Behavior, PET and Histology in Novel Regimen of MPTP Marmoset Model of Parkinson's Disease for Long-Term Stem Cell Therapy

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Stem cell technologies are particularly attractive in Parkinson's disease (PD) research although they occasionally need long-term treatment for anti-parkinsonian activity. Unfortunately, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) widely used as a model for PD has several limitations, including the risk of dose-dependent mortality and the difficulty of maintenance of PD symptoms during the whole experiment period. Therefore, we tested if our novel MPTP regimen protocol (2 mg/kg for 2 consecutive days and 1 mg/kg for next 3 consecutive days) can be maintained stable parkinsonism without mortality for long-term stem cell therapy. For this, we used small-bodied common marmoset monkeys (*Callithrix jacchus*) among several nonhuman primates showing high anatomical, functional, and behavioral similarities to humans. Along with no mortality, the behavioral changes involved in PD symptoms were maintained for 32 weeks. Also, the loss of jumping ability of the MPTP-treated marmosets in the Tower test was not recovered by 32 weeks. Positron emission tomography (PET) analysis revealed that remarkable decreases of bindings of ¹⁸F-FP-CIT were observed at the striatum of the brains of the marmosets received MPTP during the full period of the experiment for 32 weeks. In the substantia nigra of the marmosets, the loss of tyrosine hydroxylase (TH) immunoreactivity was also observed at 32 weeks following the MPTP treatment. In conclusion, our low-dose MPTP regimen protocol was found to be stable parkinsonism without mortality as evidenced by behavior, PET, and TH immunohistochemistry. This result will be useful for evaluation of possible long-term stem cell therapy for anti-parkinsonian activity.

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INTRODUCTION

Parkinson's disease (PD), a common neurodegenerative movement disorder, is characterized by selective degeneration of dopaminergic (DA) neurons in the substantia nigra area of the brain, leading to asymmetric onset of slowness of movement,

tremor, muscular rigidity and postural instability [1]. Since the 1960's, DA drugs still remains the most effective therapy for PD condition [2]. Stem cell technology is being investigated for the treatment of PD. It has been previously shown that embryonic stem cells (ES cells) differentiate into midbrain DA neurons [3,4]. Functional motor improvement of autologous induced pluripotent stem cells (iPSC)-derived DA neurons in nonhuman primates (NHPs) has also been reported [5]. Preclinical study has shown that mesenchymal stem cells have the capability to differentiate into tyrosine hydroxylase (TH) positive cells [6]. Also, transplantation of midbrain precursor-derived DA neurons has provided behavioral recovery in animal models of

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PD [7].

PD pathogenesis is a result of complex interactions between environmental factors and individual inherited genetic factors and thus *in vitro* models cannot completely reflect all aspects of human PD, leading to the wide uses of animal models to find PD pathogenesis and promising therapeutic tools [8,9]. In particular, the characteristics of NHPs showing high anatomical, functional, and behavioral similarities to humans led us to use NHPs in the preclinical process for novel treatments on PD between rodent studies and human clinical trials despite current limitations involved in a lack of resources and skilled personnel [10-12]. Among several NHPs, a small-bodied New World monkey, common marmoset (*Callithrix jacchus*) is easily handled and live well in captivity [13]. Along with fundamental biology, pharmacology and toxicology studies, this monkey species is an appreciated model in preclinical studies of PD [14].

The marmoset models of PD include genetic models and neurotoxin-based models [particularly 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA)]. Recent introduction of exogenous genes into NHP genomes or the alteration of endogenous NHP genes [15,16] support the production of transgenic marmoset PD models using genes involved in the onset and propagation of PD in humans [9,17]. However, the long lifespan of the marmosets can make them need a relatively long time for clear PD phenotypes [18]. In contrast, it is relatively easy to obtain neurotoxic PD models by using several selective neurotoxins that destroy the DA neurons [19]. Among neurotoxins, 6-OHDA model has been known to be complicated and time-consuming to induce PD phenotype because of the necessity of stereotaxic surgery and the difficulty of direct injection into small brain structures despite several advantages including less postoperative animal care and the production of unilaterally lesioned model [13]. MPTP is also a widely used neurotoxin that induces parkinsonism in both humans [20] and NHPs [21] with the production of the classical histological and behavioral features that occur in PD [22]. After bypassing the blood-brain barrier, MPTP is metabolized by monoamine oxidase B into its active neurotoxic cation, 1-methyl-4-phenylpyridinium ion (MPP⁺), which is further taken into DA neurons of the substantia nigra pars compacta via DA transporter (DAT), resulting in selective de-

crease of mitochondrial complex I activity [9].

Although the MPTP-lesioned marmosets have been widely used to evaluate the efficacy of anti-parkinsonian therapy with several MPTP dosing regimens, there is a common problem with the risk of dose-dependent mortality associated with a relatively high dose or overdose of MPTP [23]. Importantly, biotechnology-derived products such as stem cell therapy and gene therapy occasionally need long-term treatment for anti-parkinsonian activity. Therefore, we tested if our novel MPTP regimen protocol can be maintained stable parkinsonism without mortality in the marmosets.

MATERIALS AND METHODS

Animals and experiments

Four male common marmoset (3 MPTP-exposed and 1 healthy control) weighing 350–450 g (Table 1) were obtained from CLEA Japan Inc. (Tokyo, Japan). During the studies, the animals were maintained in cages measuring 76.8×39.4×59 cm (height×width×depth) with a squeeze-back mechanism in a temperature- and light-controlled room (23–28°C, 45–70% humidity, and 12 h light/dark cycle). They were allowed to have access to a commercial pelleted diet supplemented with vitamin D₃ (New World Primate Diet, Harlan Teklad, Madsion, WI, USA) and fresh fruit with tap water *ad libitum*, and remained under intensive veterinary supervision to monitor good health and body condition including attitude, appetite, or behavior. As shown in Figure 1, the marmosets were given once-daily subcutaneous injections of MPTP hydrochloride (Sigma, St. Louis, MO, USA) for 5 consecutive days (2 mg/kg for 2 days and 1 mg/kg for next 3 days). Injections were made through the subcutaneous layer of the cervicodorsal part of the marmosets. All the procedures with the MPTP were conducted by trained researchers with personal protection in a special room with enclosed cages under negative-pressure (Fig. 2). During the study period, euthanasia is recommended for the marmosets showing significant body weight loss (>20%) with severe anorexia. At 4 weeks after MPTP treatment, all marmosets were subcutaneously treated with L-3,4-dihydroxyphenylalanine (L-Dopa) methyl ester (Sigma; 15.6 mg/kg equivalent to 12.5 mg/kg L-Dopa) to confirm the induction of parkinsonism in MPTP model. Dur-

Table 1. Information on the common marmosets and experimental design

Marmoset		MPTP dosing regimen	Sacrifice after MPTP treatment	Mortality
C1	Male, 61 m	-	-	No
M1	Male, 60 m	2, 2, 1, 1, 1 mg/kg 5 s.c. injections; once daily for 5 days	10 w	No
M2	Male, 60 m	2, 2, 1, 1, 1 mg/kg 5 s.c. injections; once daily for 5 days	11 w	No
M3	Male, 68 m	2, 2, 1, 1, 1 mg/kg 5 s.c. injections; once daily for 5 days	32 w	No

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

Figure 1. Scheme of the experimental design. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Figure 2. An enclosed cage under negative-pressure for the common marmoset.

ing the treatment period, all animals were observed for the clinical signs and mortality at least once daily. Body weights were measured daily for 1 week and then weekly until the end of the study period. Animals were sacrificed and blood samples were collected under deep anesthesia with an intramuscular injection of ketamine (10 mg/kg) and xylazine (4 mg/kg), followed by inhalation of isoflurane (1–2%). All of the animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee of the Biomedical Research Institute at the Seoul National University Hospital (SNUH-IA-

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Behavioral assessment and tower test

As described previously [24], gross behavioral observation for the marmosets in their individual living cages was recorded with video and scored by experienced observers once daily for 1 week and then weekly until the end of the study period. The items included alertness, reaction to stimuli, blinking, checking movement, posture, motility, vocalization, tremor, and fur condition (Table 2), which were considered as signs of parkinsonism. The total number was defined as the behavioral dysfunction score associated with degrees of parkinsonism induced by MPTP treatments.

Jumping ability was measured weekly according to the previously described methods [25] with minor modifications. For this, the Tower (27×27×151 cm) contains 7 levels of horizontal crossbars varying from 8 to 32 cm. Prior to testing, all marmosets were accustomed to the Tower. And, a fresh fruit at top level was used to motivate the marmoset to jump. After entering the Tower (0 level), the marmoset could freely move around on the 7 levels for 7 min. The final marmoset's level was recorded by a camera placed in front of the Tower.

Positron emission tomography

The DAT-selective ligand ^{18}F -FP-CIT [26] was used for positron emission tomography (PET) measurements in the MPTP-treated marmosets (at 2, 4, and 8 weeks after MPTP treatments for M1 and M2 marmosets; at 2, 6, 10, 14, 18, 24, and 32 weeks after MPTP treatments for M3 marmoset). The marmosets were anesthetized with an intramuscular injection of ketamine (10 mg/kg) and xylazine (4 mg/kg), followed by inhalation of isoflurane (1–2%). The marmosets were scanned using a GE eXplore Vista PET/CT camera (GE Healthcare, Lafayette, CO, USA) at 60 min post-injection 1.5 mCi ^{18}F -FP-CIT via the saphenous vein prior to the imaging.

Table 2. Neurological evaluation scores of MPTP-treated common marmosets

Behavior	Scores
Alertness	Normal, 0; Abnormal, 1
Reaction to stimuli	Normal, 0; Reduced, 1; Slow, 2; Absent, 3
Blinking	Normal, 0; Abnormal, 1
Checking movement	Present, 0; Reduced, 1; Absent, 2
Posture	Normal, 0; Abnormal trunk, +1; Tail, +1; Limbs, +1; Grossly abnormal, 4
Motility	Normal, 0; Mild slowing, 1; Moderate bradykinesia, 2; Akinesia, 3
Vocalization	Normal, 0; Reduced, 1; Absent, 2
Tremor	Absent, 0; Present, 1
Fur condition	Normal, 0; Dirty, 1

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

TH immunohistochemistry

At necropsy, brain tissue was rapidly removed from the skull and placed were fixed in 10% neutral buffered formalin for at least 72 h. Tissue slices were embedded in paraffin, sectioned and processed immunohistochemically for TH (Anti-TH antibody, Abcam ab117112, Cambridge, UK). Immunohistochemical staining for TH was conducted with the ChromoMap DAB detection kit (Ventana, Tucson, AZ, USA) according to the manufacturer's instructions.

Statistical analysis

The statistical analysis was performed using a sample t-test and one-way ANOVA, followed by a multiple comparison procedure with a Tukey/Duncan test using SPSS software version 19 (SPSS Inc., Chicago, IL, USA). *p* values of less than 0.05 were considered to be statistically significant.

RESULTS

Mortality, body weight, behavioral assessment

During the full period of the experiment, no mortality was observed in any animals (Table 1). In general, like human patients, parkinsonian marmosets have been known to display slowness of spontaneous movements (bradykinesia), fewer spontaneous movement (akinesia), and muscular rigidity [27,28]. By gross behavioral observation, alertness, reaction to stimuli, blinking, checking movement, posture, balance, motility, vocalization, tremor, and fur condition associated with parkinsonism were scored using a behavioral assessment scale [24] in the marmosets receiving MPTP for 5 consecutive days (Fig. 3A and B) (Supplementary Video 1 in the online-only Data Supplement) in comparison to the marmosets without MPTP treatment (Supplementary Video 2 in the online-only Data Supplement). The MPTP-treated marmoset showed akinesia, rigidity, postural instability, tremor, and abnormal blinking, whereas

active movements were observed prior to MPTP treatment in the marmoset. Importantly, these behavioral changes were maintained for 32 weeks (a score >10) until the end of behavioral observations. Especially, high degree of tremor and postural instability, which are representative symptoms associated with the human PD [1], were observed in the MPTP-treated marmosets for 32 weeks. Although the marmosets had Parkinson's symptoms including behavioral disability due to MPTP treatment, they were placed in an intensive care unit with artificial feedings consisting of a highly nutritious solution made from egg white, sucrose, infant formula, banana, multivitamins, and powdered marmoset pellets to increase survival rate and body weight, leading that there were no significant changes in body weights after MPTP treatment (Fig. 3C).

Tower test

In order to examine the motor performance of parkinsonian marmosets, the Tower test has been routinely conducted by using natural behavior (jumping) and captive marmoset's height preference to be on a high level [25,29]. The average number of level changes was measured weekly. The marmosets prior to MPTP treatment reached the top level 7 within 7 min (Supplementary Video 3 in the online-only Data Supplement). In contrast, the MPTP-treated marmosets were severely impaired in their jumping ability in comparison to their own baseline measurements, and could not reach the higher levels in the Tower (Fig. 4) (Supplementary Video 4 in the online-only Data Supplement). The average top level reached was level 1 to 3 within 7 min. Importantly, this loss of jumping ability of the MPTP-treated marmoset was not recovered by 32 weeks.

L-Dopa test

Although chronic use of L-Dopa, the most effective drug for the treatment of PD, causes dyskinesia in human and NHPs with parkinsonism, single dose of L-Dopa was found to be re-

Figure 3. Time-course analysis (A) and representative snapshots (B) on behaviors and body weight changes (C) in the common marmosets treated with MPTP for 32 weeks. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

versed completely parkinsonian features associated with posture, motility, tremor, and vocalization in the MPTP-treated marmosets [30]. Administration of L-Dopa 12.5 mg/kg to MPTP-treated animals markedly improved behaviors and motor (jumping) disability associated with parkinsonism within 30 min of administration (Fig. 5). In consistent with previous report [31], this reduction in parkinsonian disability was maintained for 2–4 h and then returned to baseline levels.

PET and TH immunohistochemistry

Consistent neurochemical abnormality in PD is accompanied with a loss of TH containing nerve endings in the striatum caused by degeneration of DA neurons in the substantia nigra and, leading to deficiency of striatal DA [1]. The widely applied PET imaging technique is important for studying this de-

generation process in the MPTP-induced marmoset model [32–34]. Previous studies have analyzed PET imaging with markers of DAT for the early diagnosis of PD by identifying the decrease in the binding of DAT markers in MPTP-treated NHPs [35–37]. The DAT-selective ligand ^{18}F -FP-CIT bindings from the PET analysis of the MPTP-treated and the MPTP-free marmosets are presented in Fig. 6A. High specific bindings of ^{18}F -FP-CIT were observed at the striatum of the brains of the MPTP-free marmosets (0 w), whereas marked decreases of these bindings were observed in the brains of the marmosets received MPTP. This decreased level of radioactivity was maintained for 32 weeks after MPTP treatment.

TH observed in various brain regions is the first rate limiting enzyme for DA biosynthesis [1]. In the present study, a decrease in TH immunoreactivity in the substantia nigra in the MPTP-

Figure 4. Time-course analysis (A) and representative snapshots (B) on jumping behavior in the Tower in the common marmosets treated with MPTP for 32 weeks. Active movements were observed prior to MPTP treatment in the marmoset (dotted circle). MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

Figure 5. Time-course study on the effects of L-Dopa on parkinsonian disability in the common marmosets treated with MPTP for 4 weeks. Parkinsonian disability observation (closed circle) and the Tower test (open square). Data expressed as means $\pm$ SEM. *significantly different from baseline prior to L-dopa treatment ($p < 0.05$). MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

treated marmosets was clearly observed in comparison to the control marmoset without MPTP treatment (Fig. 6B). This loss of TH immunoreactivity in the substantia nigra of the marmoset was also found at 32 weeks following the first MPTP injection (data not shown).

DISCUSSION

The wide range of doses of MPTP and the time periods of administration are using for induction of parkinsonism in the marmosets (Table 3). Iravani et al. [38,39] have treated low doses of MPTP (1 mg/kg) for 3 consecutive days. Jackson and Jenner [40] and Philippens et al. [41] have used the protocol using the marmosets received MPTP at 1 mg/kg for 5 consecutive days and 8 consecutive days, respectively. High doses of MPTP (more than 2 mg/kg) for different time periods and routes have also been used to develop the stable parkinsonism in the marmosets [42-49]. Among several dosing regimens for MPTP, once-daily subcutaneous administration of 2 mg/kg MPTP for 5 consecutive days is widely used to induce parkinsonism [42-46]. In this regimen (2 mg/kg MPTP s.c. for 5 consecutive days), all animals showed severe parkinsonian symptoms including marked motor deficits (akinesia, abnormal posture, rigidity, loss of vocalization, tremor). Following 6-8 weeks for recovery from the acute MPTP toxicity, their parkinsonian symptoms (bradykinesia, rigidity and postural abnormalities) were stabilized [43]. Huot et al. [42] and Johnston et al. [45] also found that this regimen of MPTP treatment (2 mg/kg s.c. for 5 con-

Figure 6. ¹⁸F-FP-CIT PET time-course analysis (A) and TH immunohistochemistry (B) in the common marmosets treated with MPTP. TH-positive cells in the substantia nigra are indicated with arrows. Bar represents 200 μm. Data expressed as means±SD. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

secutive days) developed stabilized parkinsonian symptoms at 12 weeks after the last MPTP injection.

Although MPTP has been known as the best model for PD, it has some clear disadvantages with the high dose of MPTP. One of common problems is the acute death of the animals that occurs within 24 h of the first dose of MPTP administration before any meaningful investigations as a result of a peripheral cardiovascular side effect unrelated to the damage in the DA systems of the brain. This risk of unintended mortality is dose dependent [23,50] and can influence the index of success for producing the models and the behavioral phenotype in the

produced models [51]. In a previous study [52], the MPTP administration regimen (2 mg/kg s.c. for 3 consecutive days; cumulative doses of 12–20 mg/kg) was repeated four times at intervals of several months. However, the third dose of each regimen during the 3rd and 4th regimens in some of the MPTP-treated marmoset was canceled due to excessive toxicity [52]. In the present study, the marmosets received only 2 days of MPTP administration also showed excessive toxicity, leading to be placed in an intensive care unit with artificial feeding to increase survival rate. In contrast, a low dose regimen of MPTP can lower the excessive toxicity from a high MPTP dose al-

Table 3. MPTP-treatment regimens to produce PD models in the common marmosets

Dose (mg/kg)	Route	Regimen	Reference
1.0	s.c.	3 injections; once daily for 3 days	[38,39]
1.0	s.c.	5 injections; once daily for 5 days	[40]
1.0	s.c.	8 injections; once daily for 8 days	[41]
1.8	i.p.	4 injections; once daily for 4 days	[61]
2.0	s.c.	3 injections; once daily for 3 days	[62]
2.0	s.c.	5 injections; once daily for 5 days	[42-46]
2.5	s.c.	3 injections; once daily for 3 days	[47]
2.5	s.c.	5 injections; once daily for 5 days	[48]
2.5	i.v.	1 or 2 injections; once daily for 1 or 2 days	[49]

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, PD: Parkinson's disease

though a low dose regimen is possible to induce partial lesion with no motor parkinsonian symptoms since Parkinson motor signs have known to appear in case that about 80% of striatal or putaminal dopamine is lost [53]. MPTP treatments at a dose of 1 mg/kg for 3 or 5 consecutive days induced partial lesion in comparison to 2 mg/kg MPTP for 5 consecutive days developing full lesion [39,40]. Also, the marmosets with daily subcutaneous injections of low doses (1 mg/kg) MPTP for 8 consecutive days were found to be not fully incapacitated and still able to feed themselves in a PD stage with slow apoptotic cell death [41]. Therefore, in our study, the marmosets received 2 consecutive days of 2 mg/kg MPTP administration were treated 1 mg/kg MPTP for next 3 consecutive days, resulting in no mortality and no excessive toxicity in all marmosets.

The advancement in biotechnology and stem cell research enable stem cell/gene-based therapies to be considered as potential and novel therapies. The transplantation of DA neurons generated from monkey ES cells has been shown to attenuate the MPTP-induced neurological symptoms in NHP PD model [54]. And, it has also been reported that human iPSC-derived neural progenitor cells survived as DA neurons in the brain of NHP model of PD for six months [55]. Yi et al. [56] indicated that naïve or genetically modified neural stem cells differentiating into TH and/or DAT-positive cells have a great potential for treating PD. Kordower et al. [57] demonstrated that glial cell line-derived neurotrophic factor delivery into the striatum and substantia nigra can be a viable therapeutic strategy for PD patients by preventing nigrostriatal degeneration and inducing regeneration in MPTP-treated NHP PD models. Importantly, stem cell therapy and gene therapy occasionally need long-term treatment for anti-parkinsonian activity. Takagi et al. [54] reported increases in ^{18}F -fluorodopa uptake at the putamen of the ES cell-transplanted animals at 14 weeks after cell transplantation in the evaluation of the efficacy of stem cell therapy using cynomolgus monkeys exhibiting stable parkinsonism, such as

tremor, bradykinesia, and impaired balance after intravenous injections of 0.4 mg/kg MPTP twice a week. Hallett et al. [5] also reported that daytime activity in animal receiving autologous iPSC was increased by 146% at 6 months after transplantation. However, parkinsonian-like symptoms induced in our low-dose MPTP regimen protocol (2 mg/kg for 2 consecutive days and 1 mg/kg for next 3 consecutive days) can be reversed spontaneously although there was no excessive toxicity such as mortality. Acute or subacute administrations of MPTP have been reported to induce reversible loss of DA functions [58]. Eidelberg et al. [59] indicated that cynomolgus monkeys treated intravenously with MPTP developed the signs of parkinsonism. However, this parkinsonism spontaneously reversed from 4 weeks after the first dose of MPTP. Close et al. [60] reported that MPTP produced a reversible parkinsonian-like syndrome in the marmosets. Our unpublished data also indicated that cynomolgus monkeys received once-daily subcutaneous injection of 0.2 mg/kg MPTP for 14 days showed spontaneous recovery from parkinsonian symptoms, leading to additional intravenous injection of 2 mg/kg MPTP on 70th day from the first MPTP dose to maintain the level of the PD signs. Therefore, in order to use our low-dose MPTP regimen for long-term stem cell anti-parkinsonian therapy, we confirmed if parkinsonian-like symptoms induced in our low-dose MPTP regimen are maintained without spontaneous recovery, and found that parkinsonian symptoms with the loss of tyrosine hydroxylase were found to be maintained for 32 weeks after the first MPTP injection.

In conclusion, our low-dose MPTP regimen protocol (2 mg/kg for 2 consecutive days and 1 mg/kg for next 3 consecutive days) was found to be stable parkinsonism for 32 weeks without mortality as evidenced by behavior, PET, and TH immunohistochemistry. This result will be useful for long-term evaluation of possible anti-parkinsonian biotechnology-derived products, stem cell therapy and gene therapy although larger

scale studies are necessary to validate this tendency of parkinsonism in marmosets.

Supplementary Materials

The online-only Data Supplements are available with this article at <http://dx.doi.org/10.1007/s13770-015-0106-3>.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

All authors including myself have seen and approved this manuscript for publication. This manuscript has not been submitted to any other journals for publication. All aspects and protocols of the animal experiments and husbandry were carried out in compliance to the international, national and institutional rules.

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