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A Water Extract of *Mucuna pruriens* Provides Long-Term Amelioration of Parkinsonism with Reduced Risk for Dyskinesias

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Abstract

Dopaminergic anti-parkinsonian medications, such as levodopa (LD) cause drug-induced dyskinesias (DID) in majority of patients with Parkinson's disease (PD). *Mucuna pruriens*, a legume extensively used in Ayurveda to treat PD, is reputed to provide anti-parkinsonian benefits without inducing DID. We compared the behavioral effects of chronic parenteral administration of a water extract of *Mucuna pruriens* seed powder (MPE) alone without any additives, MPE combined with the peripheral dopa-decarboxylase inhibitor (DDCI) benserazide (MPE+BZ), LD +BZ and LD alone without BZ in the hemiparkinsonian rat model of PD. A battery of behavioral tests assessed by blinded investigators served as outcome measures in these randomized trials. In experiment 1, animals that received LD+BZ or MPE+BZ at high (6mg/Kg) and medium (4mg/Kg) equivalent doses demonstrated significant alleviation of parkinsonism, but, developed severe dose-dependent DID. LD+BZ at low doses (2mg/Kg) did not provide significant alleviation of parkinsonism. In contrast, MPE+BZ at an equivalent low dose significantly ameliorated parkinsonism. In experiment 2, MPE without any additives (12mg/Kg and 20mg/Kg LD equivalent dose) alleviated parkinsonism with significantly less DID compared to LD+BZ or MPE +BZ. In experiment 3, MPE without additives administered chronically provided long-term anti-parkinsonian benefits without causing DID. In experiment 4, MPE alone provided significantly more behavioral benefit when compared to the equivalent dose of synthetic LD alone without BZ. In experiment 5, MPE alone reduced the severity of DID in animals initially primed with LD+BZ. These findings suggest that *Mucuna pruriens* contains water soluble ingredients that either have an intrinsic DDCI-like activity or mitigate the need for an add-on DDCI to ameliorate parkinsonism. These unique long-term antiparkinsonian effects of a parenterally administered water extract of *Mucuna pruriens* seed powder may provide a platform for future drug discoveries and novel treatment strategies in PD.

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Keywords

basal ganglia; 6-hydroxydopamine (6-OHDA); nigrostriatal degeneration; movement disorders; complementary and alternative medicine

INTRODUCTION

Chronic use of current anti-parkinsonian medications causes disabling abnormal involuntary movements known as drug-induced dyskinesias (DID) in majority of patients with advanced Parkinson's disease (PD) that are expensive and difficult to treat [1]. Hence, newer anti-PD treatments that reduce or eliminate the risk of DID are much sought after [2]. The dried endocarp powder of the *Mucuna pruriens* bean is used to treat parkinsonism (*Kampavata*) in the Ayurvedic Indian medical system [3,4]. Review of the Ayurvedic literature and current Ayurvedic practioners indicate that PD patients treated with *Mucuna pruriens* do not develop DID. *Mucuna pruriens* has been shown to contain natural levodopa (LD, [5–11]), which has been presumed to be the mechanism of its action in PD. Under this presumption, many experimental studies have concomitantly administered *Mucuna pruriens* with a dopa-decarboxylase inhibitor (DDCI) like benserazide (BZ, [12]) or carbidopa (CD, [13]); or used subjects that concurrently took a DDCI as part of their treatment regimen without adequate washout [14]. However, no DDCI is utilized in Ayurveda with *Mucuna pruriens* treatment. Therefore, in the present study, we assessed the antiparkinsonian effects of a water extract of *Mucuna pruriens* seed powder (MPE) for the amelioration of parkinsonism in the 6-hydroxydopamine (6-OHDA)-lesioned hemiparkinsonian (HP) rat model of PD. We show that this water extract has a unique anti-PD effect that does not require the addition of a DDCI and that MPE is more effective and has reduced severity of DID than conventional LD+DDCI therapy.

METHODS AND MATERIALS

Animals

Female Sprague-Dawley rats (250–400g) were used. Procedures were conducted in compliance with institutional protocols, and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1978).

6-OHDA lesion and rotational validation for HP state

Rats were unilaterally lesioned using techniques we have previously described [15]. Briefly, rats were anesthetized with ketamine/xylazine and placed in a stereotactic frame. 6-OHDA was dissolved in 0.9% saline (containing 0.2% ascorbic acid) at a concentration of 2.0mg/ml. The skull was exposed, small burr holes made, and stereotaxic injections at a rate of 1.0μl/min for 2 mins were made into the medial forebrain bundle (AP: −4.4, ML: 1.2, DV: −7.5) and the substantia nigra (SN) (AP: −5.3, ML: 2.0, DV: −7.5) in relation to bregma and dural surface. After completion of surgery, the incision was sutured and rats were allowed to recover. Apomorphine was administered at 0.2mg/Kg (s.c.) at 3 and 5 weeks after 6-OHDA exposure to measure apomorphine-induced rotations. Only HP rats with > 245 rotations over 35 minutes were used in the study.

Treatment with LD and MPE

A simple water extract of *Mucuna pruriens* endocarp powder was developed based on the presence of approximately 5% LD content in *Mucuna pruriens* [8] and HPLC estimates of the *Mucuna pruriens* endocarp powder that we used in this study (HPLC data on file, Zandu Pharmaceuticals, Mumbai, India). *Mucuna pruriens* endocarp powder (Zandu

Pharmaceuticals, Mumbai, India) was mixed in sterile water for 30 minutes then centrifuged at 15,000 RPM for 15 minutes. The supernatant was extracted, filtered and stored in sterile containers at 4°C for up to 1 week and dispensed daily. MPE was agitated briefly before each exposure and remained stable and clinically efficacious as a refrigerated solution for 1 week. Treatments were given IP on alternating sides of the abdomen. In experiment 1, we administered BZ at a dose of 15mg/Kg along with LD (levodopa methyl ester) or MPE. In experiment 2 and 3, we used parenteral MPE alone without any additives. In experiment 4, we used LD alone, MPE alone and LD+BZ. In experiment 5, we used LD+BZ and MPE alone.

Experimental Design

Experiment #1 – Comparison between equivalent doses of LD+BZ and MPE +BZ—Twenty-one HP rats separated into three groups received LD for 10 days b.i.d. at 6mg/Kg (Group 1), 4mg/Kg (Group 2), and 2mg/Kg (Group 3) with 15mg/Kg of BZ (LD +BZ). After 7 day drug-washout, animals received the equivalent LD dose of 120mg/Kg MPE (Group 1), 80mg/Kg MPE (Group 2), and 40mg/Kg MPE (Group 3) with 15mg/Kg of BZ (MPE+BZ) for 10 days b.i.d. Behavioral assessments (stepping test and DID ratings) were taken 30 mins after treatment.

Experiment #2 – Exposure to high doses of MPE alone—HP rats (N = 7) were exposed to MPE alone at 240mg/Kg (equivalent to 12mg/Kg LD) for 10 days b.i.d. After drug-washout for 7 days, rats were exposed to 400mg/Kg (equivalent to 20mg/Kg LD) for 10 days b.i.d. Behavioral assessments (stepping test and DID ratings) were taken 30 mins after treatment.

Experiment #3 – Long term efficacy of MPE alone—HP rats (N = 6) received MPE alone at 400mg/Kg (equivalent to 20mg/Kg LD) b.i.d. for 10 days. Behavioral assessments for amelioration of parkinsonism (stepping test, vibrissae-evoked forelimb placement test, body axis bias test and cylinder test) were taken at 30 mins, 60 mins, and 90 mins after treatment. DID and contralateral turning was assessed at 5, 15, 30, 60, and 90 mins after treatment.

Experiment #4 – Long-term anti-parkinsonian efficacy comparison for LD alone, MPE alone and LD+BZ—In the following experiment, we directly assessed whether equivalent doses of LD alone or MPE alone without BZ could induce equivalent behavioral benefit. HP rats were treated with LD alone (24mg/Kg), MPE alone at 480mg/Kg (equivalent to 24mg/Kg LD), or LD+BZ (6mg/Kg LD + 15mg/Kg BZ) for an average of 4 days b.i.d. (N = 5 per group). Behavioral assessments for parkinsonism alleviation (stepping test and vibrissae-evoked forelimb placement test) were taken at 30 mins, 60 mins, and 90 mins after treatment exposure. Our preliminary blinded studies showed that LD+BZ treatment induced DID that appeared to interfere with the animal's performance of the cylinder and body axis bias tests at 60 mins. The stepping test and vibrissae-evoked forelimb placement tests were not significantly affected by DID. Therefore, we excluded the cylinder and body axis bias tests in this experiment.

Experiment #5 – Long-term drug-induced dyskinesia priming effects of LD+BZ and MPE alone—In a cross-over experimental design to examine dyskinesia priming effects, Group A was first treated with LD+BZ (6mg/Kg LD + 15mg/Kg BZ) then immediately treated with MPE alone at 480mg/Kg (equivalent to 24mg/Kg LD) (N = 5). Group B was first treated with MPE alone at 480mg/Kg (equivalent to 24mg/Kg LD) then immediately treated with LD+BZ (6mg/Kg LD + 15mg/Kg BZ) (N = 5). DID ratings were

taken at 5, 15, 30, 60, and 90 mins after treatment. Each treatment was given for an average of 10 days b.i.d.

Behavioral Assessment

Stepping Test—A modified version of the stepping test [16] was used to measure forelimb akinesia and rigidity. The animal is held by the experimenter restraining both hindlimbs and one forelimb. The unrestrained forelimb touches the tabletop. The number of adjusting steps was counted while the rat is moved sideways along the surface at a rate of 90cm/5s in the direction of the unrestrained forelimb. The test was done separately for both forelimbs. Stepping test scores are expressed as the difference between the unaffected (ipsilateral to lesion) and affected forelimb (contralateral to lesion). Trials where animals struggled were not recorded.

Vibrissae-Evoked Forelimb Placement Test—This test was performed as described by Dr. Schallert [17,18]. Animals were gently held at the torso. The hind limbs and forelimb being tested were allowed to hang freely while the forelimb not being tested was carefully restrained. Each forelimb was evaluated by bringing the rat towards the edge of the table top to elicit a forelimb reaching behavior towards the surface. Each forelimb was tested independently for 10 trials. The number of successful forelimb placements for each forelimb onto the tabletop was scored.

Body Axis Bias Test—A modified version of the body axis bias test (curling behavior) was used to measure posturing activity [19,20]. The rat was placed individually in a plastic testing cage. After attaining a neutral position with all four limbs touching the bottom of the cage, the rat was vertically lifted by the base of the tail so its head was approximately 1 inch from the cage bottom. The first direction of body deviation away from vertical axis of ≥ 10° was recorded during a 5s interval. An ipsilateral turn towards the lesioned side was scored with a +1, contralateral turn away from lesioned side scored as −1, and no bias as 0 during the 5s interval. This behavior was recorded twice at each time point.

Cylinder Test—A modified version of the cylinder test was used to measure forelimb usage preference in spontaneous, exploratory behavior [17,18]. Rats were placed into a clear cylinder (25 cm tall, 16 cm ID) and videotaped for 3–10 minutes depending on the activity of each animal. Time spent in the cylinder was either limited to approximately 20 total contacts or scored for the entire 10 minute trial period. The number of unaffected, affected, and both forelimb placements were counted. Limb asymmetry scores were calculated using the following formula: (# of unaffected limb contacts + 0.5 × both forelimb contacts)/total contacts × 100.

Drug-induced Dyskinesias Ratings—DID were rated using a scale similar to previous reports [21,22]. The term “dyskinesia” is used to describe abnormal involuntary movements of the forelimb, neck, and trunk characterized by dystonic posturing, hyperkinesias and stereotypies. Animals were placed in individual cages and sessions videotaped for 2 minutes. Intensity rating scores were 0-none, 1-mild, 2-moderate, and 3-severe. Frequency ratings ranged from 0-none, 1-present < 50% of observation session, 2-present > 50% of observation session, and 3-present for entire session. Frequency and intensity were multiplied to attain individual severity scores. Profiles are the sum of limb, neck and trunk severity scores. Behavioral assessments were done in a blinded fashion such that the investigators were unaware of treatment in the animals.

Tyrosine Hydroxylase (TH) Immunohistochemistry

Animals were deeply anesthetized with pentobarbital and euthanized via transcardial perfusion with heparinized saline and paraformaldehyde-lysine-periodate fixative [23]. Brains were removed, cryoprotected and sectioned coronally at 60μm. Sections were processed using a modified procedure previously described for TH immunohistochemistry [15,24]. Briefly, sections were treated with anti-TH antibody (1:350, Pel-freeze) for 72 hours, washed and treated with biotin-donkey anti-rabbit IgG (1:300). Subsequently, sections were treated with Avidin-HRP (1:500), developed through a DAB reaction, mounted and coverslipped before microscopic evaluation and photography. Stereological counts using optical fractionator was used to estimate TH+ SN pars compacta (SNpc) neurons to confirm that there was >92% loss on the lesioned hemisphere.

Statistical Analysis

Repeated measures or one-factor ANOVA was used with post-hoc Tukey-Kramer Multiple Comparisons Test for behavioral comparisons. Data are expressed as mean ± SEM. Significance was set at $p < 0.05$.

RESULTS

Experiment #1- Comparison between equivalent doses of LD+BZ and MPE+BZ

Stepping Test—Scores for Group 1 (equivalent to 6mg/Kg LD) were 10.3 ± 2.9 post-lesion which significantly decreased to −1.9 ± 2.3 with LD+BZ ($p < 0.05$ vs. post-lesion) and −1.2 ± 2.5 with MPE+BZ ($p < 0.05$ vs. post-lesion). Group 2 (equivalent to 4mg/Kg LD) post-lesion scores were 11.6 ± 3.3 which decreased to 1.7 ± 2.1 with LD+BZ ($p < 0.05$ vs. post-lesion) and −1.8 ± 2.5 with MPE+BZ ($p < 0.01$ vs. post-lesion). The scores for Group 3 (equivalent to 2mg/Kg LD) were 6.9 ± 2.0 post-lesion. Scores decreased to 3.0 ± 0.96 with LD+BZ but were not significant ($p > 0.05$ vs. post-lesion). However, there was a significant decrease to 1.1 ± 1.8 with MPE+BZ ($p < 0.05$ vs. post-lesion) in these animals (Fig. 1A).

Dyskinesia Profile—Profiles for Group 1 were 18.3 ± 2.3 for LD+BZ and 19.0 ± 2.1 for MPE+BZ. Dyskinesia profiles for Group 2 were 11.7 ± 3.5 for LD+BZ and 12.9 ± 3.4 for MPE+BZ. Profile scores for Group 3 with LD+BZ were 0.93 ± 0.24 and 2.4 ± 0.87 for MPE +BZ. Group 3 showed significantly less DID than Group 1 and 2 for both LD+BZ and MPE +BZ ($p < 0.05$) (Fig. 1B).

These results show that LD+BZ and MPE+BZ at high and medium doses provide significant anti-parkinsonian effects but induce severe dose-dependent DID. However, only the lowest MPE+BZ dose was able to significantly ameliorate parkinsonism, whereas the equivalent synthetic LD+BZ dose did not provide significant behavioral benefit.

Experiment #2 – Comparison between high doses of MPE alone

Stepping Test—Scores were 13.1 ± 1.1 at post-lesion. Scores after treatment with 240mg/Kg of MPE significantly decreased to 5.0 ± 1.3 ($p < 0.01$ vs. post-lesion) and 4.1 ± 1.6 ($p < 0.01$ vs. post-lesion) with 400mg/Kg of MPE (Fig. 2A).

Dyskinesia Profile—Dyskinesia profiles for animals exposed to 240mg/Kg of MPE were 0.69 ± 0.16. Profiles for exposure to 400mg/Kg of MPE were 1.6 ± 0.87. These scores were significantly different from dyskinesia profiles of Group 1 (LD+BZ and MPE+BZ) from experiment #1 ($p < 0.001$) (Fig 2B).

These results suggest that MPE without any add-on DDCI (BZ) can provide behavioral benefit with a reduction in DID severity.

Experiment #3 – Long term efficacy of MPE alone

Stepping Test—Scores at post-lesion were 15.6 ± 1.5 which significantly improved after treatment with 400mg/Kg of MPE alone with scores of 6.9 ± 1.2 at 30 mins, 5.1 ± 0.98 at 60 mins, and 8.7 ± 0.71 at 90 mins ($p < 0.001$ vs. post-lesion) (Fig. 3A).

Vibrissae-Evoked Forelimb Placement Test—Post-lesion scores showed significant deficit with values of 0.67 ± 0.40. The unaffected forelimb showed no behavioral deficits. However, significant amelioration of parkinsonism was evident after MPE alone at 30 mins with a score of 7.1 ± 0.77, 9.1 ± 0.36 at 60 mins and 9.9 ± 0.06 at 90 mins after treatment ($p < 0.001$ vs. post-lesion) (Fig. 3B).

Body Axis Bias Test—Body axis bias significantly decreased after administration of MPE from post-lesion to 0.28 ± 0.48 at 30 mins, 0.56 ± 0.61 at 60 mins, and 0.33 ± 0.49 at 90 mins after MPE treatment ($p < 0.05$ vs. post-lesion) (Fig. 3C).

Cylinder Test—Post-lesion cylinder test scores were 87.7 ± 2.4. There was a significant decrease with MPE alone with scores at 53.3 ± 7.7 at 30 mins after drug exposure ($p < 0.01$ vs. post-lesion) showing almost equivalent usage of both forelimbs. Scores at 60 mins (74.2 ± 3.7) and 90 mins (85.4 ± 4.3) showed a decreasing trend but were not significant from post-lesion scores ($p > 0.05$) (Fig. 3D).

Dyskinesia Profile and Contralateral Turning—At 5 and 15 minutes after treatment, animals displayed both contralateral turning and DID. Four animals displayed consistent contralateral rotations with an average of 49.9 ± 5.8 rotations at 5 mins and 22.0 ± 4.9 at 15 mins after treatment. Dyskinesia profiles were 7.3 ± 1.3 at 5 mins, 4.8 ± 1.8 at 15 mins and 2.6 ± 0.58 at 30 mins after treatment. These profiles were significantly less than Group 1 dyskinesia profiles in experiment #1 with BZ ($p < 0.01$ and $p < 0.001$) (Fig. 3E). There was no contralateral turning at 30 mins, 60 mins, and 90 mins and DID were non-existent at 60 and 90 mins after treatment.

The results of this experiment suggest that MPE alone can provide significant long-term behavioral benefit while reducing the severity of DID.

Experiment #4 – Long-term anti-parkinsonian efficacy comparison for LD alone, MPE alone and LD+BZ

Stepping Test

LD Alone (24mg/Kg): Scores were 15.0 ± 0.71 at post-lesion. LD alone treatment did not provide significant long-term amelioration of parkinsonism with scores of 8.2 ± 2.87 at 30 mins, 9.4 ± 1.40 at 60 mins and 8.6 ± 1.17 at 90 mins ($p > 0.05$ vs. post-lesion).

MPE Alone (480mg/Kg - equivalent to 24mg/Kg LD): Post-lesion scores were 15.6 ± 0.60. There was significant improvement after treatment with MPE alone with scores of 1.0 ± 0.45 at 30 mins, 3.2 ± 1.28 at 60 mins and 3.4 ± 1.81 at 90 mins ($p < 0.001$ vs. post-lesion).

LD+BZ (6mg/Kg LD + 15mg/Kg BZ): Scores were 15.0 ± 0.71 at post-lesion. After treatment with LD+BZ, there was significant alleviation with scores of 1.2 ± 0.97 at 30 mins, 6.8 ± 2.08 at 60 mins and 0.2 ± 3.22 at 90 mins ($p < 0.05$ vs. post-lesion). Results are shown in Fig. 4A.

Vibrissae-Evoked Forelimb Placement Test

LD Alone (24mg/Kg): There was significant deficit at post-lesion with scores of 0.10 ± 0.10. The unaffected forelimb had no behavioral deficits. There was no significant amelioration of parkinsonism with LD alone at 30 mins with scores of 5.4 ± 2.27, 2.2 ± 1.36 at 60 mins and 4.0 ± 2.45 at 90 mins after treatment ($p > 0.05$ vs. post-lesion)

MPE Alone (480mg/Kg - equivalent to 24mg/Kg LD): Post-lesion scores showed significant deficit with values of 0.20 ± 0.20. The unaffected forelimb was normal with this test and had no deficits. However, significant amelioration of parkinsonism was evident after MPE alone at 30 mins with a score of 8.0 ± 1.14, 9.0 ± 0.63 at 60 mins and 10.0 ± 0.00 at 90 mins after treatment ($p < 0.001$ vs. post-lesion).

LD+BZ (6mg/Kg LD + 15mg/Kg BZ): Post-lesion scores had significant deficit with values of 0.10 ± 0.10. The unaffected forelimb was normal. There was significant amelioration of parkinsonism after LD+BZ only at 30 mins (6.8 ± 1.77; $p < 0.05$ vs. post-lesion) and 90 mins (8.0 ± 2.0; $p < 0.01$ vs. post-lesion). Scores at 60 mins were not significant when compared to post-lesion (4.8 ± 2.17; $p > 0.05$ vs. post-lesion). Results are shown in Fig. 4B.

Taken together, these results demonstrate that MPE alone provides significant behavioral benefit and that the equivalent synthetic LD dose when given without the DDCI BZ is unable to provide significant equivalent anti-parkinsonian effects.

Experiment #5 – Long- term drug-induced dyskinesia priming effects of LD+BZ and MPE alone

Group A: First Treatment – LD+BZ, Second Treatment – MPE alone—Group A first received LD+BZ (6mg/Kg LD + 15mg/Kg BZ) then MPE alone (480mg/Kg - equivalent to 24mg/Kg LD). LD+BZ dyskinesia profile scores were 0.05 ± 0.05 at 5 mins, 3.7 ± 2.06 at 15 mins, 14.3 ± 5.48 at 30 mins, 12.0 ± 4.25 at 60 mins and 11.1 ± 4.55 at 90 mins. MPE alone dyskinesia profile scores were 5.0 ± 2.48 at 5 mins, 3.7 ± 1.81 at 15 mins, 1.3 ± 0.58 at 30 mins and no DID at 60 and 90 mins. Comparisons between various treatment time points showed significant differences between MPE alone and LD+BZ treatments ($p < 0.05$) (Fig. 5A). These results show that MPE alone substantially ameliorates the occurrence of DID in animals that had previously exhibited robust DID in response to intermittent LD+BZ treatments.

Group B: First Treatment – MPE alone, Second Treatment – LD+BZ—Group B first received MPE alone (480mg/Kg – equivalent to 24mg/Kg LD) then LD+BZ (6mg/Kg LD + 15mg/Kg BZ). MPE alone dyskinesia profile scores were 2.0 ± 0.75 at 5 mins, 1.5 ± 0.97 at 15 mins, 1.1 ± 0.48 at 30 mins, 0.25 ± 0.25 at 60 mins and 0.05 ± 0.05 at 90 mins. LD+BZ dyskinesia profile scores were 0.0 at 5 mins, 0.85 ± 0.56 at 15 mins, 12.5 ± 1.60 at 30 mins, 19.2 ± 3.23 at 60 mins and 12.9 ± 4.72 at 90 mins. Comparisons between various treatment time points showed significant differences between MPE alone and LD+BZ treatments ($p < 0.01$) (Fig. 5B). MPE alone pretreatment did not appear to ameliorate the intensity of LD+BZ-induced dyskinesias.

Confirmation of HP nigrostriatal degeneration

Apomorphine-induced rotations, TH immunohistochemistry and unbiased stereological counts of TH+ SNpc neurons confirmed the HP state and unilateral degeneration of TH+ nigrostriatal neurons.

DISCUSSION

Our study is the first to demonstrate that a simple water extract of *Mucuna pruriens* endocarp powder with no additives has a superior effect to the combination of MPE+BZ on parkinsonism and that MPE alone is superior to LD alone or LD+BZ combinational therapy in terms of efficacy of ameliorating parkinsonism with dramatically reduced risk for DID. This result is consistent with the observations by Ayurvedic practitioners that PD patients treated with *Mucuna pruriens* alone do not exhibit any significant DID. Interestingly, we found that addition of BZ to MPE induced severe DID. The most probable explanation for our findings is that the inhibitory effect of BZ on peripheral DDC allowed the abrupt and rapid increased transport of natural LD contained in MPE across the blood brain barrier without being inactivated in the peripheral blood. This increased availability of LD to the brain is the most likely cause of the severity of DID in MPE+BZ treated animals. An alternative explanation is that the natural form of LD contained in MPE is in combination with one or more natural agents that protect it from rapid decarboxylation by DDC and allow gradual protected transport across the blood brain barrier. A third possibility is that MPE may have natural anti-dyskinetic agents that prevents or mitigates DID and that the addition of BZ to MPE negates these beneficial anti-DID compounds. These hypotheses are the topic of ongoing fractionation studies using MPE. Previous reports in the rat have suggested that chronic *Mucuna pruriens* treatment has no significant effect on LD content or dopamine and its metabolites in the nigra or striatum [25]. However, these experiments were performed in a manner that did not directly test whether LD levels in the brain were acutely altered after administration of *Mucuna pruriens*. In these studies, animals were fed orally *Mucuna pruriens* powder mixed with rat chow nightly for 52 weeks then sacrificed in the morning several hours after the last drug exposure. Therefore, it is possible that nigrostriatal catecholamine content was higher immediately after exposure than it was at the time of brain examination. Future studies that measure nigrostriatal LD and dopamine content in a strict time course post-drug administration will be necessary to further delineate whether addition of BZ or other DDCI to MPE will cause a rapid increase of LD and dopamine in the striatum. Moreover, unlike other preparations derived from *Mucuna pruriens* that required significant proprietary processing, we utilized a simple water extract of *Mucuna pruriens* dried endocarp that can be stored up to 1 week in a refrigerator and show that such an extract can provide significant behavioral amelioration of parkinsonism with minimal risk for DID in a sustained fashion.

We observed an apparent bimodal time course of behavioral benefit in the stepping and vibrissae-evoked forelimb placement tests when HP rats were treated with LD+BZ (Fig. 4A and B) and LD alone (Fig. 4B) with an initial benefit noted at 30 mins post-treatment, followed by a reduction in benefit at 60 mins post-treatment and resumption of benefit at 90 mins post-treatment. The behavioral rater and independent blinded evaluations of the tapes did not reveal any DID that significantly interfered with the ability of the animals to perform these behavioral tasks. A previous study evaluating adjusting steps (modified step test) in 6-OHDA lesioned rats at a time course of 15 and 45 mins post-LD (6mg/Kg) treatments reported more behavioral benefit at the 45 mins time point than the 15 mins time point [12]. The differences in results between this study and the present study may be due to the use of different evaluation time points (45 mins vs. 60 mins), shorter stepping test procedure (70cm/4s vs. 90cm/5s) and/or a lower dose of BZ (6mg/Kg vs. 15mg/Kg). Thus, the reasons for this apparent bimodal time course are unclear. One possibility is that neurocognitive toxic effects of LD and LD+BZ that we did not specifically test interfered with the ability of these animals to perform the stepping and vibrissae-evoked forelimb placement tests at 60 minutes. Future studies that include neurocognitive testing may help with comparison of MPE to LD and LD+BZ in the non-motor aspects of PD.

Previous studies using a variety of formulations of *Mucuna pruriens* have suggested that there are anti-PD compounds besides LD in this naturally occurring seed [4,12–14,25–28]. However, these previous studies did not recognize the water soluble nature of these compounds and the notion that MPE is best when used by itself with no additives of DDCI. Early clinical trials of *Mucuna pruriens* for PD [26,28] suggested the presence of other compounds besides LD in *Mucuna pruriens* endocarp powder provide anti-PD effects. In the HP-200 (a proprietary *Mucuna pruriens* formulation) study, two separate and distinctive populations of PD patients were recruited; the first group of patients that had PD for several years and had used anti-PD medications for several years and a second group of patients who were drug naïve newly diagnosed PD patients. The first group of patients did not have any drug washout prior to enrollment. Therefore, the effects of HP-200 by itself could only be assessed in the second group of drug naïve early PD patients, who do not develop DID. Hence the effects of *Mucuna pruriens* on DID could not be properly assessed in this study [26]. However, they noted that the drug naïve cohort got substantial benefits despite being on very low doses of HP-200 that contained very small quantities of natural LD, leading them to conclude that additional anti-PD agents were contained in HP-200. Vaidya et. al. also made a similar observation in their PD patients treated with *Mucuna pruriens* [28]. However, these investigators bought *Mucuna pruriens* from multiple local Ayurvedic drug providers that are known to vary in quality and used a clinical rating method that has subsequently been shown to be deficient. Moreover, these investigators allowed the use of concomitant medications without any restrictions. Therefore, critics had attributed the anti-PD effects of *Mucuna pruriens* in these studies mainly to the naturally present LD in the seed and not to other compounds found in *Mucuna pruriens*.

A subsequent preclinical behavioral study evaluated the rotational effects of *Mucuna pruriens* in the 6-OHDA lesioned HP rat by exposing animals orally to equivalent doses of *Mucuna pruriens* and LD with and without CD [13]. In this study, *Mucuna pruriens* + CD treated HP rats displayed significantly more contralateral rotations than equivalent doses of synthetic LD+CD. When animals were exposed to *Mucuna pruriens* alone, minor contralateral rotations were noted. However, these investigators did not evaluate any other behavioral effects of *Mucuna pruriens*. A recent study of a proprietary formulation of *Mucuna pruriens* that reportedly contains a higher (12.5%) concentration of natural LD was tested in combination with BZ in HP rats using behavioral tests. These investigators did not test spontaneous behaviors (e.g. cylinder test) and did not use standardized DID scales. These investigators noted that their *Mucuna pruriens* formulation (in combination with BZ) was more efficacious and produced a lower incidence of involuntary movements than the equivalent LD+BZ [12]. However, they did not test their formulation without added BZ.

Nagashayana et. al. evaluated *Mucuna pruriens* mixed with other Ayurvedic treatments in PD patients [27] who were taken off all medications 15 days prior to enrollment. The first group of patients underwent cleansing and palliative therapy while the second group underwent only palliative treatment. Only the first group exhibited significant symptomatic improvement. These findings support our results that *Mucuna pruriens* treatment may be more effective when the patients do not have other competing DDCI in the system and a complete washout of any DDCI may enhance the effectiveness of *Mucuna pruriens*. Katzenschlager and colleagues also report effectiveness of a proprietary formulation of *Mucuna pruriens* in 8 advanced PD patients [14]. They showed that *Mucuna pruriens* treated patients had a more rapid onset of action and longer “on” time when compared to LD +CD treatment of comparative doses. This single dose study was a 4-hour evaluation of this formulation of *Mucuna pruriens* that contained several additives. These patients were not completely washed off their existing anti-PD medications that included DDCI. Thus, this 4-hour study could not truly evaluate the effects of *Mucuna pruriens* (without any DDCI) on PD symptoms including the risk of causing DID. This could be an explanation for why they

did not find any differences in DID between *Mucuna pruriens* treatment and the LD+CD treatment in their patients. Moreover, withdrawal of anti-PD medications in patients with advanced disease has morbidity and can lead to potential fatality. Therefore, a clinical research trial of *Mucuna pruriens* or MPE in advanced PD patients without any confounding DDCI would be extremely difficult to execute.

The present study addresses several drawbacks of previous studies by using a well-characterized animal model of PD, treatment dosing similar to what is used in PD patient population, single drug treatment with no confounding concomitant medications or additives, a full battery of validated behavioral tests including DID assessments, and histological confirmation of uniform nigrostriatal deficits in all animals. Furthermore, we demonstrate that MPE is effective with duration of action that exceed oral *Mucuna pruriens* and is advantageous over conventional oral anti-parkinsonian medications. Gastrointestinal dysfunction is a common problem in PD and may cause issues with absorption of oral treatments [29]. To avoid problems with oral consumption and potential issues with gastrointestinal absorption, we used a water extract of the *Mucuna pruriens* seed powder parenterally administered. Future studies are necessary to identify the water-soluble anti-dyskinetic and anti-parkinsonian compounds that are present in MPE.

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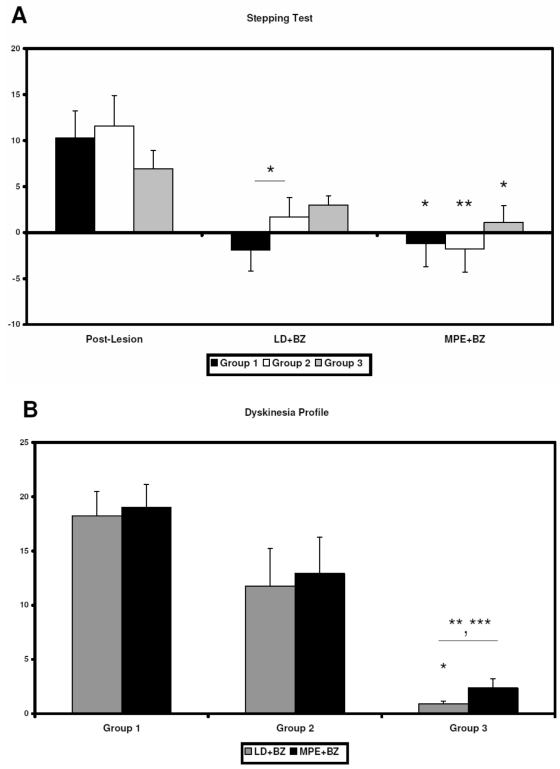

Fig. 1.

Comparison of LD+BZ and MPE+BZ. (A) Group 1 (equivalent to 6mg/Kg LD) and Group 2 (equivalent to 4mg/Kg LD) showed significant amelioration of parkinsonism as shown by the stepping test when exposed to both LD+BZ and MPE+BZ (*$p < 0.05$ vs. post-lesion, **$p < 0.01$ vs. post-lesion). However, doses given to Group 3 (equivalent to 2mg/Kg LD) indicate that only MPE+BZ had significant relief of parkinsonism. (B) There was a dose-dependent severity of DID among the 3 groups. There was no significant difference within groups (***$p < 0.001$ Group 1 vs. Group 3; **$p < 0.01$ Group 2 MPE+BZ vs. Group 3; *$p < 0.05$ Group 2 LD+BZ vs. Group 3 LD+BZ).

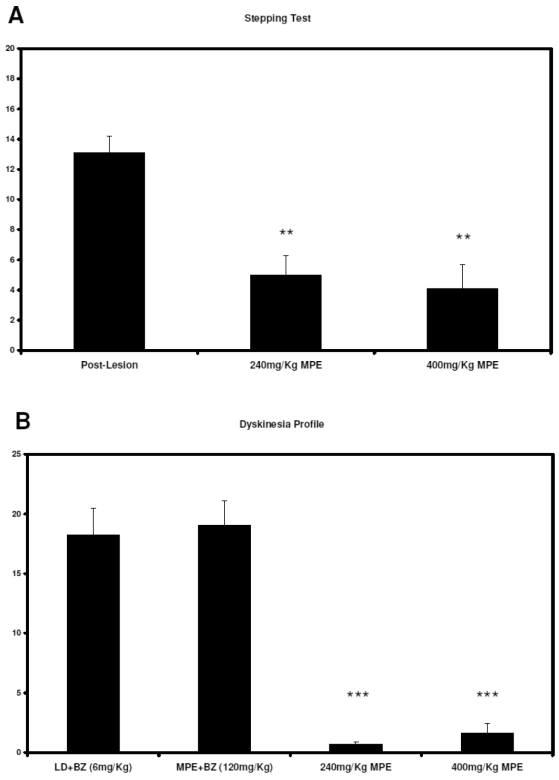

Fig. 2.

High doses of MPE alone. (A) Animals treated with MPE alone at 240mg/Kg MPE and 400mg/Kg MPE (equivalent to 12 mg/Kg and 20 mg/Kg LD) showed significant amelioration of parkinsonism in the stepping test (**$p < 0.01$ vs. post-lesion). (B) MPE caused significantly less DID than doses of LD+BZ and MPE+BZ (***$p < 0.001$ vs. LD +BZ and MPE+BZ).

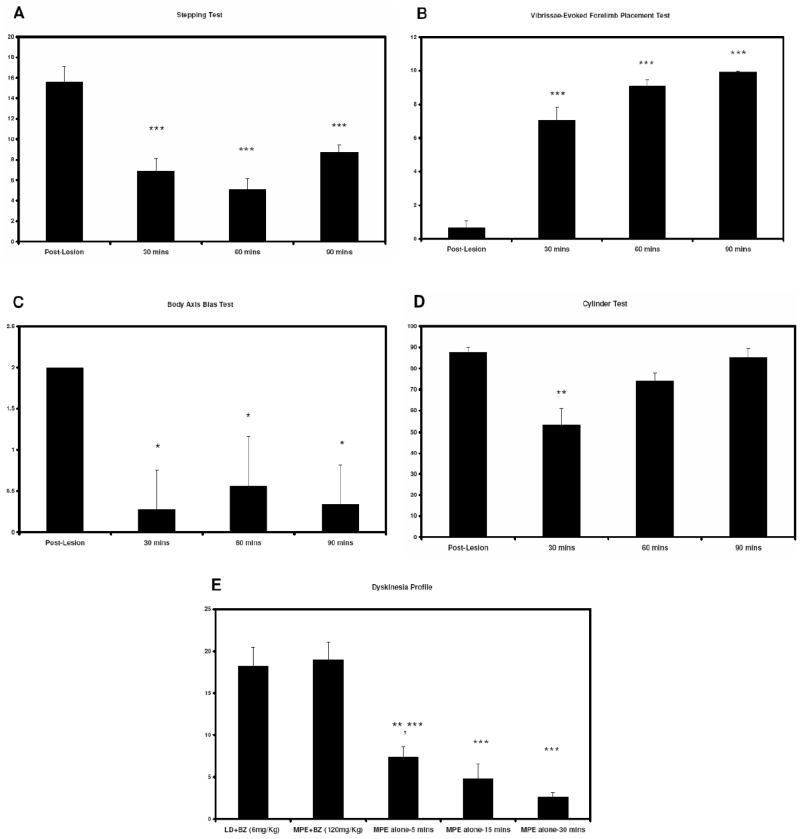

Fig. 3.

Long term effects of MPE alone at 400mg/Kg (equivalent to 20mg/Kg LD). (A) Significant amelioration of parkinsonism up to 90 mins post treatment in the stepping test (***$p < 0.001$ vs. post-lesion). (B) Almost complete restoration of forelimb usage with the vibrissae-evoked forelimb placement test after treatment (***$p < 0.001$ vs. post-lesion). (C) Posturing behavior as measured by the body axis bias test show less bias after drug exposure (*$p < 0.05$ vs. post-lesion). (D) Spontaneous forelimb usage bias was significantly decreased as shown by the cylinder test at 30 mins but not at 60 and 90 mins (**$p < 0.01$ vs. post-lesion). (E) Dyskinesia profiles were significantly less with MPE alone at 5 mins, 15 mins, and 30 mins after drug exposure compared to LD+BZ and MPE+BZ (**$p < 0.01$ vs. LD+BZ, ***$p < 0.001$ vs. MPE+BZ and LD+BZ at 15 and 30 mins).

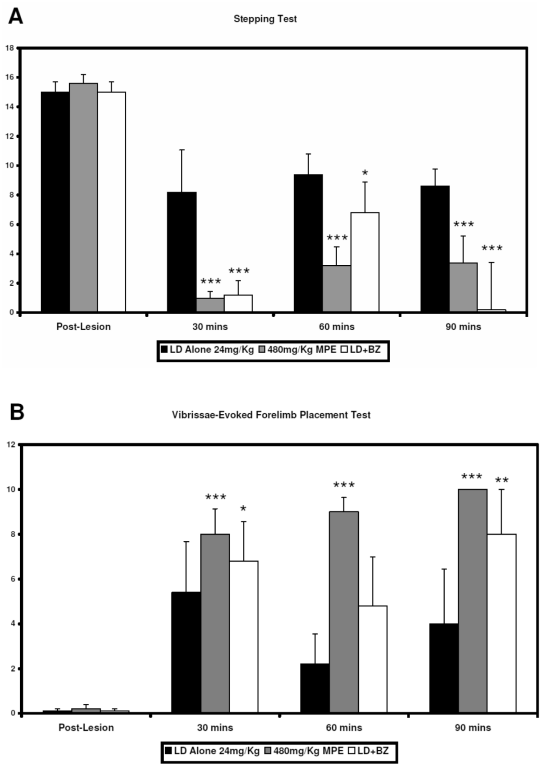

Fig. 4.

Comparison of LD alone (24mg/Kg), MPE alone at 480mg/Kg (equivalent to 24mg/Kg LD) and LD+BZ (6mg/Kg LD + 15mg/Kg BZ). (A) Significant amelioration of parkinsonism in the stepping test for MPE alone and LD+BZ (*$p < 0.05$ vs. post-lesion, ***$p < 0.001$ vs. post-lesion) but not with LD alone. (B) Significant restoration of forelimb usage with the vibrissae-evoked forelimb placement test after MPE alone and LD+BZ but not with LD alone (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. post-lesion).

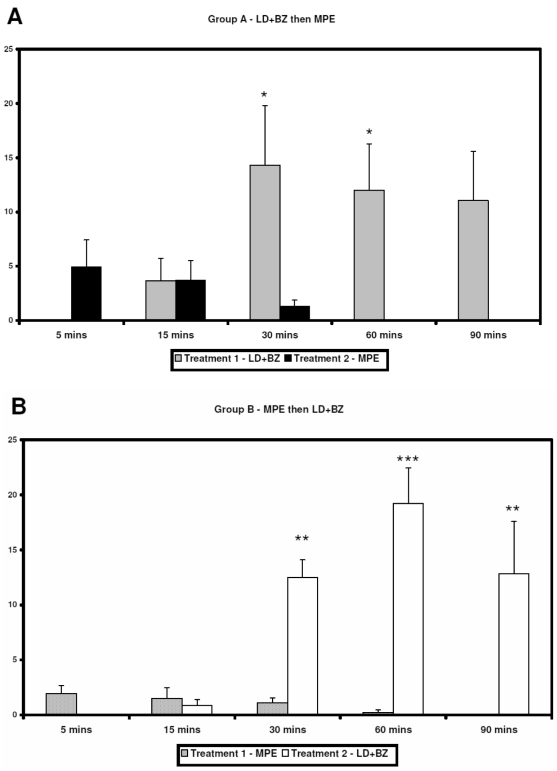

Fig. 5.

Dyskinesia priming effects of LD+BZ and MPE alone. (A) Group A first received LD+BZ (6mg/Kg LD + 15mg/Kg BZ) then MPE alone (480mg/Kg - equivalent to 24mg/Kg LD). (B) Group B was first treated with MPE alone at 480mg/Kg (equivalent to 24mg/Kg LD) then immediately treated with LD+BZ (6mg/kg LD + 15mg/Kg BZ)(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ between treatment time points).