Chapter 8

Differences Between Intact and Ovariectomized Hemiparkinsonian Rats in Response to L-DOPA, Melatonin, and L-DOPA/Melatonin Coadministration on Motor Behavior and Cytological Alterations

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Abstract

Parkinson's disease (PD) higher incidence has been observed in postmenopausal women compared to premenopausal women, suggesting estrogen neuroprotective effect. L-DOPA (LD) chronic treatment causes dyskinesia; evidences indicate that LD increases the preexisting oxidative stress condition. This study determines melatonin ability, alone or in combination with LD (LD/Mel) to protect dopaminergic loss induced by 6-OHDA in a rat PD model in ovariectomized (OVX) and intact (with ovaries (W/OV)) rats on motor behavior and cytological alterations, comparing with LD-only treated rats. LD/Mel-treated rats showed dyskinesia decrease (score 5–7.5) and had the best performance in the staircase test (five pellets) throughout all studies. The beam walking time was 20–35 s, showing good coordination (as control group (20–38 s)), dopaminergic cells increase of 22.8% (W/OV rats) and 27.2% (OVX rats) in the contralateral side as well as 100% conservation in the contralateral dendritic spines. Our results suggest that LD/Mel co-administration and estrogen presence result in an efficient treatment to reduce dyskinesia through the conservation of some dopaminergic cells, which imply a well-preserved

neuropil of a less denervated striatum. We assume that these results are because of a synergistic effect between LD, melatonin and estrogens.

Keywords: L-DOPA/melatonin dyskinesia, estrogen, Parkinson's disease experimental model, rat

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the loss of dopamine-containing neurons in the substantia nigra compacta (SNc) and by Lewy body presence. The subsequent striatal dopamine (DA) deficiency leads to the parkinsonian condition of bradykinesia, rigidity, tremor, and motor and postural instability [1, 2]. Some efficient drugs, including L-DOPA (LD), dopamine agonists, and inhibitors of dopamine-metabolizing enzymes, have been used for the clinical treatment of LD [3]. Unfortunately, chronic LD therapy is compromised by numerous side effects, the most evident LD-induced dyskinesia (LID) that is abnormal involuntary movements (AIMs), which severely compromise patients' lifestyle [4]. LID usually increases when DA reaches the maximum concentration in the brain *per* LD dose (peak-dose dyskinesias), and dystonia ("off" dystonia) can occur when the level of LD is very low [5]. Risk factors for LID include duration and dose of LD treatment, and consist of asymmetric choreiform movements, athetosis, and dystonia of facial muscles, jaw, tongue, neck, limbs, and toes [6]. Similarly, rats with unilateral 6-hydroxydopamine (6-OHDA) lesion, LD-treatment produces abnormal involuntary movements (AIMs), which are displayed as asymmetric and purposeless movements affecting the limbs, orofacial muscles, and trunk [7]. AIMs evaluation maintains prognostic validity for the preclinical screening of novel antidyskinetic PD treatments [8, 9]. Therefore, the identification of neurochemical features involved in the regulation of motor function may enable the discovery of new potential targets that perform together with LD, improving the effectiveness of these drugs and decreasing the incidence and severity of AIMs and response fluctuations [10].

The etiology of sporadic PD, which is most PD cases, is still unclear. Numerous results have been accumulated from pharmacological and pathological studies on PD and animal or *in vitro* reports using dopaminergic toxins, which cause Parkinsonism in animals [11]. These reports have revealed that oxidative stress [12], inflammation [13], and mitochondrial dysfunction [2, 14] play essential roles in the progress and pathogenesis of sporadic PD. Nevertheless, the mechanisms of dopaminergic neuron cell loss have not been entirely elucidated. However, some data suggest oxidative stress as the main candidate to mediate in the primary unknown cell death cause. Studies on PD brains have given evidence to support this hypothesis [15–17]. The free radical formation has been confirmed in lipids [18], proteins, and SNc nucleic acids of PD patients [19]. Therefore, the reactive oxygen species (ROS) production induced by oxidative stress, the basal ganglia and SNc lack of antioxidant defenses, is commonly considered [20] the final cause of neuronal death [21, 22]. On the other hand, previous studies have

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investigated the reasons for LD long-term problems. Some proposed mechanism that describes LD to induce oxidative damage, perpetuating the cell death [23–25], and it seems that LD produces 6-OHDA in the mouse striatum, generating more ROS formation [26, 27]. It has been proven that dopaminergic nuclei are full with DA following LD acute, subacute, or chronic administration [26], and the augmented DA can stimulate the 6-OHDA production in the brain [27]. Hence, we assumed that parkinsonian neurotoxins that generate free radicals in a DA-enriched milieu would promote oxidative stress production, and it is possible that melatonin might be a free radical scavenger protecting against ROS formation preventing the cell death.

Melatonin is an indoleamine first described in 1993 by Tan et al. [28] as an effective antioxidant. This indoleamine possesses unique benefits. First, its solubility in both water and lipids allows it to be efficiently allocated to the cell. Second, its capacity to cross the blood-brain barrier allows it to reach the central nervous system [29]. There are reports which mention that melatonin protects neurons from neurotoxin-induced damage in a wide range of neuronal culture systems serving as PD experimental models (for review, see [30]). Previous studies have shown that short-term treatment with melatonin does not exert a neuroprotective effect in DA-depleted animals, probably because the levels of this neurohormone are low in the brain [29]; in this sense, it is suggested that melatonin level has to be high and continuously maintained for a long time in the brain to guarantee its neuroprotective effect [30, 31]. It is important to note that *in vivo* experiments are still uncommon, and most of them have been done in acute models of the disease. These studies show melatonin protective effects in both the striatum dopaminergic terminals [31] and midbrain neurons [32]. However, there are insufficient reports about its effects on the initial stages of neurodegeneration.

On the other hand, it is known that the prevalence of several neurodegenerative diseases, such as PD, correlates with gender [33]. Therefore, PD happens 1.5 times more frequently in men than in women [34–37]. In women, the onset age of PD relates to the fertile life duration [38, 39].

It seems that there are several mechanisms of estrogen protection on the nigrostriatal pathway [39]. It has been reported that estrogen has neuroprotective effects in PD animal models utilizing the neurotoxins MPTP [40, 41], 6-OHDA [42, 43], or methamphetamine [44, 45]. The foundations for these sex/gender differences in SNc DA cell death are not known. Nevertheless, the gonadal steroid hormone estrogen seems to be a critical aspect responsible for these differences [39]. *In vivo* confirmation of the neuroprotective effects of estrogens has been reported since estrogen treatment in female ovariectomized (OVX) rodents protects against neurotoxin-induced depletion of striatal dopamine [46, 47]. However, it is not well known whether this neuroprotective effect prevents SNc dopaminergic cell death. Besides, it is not known whether L-DOPA/melatonin (LD/Mel) cotreatment can influence the neuroprotection degree. Therefore, the present study tries to investigate the capacity of melatonin or LD/Mel to protect striatal dopaminergic denervation induced by 6-OHDA in a hemiparkinsonian rat model, comparing the results with LD-only treated rats. The treatments were administered

4 days after lesioning, daily for 6 months at doses suitable to improve motor performance, and their effects were assessed using measures of skilled forelimb use, stepping ability, and AIMs. At the cellular level, the treatment response has been evaluated using tyrosine hydroxylase (TH) immunoreactivity and estimating the number of dendritic spines in the striatal medium-sized spiny neurons, all in female rats, to examine estrogen's presence or absence.

2. Experimental procedures

The experiments were conducted in 50 female Wistar rats weighing 180 ± 20 g at the start of the study. The animals were individually placed in plastic cages under controlled light conditions (12:12-h light-dark regime) and fed with Purina Rat Chow® and water *ad libitum*. Body weight was registered daily. The experimental protocol was conducted out in agreement with the National Institutes of Health, Guide for Care and Use of Laboratory Animals certificated by the Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA) (NOM-062-ZOO-1999, Mexico) and approved by UNAM institutional animal care committee. All attempts were made to reduce the number of animals used and their suffering.

2.1. Motor behavior

Before ovariectomy and 6-OHDA surgery, all animals were trained for 1 week in the beam walking and in the staircase tasks to evaluate motor performance. Training and testing were performed during the light part/period of the cycle, at the same hour every day. For the staircase test, rats were food-deprived for 24 h. Afterward, they received a restricted diet of ~10-g/kg body weight adjusted to keep their weight constant. Food restriction considered the natural gain in body weight during the training period, which prevented excessive weight reduction. After the 6-OHDA surgery, each rat was tested once a week, a different day for each test. Two observers blind to the rats' condition perform all behavioral assessments.

2.1.1. Staircase test

Rats were trained in the staircase test, which measures the independent use of forelimbs in skilled reaching and grasping tasks [48]. Briefly, each rat is placed into a clear plexiglass case (length 30 cm, width 6.8 cm, and height 12 cm) in which the rat rests on a central elevated platform with six stairs descending on each side. Each stair contained one food pellet. Food pellets on the left stairs may be retrieved only using the left paw, whereas pellets on the right stairs must be obtained using the right paw (**Figure 1**). Rats were trained for a week (2/15 min sessions/day) and were excluded from this test if they did not retrieve at least six pellets/side [49]. The last 5 days of training were used to calculate baseline performance. The skilled reaching ability was quantified by recording the number of food pellets retrieved with each paw. The qualitative analysis of this test comprises the appropriate movements to take the pellets: (1) prepared to take food, (2) stretched the forelimb, (3) took the pellet (pronation movement), (4) paw rotation around the wrist (supination), and (5) eat the food [49].

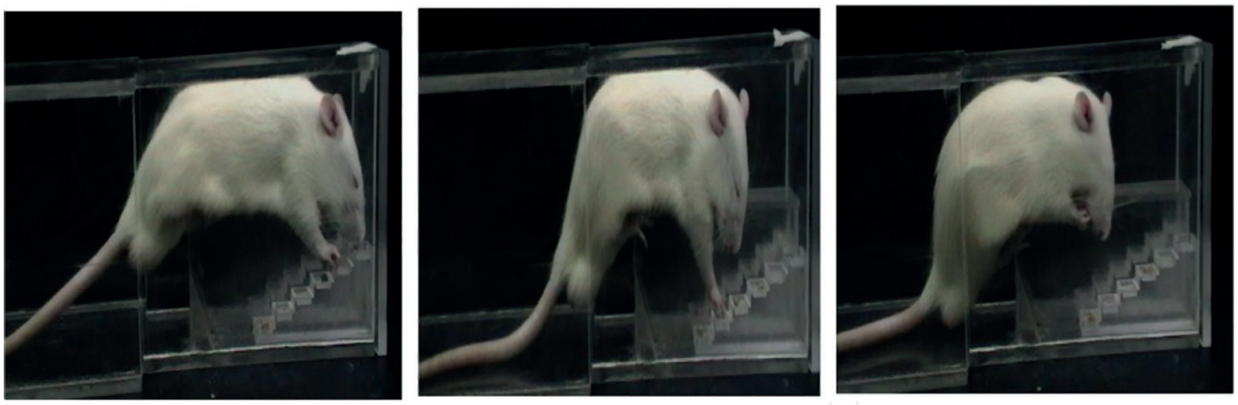

Figure 1. Staircase test used to assess skilled reaching deficits after 6-OHDA lesion.

2.1.2. Beam walking task

The additional test to measure motor coordination was evaluating the ability of the animals to traverse a narrow beam (12 mm wide) to reach an enclosed safety platform [50]. The rats were trained for 1 week to cross the wooden beam. The beam measured 2 m long and was elevated to a height of 1 m over the floor with wood supports with 15° inclination. Each test session consisted of four trials in which latency to cross the beam was recorded (establishing a maximum range of 120 s; if the animal did not pass at that time, the activity was terminated and assigned the value of 120 s for that evaluation). Five trials were averaged to give a mean latency [51]. The testing was done every week after 6-OHDA lesion during the first month and after that every 15 days.

2.2. Surgery

2.2.1. Ovariectomy

Bilateral ovariectomy (OVX) was performed through two lateral incisions of the abdominal wall under Isoflurane anesthesia (n = 25).

2.2.2. Stereotactic surgery and treatments

The rats were anesthetized with Isoflurane and placed in a stereotaxic apparatus. The rats (n = 20 OVX and 20 with ovaries (W/OV)) were infused with 4 μl saline solution carrying 8 μg of 6-OHDA (Sigma Chemical, USA) and 0.2 mg of ascorbic acid into the left medial forebrain bundle (MFB) (n = 40), and sham lesion was made with vehicle (n = 10; 5 OVX and 5 W/OV (control group) [7]. The injections were given over a 5-min period with a Hamilton syringe attached to a glass micropipette with a tip diameter of 20–50 μm. The stereotaxic coordinates were as follows: AP = −3 mm anterior of the ear bar; L = 1.6 mm lateral of bregma; V = −8 mm vertical of the Dura (according to [52]). After anesthesia recovery, the animals were returned to their cages. Apomorphine (Sigma Chemical, USA; 0.25 mg/kg i.p.) provoked contralateral rotational behavior was tested 2 days after lesioning. Only those animals displaying more than 200 full turns in a 30-min period were used [53]. Two days after the rotational behavior test, we began the treatments as follows: 5 OVX and 5 W/OV lesioned rats were treated with 7.5 mg/kg

LD (Sinemet® (Carbidopa-L-DOPA 25/250)), 5 OVX and 5 W/OV lesioned rats were treated with 10 mg/kg melatonin (Sigma Chemical, USA), and 5 OVX and 5 W/OV lesioned rats were treated with 7.5 mg/kg LD/10 mg/kg Mel. The drugs were dissolved in 10 ml distilled water and given orally with an insulin syringe for 6 months during the light period (at 10:00 AM every day) [7]. The other 10 (5 OVX and 5 W/OV) lesioned rats without treatment, as well as the control animals (5 OVX and 5 W/OV), were kept for the same time. The motor performance was evaluated weekly during the first month and after every 15 days; the rats were tested during the light period at 14:00 h, a different day for each test.

2.3. AIMs rating

LD-induced AIMs were calculated at day 30 according to a rat dyskinesia scale [54–56]. Rats were placed individually in transparent cages and observed every 20th min, from 20 min before to 180 min after giving the treatments (10 monitoring periods of 1 min each). Four AIM subtypes were classified according to their topographic distribution as locomotive, axial, forelimb, or orolingual (for details, see **Figure 2**). Signs of otherwise normal behaviors, such as rearing, sniffing, grooming, and gnawing, were not included in the evaluation [57]. AIM severity was assessed using the method of Cenci et al. [55], and Lundblad et al. [9], which designates a score from 0 to 4 to each of the four AIM subtypes mentioned before according to the proportion of time/monitoring period through whichever AIM is observed. Borderline scores, such as 0.5, 1.5, 2.5, and 3.5, were allowed to increase the sensitivity of the evaluation [7, 57].

2.4. Video recording

Performance during motor tests and AIM analysis was video-recorded (Panasonic camcorder DR-H80 model). Representative still frames were captured from digital video recordings with the video-editing software Final Cut Pro. Pictures were cropped and adjusted for color and brightness contrast in Adobe Photoshop but were not altered in any other way [57].

2.5. Cytological analysis

All animals were perfused under sodium pentobarbital anesthesia immediately after the 6-month treatments via the aorta, with saline solution followed by fixative including 0.2% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and deposited in the fixative solution for 1 h. For the TH Immunocytochemistry, coronal sections (50 μm) were collected on a vibrating microtome through the mesencephalon. Tyrosine hydroxylase (Chemicon International, Inc., CA, USA; 1:1000) immunostaining with the ABC detection method (Vector Lab MI, USA) was conducted for light microscope analysis. The analysis was performed with a computer-assisted system (Image-Pro Plus, Media Cybernetics, L.P. Del Mar, CA, USA) connected to a CCD camera to Optiphot 2 microscope (Nikon, Japan). The number of TH-positive neurons was calculated in 1500 μm^2 from seven SNc sections of each animal [58]. The dendritic spines analysis was performed by the Golgi method. Blocks from the striatum were cut into 90-μm-thick sections and processed

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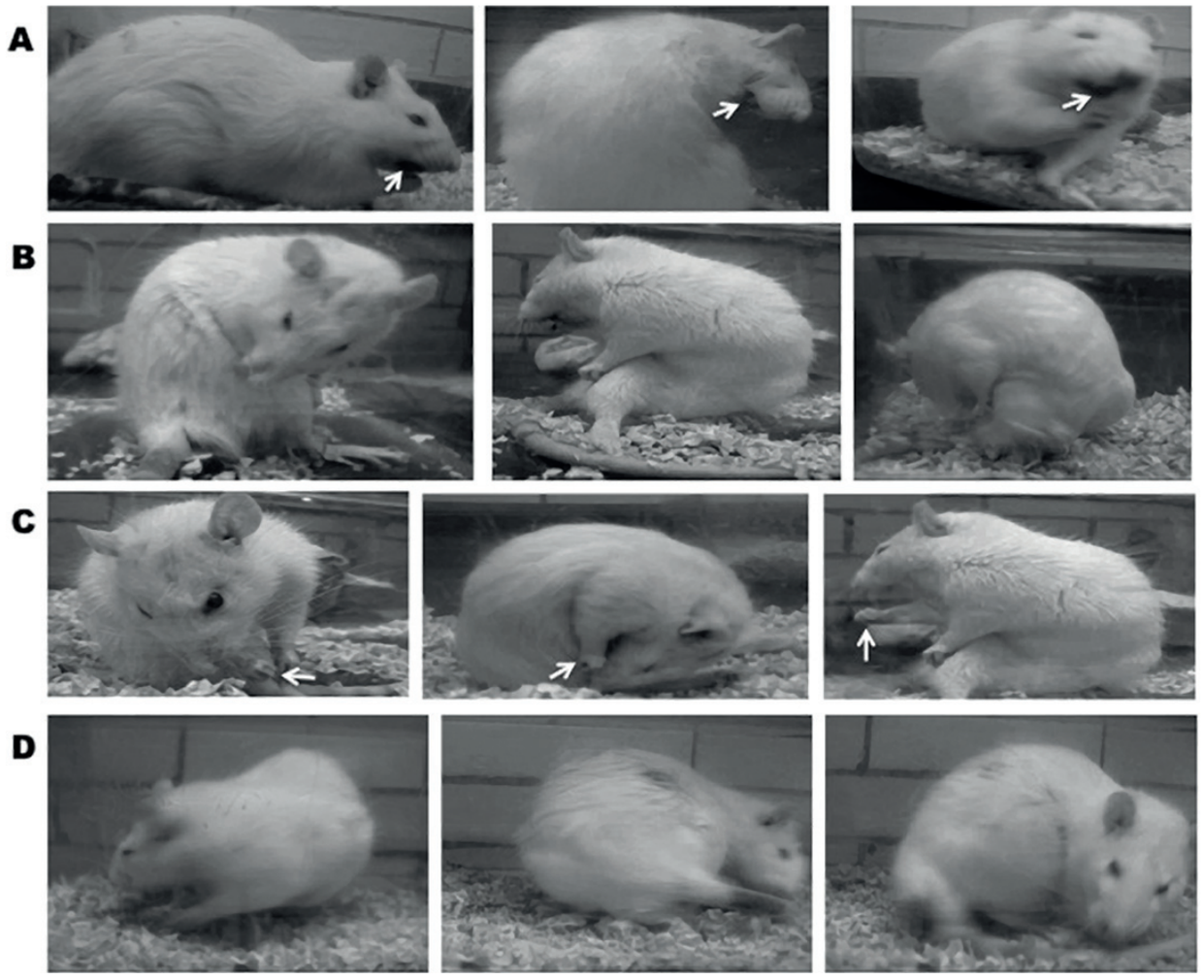

Figure 2. Video recording sequences from rats affected by orolingual (A), axial (B), forelimb (C), and locomotive (D) AIMs. Orolingual AIMs (A) include opening and closing of the jaws and tongue protrusion toward the side contralateral to the lesion (arrow). The series in (B) displays a neck and upper trunk torsion action toward the contralateral side to the lesion. Body torsion is maximally critical (>90°), causing the rat to lose equilibrium. Forelimb AIMs (C) include purposeless up and down translocation of the Parkinsonian (right) forelimb (arrow). Locomotive AIMs (D) comprise circular movement toward the contralateral side to the lesion. Only locomotive movements involving all four limbs are considered under this AIM category.

for the rapid Golgi method. The analysis consisted in counting the number of dendritic spines in a 10-μm-long section from 5 secondary dendrites to 20 striatal medium-sized spiny neurons [58].

2.6. Statistical analysis

Two-way ANOVA was used to analyze the number of TH-immunoreactive cells, the number of dendritic spines, and the behavioral data. Group differences were considered statistically significant at $P < 0.05$. When appropriate, *post hoc* comparisons were made with Tukey test. All analyses were conducted with GraphPad Prism 7 for Mac Software.

3. Results

After 6 months, neither clinical alterations nor significant weight changes were detected in the experimental animals compared to controls.

3.1. Staircase test

It is well known that motor behavior is crossed; We lesioned the left MFB (ipsilateral) affecting the right side (contralateral), so we only show the contralateral paw data. For the treatment's effect analysis, data from OVX and W/OV rats were plotted separately (**Figure 3A** and **B**, respectively). Those graphs show that all control rats maintained the same number of reaching success through all the study (5.2 ± 0.20–6) comparing to the baseline. In contrast, the 6-OHDA-lesioned rats showed significant motor alterations during the whole study showing a drastic decrease in the number of pellets reached (1.5 ± 0.28–2.2 ± 0.750). 6-OHDA + LD treatment animals presented motor behavior recovery until 21–28 days after treatment, and then, the rats failed in the task (1.8 ± 0.12–2.4 ± 0.47 pellets), behaving very similar to the untreated lesioned animals (**Figure 3A** and **B**). Unlike the 6-OHDA + melatonin rat's motor performance, initially they had similar values to untreated 6-OHDA animals (2.2 ± 0.47) and subsequently had a gradual recovery of reaching values (5.8 ± 0.10) as control animals (5.2 ± 0.2). The 6-OHDA + LD/Mel rats showed improvement in the performance from the start, lasting this effect until the end of the study; the number of successes (4.2 ± 0.25–5 ± 0.40) was similar to control animals (5.4 ± 0.24–5.8 ± 0.20) (**Figure 3A** and **B**).

To compare estrogen protection data from W/OV and OVX, rats were plotted by treatment (**Figure 4**). We can observe that 6-OHDA + LD OVX rats at 21 days decrease the reaching values (3 ± 0.49) similar to 6-OHDA animals (2.22 ± 0.27), unlike 6-OHDA + LD W/OV rats presented motor impairment until 42 days (2.2 ± 0.026 pellets), this group exhibited delayed deterioration (**Figure 4A**). W/OV 6-OHDA + melatonin rats, reaching values (5.8 ± 0.10), were

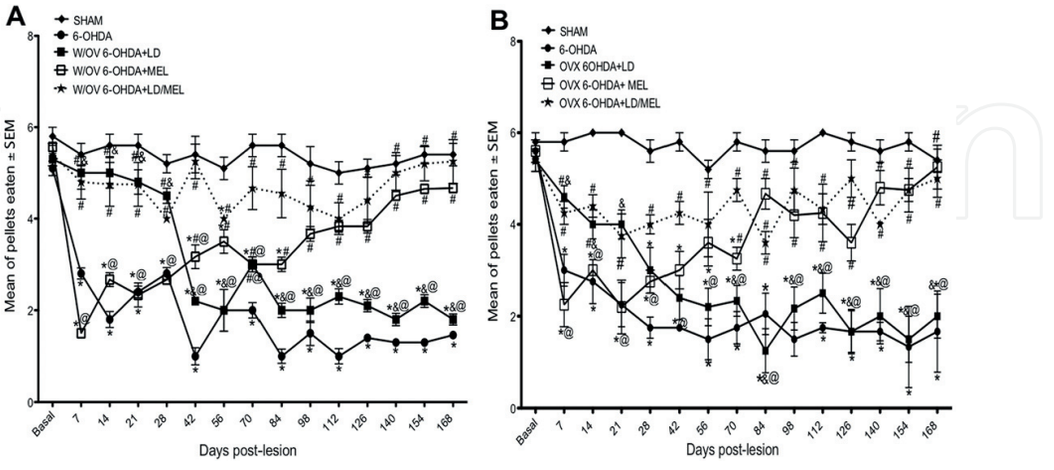

Figure 3. Contralateral forelimb staircase test results. The number of reaching successes recorded in W/OV (A) and OVX (B), with the different treatments. * = P < 0.05 experimental groups vs. control groups; # = P < 0.05 treatments vs. untreated 6-OHDA; & = P < 0.05 6-OHDA + LD vs. 6-OHDA + melatonin; @ = P < 0.05 6-OHDA + LD and 6-OHDA + melatonin vs. 6-OHDA + LD/Mel.

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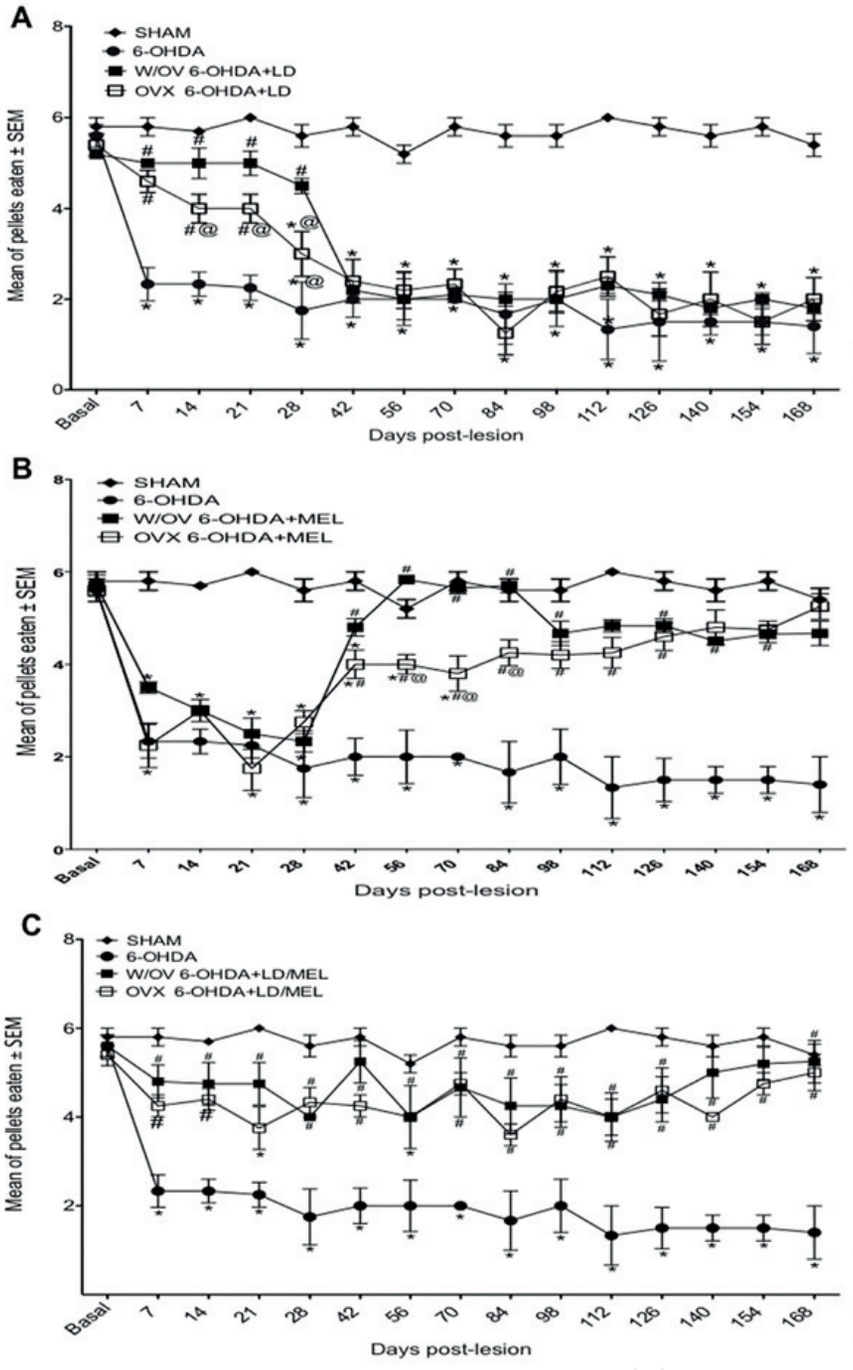

Figure 4. Estrogen protection in the staircase test contralateral forelimb. The number of reaching successes recorded in W/OV and OVX. 6-OHDA + LD (A), 6-OHDA+ melatonin (B) and 6-OHDA + LD/Mel (C). * = P < 0.05 experimental groups vs. control groups; # = P < 0.05 treatments vs. untreated 6-OHDA; @ = P < 0.05 W/OV vs. OVX rats.

similar to the control animals (5.8 ± 0.20) after 42 days of treatment, unlike OVX rats who reached control group values (4.25 ± 0.27) at 84 days of treatment (**Figure 4B**).

All animals receiving 6-OHDA + LD/Mel perform similarly to control group animals during the 6 months of treatment and displayed no statistically significant differences between them (**Figure 4C**).

3.2. Beam walking test

Figure 5A and **B** illustrates the mean numbers of total time to cross the beam, and the treatments' effect in W/OV and OVX rats. 6-OHDA animals significantly increased the time (120 s) compared to control animals (30 s ± 2.71–40 s ± 1.3), remaining these values throughout the study. The 6-OHDA + LD group showed statistically significant improvement for about 21–28 days (25 ± 4.26 and 38.5 ± 2.7), displaying scores like the control group (24.8 ± 1.31 and 20.4 ± 2.24 s). Afterward, these rats increased the time (62 ± 6.1), behaving similarly to untreated 6-OHDA group (100 ± 6.7). 6-OHDA + melatonin rats, at the beginning of the treatment, showed increased time to cross the beam (72.7 ± 4.71) to approximately 28 days, with values like 6-OHDA-untreated animals (112.8 ± 7.2), and then, at 42 days, the animals improved their motor activity (46.6 ± 1.83 s), reaching values of control animals (25.6 ± 2.48 s). 6-OHDA + LD/Mel rats presented values (30.40 ± 2.71 to 40.4 ± 1.37 s) similar to control animals during the entire study (**Figure 5A** and **B**).

Regarding the comparison between estrogen status, we observed that OVX 6-OHDA + LD decreased the time to cross the beam. They have reached values (62 ± 6.1 s) similar to 6-OHDA untreated group (100 ± 6.78) to day 42, and unlike 6-OHDA + LD W/OV showed similar (67 ± 2.14 s) values to 6-OHDA untreated animals (104.5 ± 9.17) from 126 days of treatment, again, we observed that W/OV rats showed delayed motor impairment compared to 6-OHDA + LD OVX rats (**Figure 6A**). OVX 6-OHDA + melatonin rats increased the time to cross the beam (107.8 ± 2.2 s) as 6-OHDA untreated animals (105.2 ± 9.82 s) until 21 days of treatment; unlike 6-OHDA + melatonin W/OV rats increased the time (60.83 ± 3.95 s); subsequently, after 28 days of treatment, 6-OHDA + melatonin W/OV animals had similar values (46.66 ± 1.86 s) to the control group (25.60 ± 2.48 s), while OVX rats reached values (50 ± 5.6 s) as control animals (30.2 ± 6.55 s) until 70 days of treatment. W/OV 6-OHDA + melatonin rats

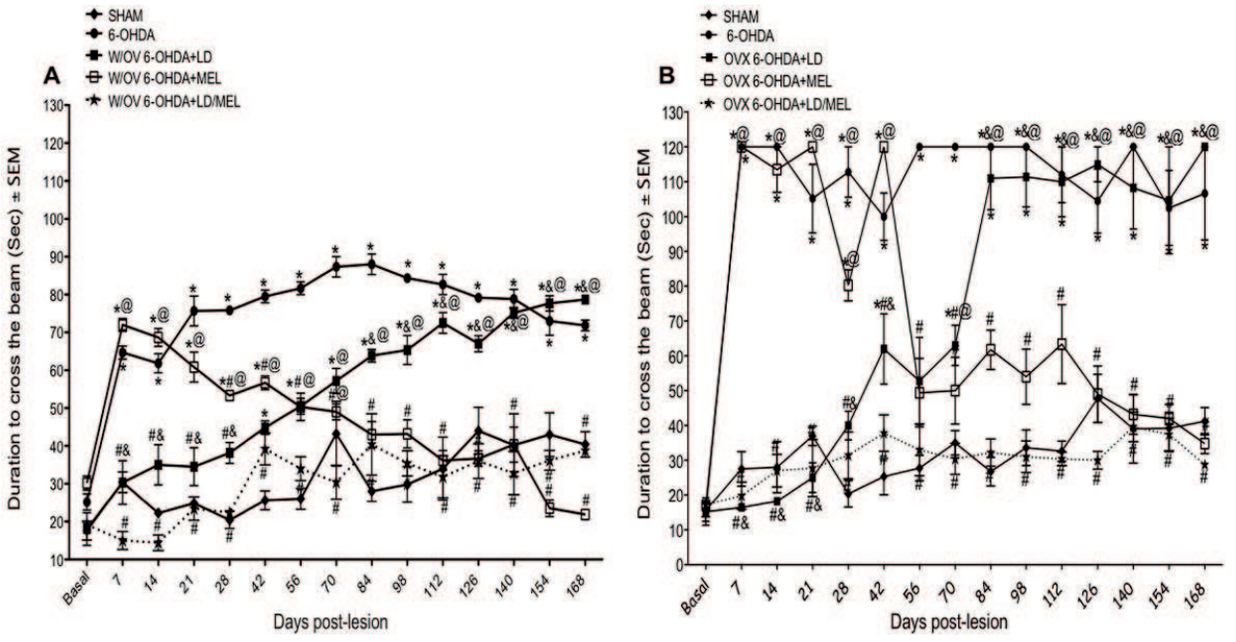

Figure 5. Beam walking test evaluation W/OV (A) and OVX (B) rats, with the different treatments. * = P < 0.05 experimental groups vs. control groups; # = P < 0.05 treatments vs. untreated 6-OHDA; & = P < 0.05 6-OHDA + LD vs. 6-OHDA + melatonin; @ = P < 0.05 6-OHDA + LD and 6-OHDA + melatonin vs. 6-OHDA + LD/Mel.

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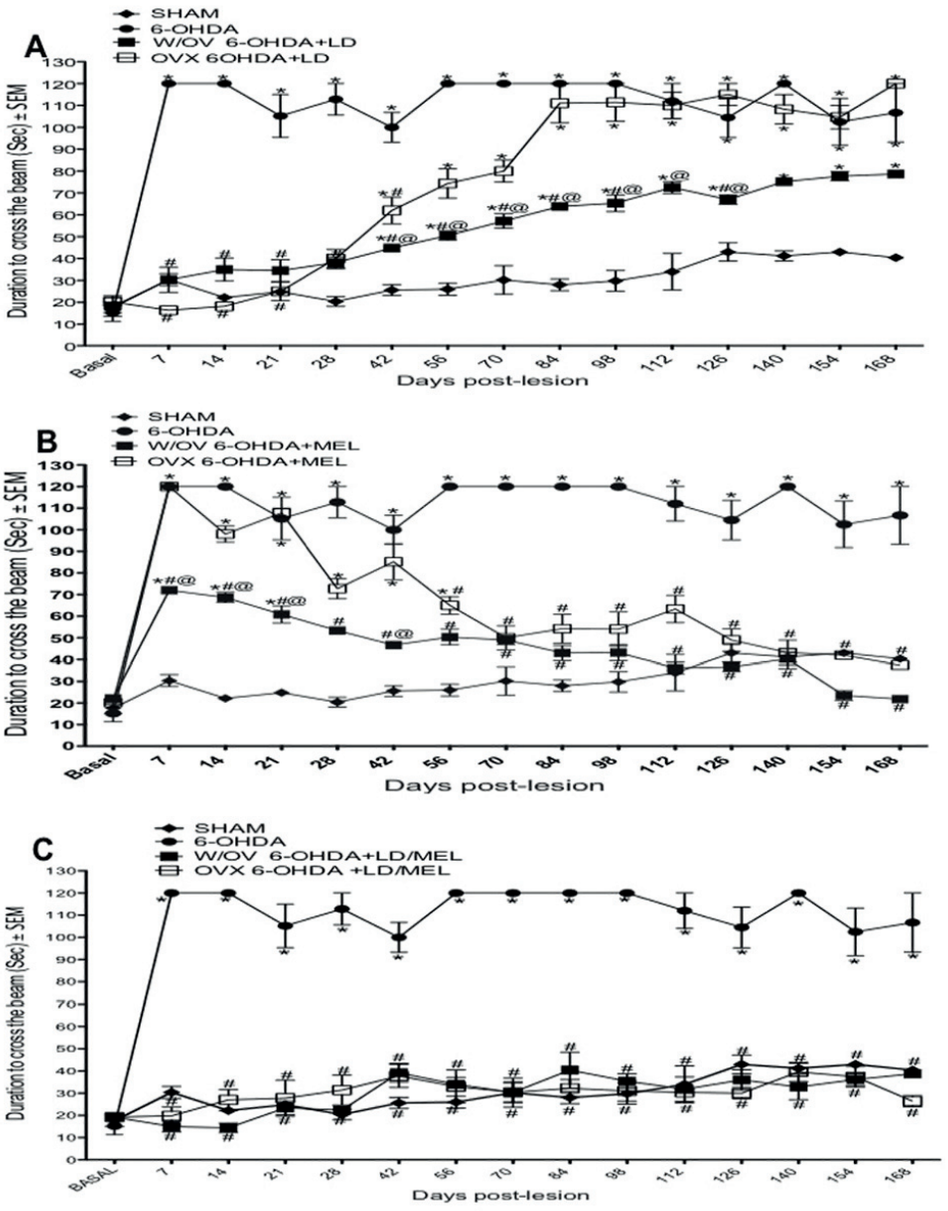

Figure 6. Estrogen protection on the beam walking test. The time to cross the beam recorded in W/OV and OVX rats. 6-OHDA + LD (A), 6-OHDA+ melatonin (B) and 6-OHDA + LD/Mel (C). * = P < 0.05 experimental groups vs. control groups; # = P < 0.05 treatments vs. untreated 6-OHDA; @ = P < 0.05 W/OV vs. OVX.

recovered faster compared to OVX rats (**Figure 6B**). It is important to note that all 6-OHDA + LD/Mel animals displayed similar (values 19.8 ± 0.97 to 38.75 ± 1.03) to the control animals (30.4 ± 2.71 to 40.4 ± 3.37) over the 6 months of treatment and no statistically significant differences between groups (**Figure 6C**).

3.3. Abnormal involuntary movements

3.3.1. Time course and overall incidence AIMs

To get an overview of the development of dyskinesia in the different groups, we carried out the summation of all AIMs subtypes (axial + locomotive + limb + orolingual). As shown in

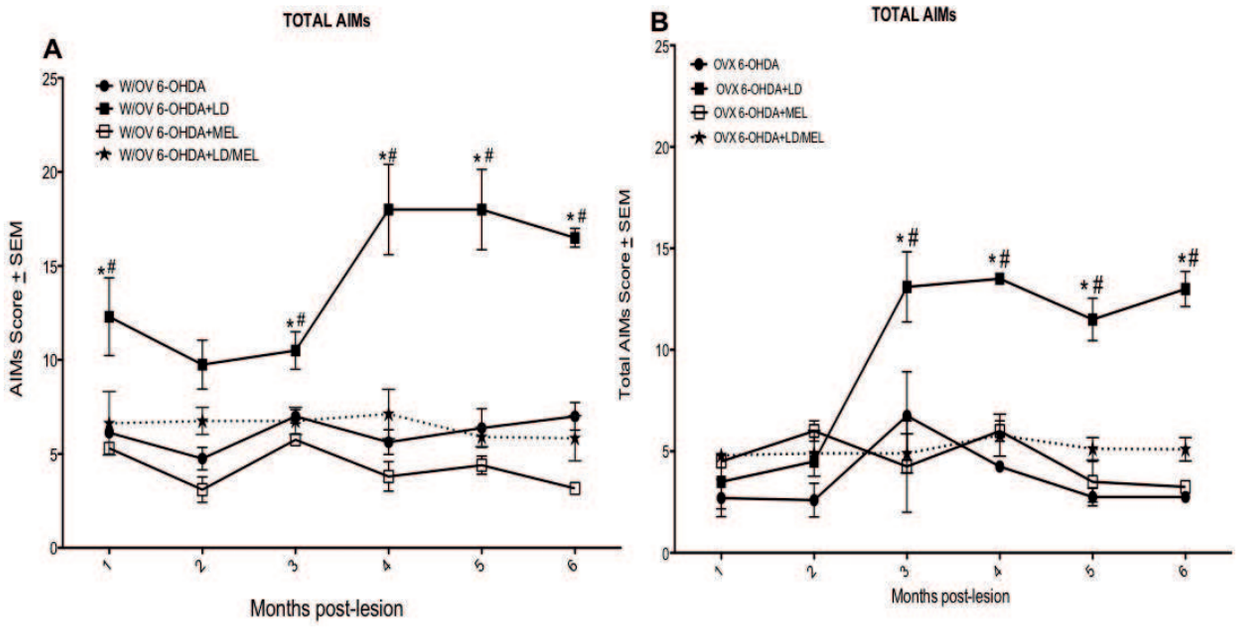

Figure 7. Total AIMs (orolingual, axial, forelimb, and locomotive) within 6 months of treatment of W/OV (A) and OVX (B) rats with the different treatments. * 6-OHDA + LD vs. untreated 6-OHDA and 6-OHDA + melatonin; # 6-OHDA + LD vs. 6-OHDA + LD/Mel. $P < 0.05$.

Figure 7 A and **B**, repeated-measures ANOVA revealed significant overall differences between untreated 6-OHDA-lesioned (2.5 ± 0.80 to 2.75 ± 0.14) and melatonin-treated groups (5.3 ± 0.30 to 3.16 ± 0.17) comparing to LD-treated groups. 6-OHDA + LD rats from the first month of the evaluation showed high values (12.30 ± 2.068) of MIAs (**Figure 7A** and **B**).

Remarkably, all 6-OHDA animals receiving LD/Mel coadministration developed MIAs scores (4.80 ± 0.25 to 5.83 ± 1.20) similar to untreated 6-OHDA (2.5 ± 0.80 to 2.75 ± 0.14) and 6-OHDA + melatonin animals (5.3 ± 0.30 to 3.16 ± 0.17) (**Figure 7A** and **B**).

Concerning total LIDs and the comparison between estrogen status, we observed that OVX 6-OHDA+ LD began to develop LIDs after 3 months of treatment unlike W/OV 6-OHDA + LD rats, which showed LIDs from the first month, and the OVX 6-OHDA + LD group showed delay in LIDs development, the two groups subsequently, had similar scores (18 ± 2.40 for W/OV 6-OHDA + LD rats and 13.5 ± 0.28 OVX 6-OHDA+ LD), and showed no statistically significant differences between them (**Figure 8A**). W/OV and OVX 6-OHDA + melatonin rats developed low MIAs scores (5.3 ± 0.30–3.16 ± 0.16 and 4.5 ± 0.1–3.25 ± 0.25, respectively) like the untreated 6-OHDA animals (2.5 ± 0.80–2.70 ± 0.14) and showed no statistically significant differences (**Figure 8B**). It is important to note that all 6-OHDA + LD/Mel rats, since the first evaluation, showed small LID scores (4.80 ± 0.25–5.83 ± 1.20) and no statistically significant differences (**Figure 8C**).

3.4. TH immunocytochemistry

Our results show that W/OV and OVX control rats had similar values in the number of TH-immunopositive cells, in both ipsilateral and contralateral sides (**Figures 9A** and **B** and **10**),

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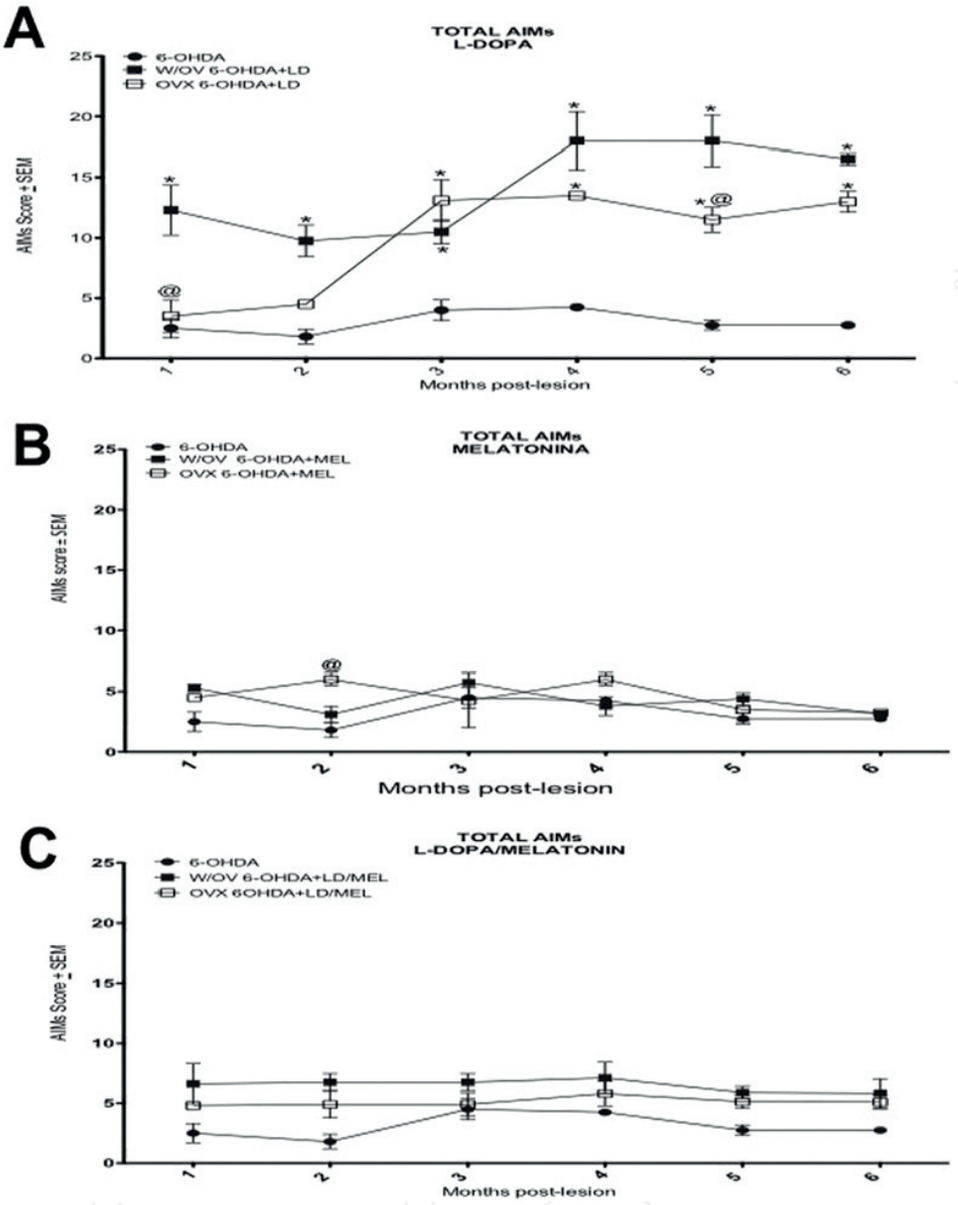

Figure 8. W/OV and OVX rats' comparison in total AIMs during 6 months of treatment. 6-OHDA + LD (A), 6-OHDA + melatonin (B), 6-OHDA + LD/Mel (C). *6-OHDA + LD vs. untreated 6-OHDA; @ OVX vs. W/OV rats, $P < 0.05$.

and display no statistically significant differences between groups. In **Figure 9A**, it can be observed a drastic dopaminergic neuronal loss in the ipsilateral SNc, W/OV and OVX 6-OHDA-lesioned rats had neuronal survival of 3.97% and 6.14%, respectively, like W/OV and OVX 6-OHDA + LD (2.2% and 3.46%) and 6-OHDA + melatonin (3.45% and 5.9%). Note that both W/OV and OVX rats who received 6-OHD + LD/Mel had a higher percentage of cells 7.67% and 10.46%, respectively; however, we found no statistically significant differences between groups.

Regarding contralateral SNc, **Figure 9B** shows that W/OV and OVX 6-OHDA-lesioned groups and all animals with 6-OHDA + LD showed a decline of approximately 20% neuronal loss, compared to control groups, and showed no statistically significant differences between

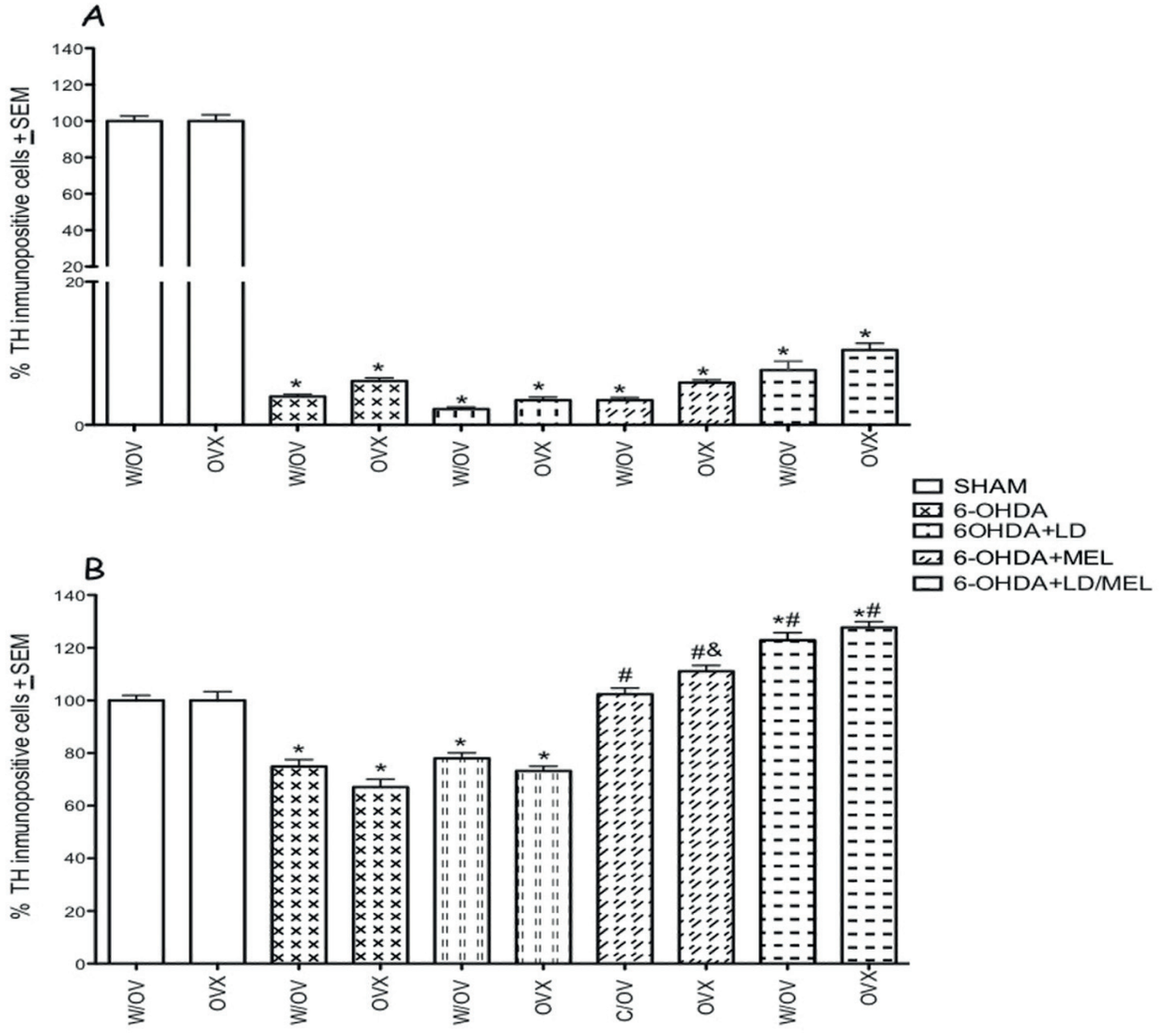

Figure 9. TH-immunoreactive cell percentages from the ipsilateral (A) and contralateral (B) SNc in the control and experimental groups. The data are depicted as mean ± SEM. * Experimental vs. control; # 6-OHDA + melatonin and 6-OHDA + LD/Mel vs. untreated 6-OHDA and 6-OHDA + LD; & 6-OHDA + melatonin vs. 6-OHDA + LD/Mel; $P < 0.05$.

groups. W/OV and OVX rats 6-OHDA + melatonin-treated had values (values to 102% and 111%, respectively) similar to the control group. Surprisingly, the W/OV and OVX 6-OHDA + LD/Mel rats showed a higher percentage of TH-immunopositive cells (22.8% and 27.2%, respectively) compared to control group and no statistically significant differences.

3.5. Dendritic spines

When performing dendritic spines counting, we observed that control W/OV rats displayed a mean of 7.94 ± 3.23 in the ipsilateral striatum and 7.97 ± 3.47 in the contralateral side; these values were taken as 100%. OVX rats showed a decreased of 21.3% dendritic spines in the ipsilateral striatum and 20.94% in the contralateral side compared to control W/OV (**Figure 11A** and **B**),

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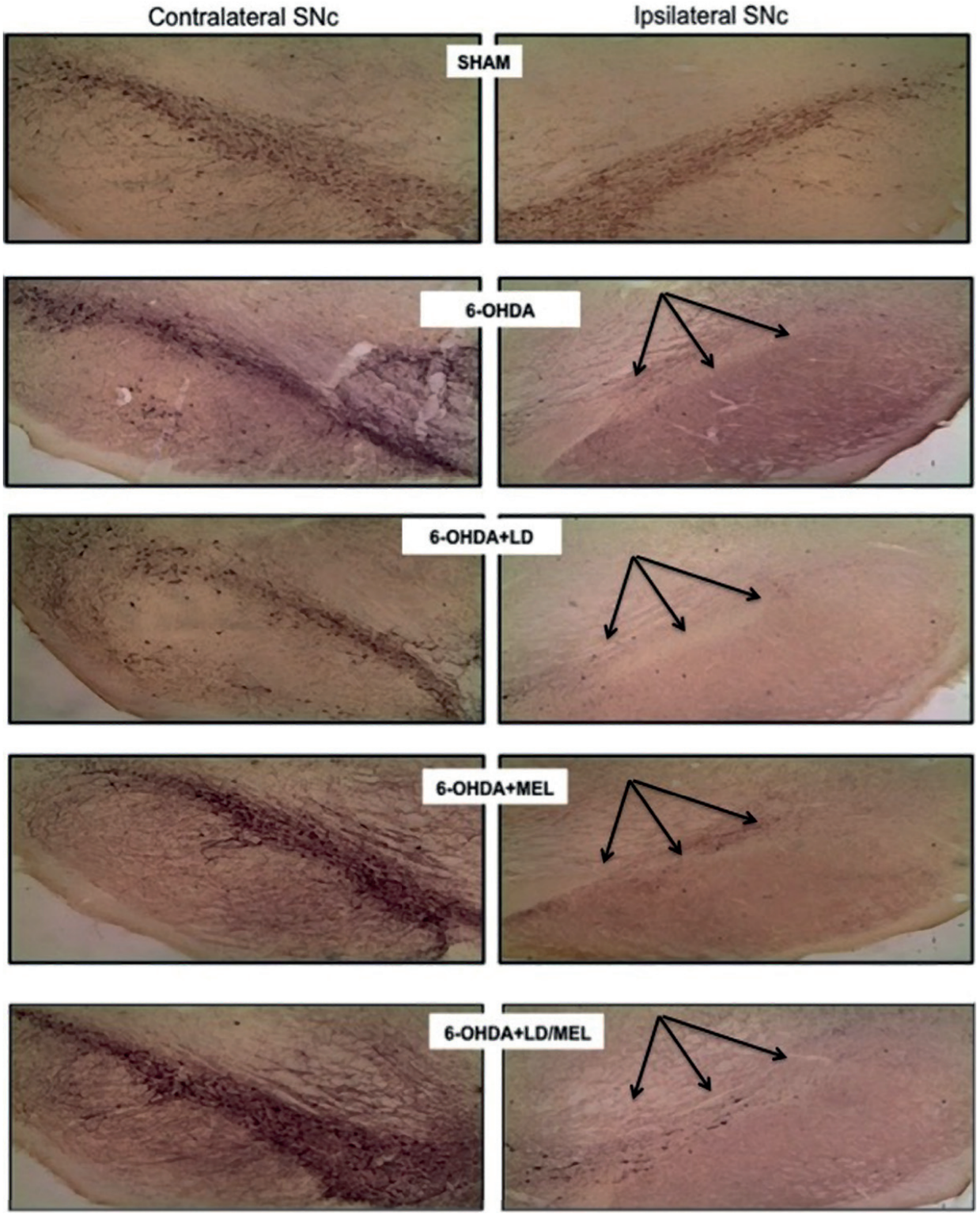

Figure 10. Representative tyrosine hydroxylase immunostained from coronal sections containing the SNc of control, 6-OHDA-untreated, 6-OHDA + LD, 6-OHDA + melatonin and 6-OHDA + LD/Mel-treated rats. Note the significant cell loss in the ipsilateral SNc in the four experimental groups (arrows), being more evident in the untreated 6-OHDA and LD treated ones; also, the contralateral SNc of melatonin and LD/Mel-treated rats lost fewer neurons than the other two experimental groups, and LD/Mel-treated rats had more neurons than control rats (magnification 4×).

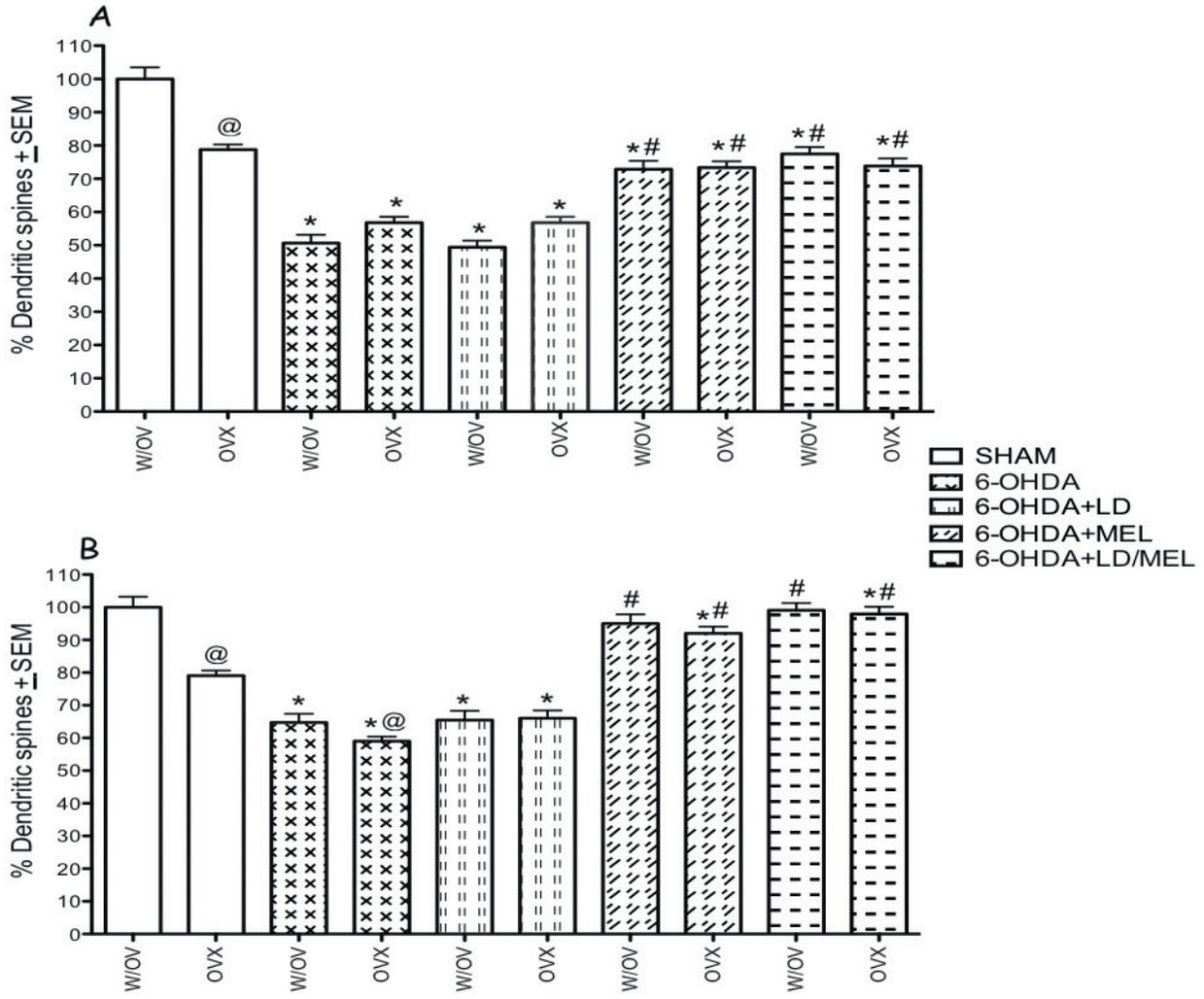

Figure 11. Striatal medium-sized spiny neurons dendritic spine percentage ipsilateral (A) and contralateral (B). * Experimental vs. control; # 6-OHDA + melatonin and 6-OHDA + LD/Mel vs. untreated 6-OHDA and 6-OHDA + LD; @ untreated 6-OHDA OVX vs. untreated 6-OHDA W/OV, $P < 0.05$.

In the ipsilateral striatum, W/OV and OVX 6-OHDA-lesioned rats and 6-OHDA + LD-treated rats presented severe dendritic spines loss (50, 44, 49, and 51%, respectively), unlike 6-OHDA + melatonin-treated (72 and 73%) and 6-OHDA + LD/Mel coadministration rats (77 and 73%), which showed a greater number of dendritic spines and showed no significant differences between groups (**Figures 11A** and **12**). Regarding contralateral striatum, we observed that OVX untreated 6-OHDA rats displayed higher dendritic spines loss (41%) compared to W/OV 6-OHDA untreated animals (36%), showing statistically significant differences. W/OV and OVX all 6-OHDA + LD groups showed significant dendritic spines loss (35% and 34%), showing similar values with W/OV and OVX untreated 6-OHDA rats (36% and 41%), with no statistically significant differences between groups. W/OV 6-OHDA + melatonin (95%) and W/OV 6-OHDA + LD/Mel (99%) rats had similar values for the number of dendritic spines to control group. OVX 6-OHDA + melatonin (92%) and OVX 6-OHDA + LD/Mel-treated rats (97%) had a higher percentage of dendritic spines compared to control group (76%), showing increased number of dendritic spines similar to control W/OV group (**Figures 11B** and **12**).

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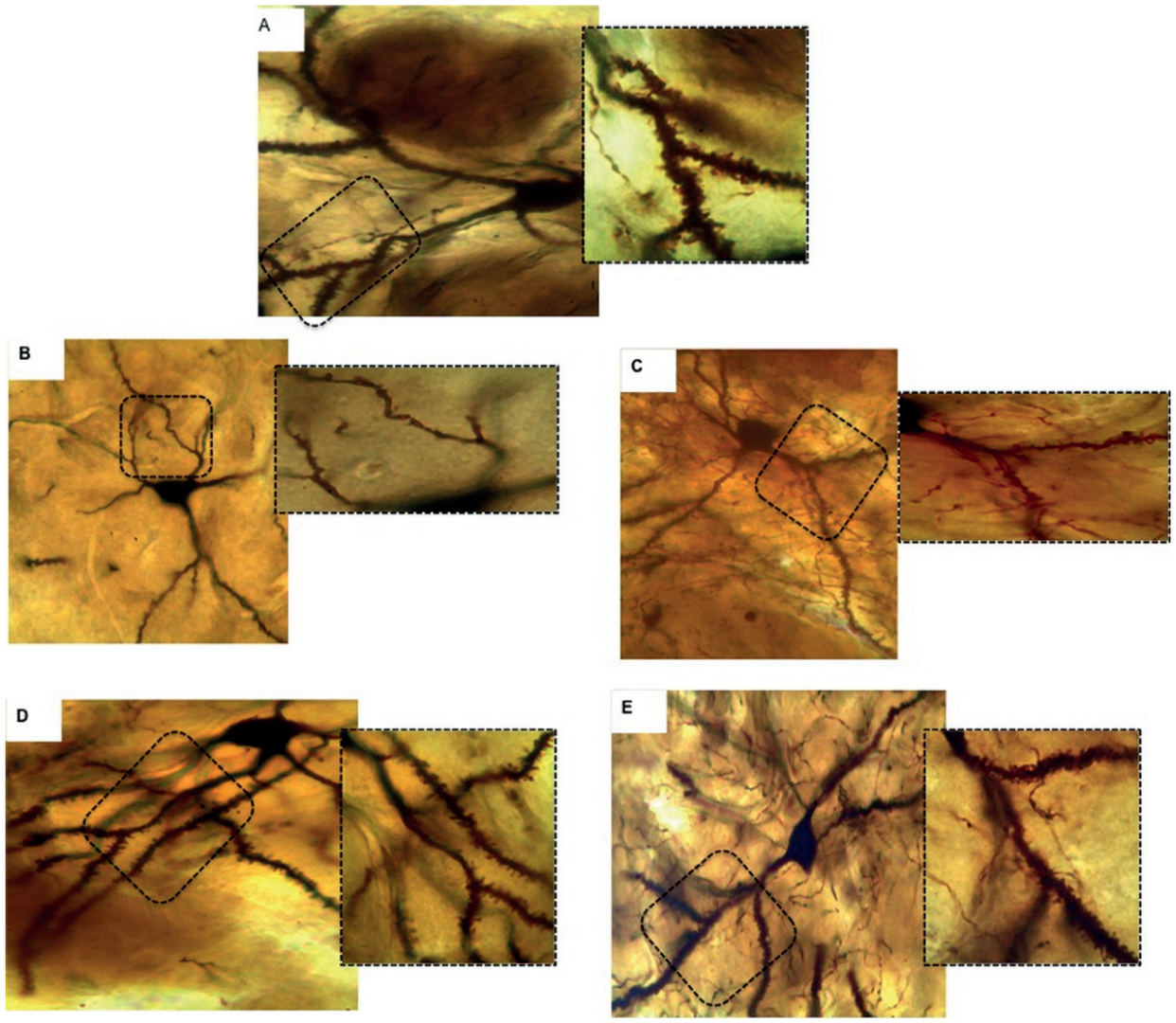

Figure 12. Representative micrographs of Golgi-stained medium-sized spiny neurons of the ipsilateral striatum with an illustrative box of dendritic spine densities from the control group (A), untreated 6-OHDA group (B), 6-OHDA + LD (C), 6-OHDA + melatonin (D), and 6OHDA + LD/Mel (E). Both untreated 6-OHDA and LD-treated induced a marked decrease in the total number of spines mainly in the ipsilateral striatum. In contrast, melatonin and LD/Mel-treated groups showed a well-preserved dendritic spine density (magnification, 40× and 100×).

4. Discussion

Our data show that the LD/Mel coadministration and the estrogen presence appear to be a very effective combination to reduce AIMs through the conservation of some functional SNc dopaminergic cells, which in turn imply a well-preserved neuropil of a less denervated striatum. We assume that these results are probably because of a synergistic effect between LD, melatonin, and the estrogen presence.

4.1. Staircase test

It has been reported that PD patients have poor manual skills that worsen as the disease progresses, and patients have difficulty performing tasks that require sequential movements,

for example, when performing repetitive movements of forearm pronation and supination, openness and closing hand and reaching for objects [59]. The rats' movements in the staircase test are very like humans, so that test allows evaluating DA deficiency and treatments effectiveness [48, 59]. The rats' movement in the staircase test by using the forelimb to keep the pellet and eat it is a complex and anomalous activity for animals with 6-OHDA unilateral lesion. According to our results, all untreated 6-OHDA animals showed severe motor damage mainly affecting the contralateral side, expressed by the drastic reduction in the number of pellets eaten, which is consistent with other authors [60, 61]; the performance of this activity was abnormal, and although sometimes the rats obtained the food pellet successfully, supination and pronation movements were limited compared to control animals. Some rats also use compensatory strategies such as increasing their digit pressure and frequently used tongue and teeth to achieve the pellet [59]. In this respect, it is known that motor alterations in the staircase test depend on the striatonigral dopaminergic system integrity [60, 62, 63]. Besides, several authors have reported that animals with this motor impairments display severe SNc TH-immunopositive neuronal loss and fewer DA fibers in the striatum [60, 64].

4.2. LD treatment

As our results show, all 6-OHDA + LD animals showed recovery since the first day to 21–28 days of treatment. Subsequently, they displayed notorious motor alterations. Thus, our results are consistent with previous studies where it has been observed that PD-experimental animals LD-treatment therapeutic benefit in rodents are approximately 3 weeks [57, 65]. In this respect, it has also been reported that LD-treated PD patients improve the motor response in tests that include taking objects on a surface, display greater coordination, and recover the movement initiation [66, 67]. However, when LD treatment is chronically administered (6–13 months), patients do not improve and show alteration in elbow flexion, supination, pronation, and bradykinesia [68]. It is suggested that, after a while, LD treatment is no longer effective [66, 67, 69, 70]. In our results, we also observed 6-OHDA + LD animals when they used the contralateral forelimb, the movement was limited, and the limb tended to remain flexed, which are clear signs of hypokinesia and rigidity. It is important to note that with chronic LD treatment, the animals showed mainly orolingual, axial, and limb-type dyskinesia at the time they were evaluated in the staircase test. Therefore, the pellets were harder to take, corresponding with Winkler et al. [71] results. Besides, it is considered that the motivation that leads the animal to get the food pellets is the food restriction [48], generating anxiety and promoting the realization of quick and inaccurate limb movements [72].

4.3. Melatonin treatment

All 6-OHDA + melatonin-treated animals behaved very similarly to untreated 6-OHDA animals at the beginning of the treatment; later at approximately 21 days, they showed gradual improvement. In a study conducted by Singh et al. [73], they show that 35 days of melatonin treatment in 6-OHDA-lesioned animals, they display improvement in posture and ability to take the food pellets in the staircase test with the contralateral forelimb, coinciding with our data, since we found improvement in the animals between days 28 and 42. These authors propose that melatonin neuroprotective effect is due to its ability to stimulate antioxidant

enzymes, which act on the 6-OHDA-free radicals; in addition, it is known that these enzymes are diminished in the DA-depleted brain [74, 75]. Previous studies have shown that short-term melatonin treatment does not exert a neuroprotective effect in DA-depleted animals [76, 77], probably due to the fact that this neurohormone levels are low in the brain [76]. In this sense, it is suggested that melatonin level has to be high and continuously maintained for a long time in the brain to guarantee its neuroprotective effect [76, 78, 79].

4.4. LD/Mel treatment

Remarkably, as shown in our results, all animals treated with 6-OHDA + LD/Mel coadministration showed improvement in their motor performance in the staircase test from the beginning of treatment. We also observed that these animals improved the digit contraction and projection movements, pronation, and supination, in comparison with the other groups. The neuroprotective effect we observed is probably due to the melatonin's characteristic as an antioxidant, avoiding LD autoxidation and the consequent ROS formation, thus restoring LD levels and increasing striatal DA bioavailability [26, 27].

4.5. Beam walking test

This test evaluates stereotyped movements, coordination, and motor alterations characteristic of PD in this animal model [80]. The device we used implied greater difficulty in its execution due to the thickness of the beam (12 mm). Besides, when placed diagonally to 15° to the floor, it required more effort to maintain a stable position. In humans, balance deterioration occurs when the loss of dopaminergic neurons is >70% [81]. Bracha et al. [82] report that PD patients, when tested showed asymmetry toward the hemisphere containing less dopaminergic activity, decreased movement initiation (akinesia), and walking was slow and presented postural changes. So that it is suggested that these changes may be similar in hemiparkinsonian rats, which may contribute to motor deficit observed in the beam walking test [50].

4.6. LD treatment

The data obtained from the 6-OHDA + LD animals are consistent with data previously reported in our laboratory, where 6-OHDA LD-treated rats show motor activity recovery in the first days of treatment, but after 28 days dramatically increased the time to pass the beam [57]. PD patients' studies treated with LD showed a significant increase in walking speed and balance [83]. In our study, we observed that the animals frequently interrupted their ascent and slipped due to the low digit clamping force produced by the lesion, which is not reversed by LD treatment [68]. The SNc degeneration produced by 6-OHDA lesion considerably decreases LD therapeutic benefit [71] probably because this drug produces oxidative stress and therefore increases the neurodegeneration of the remaining dopaminergic cells [84]. In addition, when animals attempted to traverse the beam, they stopped because they had axial and limb-type AIMs.

4.7. Melatonin treatment

6-OHDA + melatonin-treated animals, after 42 days, showed gradual motor activity recovery, suggesting that somehow melatonin contributed to the improvement motor coordination [57];

so the animals were probably able to make optimal postural adjustments to maintain the balance and move on the beams. Patki and Lau [85] performed a study on DA-depleted animals, which were continuously melatonin-treated for 18 weeks, and when evaluating the animals in the beam walking test, they observed improvement in motor coordination compared to animals that did not receive treatment. In addition, chronic melatonin treatment increased striatal DA levels, so the authors conclude that long-term melatonin treatment has a neuroprotective potential to preserve nigrostriatal dopaminergic function. Probably because during the treatment, high and constant melatonin levels were maintained in the brain [79].

4.8. LD/Mel treatment

Animals receiving chronic LD/Mel coadministration showed recovery of motor coordination; the animals cross the beam alternating the limbs, which made the movement faster and better so that they presented similar times to pass the beam to the control group animals throughout the experiment. In this regard, recent studies show that melatonin, given in conjunction with LD in MPTP mice, reverses akinesia by restoring the number of dendritic spines in medium-sized spiny neurons and attenuating striatal DA loss. Proposing that melatonin could be an ideal LD adjuvant in PD therapy [77]. In this sense, our data also showed that animals receiving LD/Mel treatment had preservation of dendritic spines and more dopaminergic neurons on the contralateral SNc, so it is feasible to think that maintaining the nigrostriatal connections would allow the animals to make optimal adjustments in their movements to maintain the balance and move better over the beam.

4.9. Abnormal involuntary movements

As shown in **Figures 7** and **8**, untreated DA-depleted animals had small AIM scores compared to those receiving LD treatment, which is consistent with results of other authors [71, 86]. Also, animals receiving melatonin treatment showed similar behavior, corroborating these data with those previously reported by our group [57]. These groups of animals are primarily characterized by having contralateral and orolingual AIMs (considered as resting tremor [71]). Previous studies suggest that rat AIMs, regarding severity and topographical distribution, are related to striatal dopaminergic denervation [71], and this can be explained by the somatotopic organization of this structure. According to this, the dorsolateral striatum controls jaw and limb movements. Abnormal function of this region is correlated with the presence of orolingual AIMs [87, 88]. Some studies have shown that the response to LD changes with the progression of the disease. Deogaonkar and Subramanian [89] demonstrated that LD minimal dose produces dyskinesias in PD patients in advanced stages compared to patients in early stages, suggesting that the LD therapeutic window is lost in advanced stages of the disease. The DA fluctuations are closely related to the development of LIDs [90]. Furthermore, LD treatment triggers LIDs via signaling pathways in striatonigral neurons, probably by D1 and D2 receptors' stimulation [91]. On the other hand, there are data which sustained that dopaminergic depletion can generate changes in the postsynaptic neurons, which involve modifications in the neuronal morphology and striatal dendritic spines loss, which would result in a decrease in synaptic connections [92–97].

On the other hand, 6-OHDA + LD/Mel animals displayed low AIM scores compared to those receiving exclusively LD, showing that somehow melatonin has some influence on LIDs. It is important to stand out that there are no studies on the effect of LD/Mel coadministration on LIDs in PD. However, several authors suggest that melatonin may have a beneficial effect on LIDs because of its antioxidant properties [27, 98] and its ability to stimulate antioxidant enzymes [99]. Rocchitta et al. [100] reported that LD/Mel coadministration inhibits LD autoxidation, thereby increasing striatal DA bioavailability, and then, melatonin appears to be the most suitable antioxidant drug to be used as LD adjuvant to avoid LD and DA nonenzymatic autoxidation. According to these studies, it is feasible to think that with LD/Mel coadministration the DA concentration fluctuations are avoided, thus reducing LIDs.

4.10. TH immunocytochemistry

As expected, MFB 6-OHDA lesion drastically reduced the number of TH-immunopositive neurons in the SNc, coinciding with previous works in PD patients [101] and 6-OHDA model [80, 102–105]. Thus, it is suggested that this model simulates PD advanced stages. The precise 6-OHDA cytotoxicity molecular mechanism remains under discussion. Several hypotheses have been proposed. One of which is related to free radical formation, in addition to decreasing mitochondrial complex I activity with the consequent ATP decrease and cell death [106], which has also been reported in PD *postmortem* studies [107]. Moreover, LD-treated animals showed a dramatic loss of TH-immunopositive cells and both, the ipsilateral and contralateral SNc, similar to untreated 6-OHDA-lesioned rats, features are also reported by Smith et al. [108] and by our group [57]. *In vivo* and *in vitro* studies confirm that LD-treatment decreases TH-immunopositive cells; these results suggest that LD induces cell death due primarily to the ROS generation [12, 25, 27], which may increase oxidative stress in the nigrostriatal pathway [109, 110]. In addition, previous studies in our laboratory showed that hemiparkinsonian LD-treated animals displayed increased levels of lipid peroxidation, which is the principal oxidative stress characteristic [57].

Melatonin treatment favored SNc dopaminergic neuron preservation compared to untreated rats, consistent with previous studies [57, 85]. It is proposed that melatonin protection may be by direct antioxidant action [57, 111, 112] or by indirect stimulating antioxidant enzymes [112, 113]. LD/Mel-treated animals had lower SNc TH-immunopositive cell death compared to the other groups, although no significant differences; so, it is feasible to think that this small percentage of cells could be involved in improving motor tests and decreased dyskinesia. Surprisingly, on the contralateral SNc, the animals showed dopaminergic neurons increase, probably trying to compensate ipsilateral SNc damage. In this regard, it has been reported that DA is essential for neurogenesis, which was evidenced in DA-depleted animals [114, 115], and this effect was reversed when given LD [116]. Apparently, the neurogenic effect is modulated by activation of DA receptors [115].

4.11. Dendritic spines

Our results show that dopaminergic denervation produced by 6-OHDA results in loss of striatal neuron dendritic spines. PD patients' *postmortem* studies have shown 30% decrease, and this loss can reach 50% in dendritic spine density, and the reduction in the size of dendritic

trees [92, 117]. Similarly, MPTP nonhuman primates and 6-OHDA-lesioned rodents show drastic loss of these structures [7, 57], suggesting nigrostriatal system importance in morphological regulation and plasticity of dendritic spines in the striatum [104]. We have observed that LD chronic treatment does not restore striatal dendritic spine density, which is consistent with previous PD *postmortem* studies that show that the loss of dendritic spines was present even though all patients were treated with LD for several years [117]. Deutch et al. [92] propose that LD may be ineffective in PD advanced stages, probably due to dendritic spine loss. In rodents with different models of PD LD-treated, the number of dendritic spines [7, 77, 92] is not restored. We also observed that melatonin treatment helped the conservation of dendritic spines. In this regard, it is reported that melatonin prevents cytoskeletal damage by reducing oxidative stress [118].

LD/Mel coadministration significantly restored the dendritic spine density of both ipsilateral and contralateral striatum. Recent studies show that the presence of dopaminergic neurons enhances dendritic spine formation in medium spiny neurons in culture. So it is possible that dopaminergic neurons have neurotrophic effect [119]. In this context, it is feasible to think that as LD/Mel coadministration increases the number of dopaminergic neurons in contralateral SNc and exerts a neurotrophic effect, promoting the formation of new dendritic spines. Our results are also consistent with those described by Naskar et al. [77], who show that MPTP-exposed rodents and LD/Mel-treated for 2 days have restored the morphology and density of dendritic spines of medium-sized spiny neurons, suggesting that melatonin primarily regulates this effect due to its characteristics of reducing excessive calcium flow.

4.12. W/OV and OVX comparison

In our study, we show that W/OV rats, which were 6-OHDA-lesioned and received different treatments, have greater neuroprotection compared to OVX females, confirming the estrogen protection, besides the neurodegeneration delay difference, suggesting beneficial estrogen effect in the development and progression of the disease. It has been observed that estrogen has a neuroprotective effect on the nigrostriatal system. Recent studies suggest that PD women tend to have a delay in the appearance of certain motor symptoms compared with men [33, 120]. Furthermore, PD postmenopausal women treated with estrogen showed improvement in their motor performance [121, 122], suggesting estrogen symptomatic role [123]. As our results indicate, 6-OHDA-untreated W/OV and OVX rats showed no significant difference from both ipsilateral and contralateral SNc dopaminergic cells. This could be because the neurotoxin is very aggressive, which somehow does not allow the cell survival. It is also proposed that estrogens protect dopaminergic neurons surviving for a short time, but subsequently the cells could die as a result of neurotoxin action [124]. Previous studies showed the effect of replacement estrogen therapy in physiological doses in OVX rats after 6-OHDA injection in the nigrostriatal pathway, reporting that estrogen treatment showed no effect on survival of TH-immunopositive cells. Nonetheless, estrogen attenuated the striaral DA loss; the authors suggest that estrogens can somehow promote an adaptive DA mechanism synthesis, release, and metabolism in the surviving cells, so probably the females may be able to resist the onset and progression of neurodegenerative lesions compared with males [125].

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Interestingly, our results also show that there is a difference between the estrogen condition regarding dyskinesias and motor behavior, noting that W/OV 6-OHDA + LD have motor impairment delayed but are more likely to develop dyskinesias compared to OVX 6-OHDA + LD, which is consistent with previous studies, which shows that there are sex differences in LD treatment, showing that women performed better in the UPDRS test (unified Parkinson's disease Rating Scale) and presented longer "on"-LD state compared to men. However, women had a higher prevalence to develop dyskinesias. It is still uncertain why women are more prone than men to develop dyskinesias, but it is suggested that estrogen may be the basis of this susceptibility [123, 126, 127]. One possible explanation for such proneness is the fact that humans and rats have similar expression characteristics of the catechol-o-methyl transferase (COMT) [128], which is a catecholamine degrading enzyme, and that women have 20–30% decrease in COMT activity compared to men [129]. In this regard, it has been demonstrated that estrogen can decrease the regulation of COMT gene [130]. Therefore, if estrogens decline, the COMT system could have a pharmacological potential to increase the LD striatal availability and prolong LD-"on" state as well as dyskinesias [130].

On the other hand, W/OV melatonin-treated rats recover faster in behavioral tests compared to OVX rats, and in the last month of treatment, all animals had similar control values; cytologically W/OV rats exhibited contralateral SNc dopaminergic cell protection and dendritic spine recovery of both ipsilateral and contralateral striatum. Studies of melatonin and estrogen therapy in neurodegenerative models are few, so this work provides new knowledge about it. In a study of W/OV rats that were subjected to a stroke model and receiving melatonin, it was observed that estrogen and melatonin exhibit synergistic effect to decrease the levels of lipid peroxidation, increasing the activity of free radical scavenger and the number of surviving neurons in the cortex, and improve sensorimotor behaviors [131]. According to these studies, we can expect that after 6-OHDA injection melatonin treatment and estrogen presence in W/OV rats work together to activate different signaling pathways to reduce oxidative stress and thus protect the dopaminergic neurons (at least the contralateral SNc) and the number of striatal dendritic spines and thus improve motor capacity; this could be a possible explanation of why W/OV rats tend to recover faster in motor performance compared to OVX rats.

Interestingly, animals receiving LD/Mel treatment showed behavioral recovery from the start of the treatment, increase in the number of dopaminergic neurons in the contralateral SNc and striatal ipsi and contralateral dendritic spine protection; these results were estrogen independent. So, we suggest that LD/Mel cotreatment could improve the LD efficacy by increasing striatal DA levels [77]. Also, it has been suggested that these drugs could act synergistically to exert a modulatory role in nigrostriatal transmission pathway, which may be responsible for many of the beneficial effects, such as biochemical alterations, regulation of dendritic spines and cell survival [77, 131], and motor disorders such as dyskinesias.

Finally, it is important to mention that estrogens act as neuroprotectors in neurodegenerative diseases such as PD and Alzheimer disease, improving women quality of life [126]. But it should be noted that the use of estrogen also involves risks. Women who take hormone replacement treatment are more likely to suffer cancer [132] if there is a family history of these diseases, so careful and controlled administration of estrogens is required.

5. Conclusion

According to our results, we can conclude that regardless of the estrogen situation, LD/Mel coadministration was the most effective in reducing motor alterations. So, it is feasible to think that the combination of these drugs exerts a modulatory role in the nigrostriatal transmission involving motor activity and dyskinesias by protecting dendritic spines and dopaminergic neurons. Therefore, we consider that the LD/Mel coadministration may be a possible candidate for PD treatment.

Furthermore, our data show that W/OV rats have a better response to LD or melatonin treatment, being less motor and cytological damage than in OVX rats, suggesting that estrogens have a beneficial effect on the development and progression of the disease. Those facts could lead us to think about the importance of taking into consideration the estrogens-based therapies for PD as a possible adjunct in women. So it is suggested to study the effect that could have estrogen in males in subsequent studies.

The study of estrogens mechanisms of action in the basal ganglia and their role in movement disorders will become stronger. It is recognized that estrogen may have neuroprotective effects in many neurodegenerative processes, including PD. The neurodegenerative diseases field is in great need of therapies that can prevent or slow the disease progression. Thus, the introduction of Melatonin combined with LD treatment is a promising therapeutic strategy. So, we suggest the use of such drug combination plus the estrogen replacement therapy as useful PD treatments.

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