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The 6-hydroxydopamine rat model of Parkinson's disease

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TITLE:

The 6-hydroxydopamine rat model of Parkinson's disease

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SUMMARY:

The 6-hydroxydopamine (6-OHDA) model has been used for decades to advance the understanding of Parkinson's Disease. In this protocol, we demonstrate how to perform unilateral nigrostriatal lesions in the rat by injecting 6-OHDA in the medial forebrain bundle, assess motor deficits, and predict lesions using the stepping test.

ABSTRACT:

Motor symptoms of Parkinson's disease (PD)—bradykinesia, akinesia, and tremor at rest—are consequences of the neurodegeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and dopaminergic striatal deficit. Animal models have been widely used to simulate human pathology in the laboratory. Rodents are the most used animal models for PD due to their ease of handling and maintenance. Moreover, the anatomy and molecular, cellular, and pharmacological mechanisms of PD are similar in rodents and humans. The infusion of the neurotoxin, 6-hydroxydopamine (6-OHDA), into a medial forebrain bundle (MFB) of rats reproduces the severe destruction of dopaminergic neurons and simulates PD symptoms. This protocol demonstrates how to perform the unilateral microinjection of 6-OHDA in the MFB in a rat model of PD and shows the motor deficits induced by 6-OHDA and predicted dopaminergic lesions through the stepping test. The 6-OHDA causes significant impairment in the number of steps performed with the contralateral forelimb.

INTRODUCTION:

The main neuropathological characteristics of PD are the chronic progressive neurodegeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the presence of Lewy bodies containing α-synuclein protein[1]. As SNc dopaminergic neurons project their axons into the

striatum through the nigrostriatal pathway, neurodegeneration of neurons in SNc results in a dopaminergic deficit in the striatum[2]. The absence of dopamine in the striatum causes an imbalance in the activities of the direct and indirect motor control pathways, which is responsible for the main motor symptoms of PD: akinesia (slow movement), bradykinesia (difficulty in starting movements), muscle stiffness, and tremor at rest[3–5].

As the molecular and physiological mechanisms involved in the onset of PD are still not fully understood, currently available principal treatments seek to alleviate the motor symptoms through pharmacotherapies, deep brain stimulation[6,7], genetic therapies[8], and cell transplantation[9]. Therefore, preclinical research is fundamental to elucidate the mechanisms involved in the onset of PD and discover new methodologies for the early diagnosis and new therapies to prevent or stop the degeneration of neurons affected by PD[10].

Animal models have been widely used to simulate human pathology in the laboratory, contributing to the advancement of medicine and science[11–14]. However, it is essential to emphasize that the correct choice of the animal model is fundamental for the success of the study. Therefore, the animal model must be validated in three main aspects: i) face validity, in which the animal model must have the characteristics of human pathology; ii) constructive validity, in which the animal model must have a solid theoretical basis; and iii) predictive validity, in which animal models must respond to treatments in a similar way to clinical treatment.

Currently, several animals are used as animal models for PD. The main groups include mammals, such as rodents, primates, minipigs, dogs, and cats, and other groups such as drosophila and zebrafish. Rodents are the most classic animal model for PD and the most used due to their ease of handling and maintenance. In addition, the anatomy and molecular, cellular, and pharmacological mechanisms of PD are similar in rodents and humans[15].

A review published by Kin and colleagues in 2019 analyzed the principal animal model methodologies used for PD in the 2000s and found that the most used animal model involved neurotoxins such as 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both neurotoxins cause mitochondrial dysregulation in dopaminergic neurons in the nigrostriatal pathway, leading to cell death[16]. Another widely used model involves genetic manipulation through mutation in specific genes involved in the onset of PD, causing mitochondrial dysregulation[17]. Neurotoxin models are commonly used to evaluate and compare therapeutics, whereas genetic models are used to study the development of preventive therapies and idiopathic PD[15].

The neurotoxin MPTP was discovered to cause parkinsonism in the mid-1980s after seven patients used the substance and exhibited severe PD symptoms. In addition to the symptoms, the patients responded to treatment with L-DOPA, which made the researchers link the molecule directly to PD. After the case was published in 1986, several researchers began using MPTP in preclinical PD research[18]. Researchers have found that being a lipophilic molecule, MPTP can cross the blood-brain barrier (BBB) and be converted to MPP^{+}[19]. This toxic substance accumulates inside neurons and causes damage to complex 1 of the mitochondrial respiratory chain, leading

to the death of dopaminergic neurons[20].

The 6-OHDA neurotoxin model was first used to induce the degeneration of monoamine neurons of the nigrostriatal pathway in 1968[21]. The 6-OHDA model is commonly used to cause neurodegeneration in the nigrostriatal pathway as it is a dopamine analog and toxic for catecholamine-containing cells. After 6-OHDA enters the brain, it may be taken up by the dopamine transporter (DAT) in dopaminergic neurons, leading to the most selective degeneration of the nigrostriatal pathway[22]. Because 6-OHDA does not penetrate the BBB, it must be administered directly through intracerebral stereotaxic injection[23]. A noradrenaline reuptake inhibitor is often combined with 6-OHDA microinjection to preserve noradrenergic fibers and provide a more selective degeneration of dopaminergic neurons[24].

After DAT takes up 6-OHDA, it will accumulate in the cytosol of neurons, producing reactive oxygen species (ROS) and leading to cell death[15]. Three different lesions models of 6-OHDA are frequently used: i) lesions to the SNpc[25,26]; ii) lesions to the striatum[27,28]; iii) lesions to the MFB[29,30]. Lesions caused in the striatum result in a slow and retrograde degeneration of dopaminergic neurons in SNpc. In contrast, lesions caused in SNpc and MFB result in rapid and total degeneration of neurons, leading to more advanced parkinsonian symptoms[31].

Unilateral or bilateral injection of 6-OHDA can cause neurodegeneration in dopaminergic neurons. 6-OHDA does not always cause severe damage to the neurons; sometimes, the injection results in partial damage, which is also used to simulate the early stages of PD[32]. The unilateral injection is more commonly used due to the model's ability to assess the animal's motor deficits and predict cell loss through tests such as amphetamine/apomorphine-induced rotation and the stepping test[29]. Bilateral injections are most used to evaluate spatial memory and recognition[33].

The amphetamine/apomorphine-induced rotation test is a behavioral test commonly used to predict cell loss in the nigrostriatal pathway. It is defined as a process in which repeated administration of dopamine agonists, such as L-DOPA, leads to an intensification of rotational behavior in 6-OHDA-lesioned animals[34]. Rotational behavior consists of quantifying amphetamine-induced ipsilateral rotation or apomorphine-induced contralateral turns in unilaterally lesioned rodents. Drug-induced rotational behavior has been criticized because rotation does not correspond to PD symptoms in humans and can be affected by variables such as tolerance, sensitization, and "priming"[35].

Priming is one of the most critical factors in these behavioral tests. Some cases have been reported wherein a single dose of L-DOPA led to a failure in rotational behaviors[36]. Additionally, another critical factor related to the combined application of the amphetamine-induced test and apomorphine-induced test for parallel use is that they measure different endpoints because of different mechanisms of action, reflecting the inactivation of different signaling mechanisms and pathways. Furthermore, the amphetamine-induced test is more accurate to measure nigrostriatal lesions above 50–60%, whereas the apomorphine-induced test is more accurate for lesions above 80%[37].

The stepping test has emerged as a behavioral test that indicates deficits related to dopaminergic neuron degeneration and therapeutic effects. It enables the analysis of akinesia caused by a 6-OHDA lesion in dopaminergic neurons without a drug-induced procedure. Furthermore, the test has been well established and commonly used since 1995, when it was first described by Olsson et al.[35]. In 1999, Chang et al.[38] also analyzed and compared the performance of rats in the stepping test with the level of degeneration caused by 6-OHDA and found that animals that performed worse in the stepping test also had a more significant degeneration of dopaminergic neurons.

The stepping test is an excellent method to predict severe dopaminergic nigrostriatal damage in 6-OHDA-lesioned rats. Evidence suggests that motor deficits appear in the contralateral forelimb of the 6-OHDA infusion during the stepping test when the degree of dopaminergic loss in SNc is >90%[39]. This paper describes the protocols, methodologies, and materials used to perform stereotaxic surgery for the unilateral infusion of 6-OHDA into the MFB of rats and how to predict the dopaminergic lesions caused by the toxin through the stepping test.

PROTOCOL:

All procedures involving animals followed the ethical principles of the National Council for the Control of Animal Experimentation (CONCEA) and the Arouca Law (Law 11.794/2008) and were approved by the local ethics committee (CEUA-FFCLRP/USP (18.5.35.59.5).

1. Preparation of drugs

1.1. Anesthesia with Ketamine/Xylazine

NOTE: The dose of ketamine used is 70 mg/kg, and the dose of xylazine is 10 mg/kg.

1.1.1. To prepare 1 mL of anesthetic using ketamine 100 mg/mL solution and xylazine 20 mg/mL solution, combine 0.35 mL of ketamine solution, 0.25 mL of xylazine solution, and 0.4 mL of 0.9% sterile saline solution. Administer the anesthetic solution at a final volume of 2 mL/kg.

NOTE: Ketamine along with xylazine can produce sedation for 60–80 min. If the animal still has reflexes (e.g., hind leg pitching and/or blinking reflex), administer an additional 10% of the individual dose.

1.2. Imipramine

NOTE: The individual dose of imipramine used is 20 mg/kg.

1.2.1. To prepare 1 mL of imipramine 20 mg/mL solution, combine 20 mg of imipramine and 1 mL of 0.9% sterile saline solution. Administer the imipramine solution at a final volume of 1 mL/kg.

1.3. Meloxicam

NOTE: The individual dose of meloxicam used is 1 mg/kg.

1.3.1. To prepare 1 mL of meloxicam 1 mg/mL solution, combine 0.05 mL of meloxicam 2% and 0.95 mL of 0.9% sterile saline solution. Administer the meloxicam solution at a final volume of 1 mL/kg once a day for two days.

1.4. Ascorbic acid 0.1%

1.4.1. To prepare 1 mL of 0.1% ascorbic acid, combine 1 mg of ascorbic acid and 1 mL of 0.9% sterile saline solution.

1.5. 6-hydroxydopamine (6-OHDA)

NOTE: 6-OHDA is a neurotoxin used to selectively destroy dopaminergic and noradrenergic neurons in the brain. Avoid direct contact with skin and mucous membranes of the eyes, nose, and mouth. When handling 6-OHDA, wear double nitrile gloves, lab coat, disposable gown, eye protection, and surgical mask or face shield. The total infusion volume of the toxin is 4 mL/animal, and the individual amount is 10 mg of 6-OHDA/animal.

1.5.1. To prepare 1 mL of 6-OHDA at a final concentration of 2.5 mg/mL, mix 2.5 mg of 6-OHDA and 1 mL of 0.9% saline solution containing 0.1% ascorbic acid (described above).

NOTE: 6-OHDA is light-sensitive and degrades faster when exposed to bright light. It must be properly handled and stored in an environment protected from light. If the color of the solution is reddish, discard it.

1.6. Lidocaine hydrochloride (2%)

1.6.1. Prepare 2% lidocaine solution for local application to the animal.

NOTE: The maximum dose that can be applied is 7 mg/kg.

1.7. Poly-antibiotic suspension

NOTE: The poly-antibiotic suspension with streptomycins and penicillins (see the **Table of Materials**) must be prepared at the time of application with the entire volume of diluent, whose ampoule accompanies the vial with the powder.

1.7.1. Remove the metallic disc on the rubber stopper. Disinfect the rubber stopper with alcohol.

1.7.2. Using a syringe with a needle of 23 G, inject the diluent into the vial. Remove the needle and shake the vial vigorously until the suspension is entirely homogenized. Inject a little air into

the vial and withdraw the desired volume of suspension.

1.7.3. Administer a deep intramuscular injection, pulling the plunger before injecting the drug to ensure that no blood vessel is reached.

NOTE: The final volume of suspension to be applied is 0.5 mL/kg.

2. Preparation of materials

NOTE: Always follow instructions provided with the material safety data sheet when handling chemicals.

2.1. Stereotaxic apparatus

2.1.1. Place the stereotaxic device on a stable and clean bench with proper illumination to perform the surgery. Sterilize the apparatus with 70% ethanol.

2.1.2. Check if the ear and incisor bars of the device are correctly aligned. Place a thermal blanket where the animal will be placed during surgery to stay warm during the procedure. Monitor the animal's temperature with an accurate rectal probe.

NOTE: The thermal blanket should be at 37.5 °C so that the animal maintains a body temperature of 37 °C body.

2.2. Microinfusion system

2.2.1. Fill (70–80%) a Hamilton syringe (50 mL or as desired) attached to a medical-grade polyethylene microtubing and a needle with double distilled water (ddH_2O) and check for leaks through the system.

2.2.2. Pull air through the system so that a single air bubble separates the ddH_2O in the syringe from the 6-OHDA solution in the microtube.

NOTE: This procedure avoids contaminating the Hamilton syringe with 6-OHDA and allows the use of several rats on the same experimental day.

2.2.3. Position the Hamilton syringe on the infusion pump so that it is firmly attached and the plunger of the syringe is parallel to the frame that will move to push it. Set the infusion pump to a speed of 0.5 mL/min so that the total application of 4 mL of 6-OHDA lasts for 8 min. Test the infusion system by confirming that there are no leaks and that the infusion occurs according to the previously set time and volume.

2.2.4. Attach the needle of infusion attached to the microtube to the apparatus at the end of the stereotactic arm and check that the needle is positioned at a 180° angle to the surface. Ensure

that the needle is straight and not bent.

NOTE: Check all the described procedures carefully because if any of the items in the infusion system do not work correctly, it may jeopardize the success of the surgery.

2.3. Suture

2.3.1. Use a sterile nylon non-absorbable suture with a 3/8 circle needle to suture the incision after surgery.

2.4. Postsurgical recovery site

2.4.1. Place a clean and sterilized housing box where animals can be monitored until fully recovered (responsive to touch and manipulation). Put a thermal blanket in the box for thermoregulation.

NOTE: As thermoregulation is important, include a supplemental heat source to maintain body temperature if necessary.

3. Surgical procedure

NOTE: In this protocol, adult male Sprague-Dawley rats (200–250 g) were kept under controlled conditions of temperature (22 ± 2 °C), air exchange (15–20 exchanges/hour), and light-dark cycles (12 h/12 h), grouped in boxes with 3 or 4 animals, with free access to food and water.

3.1. Weigh the animals to monitor weight changes in the days following the surgery. Calculate the dose of drugs to be administered.

3.2. Administer imipramine intraperitoneally 30 min before surgery (~10–15 min before administering anesthesia), using a 27 G needle and a 1 mL syringe.

NOTE: The imipramine will block the noradrenaline transporter (NAT) and prevent 6-OHDA uptake by noradrenergic neurons, making the lesion more selective to the dopaminergic neurons[40].

3.3. After 10–15 min of the administration of imipramine, administer the intraperitoneal ketamine/xylazine anesthesia using a 27 G needle and a 1 mL syringe. Wait until the animal is completely anesthetized. Verify that the animal is under deep anesthesia when the animal does not respond to hind leg pinching and does not show a blink reflex.

3.4. Shave the rat's fur in the region of the head where the incision will occur.

3.5. Position the rat in the stereotaxic apparatus.

3.5.1. Position the head over the incisor bar and fix the bar 3.3 mm below the interaural line.

3.5.2. Position the ear bars, one side at a time. Position the incisor bar and the ear bars so that the top of the skull is straight and parallel to the surface.

3.5.3. Adjust the nose clamp and test that the head is firm and does not move to either side.

3.6. Apply sterile ophthalmic ointment to the rat eyes to prevent corneas from drying out.

3.7. Apply povidone-iodine to the area to be incised to sterilize the site.

3.8. Apply local lidocaine for analgesia of the incision region; do not exceed 7 mg/kg.

3.9. Administer the meloxicam subcutaneously using a 27 G needle and a 1 mL syringe.

NOTE: Meloxicam is a nonsteroidal anti-inflammatory analgesic that will help the animal recover post surgery.

3.10. Administer the poly-antibiotic suspension intramuscularly using a 23 G needle and a 1 mL syringe.

NOTE: The poly-antibiotic suspension is administered as a prophylactic treatment to avoid possible bacterial infections in the postsurgery recovery.

3.11. Check that the animal is in a state of deep anesthesia by checking for blink reflexes or hind limb reflexes by pinching the hind paw with tweezers.

3.12. With a scalpel, make an incision of ~1.5 cm in the region where the microinjection will occur.

3.13. Clean the skull region with cotton swabs and cotton buds until the Bregma and Lambda can be seen. Mark the Bregma and the Lambda with a fine pen.

3.14. Check that the dorsal–ventral (DV) coordinates of Bregma and Lambda are similar. If they are different, readjust the rat in the stereotaxic apparatus as the rat's head is not correctly positioned.

3.15. Note down the anteroposterior (AP) and mediolateral (ML) coordinates of the Bregma.

3.16. Move to the AP and ML coordinates of the right MFB according to [41]: AP: -4.3 mm, ML: 1.6 mm from Bregma.

3.17. Mark the region of the trepanation with a fine pen.

3.18. With a drill, slowly pierce the animal's skull, taking care not to injure the dura mater.

3.19. Position the microinjection needle on the dura mater and note the DV coordinates. Take a thin needle and gently rupture the dura mater. Insert the needle to the DV coordinate (8.3 mm ventral) of the MFB, where the microinjection will take place.

3.20. Operate the microinjection pump to release the 6-OHDA solution into the MFB. When the microinjection is finished, check the Hamilton syringe to see if 4 mL of 6-OHDA has been injected.

NOTE: The microinjection should last 8 min.

3.21. After administration of the 6-OHDA, wait for 10 min before removing the needle to avoid backflow of the drug. Remove the microinjection needle slowly from the animal's brain.

3.22. Clean the incision region again with povidone-iodine.

3.23. Suture the incision area with ~3–4 surgical knots.

NOTE: The knot should not be too strong or too loose.

3.24. Remove the rat from the stereotaxic apparatus and place it in a clean box for recovery on the thermal blanket until the animal has fully recovered from anesthesia. Observe the animal every 15 min until it is fully awake from anesthesia.

4. Postoperative procedures

4.1. Monitor the weight of the animals over the next four days after surgery. Treat them with meloxicam subcutaneously once a day for two days after surgery, adjusting the dose for each day's weight.

4.2. Check the incisions daily for at least four days to ensure they are not infected. Look for heat, swelling, pain, discharge, and redness until the incisions heal.

4.3. Check the appetite and water consumption by monitoring the animal's body weight. Give wet feed to encourage the animals to eat. Observe the general body condition, attitude, and mobility daily for at least four days after surgery. Remove the sutures 7–10 days after surgery.

NOTE: Animals should be euthanized if the endpoints defined in the ethical procedures are reached.

5. Stepping test

5.1. Training

NOTE: The animals should be trained for three days before the test. According to the protocol described below, training should occur twice a day, once in the morning and once in the afternoon, or with an interval of at least 2 h between sessions. Track the time using a timer.

5.1.1. Day 1

5.1.1.1. In the first session, handle the rat by holding it in gloves for ~1–2 min to allow the rat to familiarize itself with the handler/experimenter.

5.1.1.2. In the second session, alternate between holding the rat for 20 s and placing it on the protocol table for 20 s. Repeat this training step for 3 min to familiarize the rat with the experimental setup for the stepping test.

5.1.2. Day 2

5.1.2.1. In the first session, place both forepaws of the rat on the protocol table by holding its hind paws and back with one hand. Tilt the rat downwards headfirst at an angle of 45° to the flat surface of the protocol table. Move horizontally on the table from end to end, allowing the rat to step on the table with both paws (cover 90 cm in 4 s). Hold the rat in gloves for 10 s, allowing it to rest; repeat this pattern for 3 min.

5.1.2.2. In the second session, place one forepaw of the rat on the protocol table by holding the other forepaw back with one hand and hold the rat's back and hind paws with the other hand (see step 5.1.2.1). Move horizontally on the table from end to end in 4 s, allowing the rat to step with its free paw. Hold the rat in gloves for 10 s, allowing it to rest, and repeat with another forepaw, followed by the rest period. Repeat this pattern, alternating between the two forepaws, and rest for 3 min.

5.1.2.3. Repeat the training step 3 times for 1 min each.

5.1.3. Day 3

5.1.3.1. In the first session, follow the procedure described in step 5.1.2.2 for one forepaw. Repeat with another forepaw, followed by the rest period. Repeat this pattern, alternating between the two forepaws, and rest for 3 min.

5.1.3.2. In the second session, follow the procedure described in step 5.1.2.2.

5.2. Test

NOTE: The stepping test is performed before surgery, 2 and 4 weeks after stereotaxic surgery, to evaluate the akinesia of the contralateral forelimb and the possible injury caused by 6-OHDA.

5.2.1. Hold the rat at an angle of 45° to the surface, immobilizing its hind limbs and allowing

only one of the forelimbs to rest on the platform, as explained above, for day 3 of training.

5.2.2. Drag the rat forward over a distance of 90 cm in 4 s, with the right or left paw resting on the surface.

5.2.3. Take notes and quantify the number of forehand-adjusting steps taken with each paw in each direction.

REPRESENTATIVE RESULTS:

Dopaminergic lesion assessment

The stepping test enables the assessment of the akinesia of the anterior limb contralateral to the lesion and the selection of animals with a possible lesion of the nigrostriatal pathway induced by 6-OHDA infusion (**Figure 1**). The comparison of the performance of the contralateral forelimb stepping test presurgery and 2 weeks and 4 weeks after surgery revealed interaction ($F_{2,74}$ = 93.63; $p < 0.0001$; two-way repeated-measures ANOVA) between time (pre, 2, and 4 weeks after surgery) and treatment (sham-operated and 6-OHDA-lesioned). Bonferroni's *post-hoc* test showed a significant decrease in the number of steps contralateral to the lesion in animals receiving 6-OHDA in the right MFB compared to the sham-operated animals at the second and fourth week after surgery ($p < 0.0001$) (**Figure 1**). The results were consistent with those of previous studies[35].

It is important to note that when the dopaminergic lesion is not complete, the results of the stepping test will not reach the degree of success of the results presented in this study. A previously published study performed the stepping test and immunohistochemistry of tyrosine hydroxylase (TH) with animals with a partial dopaminergic lesion after performing surgery for microinjection of 6-OHDA following the same protocol used in this study. Their finding of a partial deficit in the stepping test (4–8 steps) is the result of a partial dopaminergic lesion of ~60% of the neurons[39].

[INSERT FIGURE 1 HERE]

The comparison of the performance of the ipsilateral forelimb stepping test presurgery and 2 weeks and 4 weeks after surgery did not reveal any interaction ($F_{2,74}$ = 0.4492; $p = 0.6399$; two-way repeated-measures ANOVA) between time (pre, 2, and 4 weeks after surgery) and treatment (sham-operated and 6-OHDA-lesioned). Bonferroni's *post-hoc* test did not show any significant difference in the number of steps ipsilateral to the lesion in animals receiving 6-OHDA in the right MFB compared to sham animals (**Figure 2**).

[INSERT FIGURE 2 HERE]

Consistent with previous studies on 6-OHDA-lesioned animals[42], histological analysis (**Figure 3**) comparing TH of the striatum of both hemispheres allows a reliable assessment of the DA deficit in the striatum. Therefore, this behavioral protocol can be used in combination with immunohistochemical methods in studies involving experimental models of PD.

[INSERT FIGURE 3 HERE]

FIGURE AND TABLE LEGENDS:

Figure 1: Assessment of contralateral stepping test pre- and postsurgery for unilateral infusion of 6-OHDA or vehicle into the right MFB. Data show that animals receiving 6-OHDA had a significant decrease in the number of steps with the anterior forelimb contralateral to the lesion at the second and fourth weeks after surgery (****$p < 0.0001$ vs. sham postsurgery; two-way repeated-measures ANOVA, Bonferroni *post-hoc*). Data expressed as mean ± standard error of the mean. Vehicle is 0.9% saline solution containing 0.1% ascorbic acid. Results are based on 14 animals in the sham group and 25 animals in the 6-OHDA group. Abbreviations: P = presurgery. 2 = two weeks after surgery. 4 = four weeks after surgery; 6-OHDA = 6-hydroxydopamine; MFB = medial forebrain bundle.

Figure 2: Assessment of ipsilateral stepping test pre- and postsurgery for unilateral infusion of 6-OHDA or vehicle into the right MFB. Data show that animals receiving 6-OHDA did not significantly decrease the number of steps with the anterior forelimb ipsilateral to the lesion at the second and fourth weeks after surgery ($p > 0.05$ vs. sham postsurgery; two-way repeated-measures ANOVA, Bonferroni *post-hoc*). Data expressed as mean ± standard error of the mean. Vehicle is 0.9% saline solution containing 0.1% ascorbic acid. Results are based on 14 animals in the sham group and 25 animals in the 6-OHDA group. Abbreviations: P = presurgery. 2 = two weeks after surgery. 4 = four weeks after surgery; 6-OHDA = 6-hydroxydopamine; MFB = medial forebrain bundle.

Figure 3: Representative images of TH labeling in the 6-OHDA experimental model of PD, including anterior striatum and substantia nigra compacta. The panoramic image demonstrates the extension of the lesion, and inset zooms depict innervation e cell bodies immunostained. (**A**) Image of the striatal coronal section showing a partial injury induced by 6-OHDA in the right hemisphere. (**B**) Image of substantia nigra and ventral tegmental area coronal section from the same animal also showing the lesion extension. (**C**) Image of the striatal coronal section showing a complete induced injury by 6-OHDA in the right hemisphere. (**D**) Image of substantia nigra and ventral tegmental area coronal section from the same animal also showing the lesion extension. Scale bar = 1.3 mm in panoramic view and 65 µm in inset zooms. Abbreviations: 6-OHDA = 6-hydroxydopamine; TH = tyrosine hydroxylase; PD = Parkinson's disease.

DISCUSSION:

This paper describes a protocol for performing surgery for unilateral microinfusion of 6-OHDA in the MFB, capable of causing robust lesions in the neurons of the nigrostriatal pathway and generating akinesia in the animal. Also described is the protocol for performing the stepping test, an easily applicable and noninvasive test that can be used to prove the success of the lesions and assess forelimb akinesia. As presented in the representative results, animals receiving 6-OHDA showed a reduction in the number of adjusting steps contralateral to injury, which means that 6-OHDA-injured animals exhibit strong akinesia from 2 weeks after infusion surgery. Akinesia—the

focus of several treatments for the disease—is one of the main motor symptoms of PD. The development of akinesia in an animal model is significant for preclinical studies of PD. Moreover, these results resemble those reported by Chang et al.[37], who confirmed that animals presenting a lower number of steps had a higher percentage of dopaminergic neuron death by immunohistochemistry. Therefore, animals that presented a lower number of contralateral adjusting steps are more likely to have a dopaminergic injury.

Assessment of the success of the surgery and the lesions can also be confirmed by other behavioral tests such as amphetamine/apomorphine-induced rotation[43], elevated body swing test (EBST), corridor test, cylinder test, tissue labeling techniques such as TH immunohistochemistry, or even quantification of dopamine in the striatum by HPLC[42]. Other methodologies differ in the injected dose of 6-OHDA and postsurgery time interval for behavioral assessment. A recent review[43] summarizes the most recent articles using this methodology and the difference in dose, behavioral testing, and postsurgery interval between them. The model of PD induced by 6-OHDA does not mimic all the pathological processes related to the disease, such as the accumulation of Lewy bodies, but simulates the death of dopaminergic neurons of the striatal-nigral pathway. This enables the study of new therapies for the symptoms of the disease, which could lead to an improvement in the quality of life of patients affected by this disease.

Despite being the most widely used model, the 6-OHDA model has its limitations like all current PD models. The model has the disadvantage of not fully representing the molecular mechanisms involved in the pathology of the disease, such as the accumulation of alfa-synuclein proteins and the formation of Lewy bodies. The model simulates the death of dopaminergic neurons of the nigrostriatal pathway, corresponding to a late stage of the disease and leading to the onset of motor symptoms only. This makes it unsuitable for studying its natural development[15,32]. The 6-OHDA model described in this article is usually characterized by low mortality rates. Postsurgery recovery is crucial to prevent high mortality rates due to the union of an invasive procedure and the neurodegenerative lesion[44]. It is possible to reduce mortality by taking extra care during the postsurgery recovery period with nutritional supplementation, rehydration, and external temperature control[45]. The combination of such measures has been shown to reduce or even eliminate the mortality rate drastically[30,46]. A common cause of death is the insertion of the needle at the wrong coordinate in the brain. It is crucial to carefully check the coordinates during this delicate surgical procedure. This will avoid damage to other brain structures (e.g., the hypothalamus) by the needle, which can impair the animal's eating and drinking actions, leading to malnutrition and dehydration[47].

Finally, it is essential to highlight that although the ketamine–xylazine anesthesia protocol is well established and used in rodent experiments[48], some evidence suggests that the combination of these anesthetics may be insufficient for an extended period of surgery. Additionally, ketamine-xylazine sensitivity might vary according to different strains of mice and rats[49,50]. An alternative may be to induce anesthesia by isoflurane inhalation. One study demonstrated faster loss of the righting reflex with isoflurane-induced anesthesia than with ketamine–xylazine. Moreover, 60% of the rats anesthetized with ketamine–xylazine presented consecutive toe pinch reflexes during the surgical procedure, even with dose supplementation. In contrast, animals anesthetized with

isoflurane presented isolated cases of TP reflexes that disappeared after volume adjustment[51].

ACKNOWLEDGMENTS:

This work was supported by São Paulo Research Foundation (FAPESP, grant 2017/00003-0). We are grateful for the Coordination for the Improvement of Higher Education Personnel (CAPES). We thank Dr. Anthony R. West, Dr. Heinz Steiner, and Dr. Kuei Y. Tseng for support and mentoring.

DISCLOSURES:

The authors have no conflicts of interest to declare.

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Figure 1

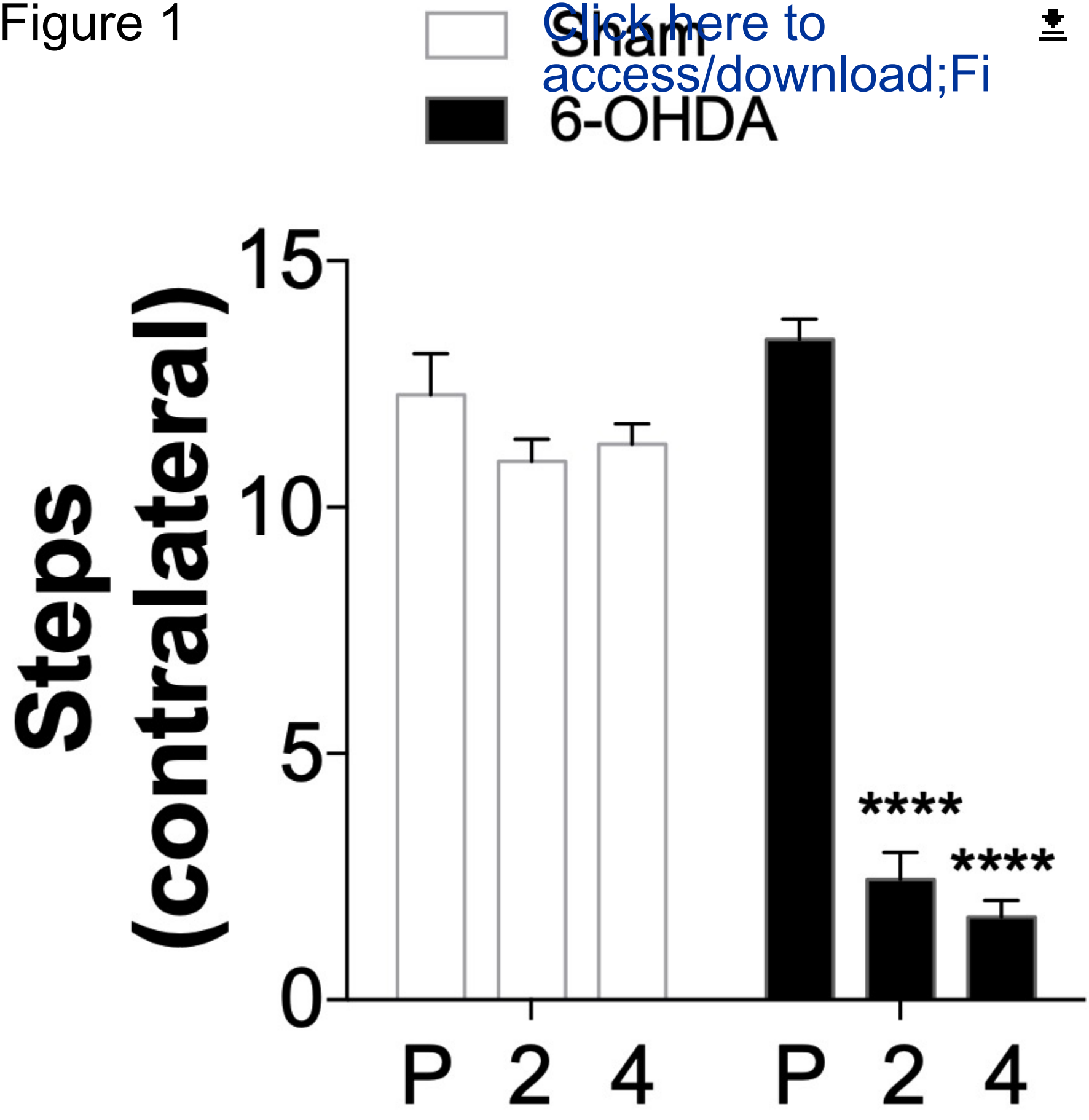

Figure 2

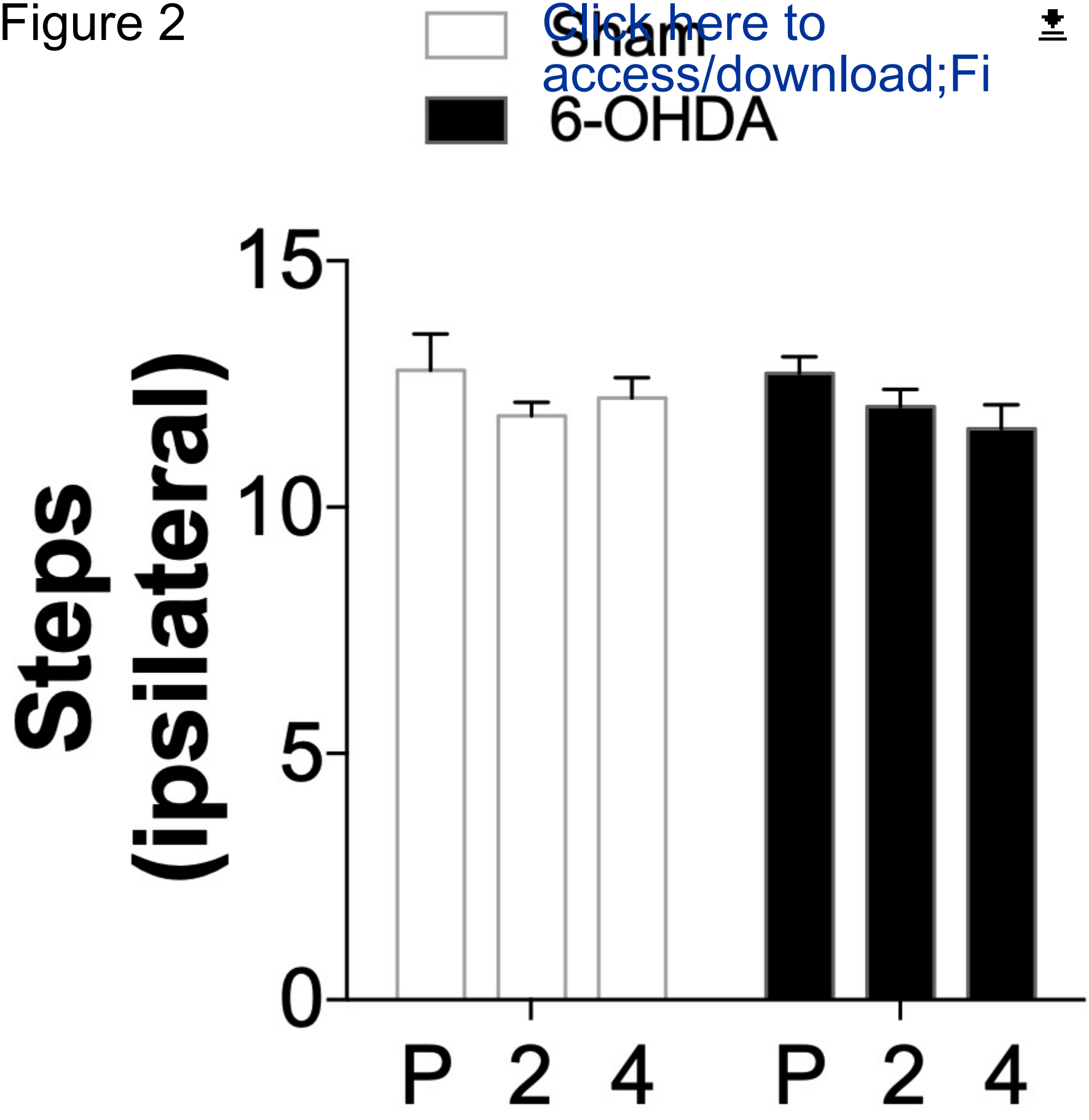

Figure 3

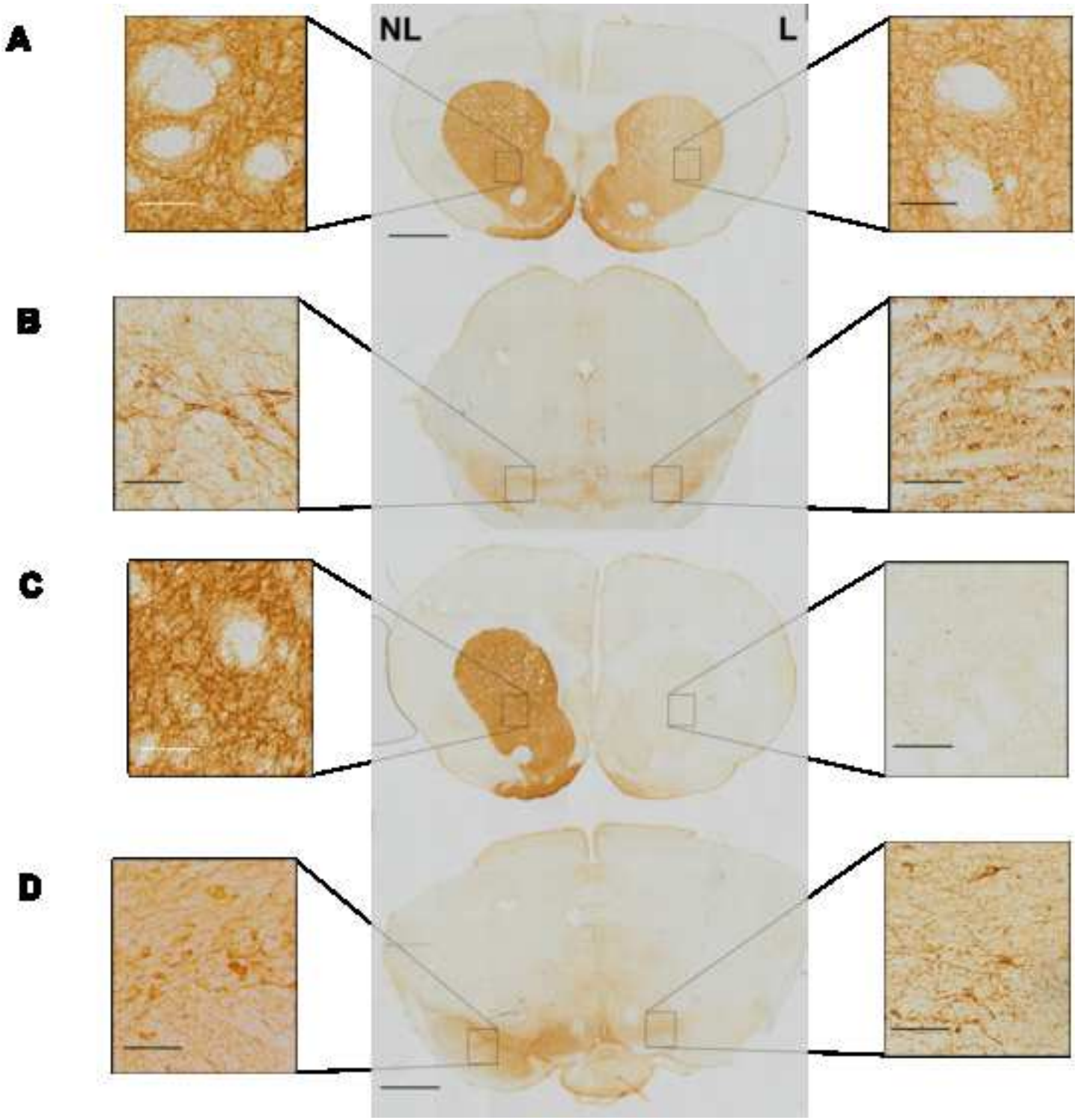

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Table of Materials

Materials_FN.xls

We thank the reviewer for his comments on the manuscript, which we have addressed extensively here and throughout the manuscript's main text as indicated. Original reviewer comments are in blue text, our responses are in black text, and indications for where the manuscript has been subsequently altered are highlighted in yellow.

Reviewer#1:

Manuscript Summary:
This manuscript aims at describing a protocol for stereotaxic surgery for 6-OHDA infusion in the medial forebrain bundle of rats and for dopaminergic lesion evaluation with the stepping test.

Major Concerns:
According to the JoVE guidelines, which states that the work should "Include data from successful experiments and data from suboptimal experiments to demonstrate the range of outcomes possible", the data from suboptimal experiments are missing and not discussed.

Although we do not have enough data to correlate suboptimal results of 6-OHDA lesions with stepping test results, we added a comment to the end of the representative results topic. Note that the article we cite uses a very similar protocol to the one we describe in our article:

"*It is important to note that when the dopaminergic lesion is not complete, the results of the stepping test will not reach the degree of success of the results presented in this study. A previously published study performed stepping test and immunohistochemistry of TH with animals that had partial dopaminergic lesion after performing surgery for microinjection of 6-OHDA following the same protocol used in this study, finding that a partial deficit in the stepping test (4 to 8 steps), is the result of a partial dopaminergic lesion of about 60% of the neurons*[39]*."*

Minor Concerns:
1. Introduction

1.1. Line 56: For DBS treatments, please cite one of the leading teams in DBS applications:
- Karachi et al. Parkinsonism Relat. Dis. 2019 May;62:91-97. "Clinical and anatomical predictors for freezing of gait and falls after subthalamic deep brain stimulation in Parkinson's disease patients". doi:10.1016/j.parkreldis.2019.01.021.
- Baizabal-Carvalo et al. Parkinsonism Relat. Dis. 2013 May;19(5):566-8. "Combined pallidal and subthalamic nucleus deep brain stimulation in secondary dystonia-parkinsonism". doi: 10.1016/j.parkreldis.2013.01.010.

Thank you for recommending the citations. We added both references to the article (they appear as citations 6 and 7).

1.2. Lines 78-79: Please add some references to the statement "Both neurotoxins cause (…) cell death".

We added the citation "Schober, A. Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP. *Cell Tissue Res.* **318**, 215–24 (2004)." It appears as citation 16.

1.3. Lines 97 and 102 are repeats, please change to improve the flow of the text.

We excluded line 102 and changed the sentence in line 97 to "*Since the 6-OHDA does not penetrate the BBB, it requires a direct administration of the toxin through intracerebral stereotaxic injection*[23]"

2. Protocol

2.1. Drug Preparation

2.1.1 Please provide the type of rats that this protocol is applicable to: Sprague Dawley? Wistar? Any? As well as the average weight or range of weights that this protocol was tested on or would be applicable to.

We added the following note before the topic Surgical Procedure:

"NOTE: In this protocol, we used adult male Sprague-Dawley rats (200-250 g) kept under controlled conditions of temperature (22 ± 2 ^{o}C), air exchange (15-20 exchanges/hour), and light-dark cycles (12/12), grouped in boxes with 3 or 4 animals, with free access to food and water."

2.1.2. Line 151: 2 ml/kg is a dose not a volume. Please change the wording. Same for lines 160, 166 and 201.

We have rewritten the sentences so that it is clearer the dose and volume used in the protocol.

2.1.3. Line 168: 0.5 ml of meloxicam + 0.95 ml of saline is not 1 ml of final solution. Please revise.

Thanks for the observation - we corrected it.

2.1.4. 6-OHDA is a neurotoxin, so please add the following information:
Indicate any toxic or harmful chemicals with the word "CAUTION" when they are first used. Describe the hazard and the appropriate handling guidelines.

We added the following sentence to the 6-OHDA topic at the Drug Preparation section:

CAUTION: 6-OHDA is a neurotoxin used to selectively destroy dopaminergic and noradrenergic neurons in the brain. Avoid direct contact with skin and mucous membranes of the eyes, nose, and mouth. When handling 6-OHDA, wear double nitrile gloves, lab coat, disposable gown, eye protection, and surgical mask or face shield."

2.2. Material Preparation

2.2.1. Please provide the drugs' full references.

We are not sure if we understood this suggestion. We might have misinterpreted the journal's guidelines that suggest adding the drug references in a separate table and not citing the text. We will be happy to fix that, though.

2.2.2. Line 220: Should 50 mL be 50 microL? Same below: line 230: 0.5 mL/min should be 0.5 microL/min? and 4 mL should be 4 microliters?

Thank you for this observation - we corrected all the wrong units mentioned above.

2.2.3. Please provide the type of sutures needed: (non-)absorbable stitches? Nylon? Type of needle?

We added the following topic to the Materials Preparation section:

"*3. Suture*

3.1. Use a sterile nylon non-absorbable suture with a 3/8 circle needle to suture the incision after surgery."

3. Surgical procedure

3.1. The statement that the research was performed in compliance with institutional guidelines at the beginning of the protocol section is missing. The ethical committee approval number should be provided.

We added the following paragraph at the begging of the Surgical Procedure:

"All procedures followed the ethical principles of the National Council for the Control of Animal Experimentation (CONCEA) and the Arouca Law (Law 11.794/2008) and were previously submitted to and approved by the local ethics committee (CEUA-FFCLRP/USP (18.5.35.59.5)."

3.2. Line 317: Please indicate the needle size and the administration route.

We appreciate the suggestion and have added the needle size and the administration route.

3.3. Line 363: 4 ml should be 4 uL (microliters).

We corrected this information.

4. Post-operative procedures

4.1. Please indicate that the animals should be removed from the study or euthanized if the end-points defined in the ethics procedures are reached.

We added the following paragraph at the end of the post-operative procedure's topic:

"*NOTE: Animals should be removed from the study or euthanized if the endpoints defined in the ethical procedures are reached.*"

5. Representative Results

5.1. TH should be defined here.

We added the definition of tyrosine hydroxylase (TH).

5.2. According to the JoVE guidelines "Include data from successful experiments and data from suboptimal experiments to demonstrate the range of outcomes possible" is missing.

We responded to this topic in "major concerns".

6. Discussion

6.1. Line 542: use the abbreviation only for TH.

We corrected.

6.2. According to the JoVE guidelines, the discussion on the protocol itself is missing and should be the main focus to avoid suboptimal experiments:
"Discuss the following with citations:
Critical steps in the protocol modifications and troubleshooting of the method limitations of the method".

We have improved the last paragraph of the discussion, addressing the critical points of this animal model. We also discuss essential steps and limitations of the protocol related to post-surgery recovery, wrong needle insertion during the surgical procedure, anesthesia protocol, and mortality rates in these steps by adding the following paragraphs:

Despite being the most widely used model, the 6-OHDA model, like all current PD models, has its limitations. The model has the disadvantage of not fully representing the molecular mechanisms involved in the pathology of the disease, such as the accumulation of alfa-synuclein proteins and the formation of Lewy bodies. The model simulates the death of dopaminergic neurons of the nigrostriatal pathway, corresponding to a late stage of the disease, leading to the onset of motor symptoms only, making it unsuitable for studying its natural development[15,32].

Also, the 6-OHDA model described in this article is usually characterized by low mortality rates. Post-surgery recovery is crucial to prevent high mortality rates due to the union of an invasive procedure and the neurodegenerative lesion[44]. *It is possible to reduce mortality by taking extra care during the post-surgery recovery period with nutritional supplementation, rehydration, and external temperature control*[45]. *The combination of such measures was able to reduce or even eliminate the mortality rate drastically*[30,46]. *A common cause of death is the insertion of the needle at the wrong coordinate in the brain. Since it is a very delicate procedure, carefully check the coordinates during the surgical procedure. This will avoid that the needle causes damage to other brain structures, such as the hypothalamus, which can impair the animal's eating and drinking actions, leading to malnutrition and dehydration*[47]

Finally, it is essential to highlight that although the ketamine-xylazine anesthesia protocol is very well established and used in rodent experiments[48], *some evidence suggests that the combination of these anesthetics may be insufficient for an extended period of surgery. Also, ketamine-xylazine sensitivity might vary according to different strains of mice and rats*[49,50]. *An alternative may be to use anesthesia by inhaling isoflurane. One study demonstrated that in comparison with ketamine-xylazine, isoflurane-induced anesthesia with faster loss of the righting reflex. Moreover, 60% of the rats anesthetized with ketamine-*

xylazine presented consecutive toe pinch (TP) reflexes during the surgical procedure, even with dose supplementation. In contrast, animals anesthetized with isoflurane presented isolated cases of TP reflexes that disappeared after volume adjustment[51].

7. Figures

7.1. Line 509: Figure 2 legend: p>0.005 is not usually indicative of a non-significant level. Did you mean p>0.05? Or did you deliberately define a very stringent level of significance?

We corrected for p>0.05.

7.2. Figure 3: Please add boxes on the panoramic views corresponding to the insert zooms. Also, please indicate the lesion side.

We appreciate the suggestion. We indicated the corresponding boxes in panoramic views, and hemispheres were labeled accordingly (L:lesion/NL:non-lesion).

Reviewer #2:

Manuscript Summary:
In the present manuscript, the authors described in details about the protocol of MFB infusion of 6-OHDA.The behaviors and neuropathological changes were assessed.

Major Concerns:
1. I would like to suggest a more detailed time-course evaluation of stepping test and dopaminergic lesions. Considering there was obvious changes at the indicated 2w and 4w, earlier timeline was suggested (e.g. 1 day, 3 days and 1 weeks)

We thank you for the observation and agree that it might be exciting for future experiments. Unfortunately, we performed the stepping test only in the second and fourth weeks in these experiments.

2. The figures showing higher magnification were only available in the lesioned groups. There should be the comparison for both sides, that is, the lesioned side and the unlesioned side.

We agree with the reviewer's suggestion, and magnification was included for both hemispheres, which were labeled accordingly (L:lesion/NL:non-lesion).

Minor Concerns:
Please check the typos. For example, "MPB" in the abstract should be "MFB". Line 545 "A review written by Prasad & Hung41 (2020)", the number 41 was not a superscript.

We have corrected the above errors.

Reviewer #3:

Manuscript Summary:
In the present manuscript the Authors described the protocols, methodologies, and materials used to perform stereotaxic surgery for unilateral infusion of 6-OHDA into the MFB of rats and how to predict the dopaminergic lesions caused by the toxin through the stepping test

Major Concerns:
There are errors concerning the volumes used i.e. line 220 ml should be ul

All the errors about de volumes were corrected, it happened due to a mistake of changing the main font of the text.

Minor Concerns:
Details about the representative results should be added. In addition, the figures regarding the TH immunostaining corona sections should have the same coordinates from Bremga. Please replace with more similar one.

We thank you for the valuable comment. As the images are merely illustrative, the slight difference will not impact the general idea to represent the lesion. In addition to that, the point of comparison is the contralateral side of the lesion. Interestingly, the lesions concentrate in caudal sections (medial and lateral portions) in substantia nigra, and the TH protocol was already further explored in another paper (Del-Bel, E., Padovan-Neto, F.E., Szawka, R.E. et al. Counteraction by Nitric Oxide Synthase Inhibitor of Neurochemical Alterations of Dopaminergic System in 6-OHDA-Lesioned Rats Under L-DOPA Treatment. Neurotox Res 25, 33–44 (2014). https://doi.org/10.1007/s12640-013-9406-3).