Dysautonomias in Parkinson’s disease: cardiovascular changes and autonomic modulation in conscious rats after infusion of bilateral 6-OHDA in substantia nigra

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PARKINSON’S DISEASE (PD) is characterized by a decrease in striatal dopamine (DA) levels due to the death of neurons in the substantia nigra pars compacta (SNpc). After an 80% decrease of DA levels in the striatum, the classic motor symptoms of PD appear (11, 38). These include bradykinesia, rigidity, tremor, and postural instability (29). Although the motor signs of PD are well studied and recognized, symptoms of dysautonomia, despite being reported at all stages of PD, are rarely considered clinically even if they are the main complaint. Dysautonomia considerably impairs the quality of life in the early stages, and it can be debilitating with advancing disease (7, 8).

Cardiovascular involvement is very common in patients with PD (21, 24). Orthostatic hypotension (OH), postprandial hypotension, as well as cardiac arrhythmias, livedo reticularis, and leg edema are commonly reported (26, 43). OH is the most reported symptom with a prevalence of up to 58% in patients with PD (18, 24). However, despite all of the speculation, the pathophysiology of OH, as well as the severity of hemodynamic compromise, remains controversial. This has been associated with a central disorder that impacts the medulla, namely, the involvement of both the central nervous system and the peripheral postganglionic pathways of the autonomic nervous system (2, 17, 19, 26, 34).

Buob et al. (6) directly determined the catecholamine-mediated chronotropic and contractile responses and provided evidence against functionally relevant sympathetic dysfunction. The authors suggest that sympathetic denervation might be incomplete and that the remaining fibers are sufficient for the maintenance of autonomic control. Moreover, they suggested that the depression of the various parameters of heart rate (HR) support significant changes in parasympathetic activity at early stages of PD with subclinical autonomic failure. In contrast, the literature suggests that cardiac dysfunction, even without OH, is due to sympathetic changes (17).

An understanding of the pathophysiology of dysautonomia in PD is very important because the symptoms of dysautonomia, especially in the advanced stages of the disease, are often responsible for limiting and contributing to a poor quality of life. This understanding is needed for the advancement of the search for new treatments. Moreover, this could facilitate a possible early diagnosis of PD.

Considering the importance of elucidating the role and involvement of autonomic function in patients with PD and the paucity of studies in animal models, this study investigated cardiovascular function by assessing autonomic modulation in an animal model of Parkinsonism, the 6-hydroxydopamine (6-OHDA) model.
MATERIALS AND METHODS

**Animal care.** All experiments were performed in adult male Wistar rats weighing 280–320 g at the beginning of the experiments. They were supplied by the central animal care facility of the State University of Londrina in Brazil. The animals were housed individually in Perspex cages in a room with a 12:12-h light/dark cycle. Food and water were freely available at all times, except during the experiments. All the experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals and the Ethical Principles for Animal Experimentation established by the Brazilian Committee for Animal Experimentation (COBEA). This investigation was approved by the animal experimentation ethics committee of the State University of Londrina (process number: 1288.2012.55), and complied with international laws.

**Stereotoxic surgery.** The animals were anesthetized intraperitoneally with tribromoethanol (250 mg/kg). With a stereotoxic apparatus (David Kopf), 6-OHDA (6 mg/ml in 0.2% ascorbic acid in sterile saline) or sterile saline (sham group) was bilaterally administered directly into the SNpc with a 30-gauge stainless-steel needle (1 μl over 3 min). All the chemicals were obtained from Sigma-Aldrich. The neurotoxin dose was chosen based on the findings of a previous study that established the dose required for effective neuronal loss after 6-OHDA administration and the subsequent generation of behavioral alterations (3, 13). The needle was kept in the infusion site for an additional 3 min to avoid reflux. The following coordinates for the procedure were adapted from the Paxinos and Watson (28) atlas: anteroposterior, −5.0 mm from the bregma; mediolateral, ±2.1 mm from the midline; dorsoventral, 8.0 mm from the skull. After the surgery, the animals were kept in a temperature-controlled chamber until they recovered from the anesthesia. At the end of the surgery, all the animals received prophylactic doses (30,000 IU) of veterinary pentabiotic and were allowed 6 days for recuperation.

**Measurement of the cardiovascular parameters.** Six days after the stereotoxic surgery and 24 h before the recording experiments, the animals were induced tribromoethanol anesthesia (250 mg/kg), and polyethylene catheters were inserted into the abdominal aorta through the femoral artery and vein. Both were dorsally externalized to record mean arterial pressure (MAP) and HR and for drug injections, respectively. The detailed methodology for the arterial and vein catheterization was recently described (10).

On the day of the experiment, basal recordings were obtained for at least 20 min before beginning the experimental protocol. MAP and HR were recorded with a MLT0380 blood pressure transducer that was connected to a Powerlab system 4/20 T (ADInstruments, Bella Vista, Australia). These recordings were done when the animals were not anesthetized and moving freely.

**Selective autonomic blockade.** After recording the baseline values, a random selection of rats from the control and 6-OHDA group were administered an intravenous injection of prazosin (1 mg/ml), atropine (1 mg/kg), or propranolol (5 mg/kg) to produce α-adrenergic, muscarinic, or β-adrenergic antagonism, respectively. MAP and HR were monitored until the drug effects were evaluated. The drug concentrations were chosen based on previous studies (14, 20, 36).

**Brain dissection.** The animals were decapitated, and the brain samples were isolated and collected after manual microdissection. The stratum was dissected, and all the isolated tissues of every animal were weighed, thus ensuring homogeneity. The samples were stored at −80°C until further analysis. During the procedure, information from the brain atlas was used for guidance (28).

**Determination of striatal DA levels by high-performance liquid chromatography (HPLC).** The samples were homogenized in 0.1 M perchloric acid. After sonication, the homogenates were centrifuged at 13,000 rpm for 10 min at 4°C and 30 μl of the supernatant were automatically injected into the chromatography system. The HPLC system consisted of a Waters Alliance 2465 chromatograph (Waters, Milford, MA) with a 2465 glassy carbon electrochemical detector and reversed-phase column (Symmetry C18, 150 mm × 4.6 mm, 5-m and 100-Å particle pore diameter; Waters). The potential difference was set at 800 mV vs. a reference electrode of Ag/AgCl. The mobile phase with a flow rate of 1 ml/min consisted of 50 mM citric acid, 2 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 1.2 mM heptanolsulfonic acid, 9.4% methanol, and 1% acetonitrile, with pH adjusted to 3.2. The mobile phase was vacuum-filtered and degassed ultrasonically before application. The concentrations of the substances were corrected by the mass of the tissue samples that were dissected and were expressed as nanograms of the substance per milligram of tissue. The animals that did not show at least a 50% decrease in striatal DA levels were excluded from the experiments.

**HR and systolic arterial pressure (SAP) variability.** The arterial pressure recordings were processed with the LabChart 7 Pro computer program (ADInstruments), which could detect the inflection points in the pressure pulses that generated a beat-by-beat time series of pulse interval (PI) and SAP. The time-frequency domain analysis (PI and SAP variability) and power spectra were performed with CardioSeries v2.4 custom software.

For the power spectral analysis of the PI and SAP variability, the beat-by-beat series of these parameters were resampled with data points every 100 ms by cubic spline interpolation (10 Hz). Next, the interpolated series were divided into half-overlapping segments of 512 points. All of the segments were visually inspected, and the data points affected by artifacts or nonstationary data were excluded from the analysis. Subsequently, a Hanning window was used to attenuate the side effects, and spectra were calculated for all of the segments with a fast Fourier transform algorithm for the discrete time series.

Finally, the spectra were divided into low- (LF; 0.2–0.75 Hz) and high-frequency (HF; 0.75–3.0 Hz) bands. The relative powers of the LF and HF bands of the PI spectra were calculated by taking into account the total power of the spectra minus the power of the very low oscillations (<0.2 Hz). To assess the cardiac sympathovagal balance, we calculated the ratio between the powers of the LF and HF bands (LF/HF) of the PI spectrum (12, 37).

**Spontaneous baroreflex analysis.** Baroreflex sensitivity (BRS) was assessed by the sequence method with the CardioSeries v2.4 computer program. The beat-to-beat time series of the PI and SAP values were used in the BRS analysis. The time series were analyzed for the sequences of four or more beats in which progressive increases in SAP were accompanied by progressive increases in PI or progressive reductions in SAP were accompanied by progressive reductions in PI. To detect the changes in SAP and PI thresholds of 0 mmHg and 0 ms, respectively, were used. After detecting a ramp SAP (sequence of 4 or more beats in which progressive increases or reductions in SAP were or were not followed by increases or reductions in the PI), the computer program sought changes in PI without any interval, such as a delay of zero beats. A baroreflex sequence was used only when the correlation coefficient (r) between SAP and PI was ≧0.8. The BRS was determined from the slope of the linear regression between the SAP and PI of each baroreflex sequence.

**Statistical analysis.** The differences between the experimental and control groups were compared with Student’s t-tests for unpaired samples. The level of significance was set at P < 0.05. Pooled data are expressed as means ± SE.

RESULTS

**Determination of striatal DA levels.** The neurochemical analysis (Fig. 1) indicated that the DA levels in the 6-OHDA group were reduced (1.12 ± 0.14; n = 30) compared with the sham group (5.22 ± 0.31, P < 0.0001; n = 32). These results indicated a decrease in DA greater than 50%, confirming the success of the lesion in 6-OHDA animals.

**Cardiovascular parameters.** Evaluations of the baseline values of blood pressure (BP) and HR (Fig. 2) in the 6-OHDA and
sham animals showed that the former exhibited decreased values for BP ($P < 0.0001$) and HR ($P = 0.028$) compared with control animals [6-OHDA: MAP, 100 ± 1 mmHg; HR, 335 ± 6 beats/min; SAP, 122 ± 1 mmHg; diastolic arterial pressure (DAP), 82 ± 1 mmHg; $n = 30$; Sham: MAP, 111 ± 1 mmHg; HR, 355 ± 6 beats/min; SAP, 137 ± 2 mmHg; DAP, 92 ± 1 mmHg; $n = 32$].

Variability analysis. Our results showed a smaller variance in the modulation of BP in the time domain (Fig. 3) in the 6-OHDA animal model (6-OHDA: 5.07 ± 0.69, $n = 30$; sham: 9.24 ± 1.25, $n = 32$; $P < 0.05$). However, the variance of PI showed no statistical difference between groups (6-OHDA: 19.35 ± 2.61, $n = 30$; sham: 15.55 ± 1.89, $n = 32$).

Fig. 1. A: dopamine (DA) concentrations in the striatum of rats after 7 days of saline (sham) or 6-hydroxydopamine (6-OHDA) microinfusion in the substantia nigra. Values are expressed as means ± SE ($n = 30–32$/group, *$P < 0.001$). Results were analyzed by Student’s-$t$ test. B: schematic representation of the coordinates and site of infusion of the toxin. Adapted from Paxinos and Watson (28). C: graphic representation of the detection of DA by HPLC-ED. DOPAC, 4-dihydroxyphenylacetic acid.

Fig. 2. Analysis of cardiovascular parameters at baseline [mean arterial pressure (MAP) and heart rate (HR)] in nonanesthetized rats after 7 days of bilateral intranigral infusion of saline (sham) or 6-OHDA. Values are expressed as means ± SE ($n = 30–32$/group, *$P < 0.05$).
The spectral SAP analysis (Fig. 4) showed that the 6-OHDA-injured animals exhibited a decrease in the LF component in the normalized units (6-OHDA: 3.06 ± 0.30, n = 30; sham: 4.74 ± 0.71, n = 32; P < 0.05). In the autonomic modulation of PI, none of the parameters was significantly different between the groups (6-OHDA: LF, 31.59 ± 2.23; HF, 70.10 ± 2.41; LF/HF, 0.55 ± 0.06; sham: LF, 29.55 ± 2.36; HF, 69 ± 2.23; LF/HF, 0.59 ± 0.10).

**Pharmacological autonomic blockade.** When the α-adrenergic blocker was administered (Fig. 5), the 6-OHDA animals exhibited a smaller drop (in mmHg) in blood pressure (ΔMAP: −25 ± 5; ΔSAP: −30 ± 6; ΔDAP: −21 ± 4; n = 7) compared with the sham animals (ΔMAP: −47 ± 4; ΔSAP: −56 ± 5; ΔDAP: −39 ± 4; n = 8, P < 0.005). There was no significant difference in the HR responses (in beats/min) between the sham (ΔHR: 43 ± 22, n = 8) and 6-OHDA groups (ΔHR: 58 ± 14, n = 7).

There was no significant difference in the HR response (in beats/min) between the 6-OHDA and sham animals in response to intravenous administration of either a β-adrenergic or muscarinic antagonist (intravenously administered propranolol in 6-OHDA rats: ΔHR, −33 ± 5; and in control rats: ΔHR, −34 ± 5;
intravenously administered atropine in injured rats: ΔHR, 129 ± 14; and in control rats: ΔHR, 123 ± 7 (Fig. 6).

Analysis of baseline spontaneous baroreflex. In the analysis of the spontaneous baroreflex and the effectiveness, no significant difference was observed between the groups (sham: 0.15 ± 0.009, n = 32; 6-OHDA: 0.17 ± 0.007, n = 30). For the baroreflex up gain, down gain, and all gain, we observed an increase in the 6-OHDA group (up gain: 1.85 ± 0.17; down gain: 1.87 ± 0.20; all gain: 1.85 ± 0.17) compared with the sham group (up gain: 1.34 ± 0.08; down gain: 1.37 ± 0.09; all gain: 1.35 ± 0.08) (Fig. 7).

DISCUSSION

In the present study, we investigated the cardiovascular alterations and autonomic modulation that occurred after lesions were made in the SNpc by the infusion of 6-OHDA, which has been shown to decrease DA levels in the striatum and which has been shown to result in features that model Parkinsonism. Our results showed that 7 days after 6-OHDA lesions of the SN, there were changes in the baseline cardiovascular measures (decreased MAP and HR), as well as alterations in autonomic modulation, and changes in cardiovascular tone after pharmacological autonomic blockade. Our data suggested the presence of a vascular sympathetic impairment in the 6-OHDA model of Parkinsonism.

An ideal model of PD should exhibit pathological and clinical features that mimic the human disease, including similar autonomic and cardiovascular changes and behavioral signs or symptoms involving the dopaminergic and noradrenergic systems, which are characteristics of changes in the central and autonomic nervous system. Moreover, it should reflect the progressive nature of PD by taking into consideration age and aging. Unfortunately, none of the current models exhibit all these features (3, 38). We chose the 6-OHDA model because it is a classical model of PD, which is associated with behavioral and motor alterations that are typical of PD (11, 13).

In general, a concentration of around 6 μg/μl of 6-OHDA injected into the SNpc (1 μl) was sufficient to produce a 70% depletion in striatal DA levels within days. This is the degree of depletion aimed for models that attempt to mimic the early stages of PD (together with the onset of motor signs) (13). Therefore, the current model of Parkinsonism was chosen to replicate the striatal DA deficiency seen in patients with PD and determine whether the cardiovascular consequences in the model correlate with those seen in such patients.

In our 6-OHDA animals, lower MAP and HR measures were observed at baseline. Another animal study using bilateral striatal infusion of 6-OHDA examined the changes in circadian rhythms; they described a loss of circadian periodicity, particularly for HR (5). Sakata et al. (31) used telemetry tests and observed lower BPs and HRs in a model with ventral tegmental area injury caused by 6-OHDA. According to Kirouac and Ciriello (22), activation of the midbrain DA system decreases BP and HR because the metabolic rate of DA in the ventrolateral medulla, which is the location of the cardiovascular center, is related to the BP level, and part of the DA fibers in this area arise from the ventral tegmental area. These findings suggest that the descending DA system has important connec-

Fig. 5. Analysis of cardiovascular parameters in nonanesthetized rats after 7 days of bilateral intranigral infusion of saline (sham) or 6-OHDA after intravenous infusion of prazosin. Values are expressed as means ± SE (n = 7–8/group, *P < 0.01).

Fig. 6. Analysis of cardiovascular parameters in nonanesthetized rats after 7 days of bilateral intranigral infusion of saline (sham) or 6-OHDA, after intravenous infusion of propanolol or atropine. Values are expressed as means ± SE (n = 8/group).
tions with the cardiovascular center and participates in BP regulation.

Seven days after injury to the SNpc and the consequent decrease in DA levels, changes in autonomic modulation were observed. Chiaravalloti et al. (9) suggested that the relationship between the sympathetic and dopaminergic systems should be further evaluated in patients with PD because of their possible roles in the physiopathology of the illness and results of a scintigraphical study that suggest that the cardiac sympathetic and nigrostriatal system are differently affected in PD. In particular, the rate of neurodegeneration in the sympathetic system is not related to that in the nigrostriatal system and vice versa. Therefore, the extent of the dopaminergic lesions does not indicate the degree of sympathetic damage. Cardiovascular dysfunction exists even with minor dopaminergic lesions.

Several invasive and noninvasive methods have been used to evaluate sympathetic and parasympathetic function in cardiovascular diseases. Among these, pharmacological autonomic antagonism can be used to evaluate the effects of the preexisting sympathetic and parasympathetic tone on the heart and vasculature. A noninvasive method called variability analysis estimates neural and nonneural activity in response to fluctuations in the short- and long-term HR [Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (1)]. A reduction in HR variability has been identified as a strong indicator of the risk related to a large number of diseases, reflecting the vital role of the autonomic nervous system in maintaining health (40).

Our results showed a decrease in BP variability in 6-OHDA animals. We also found a decrease in the LF power of SAP (systolic blood pressure), which indicated a lower sympathetic modulation of the vasculature. These results corroborate findings in humans that show that the sympathetic nervous system activity is attenuated in patients with PD and REM sleep behavior disorder, especially when the disease progresses to PD (35). No differences were seen in the heart rate variability variables that are ascribed to parasympathetic activity, indicating that the cardiac parasympathetic nervous system, which emanates from the nucleus ambiguous, may be relatively well preserved in those patients. The progressive reduction of sympathetic nervous activity is in line with the postganglionic sympathetic nervous system dysfunction that is seen in patients with early PD (35). Similarly, Valko et al. (39) reported that patients with obstructive sleep apnea syndrome had attenuated sympathetic nervous system activity compared with controls and that this was more pronounced in patients with PD. The cardiac parasympathetic nervous system seems to be relatively well preserved in patients with obstructive sleep apnea syndrome and PD.

The decreased MAP response to the α-adrenergic blocker showed that the 6-OHDA model of Parkinsonism has impaired vascular sympathetic synaptic transmission. These data corroborate the findings of other studies (27, 41, 43). Goldstein (16) proposed that the autonomic changes in patients with PD are due to sympathetic postganglionic changes.

The sympathetic neurocirculatory failure underlying OH in patients with PD involves substantial sympathetic noradrener-
Cardiovascular and Autonomic Changes in 6-OHDA Rats

The spontaneous baroreflex was used to verify the baroreflex gain and effectivity through simple techniques in which small, spontaneous fluctuations of arterial pressure generate changes in the R-R interval (32). Our data showed that 6-OHDA-lesioned rats presented increases in both the up and down gain of spontaneous baroreflex gain. Previous studies have shown that the nigrostriatal DA pathway mediates baroreflex sensitivity in rats (25). In addition, in the unilateral 6-OHDA model of PD, baroreflex gain is attenuated (42). Previous studies have shown that the baroreflex is impaired in patients with PD (30). However, no alterations in baroreflex sensitivity have been also observed in patients with PD (4, 23). We sought to explain these differences by using an animal model of PD that presents a tonic reduction in vascular sympathetic activity that might induce an increase in baroreflex sensitivity based on a possible upregulation of adrenergic receptors. This hypothesis needs to be further addressed.

Our findings suggest that the vascular sympathetic nervous system is impaired in the 6-OHDA model of PD. This effect may collaborate with the pathophysiology of OH in PD because a decrease in sympathetic tone could be one of the causes of decreased cardiac output and contribute to OH. However, further studies assessing cardiovascular function during orthostatic alterations could clarify this issue.

In conclusion, we showed cardiovascular impairments in an animal model of Parkinsonism that was induced by 6-OHDA. These impairments were due to changes in sympathetic synaptic transmission in postganglionic α-adrenergic receptors. Examining the characteristics of autonomic dysfunction is important in development of more effective therapeutic tests and studying the pathophysiology of dysautonomia in patients with PD.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.A., R.F.J., and M.C.M.-P. conception and design of research; D.A., L.S., and C.C.C. performed experiments; D.A., L.S., R.F.J., and M.C.M.-P. analyzed data; D.A., C.C.C., R.F.J., and M.C.M.-P. interpreted results of experiments; D.A. prepared figures; D.A. and M.C.M.-P. drafted manuscript; D.A., L.S., C.C.C., R.F.J., and M.C.M.-P. approved final version of manuscript; M.C.M.-P. edited and revised manuscript.

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