinical symptoms seen in human PD patients do not appear.

However, the MPTP model was created using old world monkeys such as velvet monkey (*Chlorocebus aethiops*), rhesus monkey, and cynomolgus monkey (*Macaca fasicularis*), in addition to new world monkeys such as squirrel monkey (*Saimiri sciureus*) and common marmoset, and is the most used model (77) (Figure 3). In addition, a model development method in which MPTP administration route, dosage, and frequency or duration of admin-

istration is very diverse was introduced depending on the clinical symptom progression and maintenance period of the model (Table 2). Unlike other models, in the MPTP model, it affects only the DAergic neurons in the brain (78), and leads to the accumulation of α-syn in the dopaminergic neurons associated with LBs (79).

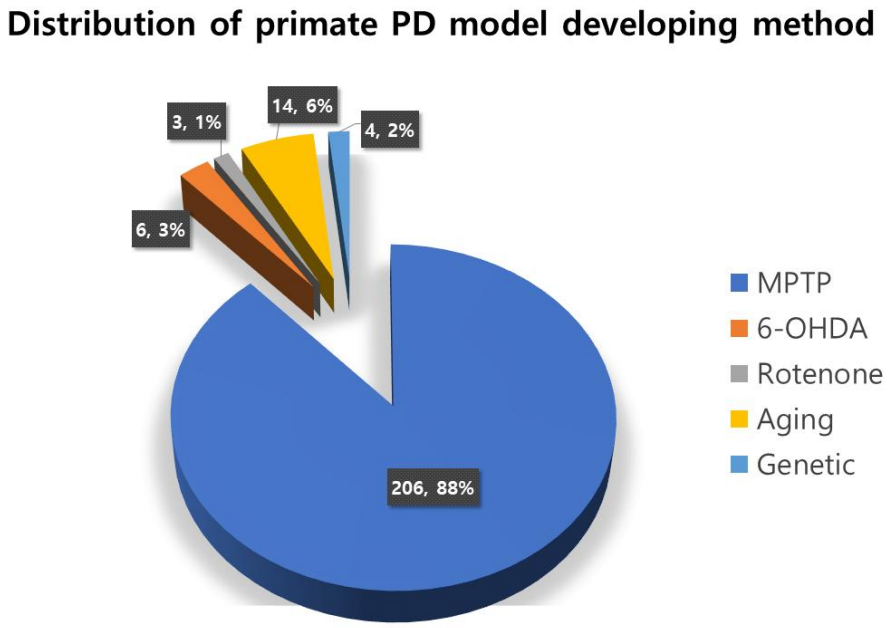


Figure 3. Methods used to create NHP model of PD from 1974 to 2019. As a result of literature search in SCOPUS with "Parkinson's disease", "animal model" and "primate" or "monkey", most studies were conducted using the MPTP treatment model (88%), followed the Aging model (6%), and the 6-OHDA treatment model (3%).

Table 2. NHP PD model characteristics according to various MPTP treatment methods

Types	Species	Routes	Doses	Duration	Study periods	References
Systemic	*M. fascicularis*	IV	A. 0.75 mg/kg B. Total 2.75-9.25 mg/kg	A. 2 wk B. 12-20 wk	A. 4 d B. 8-20 wk	Perez-Otano et al. (80)
	M. fascicularis	IV	Total 2.8-3.2 mg/kg	14-16 d	25 d	Bezard et al. (81)
	M. fascicularis	IV	0.2 mg/kg	13-17 wk	25 d	Meissner et al. (82)
	M. fascicularis	IV	Total 2.6-27.0 mg/kg	1-2 wk	4-8 wk	Crossman et al. (83)
	M. fascicularis	IV	0.6 mg/kg	18 wk	5-8 wk	Brownell et al. (84)
	M. fascicularis	IV	0.2 mg/kg	29 d	8 wk	Bezard et al. (63)
	M. fascicularis	IV	Total 12.6-15.8 mg	4-7 d	60 d	Visanji et al. (85))
	M. fascicularis	SC	Total 0.6-4.0 mg/kg	1-5 wk	4 wk	Morissette et al.* (86)
	M. fascicularis	IM	A. Total 10.7-12.3 mg/kg B. Total 1.0-6.5 mg/kg	A. 58 wk B. 4-24 wk	17 wk	Durand et el. (87)
	M. fascicularis	IM	Total 2.8-7.0 mg/kg	15-35 d	48 wk	Seo et al. (88)
	M. nemestrina	IV	Total 14.94-75.42 mg	20-52 wk	8-60 d	Schneider and Kovelowski*** (89)
	C. aethiops	IM	Total 2.8-5.3 mg/kg	2-8 wk	10 d	Jan et al.** (90)
	C. aethiops	IM	Total 3.2-13.2 mg/kg	2-8 wk	10 d	Jan et al.** (91)
	C. aethiops	IM	Total 1.3-5.2 mg/kg	2-7 wk	15 wk	Mounayar et al. (92)

(Continues)

Table 2. (continued)

Types	Species	Routes	Doses	Duration	Study periods	References
Systemic	*P. papio*	IV	A. Total 34.1-40.6 mg/kg B. Total 11.0-27.8 mg/kg	A. 68-84 wk B. 20-86 wk	A. 8-40 wk B. 28-64 wk	Hantraye et al.,**** (93)
	P. papio	IV	Total 11.0-37.6 mg/kg	20-86 wk	28-64 wk	Varastet et al. (94)
	M. mulata	IV	Total 9.9-18.6 mg	5-8 d	14 wk	Burns et al. (95)
	C. jacchus	IP	2-4 mg/kg	4 d	10 d	Jenner et al. (65)
	C. jacchus	IP	2-3 mg/kg	5 d	4-5 wk	Rose et al. (96)
	C. jacchus	IP	A. Total 6-22 mg/kg B. Total 78-83 mg/kg	A. 3-7 d B. 5 wk	12 wk	Ueki et al. (97)
	C. jacchus	SC	2 mg/kg	5 d	8 wk	Iravani et al. (98)
	C. jacchus	SC	2 mg/kg	5 d	12 wk	Fox et al. (99)
	S. sciureus	SC	A. 2 mg/kg B. Total 6.0-8.75 mg/kg	1 d	6 wk	Di Monte et al. (100)
	S. sciureus	IP	8.0-9.5 mg/kg	1-5 d	25 d	Langston et al. (101)
	S. sciureus	SC	Total 12,9-15,9 mg/kg	4-17 wk	8 wk	Stephenson et al. (102)
Unilateral	*M. mulata*	IV	0.4-1.2 mg/kg	1 inj	4-6 wk	Ovadia et al. (103)
	M. mulata	IV	3 mg	1 inj	6 wk	Emborg et al. (104)
	M. mulata	IV	3 mg/kg	1 inj	12 wk	Kordower et al. (105)

(Continues)

Table 2. (continued)

Types	Species	Routes	Doses	Duration	Study periods	References
Unilateral	*M. mulata*	IV	3-4 mg	1 inj	12 wk	Collier et al. (106)
	M. mulata	IV	0.8 mg/kg	1 inj	20-28 wk	Benazzouz et al. (107)
	M. mulata	IV	0.4 mg/kg (right) + 0.2 mg/kg (left)	each 1 inj	48 wk	Smith et al. (108)
	M. fascicularis	IV	0,05-1.6 mg/kg	1 inj	4-14 wk	Bankiewicz et al. (109)
	M. fascicularis	IV	0.3-0.6 mg/kg	1 inj	8 wk	Aebischer et al. (110)
	M. nemestrina	IV	2.5-3.5 mg/kg	1 inj	6-8 yr	Emborg-Knott and Domino***** (111)
	C. apella	IV	1.2 mg/kg	1 inj	80-88 wk	Emborg and Colombo (112)
osmotic -pump	*M. fascicularis*	SC	0.5 mg/24 h	2-4 wk	4 wk	Hadj Tahar et al. (113)
	M. fascicularis	SC	Total 3 mg	1-2 wk	48 wk	Sanchez et al. (114)
	C. jacchus	SC	Total 6.0-14.25 mg	2 wk	20 wk	Petryszyn et al. (115)
Mixed	*M. mulata*	IV	2.5 mg ICA + 0.3 mg/kg	1 inj + 4 wk	6 wk	Emborg ME et al. (116)
	M. mulata	IV	2.5 mg ICA + 0.3 mg/kg	1 inj + 2-7 wk	28-68 wk	Eberling et al. (117)

inj: injection; d: day; wk: week; yr: year

* 2-3 mg injections at weekly intervals

** 0.4 mg/kg 4 daily injections in each of the first 2 weeks followed by 1 or 2 injections per week

*** 0.010-0.175 mg/kg injections up to 3 times per week

**** 0.4 mg/kg 5 daily injections followed 5-6 months later by weekly injections of 0.2-0.5 mg/kg for 8-18 months

***** Freezing was not observed in all MPTP-infused monkeys.

Aging models

The NHP PD model can be largely classified into three types: an aging model, a neurotoxin administration model, and a genetic modification model. The aging model was developed based on the fact that PD in most human patients occurs at an older age and aging is presumed to be the cause of PD. The model was developed by several investigators using rhesus monkeys (118-126) and squirrel monkeys (127, 128). In the aging model, postural and gait abnormalities and mild tremors were detected in human PD cases, whereas histopathological degenerative changes involving nigrostriatum such as dopamine reduction in the striatum, dopamine neuronal necrosis in the SN, and deposition of lipofuscin were observed. However, aging is not a disease, but a natural change, and among NHP, it is difficult to develop a specific model because the aging period vary with each strain and individual. It is difficult to associate motor symptoms with abnormalities involving the DAergic neuropathy, as it is possible to develop not only PD symptoms, but also other aging diseases of the musculoskeletal system or metabolism, in addition to the high cost required for model management.

Genetic models

In humans, α-syn is a major component associated with the *SNCA* gene mutation in patients with hereditary familial PD and the LBs found in patients with idiopathic PD. Unfortunately, in

NHPs, no human-like spontaneous mutation is known. Therefore, a model was established by directly injecting the human *a-syn* gene into the brain, and similar to the 6-OHDA model, intracranial administration was included (129, 130). Thus, most models use marmoset monkeys instead of other strains. In the model overexpressing normal α-syn and mutant α-syn, degenerated dopamine neurons were found in the striatum, whereas DAergic neuronal necrosis in the ventral midbrain region was higher in the model over-expressing the mutant α-syn than in the model over-expressing the normal α-syn (129). In order to introduce the α-syn gene, the degree of overexpression and peaking time vary depending on the type of carrier used in the viral vector (131, 132) (Table 3). Overexpression of the introduced gene and symptom manifestation occur after a few days to weeks. In addition, it is necessary to introduce a gene directly into the brain tissue similar to the 6-OHDA model, which may lead to physical damage in the absence of adequate surgical expertise. However, the symptoms observed in human familial or idiopathic PD patients cannot be detected as the gene introduced only in one hemisphere is expressed, which is a model limitation.

Table 3. Human α-syn overexpression in NHP models

Animals		Endpoint after viral injection	References
Strains	Age		
C. jacchus	21-66 mth	52 wk	Eslamboli et al., (129)
	65-72 mth	16 wk	Kirik et al., (130)
	A. 2 yr B. 6 yr	11 wk	Bourdenx et al., (133)
M. mulata	A. 2-3 yr B. 7-8 yr C. 15-18 yr	8 wk	Yang et al., (134)
M. fascicularis	8 yr	17 wk	Koprich et al., (135)

wk: week; mth: month; yr: year

Advantages of MPTP-treated NHP models

NHP PD models involving NHPs compared with other animal species are mostly developed via MPTP administration. Various NHP models such as cynomolgus monkey model were developed (136), since Langston et al. (101) developed the squirrel monkey model and Jenner et al., (65) developed the common marmoset model in 1984 (137). Cynomolgus and rhesus monkeys, which are most frequently used as old-world monkey MPTP models, exhibit individual differences in sensitivity to MPTP. Therefore, several investigators have introduced models to showcase different routes and dosages (111, 138-140). However, most of the marmoset monkeys used as the new world monkey MPTP models were developed via subcutaneous administration for 5 consecutive days, and some researchers modified the MPTP treatment method for the desired condition (97, 141, 142) (Table 2).

The NHP MPTP model can be used to evaluate in a variety of combinations such as bradykinesia, postural abnormalities, facial expression changes, tremors, and rigidity in human PD patients. It also facilitates the evaluation of cognitive abilities, fine movements, tremors, and excessive blinking using tools similar those adopted for human PD patients, as well as the Wisconsin General Apparatus Test (89, 116, 143-145).

However, the NHP MPTP model develops dyskinesia caused by L-DOPA administration similar to that of human PD patients. The varying severity of dyskinesia in the NHP model, caused by

L-DOPA after MPTP administration, is clinically relevant as mentioned in chapter 4 of the Unified Parkinson's Disease Rating Scale (UPDRS), used to evaluate human PD patients (146, 147). In particular, 70% or more of drugs evaluated using a NHP model, compared with rodents or other animal models, have scientific and ethical advantages in predicting efficacy in phase 2 clinical trials of dyskinesia treatment (148, 149). The NHP MPTP model is useful not only for the study of motor symptoms but also for non-motor symptoms. In particular, in the case of the marmoset model, unlike other species, psychosis-like behavior was numerically evaluated (150, 151). Since non-motor symptoms such as hallucinations in human PD patients are important indicators of disease severity (152), they are invaluable in new drug development or research studies evaluating excessive body care or hallucinogenic symptoms with varying severity. In addition, rapid eye movements (REM) can be observed in the NHP MPTP model in sleep (153) and cognitive disorders (154), which are very common in human PD patients (155, 156). Further, evidence of lost cognitive ability related to the frontal lobe (157) suggests its advantage as a diagnostic tool (Table 4).

Table 4. Similar clinical symptoms of human PD and MPTP-treated NHP models

Sort	Symptoms
Motor	Delayed response
	Delayed moving
	Resting tremors
	Dyskinesia caused by *L*-DOPA
Non-motor	Mild cognitive impairment
	Sleep disorder
	Confused circadian rhythm
	Constipation

Current PD therapy

Currently, PD therapy is based on drugs such as Levodopa (*L*-3,4-dihydroxyphenylalanine; *L*-DOPA) for the management of symptoms underlying motor disorders and deep brain stimulation (DBS), which is a surgical method of electrostimulation to specific parts of the brain that control motor functions. Individual medications are used to relieve symptoms associated with non-motor abnormalities.

L-DOPA and supportive medication for PD therapy

L-DOPA has been used as a combination of *L*-DOPA and DOPA-decarboxylase inhibitor (DDCI) to increase the duration of drug efficacy for nearly 20 years since the approval by the United States Food and Drug Administration (FDA) as a PD therapy in 1970 after Gerge Cotzias confirmed its clinical usefulness in 1967 (158). Subsequently, as a treatment of advanced PD, ergoline dopamine agonists (DAs) such as pergolide or cabergoline, which directly affect the dopamine receptor alter the intrinsic neurotransmitter (159), and monoamine oxidase B (MAO-B) inhibitors such as selegiline or rasagiline, which showed efficacy in MPTP-treated animals were used with *L*-DOPA (160). In the 1990s, catecholmethyltransferase inhibitors such as entacapone or trocapone were used to prevent degradation of *L*-DOPA in blood or across blood-brain barrier (161), in addition to non-ergoline DAs such as ropinirole and pramipexole (159). *L*-DOPA is still

used as a golden standard for PD therapy. Evidence supports the use of exogenous dopamine against endogenous dopamine depletion to ameliorate symptomatic parkinsonism but not to prevent progression of disorder (162-164).

Surgical approach for PD therapy

In addition, surgical treatment is used instead of drug administration, and the first reported surgical treatment involved pallidotomy (165, 166) and thalamotomy (167), which were used in early 1950s to excised the underlying basal nucleus. Since 1980, Brice and McLellan developed DBS, a reversible, controllable, and safer surgical method has been developed to suppress the disease progression via electrical stimulation to the midbrain and basal ganglia, operating in the most common mode (168). In the early stages of DBS, electrodes were also inserted into the ventral intermediate nucleus of thalamus, which is effective both tremor and dyskinesia, without affecting other parkinsonisms (169). Thus, electrical stimulation is commonly performed in the subthalamic nucleus and globus pallidus pars interna (170) during DBS. Additional insertion of electrodes into the pedunculopontine nucleus, assists with walk and balance (171).

Limitation of current therapy and alternative trials for PD

It is reported that side effects such as “Wearing-off” and

L-DOPA-induced dyskinesia may occur when PD patients are treated with *L*-DOPA long term (172, 173). In general, after an average of 5 years of *L*-DOPA treatment, at least 40% of patients develop severe dyskinesia and motor fluctuations (174), as well as significant increases in non-motor symptoms during that period, according to several studies (19, 175). Therefore, novel and variable formulations to minimize the unexpected adverse effects associated with long-term *L*-DOPA treatment have been investigated in field conditions; however, the formulation that can be used to exclude all adverse effects is not still exist (176, 177). However, DBS may alleviate or control motor symptoms, but it can not resolve the underlying cause of PD and the mechanism of motor symptom relief is not fully understood (171). In addition, there is a limitation that not all PD patients undergo DBS surgery, but only patients who meet the criteria such as clinically defined age, duration and progression of PD, and especially, response to *L*-DOPA (178). Further, in most patients undergoing DBS surgery, the therapeutic effects on motor symptoms are recognizable; however, non-motor symptoms such as hallucinations and memory loss persist (179).

Due to limitations of current pharmacological and surgical therapies, multiple complementary or alternative therapies has been offered additionally to enhance the quality of patient's life (180). Most of the currently available pharmacological or alternative therapies may be used to managed major motor symptoms, and

molecular approaches such as neurotrophic factor therapy (181, 182), gene therapy (183), or cell replacement therapy (184-186) are emphasized. Neuromodulator therapy is based on the role of neurotrophins, peptides secreted from neuroglia, support cells, such as astrocytes or dendritic cells, in regulating growth and differentiation of nervous system (187, 188). Although neurtrophins associated with PD have been reduced or neuronal necrosis is not reported in human patients, neurotrophins may be of therapeutic value by promoting neuronal growth and function and disrupting neurotoxic processes. Neutrophins that are expected to have therapeutic effects include nerve growth factor, as well as brain-derived neurotrophic factor (BDNF), neurotrophin 3 and 4/5, glial cell-derived neurotrophic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF), and recently discovered mesencephalic astrocyte-derived neurotrophic factor (MANF) (189). Gene therapy involves a combination of methods for disease regulation and non-regulation. Most studies involves in disease control aim to control cell necrosis associated with PD and regenerate necrotic cells by overexpressing neuronal regulatory substances with neuroprotective effects in the SN region. However, non-disease regulation entails normalization of abnormal nerve signals in the basal nucleus by expressing DAergic or γ-aminobutryic acidergic enzymes, and control of motor dysfunction rather than regulating the underlying cause of PD (190) (Table 5). Although those therapies are tested pre-clinically and

clinically, no remedy is available for complete patient recovery.

Table 5. Gene therapy studies for PD

Type	Gene	References
Disease-regulating	GFL modulators - GDNF - neurturin	Ariakasinen and Saama (191)
	Non-GFL modulator - CDNF - MANF	Lindahl et al., (182)
	- BDNF	Ding et al., (192)
	- Nurr1	Dong J et al., (193)
	- vascular endothelial growth factor	Sheikh et al., (194)
Non-disease regulating	Dopamine converting enzymes - tyrosine hydroxylase - GTP cyclohydroxylase - alpha-amino acid decarboxylase	Muramatsu et al., (195), Jarraya et al., (196)

Development of stem cell therapy for PD

Among the alternative therapies introduced above, cell therapy facilitates cellular transplantation to replace necrotic or apoptotic DAergic cells affected by unknown causes and restore its function (Table 6). Since a study published in 1979 demonstrating, improved dyskinesia and graft survival and differentiation after transplanting nerve cells containing DAergic cells derived from rat fetus in a rat model (197), fetal nigral cells transplantation has been reported to be effective not only in supplying dopamine but also to induce the formation of a neural network between the transplanted cells and existing cells. However, using aborted fetuses to obtain embryo-derived cells triggered ethical and immunological concerns (198). To address these challenges, a method of transplanting autologous cells was introduced, and clinical studies using various tissues to synthesize dopamine were also conducted (199). Two main approaches to cell transplantation therapy are availabe. First, neurorestoration, in which the transplanted cells or tissues might play a direct role in existing cells or tissues, alleviating or regulating motor dysfunction caused by necrotic cells via secretion and release of neurotransmitters, synapse formation, and neural circuit formation. Second, the transplanted cells and tissues modify host environment indirectly resulting in neuroprotection or neurorescue via anti-inflammatory, angiogenic or neurogenic, and immune regulatory effects (199).

Table 6. Tissues and cells used in cell transplantation studies for PD therapy

Type	Source	Host	References
fetal ventral mesencephalon (VM) tissue	Human	Human	Lindvall et al., (200) Peschanski et al., (201)
embryonic VM tissue	Rat	Rat	Lee et al., (202)
Adipose tissue-derived stem cells	Human	Rat	Schwerk et al., (203)
		Mouse	Choi et al., (204)
Bone marrow-derived stem cells	Rat	Rat	Park et al., (205) Capitelli et al., (206)
Embryonic stem cells	Human	Rat	Brederlau et al., (207)
Induced pluripotent stem cells	Human	Rat	Kikuchi et al., (208)
Neural stem cells	Mouse	Monkey	Virley et al., (209)

Implications of stem cell therapy

Stem cells can be divided into multipotent adult stem cells, pluripotent embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) according to differentiation ability. Most of the cell transplantation therapies are expected to replace necrotic cells via differentiation or play a supplemental role in case of lost function. However, the biggest risk is that of neoplasm due to excessive differentiation or hyperproliferation (210). Cell transplantation therapy must ensure the safety of the transplanted cells. General chemical-based therapy can identify adverse reactions within a short time or days after administration, in contrast to cell transplantation. The absolute physical time for proliferation and differentiation to an appropriate number ranges from a few weeks and months to a long time until the cell is settled at an appropriate position in the recipient with functional expression. Therefore, it cannot be evaluated via a safety evaluation method based on general chemical compounds (211).

Unlike other organs or tissues, the brain, in particular, has unique anatomical and histological characteristics. The blood-brain barrier prevents the direct entry of bacteria or cells, which are polymers greater than a nm in size. Therefore, a cell transplantation method for the treatment of brain diseases including PD requires, effectively delivery of cells other than direct transplantation into a specific brain region, and has yet to developed. Several studies have reported the improvement of mo-

tor function and the presence of DAergic neurons in the NHP model used in the experiment. Other studies have reported that only some of the animals used in the experiment were less effective or not at all. In particular, the effects on survival, differentiation and proliferation, and transplanted individuals after transplantation differed depending on the origin, lineage, and degree of differentiation of the cells used in transplantation (Table 7).

Table 7. Therapeutic effects of Cell transplantation using NHP PD models

Models	Transplanted cells	Results	References
MPTP	Monkey neural progenitor cells	· motor symptoms recovery was observed · no recovery pattern was observed in PET images	Takagi et al. (212)
MPTP	Human iPSC-derived neural progenitor cells	· no recovery pattern was observed in PET images · survival and differentiation of transplanted tissues were observed at 6 months after transplantation	Kikuchi et al. (213)
MPTP	Human ESC-derived neural progenitor cells	· new tissues of D14-graft cells were observed · dopamine-PET images of D42-graft cells were observed · no motor symptoms recovery was observed	Doi et al. (214)
MPTP	Monkey iPSC-derived DAergic precursor cells	· hyperplasia or new tissues was not observed · no recovery pattern in PET images or motor symptoms were observed	Emborg et al (215)
MPTP	Monkey bone marrow-derived mesenchymal stem cells	· motor symptoms recovery was gradually observed · no new tissues were observed in biopsy and PET images	Hayashi et al. (216)
MPTP	Monkey iPSCs	· motor symptoms recovery was observed in only 1 out of 3 animals · no recovery pattern was observed in PET images	Hallett et al. (217)
6-OHDA	Monkey embryonic nigra tissue	· motor symptoms recovery was observed at 6 months after transplantation · Turning movements related to caudate nucleus and limb movements related to putamen were confirmed	Annett et al. (218)

The immune response of transplanted recipients is the most important factor in the therapeutic effect of cell transplantation. In studies using rat models to date, the survival rate of DAergic neurons was only 3-20%, suggesting it is not possible to expect the effect because it cannot produce enough dopamine to improve motor symptoms. Most of the transplanted cells die within a week after transplantation. Within 24 hours, the cells are eliminated by apoptosis due to hypoxia or lack of nutrients, or phagocytosis by neutrophils. The immune response by microglia or astorcytes leads to loss of cells from day 3 after transplantation (219, 220). Therefore, as a strategy to maximize the effect on cell transplantation, various studies have been conducted to ensure that the transplanted cells survive as much as possible, and the cell survival rate is increased or rather decreased by simultaneous treatment with GDNF, the known neurotrophic factor, or drugs that control the inflammatory response (221, 222). However, immunosuppressants administered to control the recipient's immune response can lead to infections or tumors induced by bacteria or viruses, and an increased risk of teratoma formation by transplanted cells. Therefore, it is necessary to study the optimal immunosuppression strategy that can maximize the survival of transplanted cells while reducing the risk of the recipient (223, 224). The immune response is also an important factor in the selection of the animal model used in cell therapy studies. NHP models that can confirm the survival and function of transplanted

cells without immunosuppression when transplanting human-derived cells as well as allogeneic cells, is the best choice for studying the effects of cell transplantation on motor symptoms recovery or treatment (225).

Importance of early PD diagnosis and available biomarkers

Most people with PD manifest weak clinical symptoms are diagnosed with PD after an average of 10 years since the actual onset of PD. By then, approximately 70% of DAergic neurons are already lost, resulting in poor treatment nad prognostic outcomes based on a diagnosis of clinical symptoms (226). PD is not a lethal disease, but because patients can lives for at least 20 years after the intervention, it is important to ensure that the quality of life is not deteriorated by various complications caused by motor and non-motor symptoms, and the increased familial, social and economic burdens of the patient (227). Therefore, early diagnosis of PD guarantees the patient's quality of life to the maximum extent possible, as it increases the range of options available for appropriate treatment to alleviate both movement and non-motor symptoms and delay the progress of the disease. In addition, there is a great advantage in reducing the costs of patient care (15).

The difficulty in diagnosis of PD based on motor symptoms

Current studies about treatment or prevention may be focused on finding specific early symptoms (228, 229) or biomarkers (230-232) for diagnosis and potential inhibition of disease progression. However, to date, there is no biomarker for early diagnosis, and unfortunately, motor symptoms such as resting tremor, rigidity, posture impairment in PD are found in other CNS degenerative diseases such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), dementia with LBs, essential tremor, and drug-induced Parkinsonism. Therefore, it has been reported that 6-8% of misdiagnosis is attributed to neurology specialists and 47% of misdiagnosis due to general practitioners diagnosing PD with only movement symptoms (233-238).

Current imaging diagnostics for early stage PD

Currently, techniques for early diagnosis used clinically include imaging and biomarker detection using biologically derived samples. Diagnostic methods using imaging equipment include single photon emission computed tomography (SPECT), and positron emission tomography (PET) using radioactive isotopes, and magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), and transcranial sonography (TSC) without radioactive isotopes (239).

SPECT and PET

For SPECT and PET using radioactive isotopes, changes in dopaminergic nerve pathways such as the SN and striatum are identified and used in diagnosis. SPECT is generally less expensive than PET, facilitating its application in clinical trials. Although the dopamine activity is reduced in patients at early stages of PD (240), it cannot differential diagnosis of, such as MSA, PSP, and CBD (241). However, PET has recently been approved as a method for the diagnosis of PD by the USA FDA [^{18}F]F-DOPA has been used to measure the activity of *L*-DOPA decarboxylase. Various radioisotope markers such as iodine ([^{123}I]FP-CIT) and technetium ([^{99m}Tc]TRODAT) as well as fluorine ([^{18}F]FP-CIT) related to dopamine transporter in dopamine neurons were used clinically (242-244). In particular, most studies using dopamine transporters reported a sensitivity higher than 95% and a specificity of 90-100%, and suggested that changes in intake of dopamine transporters were related to the severity of motor symptoms (245). However, about 15% of patients with early PD show normal findings in PET images of dopamine carriers (246, 247).

MRI and MRS

MRI that does not use radioactive isotopes is used for diagnosis by identifying structural changes in PD-related region such as striatum or SN in the brain. It is particularly useful for differ-

ential diagnosis of atypical PD patients, and is used as an alternative diagnostic to SPECT or PET. Recently, functional MRI with blood oxygen level dependent signals has been used as an indirect diagnostic method because the signal changes and pathological mechanisms are not fully understood (248). MRS is another diagnostic method using nuclear magnetic resonance for identifying metabolic changes in the brain. MRS is useful not only for diagnosis but also identification of effective biomarkers (249, 250). In addition, several studies have reported that it is particularly useful in differentiating PD and other neurodegenerative diseases that exhibit similar movement symptoms (251).

TCS

TCS, another diagnostic method that does not use radioactive isotopes, focuses on the discovery of hyperechoic images in the SN of PD patients. van de Loo et al. (252) reported showed a diagnostic sensitivity of 79% and a specificity of 81%. Differential diagnosis is possible with diseases showed similar motor symptoms of PD based on the change in the echo of the basal nucleus or lens nucleus except the SN (253, 254).

Biomarkers in biological fluids for early diagnosis of PD

Research is ongoing to explore the specific biomarkers for early

diagnosis of PD. However, unfortunately, biomarkers for accurate prediction of PD outbreaks in preclinical stages applicable in clinical trials have yet to be identified. In general, biomarkers are used to evaluate pathophysiology, and pharmacological responses to treatment attempts. Many studies have been conducted using cerebrospinal fluid (CSF) and blood to detect biomarkers for differential and early diagnosis of PD or other diseases with Parkinsonian symptoms, however, the results were inconsistent due to the large heterogeneity of the sample population and the method of obtaining non-standardized samples (255). Blood, saliva, and biopsy tissues are sources of realistic biomarkers, because cerebrospinal fluid is limited by the risks and costs associated with obtaining a specimen.

Biomarkers related oxidative stress in CSF and serum

Biomarkers for early diagnosis of PD to date can be divided into oxidative stress-related biomarkers, which are related to pathogenesis and progression of PD, and biomarkers related to abnormal protein accumulation and aggregation. Representative biomarkers related to oxidative stress include DJ-1 protein, uric acid, homocysteine (Hcy), and 8-hydroxydeoxyguanosine (8-OHdG) (Table 8).

DJ-1

DJ-1 protein has a neuroprotective effect against oxidative stress,

and DJ-1 dysfunction is known to induce various oxidative stress-related diseases including PD (256). In addition, several studies have been conducted in relation to the DJ-1 concentration and PD symptoms in the blood (257) or CSF (258), as mutations in the DJ-1 gene are associated with hereditary PD (259). In an early study, it was found that the concentration of DJ-1 in CSF or plasma in PD patients was higher than in the normal control group and was proportional to the Hoehn-Yahr (H & Y) score, another clinical diagnostic measure. However, studies using recent techniques have shown that the concentration of DJ-1 in plasma (260) and CSF (261) of PD patients is lower than that of normal controls, possibly due to errors in sample acquisition and purification in that most of the DJ-1 occurs in hemoglobin and platelets of red blood cells. Although the relationship between the progression of PD disease and DJ-1 is not clearly known (262), it is considered as an important potential biomarker for the diagnosis of PD based on the results showing that the DJ-1 concentration in PD patients is lower than the CSF of patients with AD or MSA, other neurodegenerative disorders (263).

Uric acid

It is known that uric acid has an antioxidant effect as the free radicals (264), such as reactive oxygen species, produced during an inflammatory reaction presumed to be the cause of PD, generate oxidative stress and necrosis of dopamine neurons in the

SN (265). *In vitro* experiments revealed that uric acid not only prevents DAergic neuronal necrosis caused by oxidative stress, but also prevents degeneration. Studies using rat (266) and mouse (267) 6-OHDA models demonstrate reduced 6-OHDA toxicity and amelioration of behavioral symptoms or histological conditions when high levels of uric acid in the body are administered externally or through genetic modification. Based on the above results, uric acid concentration and pathogenesis progression rate were inversely proportional to the results of preclinical studies, confirming that uric acid can be used as a biomarker in CSF. It was confirmed that PD patients with low blood uric acid levels scored much higher on UPDRS than normal controls (268).

Hcy

It is known that high levels of plasma Hcy are commonly associated with vascular disease or AD, and metabolic diseases such as vitamin B12 and folic acid deficiency (269-271). In particular, the high concentration of plasma Hcy was also reported in PD patients treated with *L*-DOPA and Hcy concentration in CSF was also reported to be high before treatment with *L*-DOPA or compared to the normal control group (272). However, *in vitro* and *in vivo* experiments revealed that DAergic neuronal necrosis in the SN due to the toxic effects of plasma Hcy (271, 273). The high concentrations of Hcy accelerate oxidative stress in DAergic

neurons (274). In patients with degenerative brain diseases such as mild cognitive impairment, AD, and cerebral amyloid angiopathy, as well as PD, plasma levels of Hcy were higher than in normal controls, as a screening or diagnostic indicator of PD, in which the plasma levels of Hcy are the highest (275).

8-OHdG

8-OHdG is known as the best biomarker of DNA damaged due to oxidative stress. Some studies have reported an increase in the concentration of 8-OHdG in the SN of PD patients in that oxidative damage of DNA plays a significant role in PD etiology (276). The CSF and serum 8-OHdG levels in PD patients were higher than in normal controls (277, 278). In addition to CSF and serum, the concentration of 8-OHdG in urine was also significantly higher in PD patients than in the normal control group, and correlated with the H & Y score. A very high correlation of 8-OHdG with hallucinations, one of PD non-motor symptoms, suggests its role as a reliable biomarker for early diagnosis of PD (279).

Table 8. Biomarkers related to oxidative stress in human

Item	Source	Concentration of PD patient group compared to normal group	References
DJ-1	CSF	↓	Shi et al., (260)
	Plasma		Hong et al., (261)
Uric acid	Blood	↓	Boushel et al., (269)
Hcy	Plasma	↑	Irizarry et al., (275)
8-OHdG	CSF	↑	Carcia-Moreno et al., (278)
	Serum		Gmitterová et al., (277)
	Urine		Hirayama et al., (279)

Biomarkers related to abnormal protein accumulation and aggregation in CSF and serum

Representative biomarkers related to abnormal protein accumulation and aggregation include α-synuclein (α-syn), ubiquitin C-terminal hydrolase-L1 (UCH-L1), and nerve fiber light chain protein (NFL) (Table 9).

α-syn

α-syn is the most representative biomarker. It regulates synaptic formation, although its normal physiological role is unclear. In PD, it is well known as a key element of LBs, which are aggregates of α-syn in neurons. In addition, α-syn is a result of the expression of the *SNCA* gene, known as the cause of genotype PD. It is known that phosphorylation, misfolding, and abnormal accumulation of α-syn play an important role in PD etiology. It was found that the concentration of α-syn in the CSF of PD patients was significantly lower than in the normal control group (280-282), and showed a negative correlation with the H & Y score (283). In addition, when the ratio of total α-syn and oligomeric form α-syn was compared between the PD patient and the normal control group, the results showed that the oligomeric form of α-syn in cerebrospinal fluid increased significantly in PD patients with high specificity and sensitivity (284). Phosphorylated α-syn concentrations were higher in PD patients than in normal controls (285-287). Also, phosphorylated α-syn induces the death

of neurons (288) as the disease progresses, since the concentration of soluble α-syn and non-phosphorylated α-syn in plasma decreases in PD-related brain regions. Instead, the concentration of non-soluble α-syn and phosphorylated α-syn is increased, suggesting that the phosphorylated α-syn more clearly reflects the PD condition (289). In addition, the phosphorylated α-syn concentration in CSF is also considered as a useful marker for early diagnosis of PD, based on the results of higher levels in patients with PD than MSA or PSP as well as normal controls (290).

UCH-L1

UCH-L1 is a specific protein found in the brain and is known for its role in removing abnormal proteins in the cytoplasm of neurons, and is related to α-syn metabolism, a key factor in LBs causing the death of neurons. UCH-L1 is a marker of PD diagnosis, showing high sensitivity and moderate specificity (89%, 67%, respectively). The concentration of UCH-L1 in the CSF of PD patients was reduced compared with the normal control group. In particular, when compared with other degenerative brain diseases such as MSA or PSP, the concentration was the lowest (291, 292). In addition, UCH-L1 concentration in CSF is highly correlated with α-syn concentration, suggesting its benefit in early diagnosis if two markers are used together.

NFL

Nerve fibers play a very important role in neuronal function as a key element in the axonal structure of CNS and peripheral nerve system. Abnormal phosphorylation of nerve fibers was found in PD patients (293, 294). NFL, a key factor in neuronal signal transmission and morphology, was recognized as a potential biomarker associated with myelinated axon degeneration, but the concentration of NFL in serum as well as cerebrospinal fluid in actual PD patients was similar to the normal control (295, 296). Rather, it was observed that the NFL concentration in the CSF of patients with other degenerative brain diseases such as PSP, MSA, and CBD was increased (297, 298), suggesting that the NFL concentration associated with PD was not increased since PD rarely occurred in degenerative axons unlike other degenerative brain diseases indicated above. Therefore, it is difficult to use NFL alone as an early diagnostic biomarker of PD, but it facilitates differential diagnosis of other degenerative brain diseases.

Table 9. Biomarkers related to abnormal protein accumulation and aggregation in human

Item	Source	Concentration of PD patient group compared to normal group	References
Total α-syn	CSF	↓	Mollenhauer et al., (280) Park et al., (281) Parnetti et al., (282)
Oligomeric form / total α-syn	CSF	↑	Brggink et al., (284)
Phosphorylated α-syn	CSF	↑	Wang et al., (290)
	Plasma	↑	Foulds et al., (286) He et al., (287)
soluble α-syn	Plasma	↓	Foulds et al., (289)
UCH-L1	CSF	↓	Jiménez-Jiménez et al., (291) Mondello et al., (292)
NFL	CSF	-	Constantinescu et al., (293) Hansson et al., (294)

Conclusion

PD is a degenerative disease that exhibits characteristic motor symptoms due to necrosis of DAergic neurons in the SN. The disease mechanism is attributed to mitochondrial damage caused by oxidative stress, but a clear cause has yet to be identified. *L*-DOPA had been used to treat PD traditionally. However, it alleviates movement symptoms and slows the disease progression without resolving PD. DBS is a surgical treatment; however, it is limited in application in that it cannot be used for all patients. Stem cell therapy is being studied as an alternative to previous treatments. Animal studies are needed to analyze the causes, development and progression of PD, as well as therapeutic or prognostic drugs. PD animal models have been developed using various methods such as toxin administration and genetic manipulation of various animal species. However, it is very important to develop a suitable animal model expressing the characteristics and effects of living cells used as a cell therapy agent can be expressed because most animal models provide conditions conductive to investigation of existing chemical therapeutics or preventive drugs.

CHAPTER I

Establishment of a novel Parkinson's disease model in common marmoset for cell therapy evaluation

ABSTRACT

Since animal models of Parkinson's disease (PD) are useful research tools to investigate human patients, it is most appropriate and important to select the optimal research model for treatment or prevention. Because most of the characteristics of PD patients can be expressed, MPTP is mostly used to generate various animal model of PD, including NHP. In the case of a NHP model, various methods have been introduced depending on the experimental purpose. However, acute dosing is associated with a high incidence of early deaths due to the toxicity of MPTP itself. PD symptoms and lesions do not appear completely at low doses administered long term. In addition, most of the known method using MPTP are models suitable for short-term research and not for experiments that require sufficient time, such as cell or tissue transplants. Based on these findings, a new method of subcutaneous treatment using "2-2-1-1-1" mg/kg MPTP was administered to common marmosets (*Callithrix jacchus*) with stable PD symptoms over a long-term period without animal death. After MPTP treatment, stable clinical symptoms were observed continuously based on evaluation criteria of 10 or higher in daily observation. Based on the tower test, marmosets did not show an elevation of 5.61 ± 0.72 levels compared to levels before MPTP administration. In the striatal PET image, radioactivity after treatment decreased by 33.35 ± 1.23% compared to levels before

MPTP treatment. Immunohistochemistry showed a loss of TH-positive cells and fibers in the SN after MPTP treatment. It is proposed that the marmoset model developed by the novel MPTP treatment method may be an optimal model for studies requiring long-term cell transplantation.

Keywords: Parkinson's disease; MPTP; common marmoset; nonhuman primate; animal model

INTRODUCTION

Pakrinson' disease (PD) is a representative degenerative brain disease occurring in the elderly (5). The exact cause of the onset is still unknown, but the mechanism known to date entails oxidative stress, such as inflammation caused by endogenous or exogenous factors, in the mitochondria of dopaminergic (DAergic) neurons in the substantia nigra (SN). The damage induces necrosis of nerve cells manifested by tremors, limb rigidity, bradykinesia, and gait disorder as clinical symptoms (13).

Various *in vitro* (21, 299, 300) and *in vivo* (301-304) models have been developed to evaluate therapeutic and preventive drugs as well as investigate the pathogenesis of PD. The *in vitro* model targeting DAergic neurons is highly economical and reproducible. In contrast, the patient environment is in a state of harmony with various systems rather than mediated via signaling between several types of cells and cells alone. Therefore, *in vivo* studies are essential (305,306). The PD animal model was developed by treatment with another neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or rotenone, starting with a rat model developed injected with the neurotoxin 6-hydroxydopamine (6-OHDA) in the brain in 1970. A genetically engineered animal model has been developed and used since the late 1990s. To date, about 95% of studies using PD animal models have used rodent models such as mice and rats, which are still used in several recent

studies. The rat model was used more than the mouse model to test neurotoxins such as 6-OHDA or MPTP mainly used from 1970s to 1990s. However, since 1990s until recently, genetic manipulation technology was mainly used, and the mouse model was used more than the rat model. Also, in view of the ethical aspects and the ease of managing animals, few studies used animal species other than vertebrates such as fruit flies (*Drosophila*) or *Caenorhabditis elegans* (*C. elegans*), but the progress is steady (307-309).

The nonhuman primate (NHP) model of PD, which is associated with anatomical and histological advantages and genetic similarity, has been developed and used since the 1980s to overcome the difficulties associated with existing rodent models in terms of expression, evaluation or interpretation of clinical symptoms (310, 311). The cicadian rhythm of rodents is opposite to that of humans or NHP, which complicates the evaluation of parkinsonian symptoms during the day when rodents are naturally inactive (312-314). It is difficult to identify abnormal posture or gait abnormalities in the rodent, a quadruped, compared with humans, and it is difficult to evaluate motor symptoms caused by limb tremor or rigidity, which is especially important in human patients. The NHP model is very useful in evaluating non-motor symptoms such as hallucinations and emotional relief as well as motor symptoms (315, 316). The NHP model similar to the rodent model, uses a neurotoxin, for genetic manipulation, and an aging

method, which is applicable to most of the elderly human patients with PD (317, 318).

A wide variety of strains have been used to develop NHP models, from old world monkeys such as cynomolgus monkeys (*Macaca fascicularis*) and rhesus monkeys (*M. mulata*) to new world monkeys such as common marmosets (*Callithrix jacchus*) and squirrel monkeys (*Saimiri sciureus*) (319, 320). Marmosets show a high degree of genetic similarity to humans when compared to rodents such as rats and mice, and anatomically, the rodent striatum has a single structure. In contrast, other NHP, including marmosets, show the most distinctive features of differentiation between caudate and putamen in their capsule, similar to humans, in the study of central nervous system (CNS) degenerative disorders such as PD and differences in dopamine function closely related to PD mechanism and dopamine neuron distribution in the SN (321-323). When compared with other old world monkeys such as the cynomolgus monkey, marmosets are very small (about 350-450 g) and can be easily handled. Also, it is possible to survive for up to 15 years in laboratory conditions. They generally procreate twins every 5 months even in the experimental group (324-326). In addition, it is possible to use an stereotaxic device for rodents by utilizing the already standardized marmoset brain atlas, Therefore, it can be very useful for PD research compared with the old world monkey that requires magnetic resonance image (MRI) or computed tomography (CT)

images in the 6-OHDA model or cell transplantation studies requiring intracranial injection (327-330).

The NHP model using marmoset was developed and various models were used according to the purpose of the study. Since human PD patients manifest bilateral symptoms, the MPTP treatment model, which is most similar to the unilateral 6-OHDA model, is the most common. In addition, in the absence of differences in sensitivity to MPTP between individuals compared with other NHP strains, a standardized method of acute systemic treatment for MPTP dosage and frequency has been introduced (331) (Table 10). Since the common marmoset models developed with these protocols shows clinical symptoms immediately after MPTP treatment, it was mostly used to conduct short-term studies lasting 8 weeks to develop chemical treatments, such as *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) and its derivatives as the "golden rule", which are immediately recognized upon treatment, and the development of adjuvant therapy for dyskinesia following administration of *L*-DOPA.

Table 10. Various MPTP treatment methods used in common marmoset PD models

Routes	Doses	Duration	Study periods	References
SC	1 mg/kg	8 d	2 wk	Philippens et al. (332)
	2 mg/kg	5 d	2-3 wk	Smith et al. (333)
			30 d	Fox et al. (151)
			6-8 wk	Hansard et al. (334)
			8 wk	Iravani et al. (98)
			10-12 wk	Hansard et al. (335)
			12 wk	Fox et al. (99)
			15 wk	van der Stelt et al. (336)
IP	2-4 mg/kg	4 d	10 d	Jenner et al. (65)
	2-2-3-3-3 mg/kg	5 d	4-5 wk	Rose et al. (96)
	0.5-4.5 mg/kg**	29 d	10 wk	Russ et al. (337)
	A. 6-22 mg/kg; B. 78-83 mg/kg	A. 3-7 d; B. 5 wk	12 wk	Ueki et al. (97)
	0.25-1.25 mg/kg	15 wk*	12 wk	Colosimo et al. (62)

SC: subcutaneously; IP: intraperitoneally, d: day; wk: week

* twice a week with MPTP treatment

** 15 doses of MPTP treatment (total dose 25 mg/kg)

Recent studies investigating disease treatment directly (restoration via differentiation and proliferation) or indirectly (modulation of immune reaction or differentiation and proliferation) using transplanted cells have been conducted continuously for various diseases as well as PD that are refractory to current chemical treatment methods or have side effects (338-341). Stem cells or differentiation-controlled progenitor cells, which are investigated as cell therapy products, require time to migrate, settle, proliferate, and differentiate at a site of recovery post-transplantation (342-345). A novel model is needed to stably express long-term clinical symptoms to establish the expected effect of the transplanted cells, instead of the marmoset PD model, which is suitable for short-term studies conducted before. Therefore, the purpose of this study is to establish a novel MPTP treatment protocol involving common marmosets to develop a model suitable for cell therapy by modifying the protocol used to develop the existing MPTP model.

MATERIALS AND METHODS

Animals

Four adult male common marmosets (*C. jacchus*) were obtained from CLEA Japan, Inc. During the experiment, all animals were maintained in cages (76.8 × 39.4 × 59.0 cm) individually under controlled conditions (23-28℃, 45-70% humidity, 12 h light/dark cycle). Animals were fed specialized diet supplemented with nutrients and preferably in commercial diets (Teklad New World Primate Diet 8794, Harlan, USA) and fresh fruits, with quail eggs additionally. To evaluate the animals health condition, body weight was measured daily during MPTP treatment and once a week after MPTP treatment. They were cared for and maintained according to the Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2011) in a facility accredited by AAALAC international (#001169). The animals were provided chewable rubber toys for enrichment. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (No. 13-0024).

MPTP-induced PD model

With reference to protocols for establishment MPTP-treated model, all animals were subcutaneously treated with 2 mg/kg MPTP HCl (M0896, Sigma-Aldrich, USA) dissolved in saline on the first 2 days and 1 mg/kg for 3 days (Figure 4) to develop

an appropriate model in preventing animals death during the experiment while stable and prolonged clinical symptoms were observed. In order to prevent possible damage due to environmental exposure to MPTP, the animals were managed in specially designed cages which were sealed under negative pressure after MPTP treatment. All procedures related to MPTP treatment were performed according to safety regulations. To confirm the induction of parkinsonian symptoms, all animals were subcutaneously treated with 15.6 mg/kg *L*-DOPA methyl ester HCl (D1507, Sigma-Aldrich, USA; equivalent to 12.5 mg/kg *L*-DOPA).

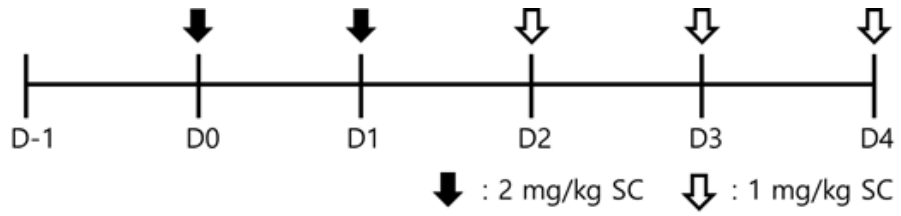


Figure 4. The scheme of MPTP treatment regimen. Black arrow: 2 mg/kg MPTP subcutaneous treatment at D0 and D1; white arrow: 1 mg/kg MPTP subcutaneous treatment at D2, D3, and D4.

Behavioral assessment

Daily observation

The behavioral assessment was conducted by recording the behavioral changes of animals in individual cages with a video camera and scoring by the observer according to the evaluation items (Table 11). The score of each item increased according to the severity of symptom expression, and the sum of the scores of each item exceeding 10 was selected to establish the PD model.

Tower test

The tower test developed by Verhave and colleagues (346) were partially modified and adapted to evaluate motor function involving head and limb movement and spatial perception of animals. The evaluation tool was made of transparent and solid acrylic material (27.0 × 27.0 × 151 cm). Seven wooden cylindrical ladders were equipped with three-dimensionally staggered positions, with the distance between each ladder ranging from 8 cm to 32 cm. All animals were fully trained until the last ladder was reached before MPTP treatment to apply the tower. During the evaluation after MPTP treatment, animals were removed from the cage and brought to the entrance at the bottom of the tower. The number of ladders climbed by the animals for 7 minutes was measured and recorded by a video camera in a separate quiet room.

Behavioral assessments after *L*-DOPA administration

To investigate the model establishment based on symptom recovery following *L*-DOPA administration, a behavioral assessment in cages was conducted and tower test was conducted at intervals of 0, 0.5, 1, 2, 4, 6, 12, and 24 h after *L*-DOPA administration.

Table 11. Evaluation items and scores of MPTP-treated common marmosets

Observation item	Score*
Alertness	0-1
Reaction to stimuli	0-3
Blinking	0-1
Checking movement	0-2
Posture	0-4
Motility	0-3
Vocalization	0-2
Tremor	0-1
Fur condition	0-1

*: 0; normal or absence, 1; mild, 2; moderate, 3 or 4; severe

PET imaging analysis

Positron emission tomography (PET) images using ^{18}F-FP-CIT, a dopamine transporter (DAT)-binding ligand, were obtained to confirm changes in striatal dopaminergic function in the common marmoset's brain over time before and after MPTP treatment. PET images were acquired before MPTP treatment and periodically after MPTP treatment (Figure 5). All animals were injected with 1.5 mCi ^{18}F-FP-CIT via the saphenous vein in the awake state, and were treated with injectable anesthetics (ketamine, 10 mg/kg) and analgesic (xylazine, 4 mg/kg) intramuscularly and inhaled gas anesthetics (isoflurane, 1-2%) while acquiring PET images with a dynamic LIST mode using the PET scanner (GE eXplore Vista PET/CT, GE Healthcare, USA) for 60 minutes. All PET images were reconstructed in advance by a PET equipment manufacturer, and processed based on information such as the type of radioisotope and CT images obtained before PET images were acquired. The radioactivity was measured by setting the striatum region as an region-of-interest (ROI) based on reconstructed PET transverse images using an analytical tool provided by the PET equipment manufacturer.

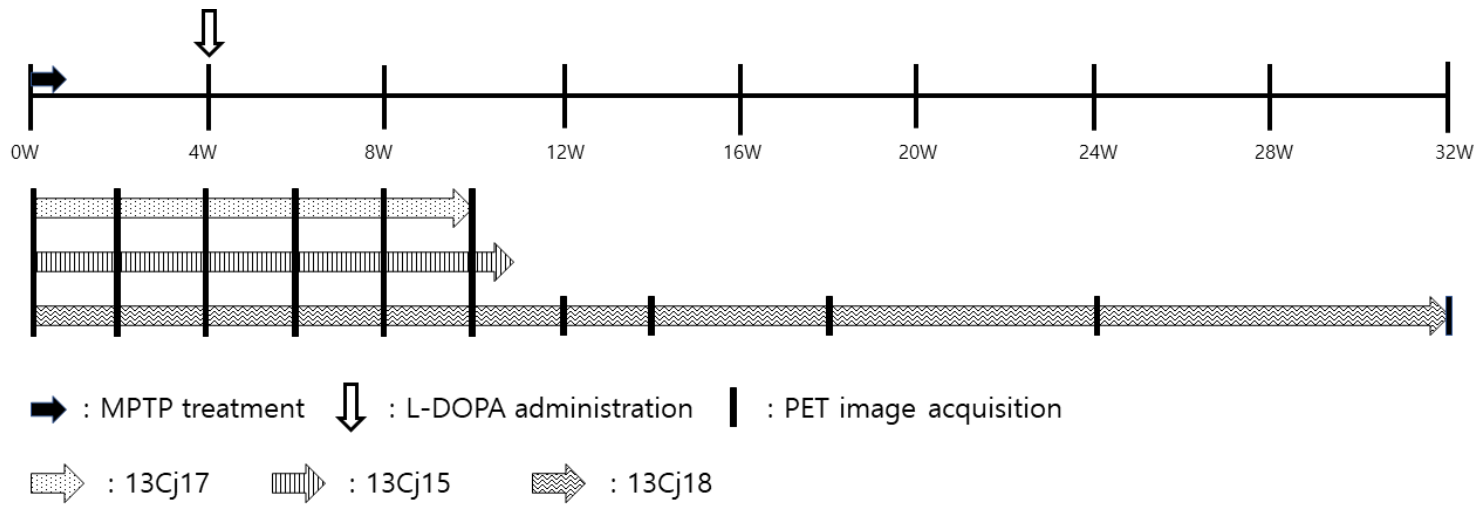


Figure 5. The experiment protocol. Black arrow: "2-2-1-1-1" mg/kg MPTP subcutaneous treatmentfor 5 days; white arrow: 12.5 mg/kg *L*-DOPA subcutaneous administration at 4 weeks after MPTP treatment; black bar: ^{18}F-FP-CIT PET image acquisition at 0, 2, 4, 6, 8, 10, 12, 14, 18, 24, and 32 weeks after MPTP treatment.

Microscopic assessment

All animal were induced to a state of shallow anesthesia by intramuscular administration of anesthetics (ketamine, 10 mg/kg) and analgesics (xylazine, 4 mg/kg) prior to blood collection. Animals were sacrificed after blood collection from the posterior vena cava to obtain brain tissue under respiratory anesthesia (isoflurane, 1-2%). The removed brain was incised into the middle and divided into left and right hemispheres, and each tissue was fixed in 10% neutral buffered formaldehyde solution for 72 h. Tissues were processed into paraffin-embedded blocks and tissue slices were sectioned. Immunohistochemistry (IHC) was performed for the detection of tyrosine hydroxylase (TH) in striatum and substantia nigra. Immunohistochemical staining was conducted with rabbit polyclonal anti-TH antibody (Abcam, ab117112, UK) and Discovery XT Automated IHC stainer using ChromoMap DAB detection kit (Ventana Medical System, USA) according to the manufacturer's protocols.

Statistical analysis

Behavioral assessment and tower test results after *L*-DOPA administration to MPTP-treated common marmosets and results of ^{18}F-FP-CIT radioactivity in the PET image of the striatum region are presented as mean ± standard deviation (SD). These results were analyzed with independent Student's *t*-test and multiple analyses with Tukey/Duncan test using SPSS 19 (IBM,

Chicago, IL, USA). The probability level for statistical significance was set to 0.05.

RESULTS

Stable parkinsonian symptoms without death for 32 weeks after MPTP treatment with novel method

The behavioral assessment of three MPTP-treated marmosets and one untreated marmoset was conducted and scored depending on: alertness, reaction to stimuli, eye blinking, checking movement, posture, motility, vocalization, tremor, and fur condition. During the MPTP treatment period, akinesia or slowness movement, rigidity, and postural abnormality were mainly observed. After the MPTP treatment, both resting and active tremors were observed characteristically, and eye blinking was also significantly increased. Importantly, no mortality was detected in any MPTP-treated marmosets during the experiment. In particular, these parkinsonian symptoms were observed continuously until the end of the experiment (up to 32 weeks), and with constant severity (a score > 10). Notably, tremors and postural instability were observed at high levels for 32 weeks in MPTP-treated marmosets, in relation to the clinical motor symptoms seen in human PD patients (Figure 6).

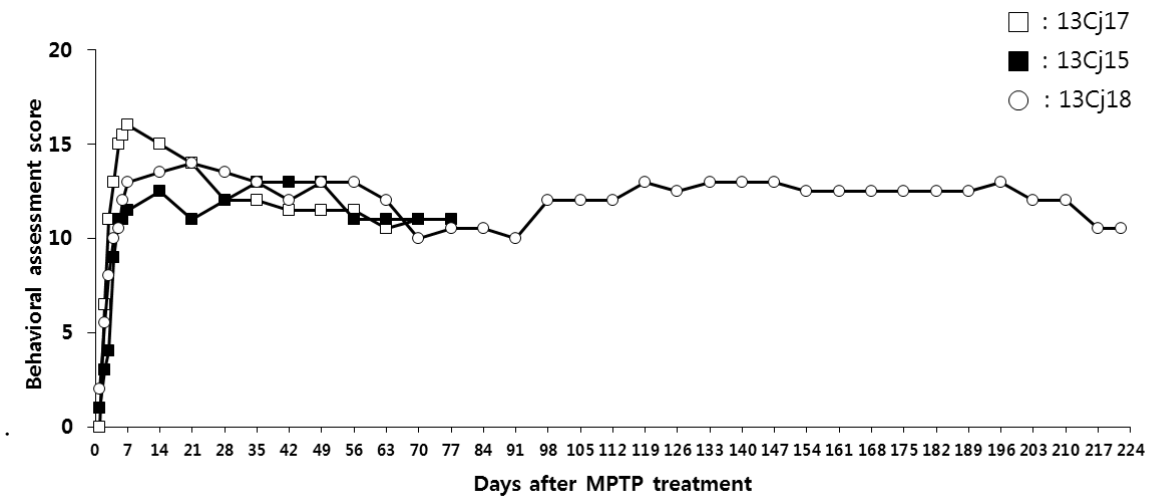


Figure 6. Behavioral assessment score of MPTP-treated common marmosets. All marmosets showed clinical symptoms immediately after MPTP treatment, and stable parkinsonian symptoms (a score > 10) without death until the end of the experiment after completing the MPTP treatment. Two marmosets (13Cj17 and 13Cj15) were euthanized at 10 and 11 weeks of MPTP administration, respectively, to confirm the establishment of the model.

In addition, these symptoms induced by MPTP treatment prevented normal intake of water and feed. Intensive management such as forced feeding was performed based on the average daily feed intake and body weight was measured daily. As a result, the body weight of the MPTP-treated marmosets was maintained without significant difference until the end of the experiment (The initial body weights: 367.0 ± 36.72 g; the final body weights: 364.7 ± 55.05 g) (Figure 7).

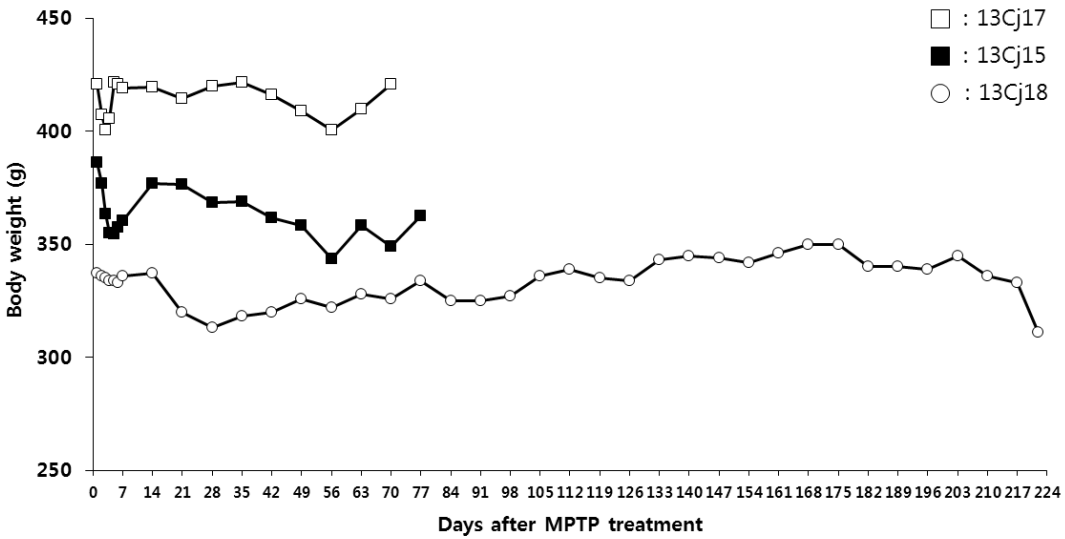


Figure 7. Changes in the body weight of MPTP-treated common marmosets. There was no significant difference between the initial and final body weights of the experiment under appropriate feed and water supply via forced feeding. Two marmosets (13Cj17 and 13Cj15) were euthanized at 10 and 11 weeks of MPTP administration, respectively, to confirm the establishment of the model.

Motor dysfunctions without recovery for 32 weeks after MPTP treatment with novel method

The tower test was conducted to evaluate changes in motor function such as limb motion and rigidity, and postural abnormality, following MPTP treatment. The tower test is a method of evaluation based on the instinct marmosets to climb to a higher place. MPTP treatment was used after confirming the highest level reached by all animals based on for about 4 weeks before MPTP treatment. After MPTP treatment, each animal was provided a week of fixed and regular time, and the highest level climbed for 7 minutes was recorded and animal movements were recorded with a video camera during evaluation. MPTP-treated animals climbed only 1 to 3 of the 7 levels on average for 7 minutes until the end of the experiment. In particular, the motor function indicated by climbing for 32 weeks was not recovered and the climbing level was maintained (Figure 8).

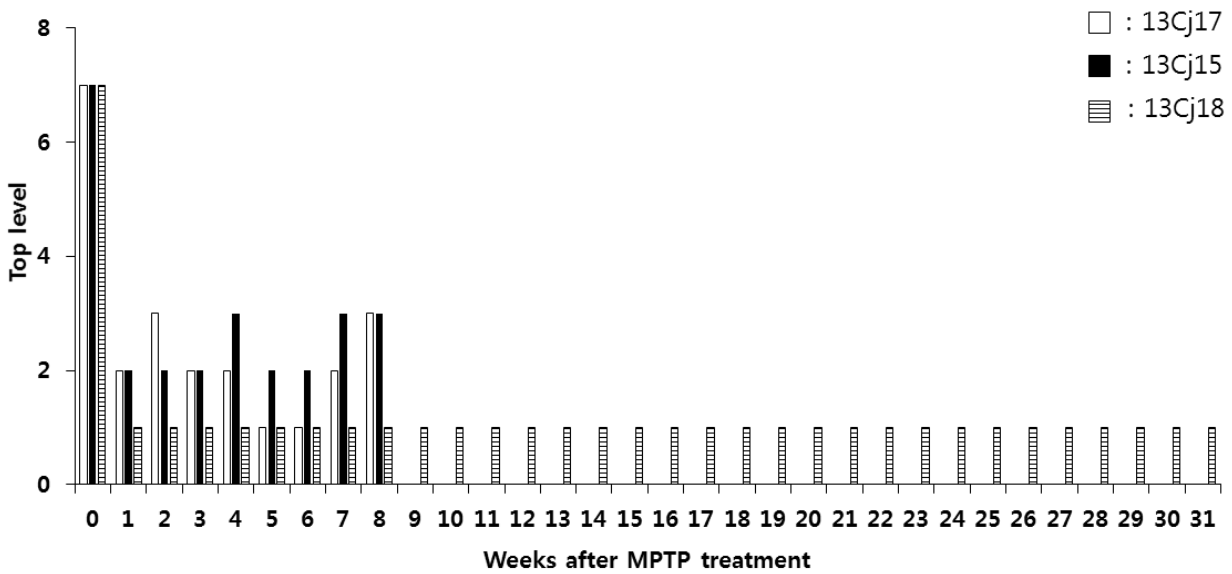


Figure 8. Tower test results of MPTP-treated common marmosets. After MPTP treatment, all marmosets failed to the high level as before due to motor dysfunction, which persisted without recovery for 32 weeks. Two marmosets (13Cj17 and 13Cj15) were euthanized at 10 and 11 weeks of MPTP administration, respectively, to confirm the establishment of the model.

Amelioration of clinical symptoms temporarily after administration of L-DOPA following MPTP treatment

In order to confirm the recovery of motor symptoms by *L*-DOPA, which is currently used widely as a standard therapy in PD patients, MPTP-treated marmosets were subjected to behavioral assessment and tower test hourly after *L*-DOPA administration. Motor dysfunction such as limb and trunk tremor and postural imbalance improved markedly between 2 and 4 h after *L*-DOPA administration. In addition, tower test results increased to an average of up to 6 levels, similar to pre-MPTP treatment. However, motor abnormality was observed again. It was confirmed that the tower test result was also lowered 1 to 2 levels from 6 to 24 h after *L*-DOPA administration (Figure 9).

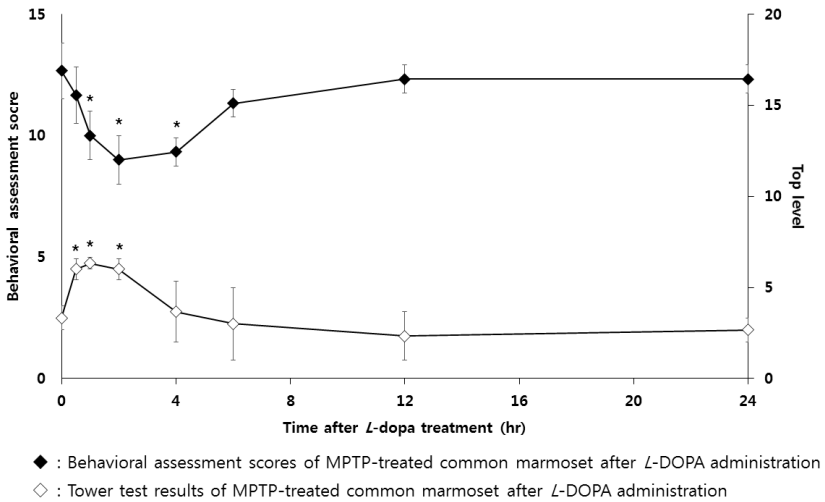


Figure 9. Behavioral assessment and tower test result of *L*-DOPA administration to MPTP-treated common marmosets. MPTP-treated marmosets (n = 3) administered with *L*-DOPA showed clinical relief from 2 to 4 hours after administration. However, clinical symptoms appeared again 6 hours after *L*-DOPA administration. Asterisks indicate significant differences between pre- and post-administration of *L*-DOPA, $*P < 0.05$.

Lower radioactivity in the striatum based on ^{18}F-FP-CIT PET images without recovery for 32 weeks after MPTP treatment with novel method

In human PD patients and MPTP-treated animal models, the DAergic pathway in the striatum and the SN was damaged, and radioisotope ligands that specifically bind to DAT were already used clinically for PD diagnosis. Among these ligands, ^{18}F-FP-CIT, which has been recently used in clinical practice, was used to obtain striatal PET images of MPTP-treated marmosets at 0, 2, 4, 6, 8, 10, 12, 14, 18, 24, and 32 weeks. Before the MPTP treatment, ^{18}F-FP-CIT showed a high affinity to the striatum, and decreased significantly after MPTP treatment. The radioactivity of ^{18}F-FP-CIT in the striatal PET image did not increase and remained low until 32 weeks (Figure 10).

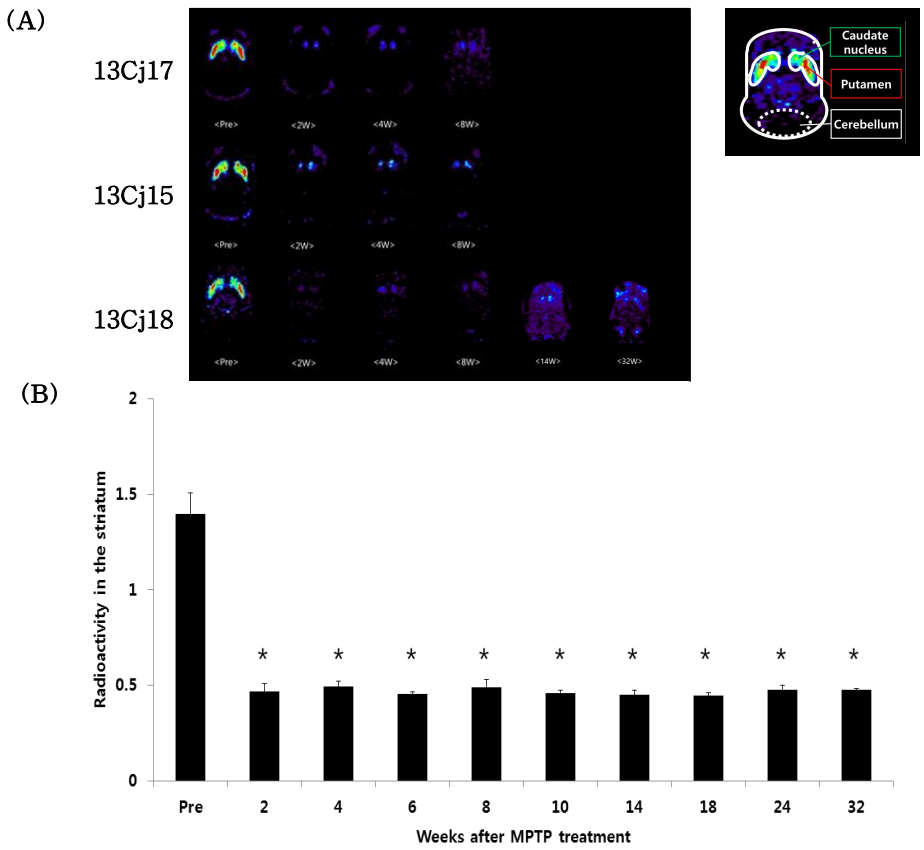


Figure 10. The striatal ^{18}F-FP-CIT PET images and schematic diagram (A) and radioactivity changes (B) after MPTP treatment in common marmosets. The radioactivity of ^{18}F-FP-CIT in the striatum was low in PET images of all MPTP-treated marmosets (n = 3), and remained low for 32 weeks without significant change. Asterisks indicate significant difference between pre- and post-MPTP treatment, $*P < 0.05$.

Loss of tyrosine hydroxylase-positive cells and fibers in the SN and striatum after MPTP treatment with novel method

It is well known that the death of DAergic neurons in the SN of human PD patients and MPTP-treated animal models, and the reduction in TH, which catalyzes the convert *L*-tyrosine to *L*-DOPA, dopamine precursor, are the histopathologic hallmarks of PD. The results of anti-TH IHC show a significant reduction in TH immunoreactivity of the SN in MPTP-treated marmosets compared with marmoset without MPTP treatment. IHC results of brain tissue obtained from marmoset after 32 weeks of MPTP treatment were also significantly reduced compared with un-treated marmosets (Figure 11).

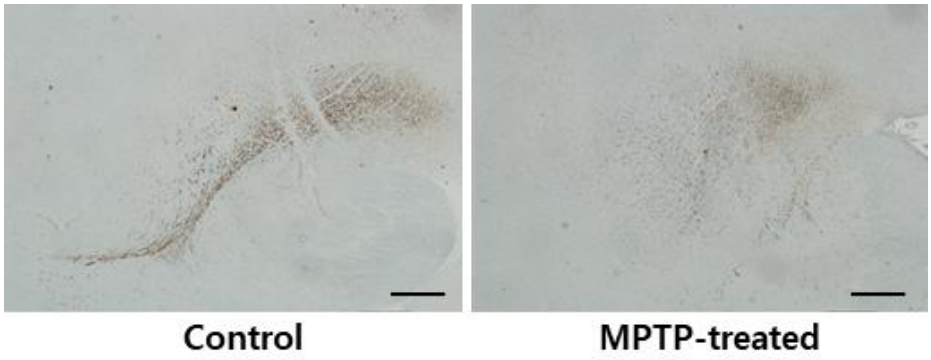


Figure 11. TH Immunohistochemistry in the SN of MPTP-treated common marmosets. TH-positive cells were significantly decreased in the SN of marmoset 32 weeks after MPTP treatment, compared to the untreated control animal; scale bar: 1 mm.

DISCUSSION

A variety of methods have been used to develop PD models of NHP by MPTP treatment, including both old and new world monkeys according to the study goals (15). Various methods to generate MPTP models using common marmosets have been introduced (Table 1). Most of the methods used to develop MPTP-treated marmoset model are acutely toxic, resulting in symptoms of motor dysfunction in severe case of human PD within a short period of time. It was a model suitable for the study of therapeutic agents designed to ameliorate motor symptoms or a prevention of dyskinesia caused by *L*-DOPA administration, which is a basic option as a PD treatment. Subcutaneous injection of 2 mg/kg for 5 consecutive days, which is a widely used method to develop the marmoset model of PD, induces various motor dysfunctions such as akinesia, rigidity, tremors (both resting and active), and postural abnormality) at the end of MPTP treatment, and these parkinsonian symptoms stabilize after 6-8 weeks of treatment completion (347). However, some reports shown that symptoms stabilize over longer periods (348, 349).

Among the various known methods used to develop animal models of PD, neurotoxins such as MPTP, 6-OHDA, and rotenone treatment are most widely used. MPTP-treated models are similar to most cases of human PD. In particular, MPTP-induced

marmoset model shows no spontaneous recovery even after a long period of time compared to old world NHP primate models such as cynomolgus monkey (*M. fascicularis*) model. Since the type and severity of symptoms expressed by various MPTP treatment regimen vary, it is the most basic task to develop and select an appropriate MPTP treatment regimen for MPTP model suitable for the experimental need. Although it is a major problem in the production of a mouse MPTP model, reports of animal death within 24 h after administration of the first high dose are attributed to a cardiovascular side effects of MPTP (350). In order to minimize mortality or other side effects, methods of dispensing MPTP treatment multiple times have been mainly used to develop the MPTP marmoset models. Although 2 mg/kg body weight is the basic daily dose over a period of several months, the model establishment may fail due to excessive toxicity during MPTP treatment when the final accumulated dose of MPTP is excessive (4 repetitions of 2 mg/kg SC for 3 consecutive days at several monthly intervals; total cumulative doses: 12-20 mg/kg) (351). However, when used at low concentrations to prevent MPTP toxicity, no or partial motor symptoms were detected in MPTP marmoset models (1 mg/kg SC for 3 or 5 consecutive days) (352,353). Even if MPTP was administered for more than a week (1 mg/kg SC for 8 consecutive days), insufficient clinical parkinsonian symptoms were associated with delayed apoptosis of DAergic neurons in the SN, suggesting the absence of defective

feed or drinking in the models (332). Based on these prior report, a novel 2-2-1-1-1 mg/kg MPTP treatment regimen was established for marmoset PD model to evaluate clinical motor symptoms over a long time because of the limited toxicity caused by MPTP. As a result, all models survived without death during the 32 weeks of experimental period, and a model was developed to monitor stable motor symptoms without signs of spontaneous recovery.

Although no animal deaths occurred during the experiment period as a result, marmoset models failed to consume adequate feed and drinking water due to severe motor symptoms, especially akinesia and bradykinesia, during the first two days of treatment with 2 mg/kg MPTP. Because parkinsonian symptoms interfere with essential physiological activities in MPTP models, intensive management is an important aspect of animal welfare and scientific strategy. Therefore, body weight was measured daily during MPTP treatment, and weekly to indirectly evaluate the health status of marmosets, and accordingly, models were intensively managed by determining the amount and frequency of feeding and drinking during the forced feeding. Feed and drinking water supplies underlying basic metabolism are essential to maintain MPTP models healthy, and can fundamentally exclude the possible causes other than MPTP treatment based on clinical symptoms observed. Basically, the forced feeding program is a liquid diet that is finely ground with a shredder after soaking fully

with double the volume of normal saline and 5% glucose fluid in a 1:1 ratio of the daily average amount of feed intake immediately before feeding. During the whole experiment, forced feeding was conducted in MPTP-treated marmosets, and the liquid diet was administered via a syringe with hand-restraint and spilled at the back of the animal tongue in small amounts to prevent aspiration pneumonia. In addition, fruits such as appels and bananas were ground finely just before feeding, placed in a syringe, and fed similarly. These forced feeding measures were implemented every four hours during the light-cycle period. Also, dehydration correction is an essential element in managing MPTP models, especially given that severe dehydration can have fatal consequences. Unlike old world monkeys such as cynomolgus and rhesus monkeys, marmosets have relatively small bodies and narrow blood vessel diameters. It is therefore very difficult to correct dehydration by parenteral methods, so a solution mixed with a normal saline and 5% glucose fluid of 1:1 was administered subcutaneously or orally.

Motor symptoms such as akinesia or abnormal posture are the main symptoms in human PD patients. However, in most PD cases, there are many non-motor symptoms such as insomnia, cognitive impairment, and hallucinations occur, in addition to constipation (354-357). Constipation was also found in rodent models treated with neurotoxins such as 6-OHDA (358) and rotenone (359, 360) and human α-syn transgenic mouse models (361 362),

especially in mouse (363, 364) and NHP (365) models treated with MPTP. Although the causes of constipation in human PD patients and MPTP animal models have yet to be determined, it is attributed to a complex set of abnormalities involving the dopamine nervous system of the digestive tract, and motor symptoms due to insufficient feed and drinking (366-368). In the previous study, MPTP-induced cynomolgus monkey models also underwent abdominal massages and treated with medication if necessary by closely monitoring the animal condition after forced feeding because of discomfort due to abdominal distention and excessive intestinal gas by constipation. Therefore, abdominal massage was used and medication if necessary in the MPTP marmoset model used in this study. Liquid diets including probiotics were administered to prevent or alleviate symptoms of constipation.

Recently, several studies including cell or gene therapies replaced conventional chemical therapy. Studies used undifferentiated cells such as autologous, allogenic, or induced pluripotent stem cells (iPSCs) to investigate musculoskeletal, cardiovascular, or neurological disorders, which are known to be difficult or impossible to regenerate or recover on their own (341). Several studies of PD therapy have also used MPTP-treated animal models, such as rodents and NHP models, to administer neural stem cells and others that can replace *L*-DOPA and other drugs-dosing methods or surgical methods such as DBS (369, 370). Since the late 1990s,

studies have been conducted on various NHP models of PD to determine the effects of iPSC transplantation as well as mesenchymal stem cells (Table 12). Most of the NHP PD models used in these cell transplantation studies w1ere treated with MPTP, and in the case of transplanted iPSCs, cells derived from humans and NHPs were used. However, Takagi et al., (212) reported the recovery of motor symptoms after 14 weeks by transplanting neural precursor cells differentiated by monkey ES cells into the cynomolgus monkey model treated with MPTP. Hallet et al., (217) reported the recovery of the motor symptoms after 6 months by transplanting autologous iPSCs derived from monkeys into a MPTP-treated cynomolgus monkey. Based on these studies, cell transplantation requires adequate time for proliferation or differentiation of transplanted cells. In addition, spontaneous recovery was observed 70 days after MPTP treatment in the cynomolgus monkey model, which was injected with a subcutaneous dose of 0.2 mg/kg every day for 14 days in a pilot study. Another study showed similar results in the MPTP-treated cynomolgus monkey model. However, reversible parkinsonian symptoms were also observed in the marmoset MPTP model. It is important to develop a primate MPTP model in which long-term symptoms are stable.

In conclusion, the novel MPTP treatment protocol of 2 mg/kg for the first 2 days followed by 1 mg/kg for 3 consecutive days, resulted in stable motor parkinsonism for 32 weeks without mortal-

ity in common marmosets based on behavioral evaluation, imaging diagnostics assessment, and histopathological investigation. Although follow-up studies are needed to corroborate the findings in this small animal study, it is a proof-of-concept study establishing an appropriate model for studies that require a longer period of time for analysis of effects such as cell transplantation or gene therapy, based on the results obtained from the marmoset MPTP model in this study. In addition, it is suggest that this study can address a maintenance methods based on animal welfare for a long term in studies using NHP PD models.

Table 12. Cell transplant studies using NHP models of PD

Models	Transplanted cells	References
MPTP	Monkey neural progenitor cells	Takagi et al. (212)
MPTP	Human iPSC-derived neural progenitor cells	Kikuchi et al. (213)
MPTP	Human embryonic stem cell-derived neural progenitor cells	Doi et al. (214)
MPTP	Monkey iPSC-derived dopaminergic precursor cells	Emborg et al (215)
MPTP	Monkey bone marrow-derived mesenchymal stem cells	Hayashi et al. (216)
MPTP	Monkey iPSC	Hallett et al. (217)
6-OHDA	Monkey Embryonic nigra tissue	Annett et al. (218)

CHAPTER Ⅱ

Evaluation of therapeutic effects of human embryonic stem cell-derived dopaminergic precursor cells transplanted into a marmoset model of Parkinson's disease

ABSTRACT

Cell transplantation is as an alternative to existing treatments for PD such as conventional *L*-DOPA administration and DBS surgery. The degree of differentiation and the homogeneity of cells after differentiation are directly linked to the recovery of clinical symptoms and the reduction of side effects in cell transplantation. Therefore, efforts to discover new markers of differentiation and homogeneous classification that are most effective in PD treatment are ongoing as transplanted cells differentiate into dopamine neurons. Accordingly, a total of 2.0×10^6 cells were implanted into striatum of the marmoset MPTP model intracranially to evaluate the therapeutic effects of dopaminergic (DAergic) precursor cells obtained using trophoblast glycoprotein, a newly discovered marker that uniquely divides into ventral midbrain DAergic neurons associated with PD clinical symptoms. Observations of daily behavior showed a significant recovery compared to the MPTP treatment group at 3 weeks after cell transplantation, resulting in a difference of up to 11.17 ± 0.83 points based on evaluation criteria. In the tower test, it was significantly higher than in the MPTP treatment group at 7 weeks after cell transplantation, confirming an average difference of up to 5.67 ± 0.33 levels. In addition, the PET image analysis of the striatum showed a significant difference from 14 weeks after cell transplantation compared with the MPTP treatment group, with

an increase of up to 0.26 ± 0.01 in SUR value. In addition, histopathologic assessment showed that no excessive inflammatory cell erosion or tumor-like tissue was observed. TH-positive cells observed were identified as those derived from the transplanted DAergic precursor cells in the cell transplant site. The results suggest that DAergic precursor cells represent a potential treatment modality for PD patients.

Keywords: Parkinson's disease; cell transplantation; dopamine precursor cells, common marmoset MPTP model

INTRODUCTION

L-*3,4*-dihydroxyphenylalanine (*L*-DOPA) is still used as a standard PD therapy, suggesting the role of exogenous dopamine against endogenous dopamine depletion in ameliorating symptomatic parkinsonism does not prevent progression of disorder (162-164). However, the side effects such as "Wearing-off" and *L*-DOPA-induced dyskinesia LID may occurr when PD patients are treated with long-term *L*-DOPA (172, 173). Therefore, novel and variable formulations which can minimize unexpected results with long-term *L*-DOPA treatment have been investigated clinically, however, therapy that is devoid of adverse effects does not exist (177, 371).

Due to the limited pharmacological therapies available, multiple complementary or alternative therapies have been suggested to ensure better quality of life (180). Whereas most of the currently available pharmacological or alternative therapies may be used to manage major motor symptoms, molecular approaches such as neurotrophic factors (181, 182), gene therapy (183), or cell replacement (184-186) are of increasing interest. Although such therapies are tested preclinically and clinically, a remedy for complete recovery is still unavailable. Among the alternative therapies introduced above, cell transplantation may be used to replace necrotic or apoptotic dopaminergic cell of unknown etiology for function. Preclinical experiments using various cells such

as fetal or embryonic ventral midbrain tissues (200, 201), mesenchymal stem cells (MSCs) (203-206), embryonic stem cells (ESCs) (372-374), and induced pluripotent stem cells (iPSCs) (375, 376) have been conducted to date, and some are undergoing clinical trials (377).

However, cell transplantation, which is expected to be effective as an alternative treatment, is asccociated with several challenges. First, since transplanted cells cannot cross the blood-brain barrier, which is an histological firewall, it is currently a common practice to transplant directly into the brain. Regardless of the skills and expertise of the medical practitioners using microinjection needles, the possibility of physical damage through repeated transplantation remains. Secondly, although the CNS has immune privilege characteristics (378), side effects include a risk of infection and neoplastic transformation following the use of immunosuppressive agents to modulate transplant rejection. Of course, when transplanting autologous MSCs or iPSCs, it is not necessary to use an immunosuppressive agent, but it is ineffective as a therapeutic agent given the time and expense to separate and purify the active ingredient (379, 380). Third, although most countries use ESCs obtained from stillborn fetuses or embryos, ESCs have yet to be completely resolved (381). However, autologous stem cel transplantation can partially address concerns, such as the side effects of using immunosuppressants. Lastly, the most important challenges include the health and

safety issues associated with cell transplantation, and tumorigenesis is one of the main considerations, associated with the overgrowth of transplanted cells, residual pluripotent cells, or mutations during cell preparation (382-385). Another main consideration is the graft-induced dyskinesia (GID), which occurs due to the difference of dopamine replacement patterns or differentiation into non-DAergic or serotoninergic cells by the graft, or immune response or abnormal plasticity by the host (386, 387). Although tumor formation or GIDs are less likely to occur using protocols to purify cells that only differentiate into the desired type, studies are being conducted to purify cells with a more homogeneous composition appropriate for clinical applications, since cells derived from ESCs or iPSCs are still at risk of heterogeneity (388-392). As a method to compensate for this problem, methods for transplanting cells differentiated into neural stem cells (NSCs), DAergic progenitor cells, and DAergic precursor cells with high probability of differentiation into DAergic neurons were performed (Table 13).

Therefore, the purpose of this study is to not only investigate the effects of recovery from parkinsonism in common marmoset (*Callithrix jacchus*) MPTP models implanted with DAergic precursor cells derived from human ESCs using a new marker that can be considered as a more homogenous, but also to obtain results of safety evaluation to determine whether the transplanted cells produce unwanted neoplasms. In addition, investigated

whether the MPTP model developed using the novel MPTP treatment method is actually amenable to cell therapy investigations.

Table 13. Cell therapy studies employing NSCs, DAergic progenitor or precursor cells

Animal model	Source cells	Implanted cells	Dose	References
Rat	mouse ESCs	Nurr1-ESCs	1.6×10^5	Kim et al., (393)
Rat	human ESCs	DAergic progenitor cells	4×10^5	Ben-Hur et al., (394)
Monkey	human ESCs	NSCs	1×10^6	Redmond et al., (395)
Rat	human ESCs	A. d10-ESCs B. d16-ESCs	A. 1.5×10^5 B. 3×10^5	Kirkeby et al., (396)
Monkey	human ESCs	NSCs	1×10^6	Daadi et al., (397)
Mouse	mouse ESCs	DAergic precursor cells	5.5×10^4	Batista et al., (398)
Rat	human iPSCs	NSCs	4×10^5	Doi et al., (388)
Monkey	human iPSCs	Neural progenitor cells	4.8×10^6	Kikuchi et al., (213)
Rat	human iPSCs	DAergic progenitor cells	$1.5\text{-}3.0 \times 10^5$	Nolbrant et al., (399)
Rat	rat ESCs	NSCs	2.5×10^6	Wu et al., (400)
Mouse	mouse ESCs	DAergic progenitor cells	3×10^5	Precious et al., (401)
Mouse	A. human iPSCs B. human ESCs	DAergic progenitor cells	1×10^5	Schweitzer et al., (402)

MATERIALS AND METHODS

Animals

Six adult male common marmosets (*Callithrix jacchus*) were obtained from CLEA Japan. During the experiment, all animals were maintained in cages (76.8 × 39.4 × 59.0 cm) individually under controlled conditions (23-28℃, 45-70% humidity, 12 h light/dark cycle). Animals were fed specialized diet supplemented with nutrients and commercial diets were preferred (Teklad New World Primate Diet 8794, Harlan, USA) in addition to fresh fruits, and quail eggs. Based on the experience gained from previous studies, the diet was soaked in a mixed fluid of normal saline and 5% dextrose. It was finely grounded, and forcibly fed in a fluid form and periodically supplied with sap to prevent dehydration. To check the animals health condition, the body weight was measured daily during the MPTP treatment and once a week thereafter. Animals were cared for and maintained according to the Guide for the Care and Use of Laboratory Animals 8th edition, NRC (2011) in the facility accredited by AAALAC international (#001169). Chewable rubber toys were provided for animal enrichment. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (No. 13-0024).

MPTP-induced PD model

Using the method established in a previous study, six animals were subcutaneously treated with 2 mg/kg MPTP HCl (M0896, Sigma-Aldrich, USA) dissolved in saline on the first 2 days, followed by and 1 mg/kg for 3 days to develop an PD model (Figure 12). To prevent MPTP exposure to the environment, animals were managed up to 48 h after administration of the final MPTP in a cage specially designed to maintain sealing and negative pressure after MPTP treatment. All procedures related to MPTP processing were carried out in accordance with safety regulations including handling and disposal of MPTP and wearing an appropriate personal protection equipment when handling.

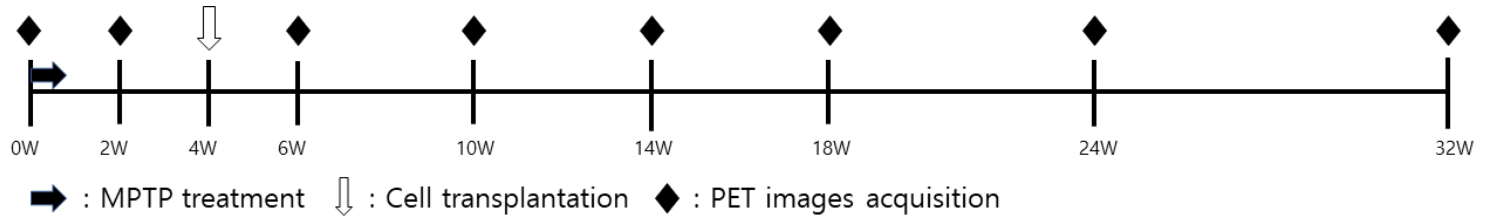


Figure 12. The experiment protocol. Black arrow: "2-2-1-1-1" mg/kg MPTP subcutaneous treatment for 5 days; white arrow: DAergic precursor cells at d20 stage following undifferentiated human ESC transplantation at 4 weeks after MPTP treatment; black rhombus: ^{18}F-FP-CIT PET images acquisition at 0, 2, 4, 6, 10, 14, 18, 24, and 32 weeks after MPTP treatment.

Cell collection

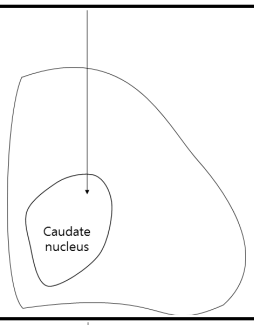
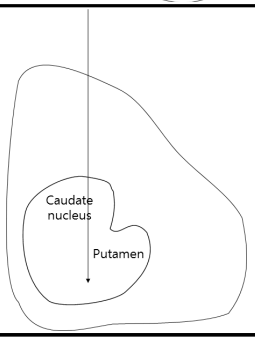
All DA precursor cells from undifferentiated human ESCs transplanted into the MPTP-treated marmoset model were kindly provided by Prof. Dong-Wook Kim, Yonsei Stem Cell Research Center (YSCRC), Yonsei University College of Medicine, Korea. DA precursor clusters obtained on day 13 after differentiation of human ESC according to the differentiation protocol were clearly separated into single cells and cultured to differentiate into neurons for 7 days. The DA precursor cell of d20 stage was separated into single cells again. A magnetic activated cell sorting assay was used to select only cells positive for cell surface marker, such as LMX1A, expressed in the midbrain dopamine neurons, EN1 and FOXA2, expressed on DAergic precursor cell, and the new marker, trophoblast glycoprotein (TPBG). The sorted DA precursor cells were used for cell transplantation after 2 days of culture.

Cell transplantation

Three marmosets were injected intracranally with DAergic precursor cells derived from human ESCs at 4 weeks after MPTP treatment (Figure 13). All animals were injected with 0.04 mg/kg atropine subcutaneously and anesthetized with 5 mg/kg ketamine and 1 mg/kg xylazine into muscle. After induction of anesthesia, animals inhaled 0.5-1.5% isoflurane (Abbvie Limited, UK) in 1 L/min of oxygen through the nose cone during the surgery,

placed on heating pad. The heart rate, oxygen saturation, body temperature parameters were monitored every 10 minutes until full recovery after surgery. Transplantation sites were defined using the marmoset brain atlas and the 4 sites of each hemisphere were determined on the putamen, and the caudate nucleus (Table 14). After surgery preparation, a skin over the target area of left and right hemisphere incised, and muscle and fascia were dissected to expose the cranial bone surface, and the burr-hole was drilled. Using the stereotaxic frame for rodents, a cell suspension of 1.0×10^5 cells/μL was prepared, 5 μL of which was injected into target sites with a 23-gauge needled Hamilton syringe driven by a microinjector at a rate of 1 μL/min. Needles were removed 5 minutes after injection. After all the injections, the site around burr-hole was washed, and the fascia and muscles were sutured with absorbable fiber, whereas the skin was sutured with non-absorbable fiber. After surgery, animals were treated with antibiotics (cefazolin, 20 mg/kg; Chong kun dang Pharmaceutical Corporation, Korea) and analgesics (meloxicam, 0.2 mg/kg; Boehringer Ingelheim, Ingelheim, Germany) for 3 days intramuscularly. No immunosuppressive agents were used until the end of the experiment.

Table 14. Cell transplant site coordinates using a stereotaxic device

Site	AP	ML	DV	Diagram
Caudate nucleus	9.9	2.3	-9.3	
			-10.6	
Putamen	8.6		-19.4	
			-20.7	

(After fixing the ear hole with ear bar, based on the bregma)

AP: Anterior-Posterior; ML: Medial-Lateral; DV: Dorsal-Ventral; mm

Behavioral assessment

Daily observation

The behavioral assessment was conducted by recording the behavioral changes of animals in the individual cage with a video camera and scoring by the observer according to the evaluation items (205) (Table 15). The score of each item was increased according to the severity of symptom expression: the normal status was scored 0 and the maximum severity score was 18. The sum of the scores of each item exceeding 10 points was selected to establish the PD model.

Table 15. Scoring scale for daily monitoring of MPTP-treated common marmoset models

Observation item	Score*
Alertness	0-1
Reaction to stimuli	0-3
Blinking	0-1
Checking movement	0-2
Posture	0-4
Motility	0-3
Vocalization	0-2
Tremor	0-1
Fur condition	0-1

*: 0; normal or absence, 1; mild, 2; moderate, 3 or 4; severe)

Tower test

To determine motor function as a natural behavior of marmosets, we modified the “Tower” apparatus (346) before using in the test. A test apparatus included a cuboid and combined 10 mm-thick transparent acryl plastic front along with a side panel and white opaque acryl plastic back panel with same thickness (270 × 270 × 1500 mm). It contained a total of seven levels for animals to step on and hang up. Each level had a distance between the front and the back for animals to turn their body and climb, and the levels varying in distance were farther at increased height. The third and sixth levels were located diagonally. All animals were trained to climb all levels in 7 min before MPTP treatment. The test was performed every week and the highest level, which the animal climbed in 7 min, was evaluated and recorded by a video camera in a separate and quiet room.

PET-CT imaging and analysis

Positron emission tomography-Computed tomography (PET-CT) images were acquired using PET-CT scanner (VISTA-CT, GE Healthcare, USA) with a dynamic list mode. We injected 1.5 mCi of ^{18}F-FP-CIT (New Korea Industrial Co., LTD, Korea) into the saphenous vein 1 h before acquiring PET-CT image (403-405). To acquire the PET-CT image, marmosets were injected with atropine and anesthetized with mixture of ketamine and xylazine

in the same protocol for cell transplantation. After induction of anesthesia, animals inhaled 0.5-1.5% isoflurane (Abbvie Limited, Berkshre, UK) in 1 L/min of oxygen through the nose cone to maintain a stable status. The heart rate, oxygen saturation, and respiratory rates of the animal were monitored every 10 min by the end of acquisition. Radioactivity was measured by setting the striatum as an ROI based on reconstructed PET transverse images using an analysis tool provided by the PET equipment manufacturer. ^{18}F-FP-CIT-specific uptake ratios (SURs) were calculated for the target striatal volume-of-interest (VOI), which was defined as (mean SUV of striatal VOI − mean SUV of cerebellum VOI/mean SUV of cerebellum VOI).

Histopathologic examination

At the end of daily observation, tower test, and PET-CT image acquisition, 32 weeks after the MPTP treatment, the abdomen was opened and blood was collected via caudal vena cava after deep anesthesia with the mixture of ketamine and xylazine. The brain was dissected immediately after bleeding by cutting the vein. The brain was divided into each hemisphere and fixed for at least 72 h in 10% neutral buffered formaldehyde. For immunofluorescence staining, the left hemisphere was frozen, whereas the right one was dissected at the site including striatum and SN and embeded in formalin-fixed paraffin. The paraffin-embedded brain tissues were serially sectioned into 4-μm-thick layers on a

microtome, deparaffinized in xylene, and rehydrated in a graded ethanol series.

H&E staining and Immunohistochemistry

Sectioned tissue slides were stained with H&E using an autostainer (Leica, Germany) and other slides were processed immunohistochemically for Tyrosine hydroxylase (TH; ab117112, Abcam, UK). Anti-TH Immunohistochemistry (IHC) was conducted with the ChromoMap DAB detection kit (Ventana, USA) according to the manufacturer's instructions.

Immunofluorescence staining

To perform immunofluorescence staining, brains were transferred sequentially at one-day intervals into 10%, 20% and 30% sucrose until they sank to the bottom of the container. The entire brain was then cut into 18 μm-thick coronal sections on a cryostat (Leica CM 3000, Leica, Solms, Germany). The sections were sampled systematically throughout the entire striatum and SN with a random start according to the stereological principles. Slices were washed with 1X phosphate-buffered saline (PBS), permeabilized with PBS containing 0.05% (vol/vol) saponin and 5% (vol/vol) normal goat serum for 30 min. Non-specific binding was blocked with 1.5% normal goat serum for 30 minutes. Section were also permeabilized for 30 min with 0.1% Triton X. For double labeling, slices were initially labeled with rabbit an-

ti-TH (1:1000; Pel-Freez Biologicals, USA) and then immunostained with mouse anti-human nuclei (1:500), mouse anti-mitochondria (1:100), rabbit anti-Lmx1 (1:1000; Merck Millipore, Germany), mouse anti-Nurr1 (1:50), and mouse anti-Ptx3 (1:50; Santa Cruz Biotechnology, Inc., USA), respectively. Slices were incubated with primary antibody overnight at 4℃. Fluorescence-labeled secondary antibodies raised against the respective hosts of the primary antibodies were used at a dilution of 1:500 and incubated for 1 hr at room temperature. All secondary antibodies were purchased from Molecular Probes (Invitrogen, Co., USA). Fluorescently immune-labeled slices were analyzed on a confocal microscope (TCS SP8; Leica Solms, Germany) equipped with three lasers (Diode 405, Argon 488, HeNe 543). Each channel was separately scanned in multitrack PMT configuration to avoid cross-talk between fluorescent labels, and to visualize labeled structures in relation to other cells.

Statistical analysis

The data were expressed as mean ± standard error of mean (SEM). Data of body weight, behavioral test, and PET images involving MPTP-treated and cell-transplanted marmosets were analyzed by independent Student *t*-test. The correlation of each item score and total score of daily observation after cell transplantation was analyzed by linear regression analysis with Spearman's ρ method using Statistical Package for Social

Sciences version 22 (IBM, Chicago, IL, USA). P values of less than 0.05 were statistically significant.

RESULTS

No significant difference in body weight between MPTP-treated and cell-transplanted marmosets due to intensive care

There was no significant difference in body weight between MPTP-treated and cell-transplanted marmosets during the entire experimental period. In particular, there was no significant difference in body weight between two groups before MPTP treatment (MPTP-treated: 339.0 ± 1.53 g; cell-transplanted: 344.0 ± 8.08 g) and cell transplantation, which was 4 weeks after MPTP treatment (MPTP-treated: 314.3 ± 5.81 g; cell-transplanted: 314.0 ± 2.31 g). As shown in a previous study, akinesia was clearly observed in MPTP-treated marmosets from an average day 2 after MPTP treatment, and consequently MPTP-treated animals failed to consume food and water voluntarily. Thus, forced feeding was performed to prevent weight loss and maintain a healthy status from day 2 after MPTP treatment. Although there was no significant difference in body weight between MPTP-treated and cell-transplanted animals after cell transplantation, voluntary feeding and water intakes were observed in cell-transplanted marmosets from 15 weeks after cell transplantation. The results showed a gradual weight gain, although it was not significant

when compared with the weight before MPTP administration (Figure 13).

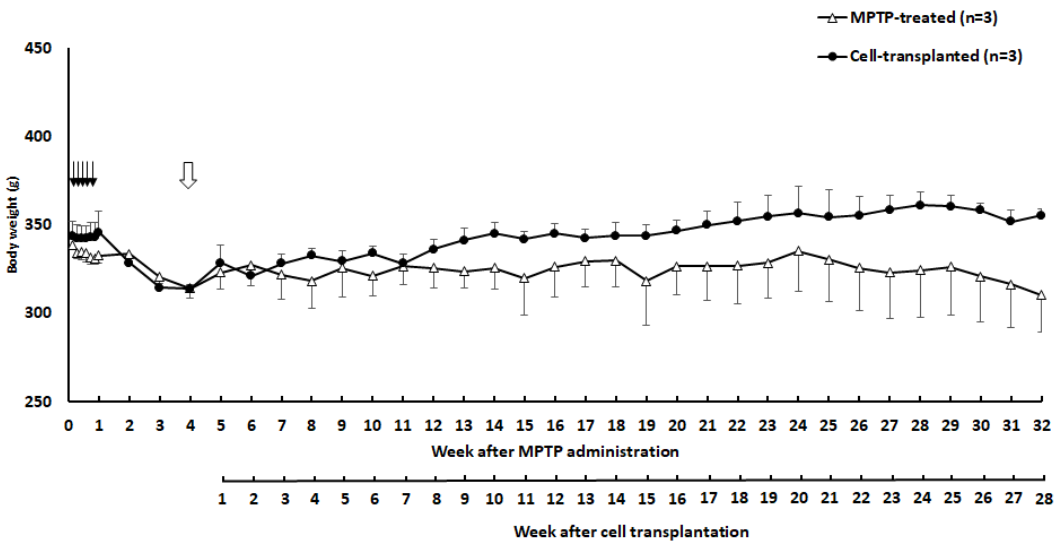


Figure 13. Monitoring body weight of MPTP-treated and cell-transplanted marmosets. Body weights of both MPTP-treated (white triangle, n = 3) and cell-transplanted marmosets (black circle, n = 3) are maintained during the study without significant change. Black arrow: MPTP treatment; white arrow: cell transplantation.

Progressive recovery of motor symptoms in MPTP pre-treated cell-transplanted marmosets compared to MPTP-treated marmosets

Immediately after administration of MPTP, akinesia or bradykinesia was observed among several PD motor symptoms in all marmosets, in addition to a few marmosets falling within the cage due to posture impairment or limb movement abnormalities (Figure 14). At the end of MPTP treatment, both resting and active tremors were observed, and excessive blinking, a characteristic motor symptom in the marmoset MPTP model, was also observed. At 3 weeks after the cells were transplanted, motor symptoms gradually began to recover, and this trend continued until the end of the experiment. As a result, the observed motor symptoms were scored, which confirmed a significantly decreased compared with the cell transplantation at 4 weeks after MPTP treatment. In particular, the motor symptoms recovering after cell transplantation were different. Alertness by observer, movement within the cage, and limb tremor were recovered from 3 to 7 weeks after cell transplantation ($r = 0.89$, 1.00, and 0.95, respectively; $p < 0.05$), and thereafter, it was observed that not only motility in the cage but also response to stimuli and postural instability were recovered ($r = 0.79$, 0.74, and 0.73, respectively; $p < 0.05$).

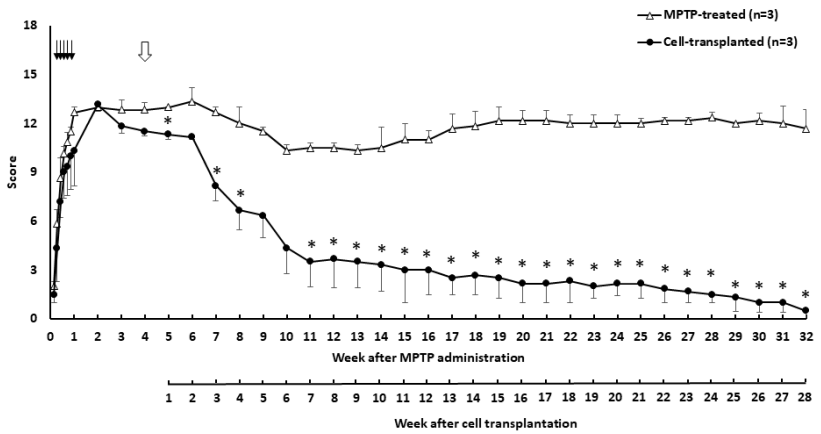


Figure 14. Behavioral assessment in MPTP-treated and cell-transplanted marmosets. Stable and no recovery of motor symptoms were observed in MPTP-treated marmosets (white triangle, n = 3) during the experimental period, whereas gradual recovery of motor symptoms occurred starting from 4 weeks after transplantation in cell-transplanted marmosets (black circle, n = 3). Black arrow: MPTP treatment; white arrow: cell transplantation. Asterisks indicate significant difference between MPTP-treated group and the cell-transplanted group, $*P < 0.05$

Significant, but not full recovery of motor function in cell-transplanted PD marmosets compared to MPTP-treated marmosets

All marmosets were tested weekly before MPTP treatment to the final day of study using the tower and completed training which by climbing to the highest level within 7 min. Both MPTP-treated and cell-transplanted marmosets did not climb even the first level from week 1 to week 4 after MPTP treatment (Figure 15). Subsequently, MPTP-treated marmosets climbed below the 1-2 level (1.4 ± 0.37) on average by the final day of the study, whereas the levels to which cell-transplanted marmosets climbed increased steadily after cell transplantation, suggesting a significant difference from week 7 after cell transplantation until the final day of study except for results at week 6-9, and the average level was the sixth (5.4 ± 0.44). Although no steady or significant difference in motor function recovery occurred from 7 weeks after cell transplantation until the end of the experiment, the motor function of the cell-transplanted marmosets recovered significantly more than that of the MPTP-treated marmosets. It recovered significantly more than the level before cell transplantation.

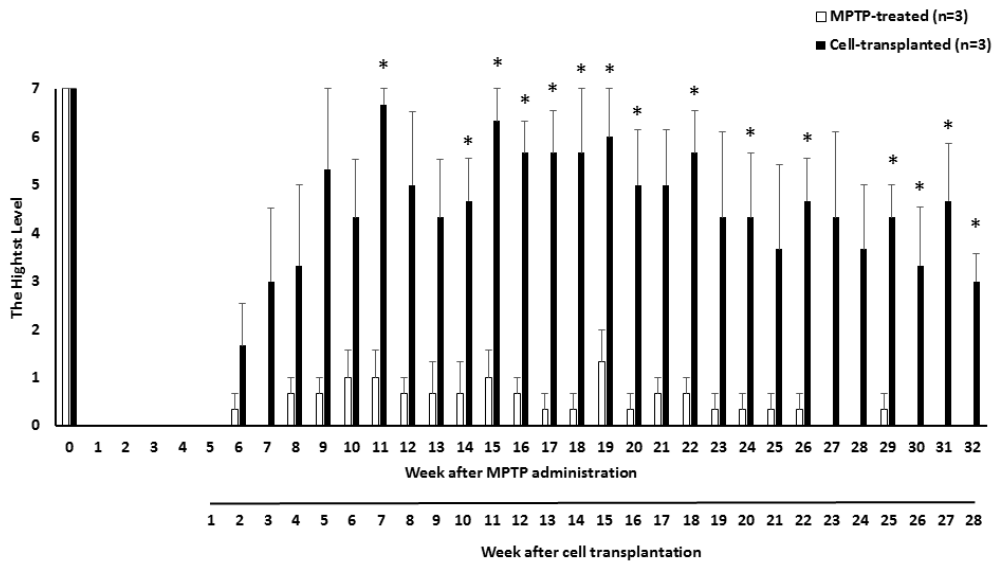


Figure 15. Comparison of jumping ability between MPTP-treated and cell-transplanted common marmosets. As a result of motor function recovery, the cell-transplanted marmosets (filled, n = 3) reached a significantly higher level than MPTP-treated marmosets (open, n = 3). Asterisks indicate significant difference between MPTP-treated group and the cell-transplanted group, $*P < 0.05$.

Weak recovery pattern in striatal PET images and SUR in cell-transplanted marmosets

^{18}F-FP-CIT-PET-CT images were obtained to confirm the baseline status in all marmosets and no significant SUR of the striatum was found between MPTP-treated and cell-transplanted marmosets (MPTP-treated: 1.27 ± 0.008; cell-transplanted: 1.25 ± 0.059) (Figure 16). The intensity of striatum in PET-CT images acquired at week 2 after MPTP treatment decreased more than the baseline intensity in both MPTP-treated and cell-transplanted group. The SUR strongly decreased in both MPTP-treated and cell-transplanted marmosets, but the difference was not significant between MPTP-treated and cell-transplanted marmosets (MPTP-treated: 0.43 ± 0.027; cell-transplanted: 0.46 ± 0.009). Subsequently, the intensity of the striatum and SUR (0.46 ± 0.010) in MPTP-treated marmosets were not significantly changed between PET-CT images at each time point obtained from week 6 after MPTP treatment to the final day of the study, whereas the intensity of the striatum and SUR (0.47 ± 0.035) in cell-transplanted marmosets were not significantly altered between PET-CT images at each time point from week 2 to week 6 after cell transplantation, compared with the intensity and SUR before cell transplantation. However, the intensity of the striatum and SUR changed significantly in PET-CT images at each time point obtained from week 14 after cell transplantation until the final day of the study (week 14: 0.9 ± 0.009; week 18: 0.73 ±

0.014; week 28: 0.74 ± 0.002, $p < 0.05$). In addition, the SUR showed a significant difference compared with SUR in MPTP-treated marmosets at the relevant time point (week 18: 0.46 ± 0.029; week 24: 0.47 ± 0.029; week 32 after MPTP treatment: 0.48 ± 0.015, $p < 0.05$)

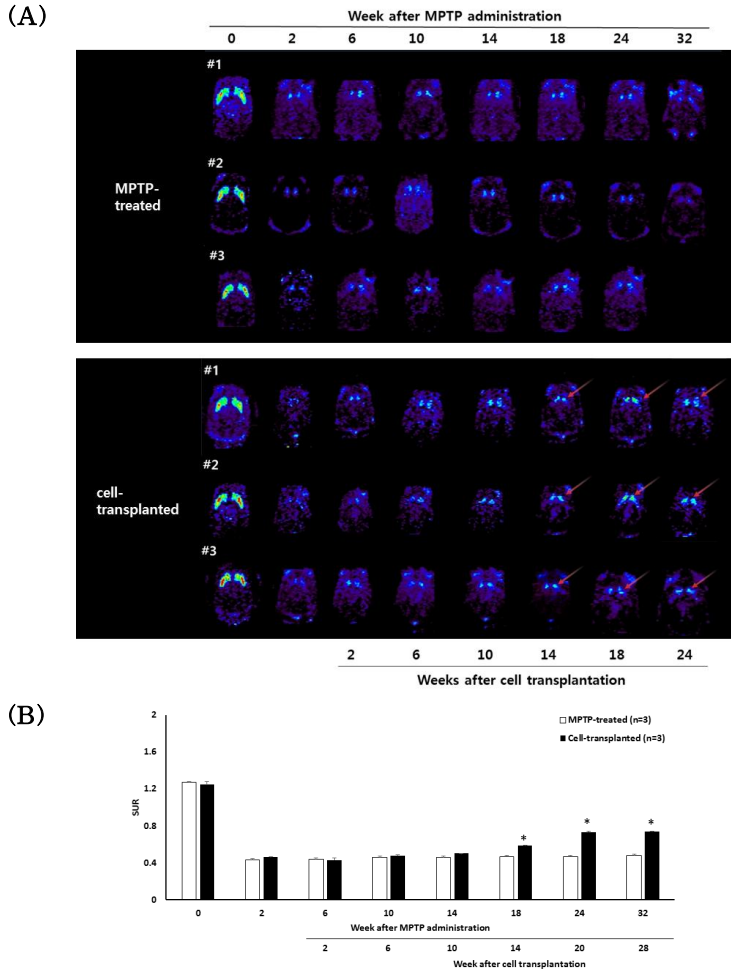


Figure 16. Striatal ^{18}F-FP-CIT-PET-CT images (A) and SUR changes (B) of MPTP-treated and cell-transplanted marmosets. ^{18}F-FP-CIT binding in the striatum and SUR of cell transplantation marmosets (filled, n = 3) were increased compared to MPTP-treated marmosets (open, n = 3). Red arrow: Augmented intensity compared with intensity level before cell transplantation. Asterisks indicate significant difference between MPTP-treated group and the cell-transplanted group, $*P < 0.05$.

No tumor-like lesions, but increased TH-positive neurons and fibers at transplant sites at 28 weeks after cell transplantation in MPTP-treated marmosets

The recovery of motor symptoms in marmosets treated with MPTP by cell transplantation was expected. However, the safety of tumor-like neoplastic tissue developed after cell transplantation required evaluation. As a result of H&E staining, no tumor-like neoplastic tissue was observed around the site of cells implantation as well as along the nigrostriatal pathway. In addition, no excessive infiltration of inflammatory cells was detected at the site of transplantation (Figure 17).

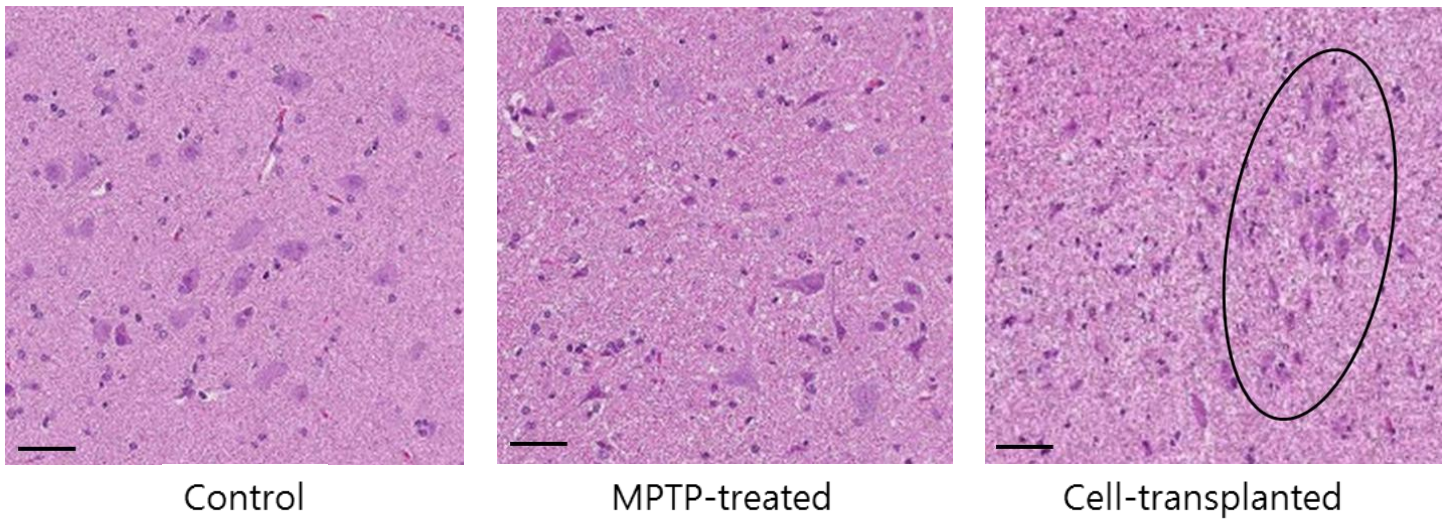


Figure 17. Comparison of H&E stain results in brain tissue of MPTP-treated and cell-transplanted marmosets. The number of neurons in the striatum of MPTP-treated and cell-transplanted marmosets was significantly reduced compared to the untreated marmoset, and no excessive inflammatory cells or un-differentiated cells were observed at the transplant site (black circle); scare bar: 50 μm.

However, as a result of anti-TH IHC, TH-positive cells and fibers in the SN region disappeared in the brain tissue obtained at the end of the experiment, 32 weeks after administration of MPTP, or 28 weeks after transplantation of cells, when compared with the marmoset not treated with MPTP. The difference between the number of TH-positive cells and fibers in the MPTP-treated marmosets and the number of TH positive cells in cell-transplanted marmosets was not significant. However, TH-positive cells and fibers in the caudate region of the striatum, the implantation site, were significantly higher in cell-transplanted marmosets than in the marmoset treated with or without MPTP (Figure 18).

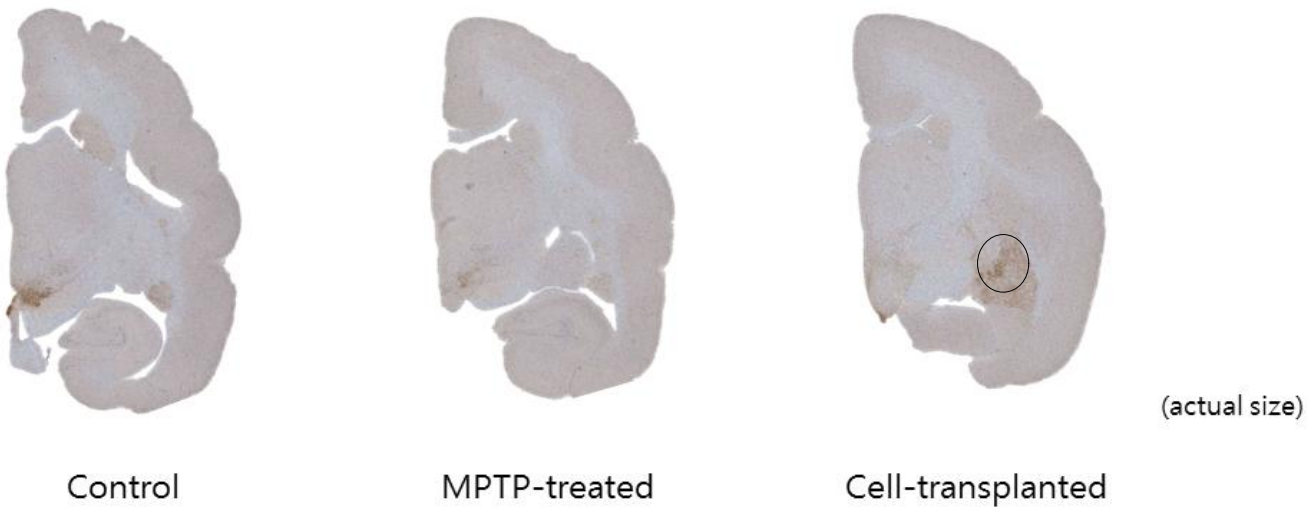


Figure 18. Comparison of anti-TH IHC results in the brain tissue of MPTP-treated and cell-transplanted marmosets. The number of TH-positive cells and fibers in the SN of MPTP-treated and cell-transplanted marmosets was significantly lower compared to the marmoset without MPTP treatment, whereas TH-positive cells and fibers in the caudate were significantly higher in cell-transplanted marmosets than in marmosets treated with or without MPTP; Black circle: cell-transplantation site.

Identification of cells expressing DAergic markers in transplanted cells at 28 weeks after cell transplantation

In order to confirm whether the increased TH-positive cells identified as anti-TH IHC were cells differentiating from the transplanted DAergic precursor cell derived from human ESCs or cells existing in the transplanted marmoset relative to the restore of motor function and recovery of motor symptoms in cell-transplanted marmosets, IF staining was performed using anti-human antinuclear (hANA) and anti-TH antibody (Figure 19A). As a result of anti-TH/anti-hANA IF staining, no hANA or TH-positive cells were detected in the caudate region of the MPTP-treated marmosets, whereas cell both positive for hANA and TH were observed in the caudate region of the transplanted marmosets. Subsequently, in order to confirm the presence of TH-positive cells in the IF-stained caudate nucleus in cell-transplanted marmosets were human-derived live and functioning cells, IF staining was performed using anti-human mitochondria (hMit) and anti-TH antibody. The results of anti-TH/anti-hMit IF staining revealed, a number of cells both positive for TH and hMit in the caudate nucleus of cell-transplanted marmosets (Figure 19B). Lastly, in order to confirm the lineage and current status of TH-positive cells in cell-transplanted marmosets, IF staining was performed using antibodies against Lmx1A, used as a midbrain progenitor cell marker, and Nurr1, Ptx3 used as a DA precursor cell marker. The results of anti-TH/anti-Lmx1A, an-

ti-Nurr1 or anti-Ptx3 IF staining showed, a number of cells both positive for TH and Lmx1A, Nurr1, or Ptx3 in the caudate nucleus of cell-transplanted marmosets (Figure 19C).

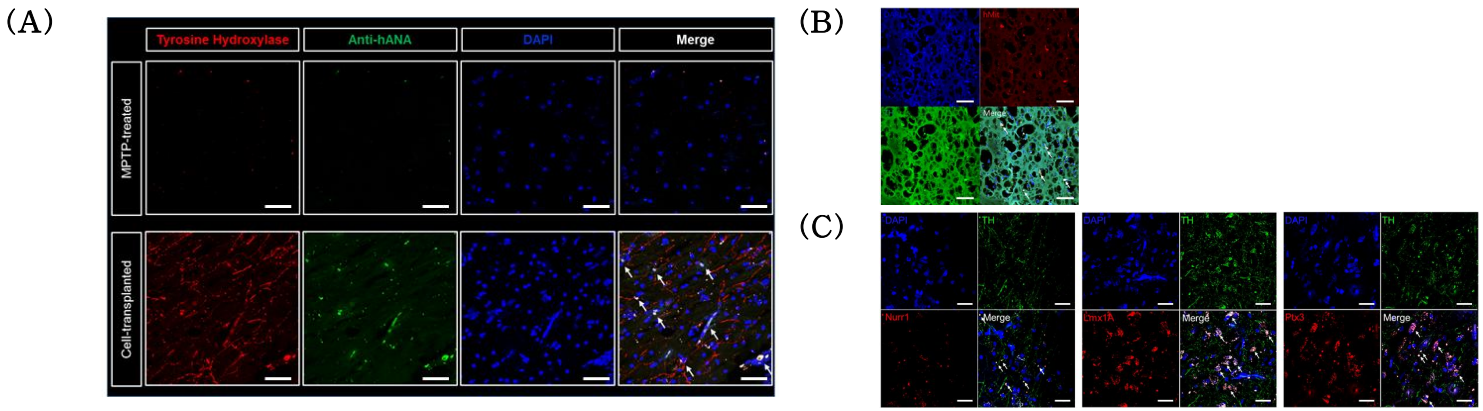


Figure 19. IF staining of the caudate nucleus in cell-transplanted marmosets to identify the origin (A), survival, function (B), and lineage (C) of the transplanted cells. (A) No hANA-positive as well as TH-positive cells were observed in the caudate nucleus of MPTP-treated marmosets, whereas cells positive for both TH and hANA were observed in the caudate nucleus of cell-transplanted marmoset (white arrows); scale bar: 50 μm. (B) A number of TH and hMit-positive cells were observed in the caudate nucleus of cell-transplanted marmosets (white arrows); scale bar: 25 μm. (C) Cells with positive TH and Lmx1A, Nurr1 and Ptx3, respectively, were observed in the caudate region of the transplanted marmoset (white arrows); scale bar: 25 μm.

DISCUSSION

MPTP is a neurotoxin that has been widely used to generate a PD model in various animal species, including rodents, primates, pigs and cats (406). Although MPTP can be introduced via different routes, such as gavage and stereotaxic injection, the systemic route, such as subcutaneous or intravenous injection, is the most universally exploited mode of administration due to its reliability and reproducibility (407). It is easy to develop a model without special equipment or technique. Importantly, defects in DAergic pathway induced by the degeneration of DAergic neurons in the SN have been observed in MPTP-induced PD models just as in PD patients (408). Such degeneration of DAergic neurons is more frequently observed in the SN than in ventral tegmental area (409, 410). However, Lewy body, one of the hallmarks of PD, is not clearly detected in MPTP-induced PD model and other pigmented nuclei area, such as locus coeruleus, which is not affected in MPTP-induced animal models (411).

Various species of MPTP-induced models manifest tremors, rigidity, dyskinesia, and abnormal posture, especially tremor at resting phase in NHP models (412). Species closer to the genetic makeups of human are more sensitive to MPTP and thus represent optimal candidates for PD models (407). Histological examination revealed that the terminus DAergic neurons in putamen was more degenerate than that of caudate nucleus in MPTP-in-

duced NHP model when injected in low concentration (413, 414). Compared with rodents such as mouse and rats, monkey is more sensitive to MPTP, and therefore clearly exhibited PD-related clinical symptoms (406). Specific symptoms depending on species investigated in a pilot study that MPTP-induced cynomolgus monkey (*Macaca fasicularis*) models showed higher rigidity, whereas, marmoset models manifested tremors at resting phase. It is meaningful that there is a difference in major motor symptoms among NHP models above in that the motor symptoms of early stage in human patients appear clinically as tremor, rigidity, and bradykinesia (415, 416). Significant amounts of data derived from marmoset models have been used for the development of drugs for PD, such as neurotrophic factors, dopamine agonist, dopamine reuptake inhibitor, and cell transplantation. Especially, ethological behaviors can be quantified and clinical scale using PD patients can be adopted to assess behavioral outcomes of MPTP-induced bilateral marmoset model (418). Although the regimen for MPTP induction method to generate a PD model varies depending on research goals (62, 65, 96-99, 151, 332-337), the standard protocol for acute regimen 2 mg/kg SC over a period of 5 days, according to Jenner and his colleagues (65) who developed such a regimen for acute MPTP-induced PD model in marmosets. Additionally, this particular model has widely been used to study dyskinesia induced by *L*-DOPA and the development of new therapeutic methods since TH mRNA was found to

decrease approximately 95% in the substantia nigra. Additionally, it has been demonstrated that nerve cells in the SN do not undergo spontaneous restoration in MPTP-induced marmoset model generated via the method described above. Recently, a few marmoset models were established by administering low dose (352) or chronic treatment (337, 351, 418) for study purpose. However, such models showed mild or moderate symptoms following approximately 50% loss of nigrostriatal DAergic neurons and spontaneous restoration eventually. Similar to a previous study, MPTP-treated marmoset models in this study showed motor symptoms clearly, although no spontaneous restoration was detected in histopathologic, behavioral and imaging studies using PET. Therefore, the marmoset model in this study is suitable for the analysis of patient stages that cause motor symptoms after treatment. In addition, by comparing the cell transplantation group with the MPTP-treated group with persistent symptoms, the correct choice of model in this study was determined.

Since the clinical symptoms of PD manifest as dopamine deficiency caused by the damaged DAergic neurons in the SN, many studies have focused on refuelling the deficient dopamine. Thus, the injection of *L*-DOPA, which is a precursor to dopamine, has been the baseline PD therapy and is currently considered as a gold standard in clinical practice. However, the administration of *L*-DOPA for a long period of time has been found to be associated with side effects, including “On-Off” phenomena and

L-DOPA-induced dyskinesia, and therefore, many studies have developed adjuvant drugs or therapies to reduce such side effects (419, 420). However, there is currently no drug that can prevent or completely reduce the side effects caused by *L*-DOPA treatment in PD patients. As an alternative to *L*-DOPA administration, the transplantation of brain tissues or cells, which when injected into the damaged region of the brain, can produce dopamine has been explored. *In vivo* experiments using the homogeneous or heterogeneous tissue or cells have shown promising results as they were associated with alleviation or improvements in PD-related clinical symptoms in animal models (370). Although significant progress has been made in the field of biotechnology, challenges in implementing the transplantation of brain tissues or cells as therapeutic methods for PD patients exist, including those involving the proliferation of neoplastic cells in the transplanted tissues or cells. Studies investigating the clinical efficacy of such a method utilized pluripotent or multipotent stem cells instead of terminally differentiated DAergic neurons (421). The transplanted tissues or cells are expected to differentiate, proliferate and eventually replace the damaged DAergic neurons in the SN. Such a therapeutic strategy is based on the idea that the undifferentiated tissue derived from the fetus or the stem cell restores the tissue by migrating to the damaged tissue.

However, undifferentiated tissue or stem cells can transform into unwanted or unexpected tissue except damaged tissue. As well,

neoplasms can develop into malignant tumors depending on the degree of differentiation or proliferation. Thus, controlling the potential for differentiation and proliferation is critical to the development of PD therapy exploiting tissue or stem cells. In addition, following clinical intervention, human PD patients survive for at least 20 years, whereas most animal models that undergo cell transplantation have the condition of the graft confirmed for a short period of time, usually within 16 weeks in rat models (422). Therefore, results of safety evaluation results for thorough tumor development over a long period of time are essential, and it is also important to develop and select animal models that show stable clinical symptoms over a long time. Although rodent models such as mice and rats are mostly used in PD studies, because animal species have different natural lifespans, studies with absolute time periods, such as cell therapy, require selection of animal species that can be monitored over a sufficiently long period of time.

Studies with iPSCs derived from each patient are increasing because of ethical concerns regarding about the source of stem cells and transplant rejection using allogenic or xenogeneic stem cells. However, uncontrollable differentiation and proliferation are critical challenges associated with iPSCs. Although the brain is a solid organ with less immune rejection and some stem cells may control immune response themselves, the immune rejection is another limitation to the transplantation of either brain tissue or

stem cells. Many studies have been and are currently underway to develop a safe and effective therapeutic modality to overcome the possible immune rejection response of the recipient to transplantation. However, no immunosuppressive drug is available to ensure the absolute safety to transplantation. Theoretically, a transplanted tissue or cell should either evade or overcome the reactions of host immune system to differentiate and proliferate so as to replace the damaged tissue. However, it is inevitable that transplanted materials encounter the host immune system, and therefore, transplanted tissue or cells undergo "apoptosis" before clinical improvement is achieved. Additional transplantation or treatment with immune suppressors could be considered to overcome such practical issues and to achieve desired therapeutic effects. However, additional transplantation requires a reliable supply of tissue or cells and resolution of economic issue related to high-degree stereotaxic procedure as well as physical damage following tissue or stem cells transplantation. Additionally, long-term treatment with immune suppressors may result in side effects associated with dysfunctional immune system of the recipient.

In this study, DAergic precursor cells obtained from differentiating human ESCs were transplanted in marmoset MPTP models, and based on behavior assessment, PET imaging analysis, and histological examination, the efficacy and safety of the transplanted DAergic precursor cells were evaluated. Any kind of

neoplastic events were not observed under both naked and microscopic analysis of the cell-transplanted areas and the pressured areas used in the needle injection. Although DAergic precursor cells were injected only once, they not only improved behavioral and clinical symptoms but also increased the number of DAergic precursor cells in the transplanted area after a long period of time even without treatment with immune suppressors. These results suggest that transplanted DAergic precursor cells differentiate and proliferate into cells that eventually replace damaged DAergic neurons and fibers in the nigrostriatal pathway. Although genetically similar but also heterogenous at the same time, the transplanted DAergic precursor cells did not elicit an immune rejection response. Therefore, DAergic precursor cells were able to survive in the transplanted area for a long period of time, allowing substantial amount of time for the damaged tissue to repair. In light of these results, it is suggested that DAergic precursor cells represent a potential therapeutic modality for PD patients as they are not associated with the major limitations previously reported in other transplantation methods.

However, challenges still remain to be overcome although the safety and efficacy of DAergic precursor cells have been demonstrated in this study. At first, the discrepancy of the therapeutic effect depending on the time of injection in acute MPTP treatment model. Based on the results of other studies, differential restoration may be minimally affected but continuous damage may

occur due to slow reaction after MPTP treatment. Therefore, in order to utilize DA precursor cells as a treatment for PD patients, further studies are needed to determine the most effective indication for transplantation after complete PD diagnosis. Furthermore, as demonstrated in previous studies (388, 393-402), the optimal number of transplanted cells should also be determined as the therapeutic efficacy has been found to vary depending on the number of transplanted cells. Second, the discrepancy between the imaging results obtained from PET scan, histological results from IHC and IF, and behavioral results from PD symptoms scale, needs to be addressed. In the absence of a study of marmoset PD model with cell transplantation, it is difficult to perform direct comparison with other studies. However, the behavioral results suggest significant differences in the behavioral results of studies involving other NHP model with varying of cell types (320, 374, 423-425). The possible reasons for such discrepancies are because the kinds and number of cells and the transplanted area vary in each published studies and also the effect of adrenergic pathway, including serotoninergic pathway compensating for the compromised dopaminergic pathway due to MPTP. Lastly, it is unclear if the improvements in clinical symptoms are due to the replacement of damaged DAergic neurons by the differentiation and proliferation of DAergic precursor cells or due to the cytokines or chemokines secreted from the transplanted DAergic precursor cells. It was possible to determine the

fate of DAergic precursor cells and observe DA precursor cells differentiated into cells with TH capable of secreting dopamine in this study. However, it was not possible to confirm whether the transplanted DAergic precursor cells showed important stem cell-like properties such as paracrine effects. As the paracrine effect of the stem cells has been reported to be a major contributor to recovery from the injured cartilaginous joint and also suppression of tumor proliferation, it will be necessary to determine if the clinical improvements following transplantation of the DAergic precursor cells are associated with other effects.

In conclusion, DAergic precursor cells derived human ESCs using new differential marker, TPBG, represent therapeutic effects in MPTP-treated common marmoset models, and it is suggest that DAergic precursor cells as a potential treatment modality can be used to ameliorate motor symptoms for PD patients.

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국문초록

파킨슨병은 가장 중요한 신경퇴행성 질환 중 하나이고, *L*-3,4-hydroxyphenylalanine 투여법이나 뇌심부자극 수술법을 현재 치료법으로 사용하고 있다. 하지만 기존 치료법으로는 완전 회복이 되지 않아 대안 치료법으로써 세포이식에 대한 연구가 활발히 진행되고 있다. 이러한 파킨슨병 치료나 예방을 위하여 많은 동물 모델이 사용되고 있고, 대부분 설치류 모델이 사용되고 있다. 파킨슨병 동물모델을 제작하는 방법으로는 1-metyl-4-phenyl-1,2,3,6-tetrahyrdopyridine (MPTP)를 투여한 모델 제작법이 가장 대표적이다. 다른 모델에 비해 영장류 MPTP 투여 모델은 파킨슨병 환자와 임상증상이 동일하다는 것과 행동학적 평가 적용이 용이하다는 장점을 가지고 있기 때문에 실험 목적에 따라 다양한 MPTP 투여법을 사용한 영장류 모델을 사용하고 있다. 하지만 대부분 영장류 MPTP 투여 모델은 세 달 이내의 단기간 연구에 최적으로 개발되어, 세포이식과 같은 장시간 연구에는 적합하지 않다. 초기 연구에서 태아 유래 중뇌조직을 이식하는 방법에서 중간엽줄기세포나 배아줄기세포를 이용한 연구가 진행되었고, 최근에는 윤리적 문제와 면역거부반응 문제를 해결할 수 있는 유도만능줄기세포를 이용한 연구가 진행되고 있다. 여러 연구를 통해 세포이식에 대한 효능 평가, 안전성 확보와 관련하여 많은 진보가 있었으나, 분화 정도와 분화 이후 세포 균질성이 임상증상 회복과 부작용 감소에 직접적으로 연관이 되어 있기 때문에 새로운 분화와 균질성 마커 발굴에 대한 연구가 꾸준히 진행되고 있다. 이러한 점들을 바탕으로 장시간 안정적인 임상증상이 발현되는 영장류 PD 모델을 제작하기 위하여 마모셋에 “2-2-1-1-1” mg/kg MPTP 피하투여법을 적용하여 새로운 영장류 PD 모델을 확립하였다. 일생행동 평가 결과 마모셋

모델은 장시간 동안 안정적인 임상증상을 보였고 tower test 결과 역시 마모셋 모델은 MPTP 투여 전에 비해 운동기능이 저하된 상태로 유지됨을 관찰하였다. 또한 마모셋 모델의 선조체 양전자방출단층촬영 (PET) 영상에서 MPTP 투여 전에 비해 유의하게 방사선 발현도가 감소함을 확인하였고, 마모셋 모델의 뇌조직 면역염색 결과 흑색질에서 티로신 수산화효소 (TH)-양성 세포와 섬유체가 소실됨을 확인하였다. 또한 새로운 분화 마커인 영양막 당단백질을 사용하여 파킨슨병 증상과 관련된 배쪽중뇌 도파민성 신경세포로 분화하는 도파민성 신경전구세포에 대한 치료 효과를 평가하기 위하여 위의 마모셋 모델의 선조체에 2.0×10^6 개 세포를 뇌내에 이식하였다. 일상행동 평가 결과 세포 이식군은 MPTP 투여군에 비해 세포이식 후 3주째부터 임상증상이 유의하게 회복됨을 관찰하였고, tower test 결과 세포 이식군은 MPTP 투여군에 비해 세포이식 후 7주째부터 올라간 계단이 유의하게 증가됨을 확인하였다. 세포 이식군의 선조체 PET 영상에서 MPTP 투여군에 비해 세포이식 후 14주째부터 specific uptake ratio 값이 유의하게 증가됨을 확인하였다. 조직병리학적 평가 결과 세포이식 부위에서 과도한 염증반응이나 종양성 신생조직은 관찰하지 못했고, 관찰된 TH-양성 세포는 뇌내에 이식한 도파민성 신경전구세포에서 유래됨을 확인하였다. 위 결과들을 종합하였을 때, 새로운 MPTP 투여법으로 제작한 마모셋 모델은 세포이식과 같은 장시간 연구에 적합하고, 도파민성 신경전구세포는 파킨슨병 치료법으로써 고려될 수 있을 것으로 제안한다.

주요어: 파킨슨병; 비인간 영장류; 세포치료제; 마모셋 원숭이; 동물질환모델; 줄기세포; 세포이식

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