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Time-Course of Brain Oxidative Damage Caused by Intrastriatal Administration of 6-Hydroxydopamine in a Rat Model of Parkinson's Disease

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Abstract
The unilateral and intrastriatal injection of 6-hydroxydopamine is commonly used to provide a partial lesion model of Parkinson's disease in the investigation of the molecular mechanisms involved in its pathogenesis and to assess new neuroprotective treatments. Its capacity to induce neurodegeneration has been related to its ability to undergo autoxidation in the presence of oxygen and consequently to generate oxidative stress. The aim of the present study was to investigate the time course of brain oxidative damage induced by 6-hydroxydopamine (6 µg in 5 µl) of sterile saline containing 0.2%

ascorbic acid) injection in the right striatum of the rat. The results of this study show that the indices of both lipid peroxidation (TBARS) and protein oxidation (carbonyl and free thiol contents) increase simultaneously in the ipsilateral striatum and ventral midbrain, reaching a peak value at 48-h post-injection for both TBARS and protein carbonyl content, and at 24 h for protein free thiol content. A lower but significant increase was also observed in the contralateral side (striatum and ventral midbrain). The indices of oxidative stress returned to values close to those found in controls at 7-day post-injection. These data show that the oxidative stress is a possible triggering factor for the neurodegenerative process and the retrograde neurodegeneration observed after 1-week post-injection is a consequence of the cell damage caused during the first days postinjection. The optimal time to assess brain indices of oxidative stress in this model is 48-h post-injection.

Keywords
Parkinson's disease • 6-Hydroxydopamine • Oxidative damage • Lipid peroxidation • Protein oxidation

Introduction

6-Hydroxydopamine (2,4,5-trihydroxyphenylethylamine; 6-OHDA) is a selective catecholaminergic neurotoxin widely used to produce dopaminergic lesions in the nigrostriatal system [1]. The main use of these lesions is to create experimental models of Parkinson's disease that provide insight into the molecular mechanisms involved in the development of Parkinson's disease in order to test new strategies for dopaminergic neuroprotection, and to experiment with transplantation approaches [2–6]. The structural similarity of 6-OHDA with both dopamine and norepinephrine makes it an appropriate ligand for the plasma membrane transporter systems of dopamine (DAT) and norepinephrine (NET) [7]. This

feature, together with the inability shown by 6-OHDA to cross the blood-brain barrier [8], makes the protocol chosen for a specific dopaminergic lesion in the nigrostriatal system extremely important. The main protocol used consists of the stereotaxic intracerebral injection of the toxin into a specific area of the nigrostriatal systems, which includes the striatum, the medial forebrain bundle or the substantia nigra [9]. The intrastriatal injection of 6-OHDA is generally performed unilaterally, using the contralateral side as a control [10]. It has been shown that the intrastriatal administration of 6-OHDA causes a rapid degeneration of nigrostriatal terminals as early as 24 h, and the loss of tyrosine hydroxylase immunoreactivity (TH-ir) increases for up to 5 days following the lesion [11].

Presumably, the neurotoxicity of 6-OHDA is attributed to its ability to generate reactive oxygen species (ROS), and it is a well-known fact that under physiological conditions this compound is rapidly oxidized by molecular oxygen to give hydrogen peroxide, hydroxyl radicals and the corresponding *p*-quinone. Although, the Fenton reaction could be involved in the formation of the hazardous hydroxyl radical during the autoxidation of 6-OHDA, this is done without the involvement of the ferrous ion or any other transition metal ion [12]. At this point, it seems interesting to highlight the reported ability of ascorbate to enhance the production of ROS by 6-OHDA [13, 14], particularly due to the fact that 6-OHDA is always administered in a saline solution containing about 2% of ascorbate. In these cases, the presence of ascorbate sets a redox-cycling, which regenerates 6-OHDA from its *p*-quinone leading to a continuous production of ROS. Moreover, the presence of the enzyme dehydroascorbate reductase in the brain may contribute to sustaining this redox-cycling. Although, this is the molecular mechanism generally accepted to explain the ability of 6-OHDA to produce oxidative stress and consequently responsible for its neurotoxicity, it has been also reported that 6-OHDA can act directly by inhibiting the mitochondrial respiratory chain at the level of complex I [15–17]. Assuming the reported capacity of complex I inhibitors to increase the leakage of superoxide anions from the electron transport chain [18], this latter

mechanism could also contribute to increase the ability of 6-OHDA to produce ROS and consequently to generate oxidative stress. Recent reports refer to the potential contribution of endoplasmic reticulum stress to the cell death induced by 6hydroxydopamine [19].

Despite the widespread use of the intrastriatal injection of 6-OHDA to obtain an experimental model of parkinsonism and an awareness of the morphological changes following its administration [20, 21], no data has been reported on the kinetics of the oxidative damage it induces in the nigrostriatal system. However, this information is crucial when this model is exploited to assess the anti-parkinsoniam properties of new drugs [22] or the benefit of transplantation or gene therapy to repair the damaged pathways [3, 23]. In the light of this, the aim of the present study was to investigate the time-course of the oxidative damage caused by unilateral and intrastriatal administration of 6-OHDA in the ipsilateral and contralateral side of both striatum and ventral midbrain, and also includes a quantification of the changes observed in the indices of lipid peroxidation (TBARS) and oxidative status of proteins (carbonyl content and free thiol content).

Experimental procedure

Materials

6-OHDA hydrochloride, ascorbic acid, thiobarbituric acid, butylated hydroxytoluene crystalline, 2,4-dinitrophenylhydrazine hydrochloride, desferrioxamine, 1,1,3,3-tetramethoypropane, 5,5'-dithiobis-(2-nitrobenzoic acid), sodium dodecylsulfate, EDTA, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Guanidine hydrochloride was from Aldrich Chemical Co. (Milwaukee, WI, USA). The

water used for the preparations of solutions was of 18.2 MW (Milli-RiOs/Q-A10 grade, Millipore Corp., Bedford, MA, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland).

Animal treatment

A total of 32 male Sprague-Dawley rats, each weighing about 200 g, were used. All the experiments were carried out in accordance with the ‘‘Principles of laboratory animal care’’ (NIH publication No. 86–23, revised 1985) and approved by the corresponding committee at the University of Santiago de Compostela. Rats were stereotaxically injected in the right striatum with 6 µg of 6-OHDA in 5 µl of sterile saline containing 0.2% ascorbic acid. Stereotaxic coordinates were 1.0 mm anterior to bregma, 2.7 mm right of midline, 5.5 mm ventral to the dura, and tooth bar at -3.3. The solution was injected with a 5 µl Hamilton syringe couple to a monitorized injector (Stoelting, Wood Dale, IL, USA) and the cannula was left in situ for 5 min after injection. All surgery was performed under equithesin anesthesia (3 ml/kg i.p.). Groups of four rats were decapitated at the following times after injection: 5 min, 1 h, 12 h, 24 h, 48 h, 3 days, and 7 days. A group of four rats (control) was sacrificed immediately after the administration of the saline.

After decapitation, the brain was removed, the striatum and ventral midbrain dissected, and the resulting samples frozen on dry ice. Each sample was immediately sonicated (250 Digital sonifer, Branson Ultrasonic Co., Danbury, CT, USA) with four volumes (w/v) a Na_2PO_4/KH_2PO_4 buffer (pH 7.4) isotonized with KCl and containing 200 µM butylated hydroxytoluene and 200 µM desferrioxamine. These compounds were used to prevent amplification of lipid peroxidation during the progression of the analysis.

Determination of TBARS

The TBARS determination was performed spectrophotometrically using a previously published method [6]. Briefly, an aliquot of the sample (200 µl) was treated with SDS (8%, w/v) followed by acetic acid (20%) and the mixture vortexed for 1 min. Then, thiobarbituric acid (0.8%) was added and the resulting mixture incubated at 95°C for 60 min. After cooling to room temperature, 3 ml of *n*-butanol were added and the mixture shaken vigorously. After centrifugation at 4,000 rpm for 5 min, the absorbance of the supernatant (organic layer) was measured at 532 nm using an UVVIS spectrophotometer, model Lambda 35 (PerkinElmer Inc., Norwalk, CT, USA). For calibration, a standard curve (5–150 nM) was generated using the malonodialdehyde (MDA) derived by the acid hydrolysis (SO_4H_2; 1.5%, v/v) of 1,1,3,3-tetraethoxypropane and the TBARS results expressed as nmol MDA/mg protein. The protein concentration of the sample was determined according to the method of Markwell et al. [24], using BSA as the standard.

Determination of protein carbonyl content

The protein carbonyl content was assessed spectrophotometrically according to a procedure previously published [25]. Briefly, an aliquot of the sample was submitted to precipitation of nucleic acids with 1% streptomycin sulfate (1:9, v/v) followed by centrifugation at 13,000 rpm. The pellet was then discarded and the supernatant treated with trichloroacetic acid (1 M) followed consecutively by sonication and centrifugation in a microcentrifuge (model E, Beckman Instruments, Palo Alto, CA, USA) at 13,000 rpm for 5 min. The resulting pellet was reconstituted in NaOH (0.5 M) with vigorous vortexing for 3 min. Then, 10 mM 2,4-dinitrophenylhydrazine in 2 M chloric acid was added and the mixture incubated at room temperature for 1 h, in darkness, and with continuous agitation. After the addition of trichloroacetic acid (1 M), the resulting mixture was

centrifuged at 13,000 rpm for 5 min. The resulting pellet was washed twice with ethyl acetate:ethanol (1:1, v/v). Then, the washed pellet was reconstituted with 6 M guanidine in a 20 mM KH_2PO_4 buffer (pH 2.3) and the absorbance of the solution measured at 370 nm. The carbonyl content was calculated from the absorbance data using as absorption coefficient for dinitrophenylhydrazone e = 22,000 M^{-1} cm^{-1} and expressing this parameter as nmol carbonyls/mg protein. Because of the numerous washing steps, protein content in the final pellet was estimated on an HCl blank pellet processed simultaneously using a BSA standard curve in 6 M guanidine, and reading the absorbance at 280 nm.

Determination of protein free thiol content

The free thiol content of proteins was estimated spectrophotometrically using a modification introduced to a standard assay [25]. Briefly, an aliquot of the sample (200 µl) was treated with trichloroacetic acid (0.5 M) for protein precipitation. After vortexing and centrifugation at 13,000 rpm for 5 min, the resulting pellet was reconstituted in an 80 mM Na_3PO_4 and 2 mM EDTA buffer (pH 8.0) containing 70 mM sodium dodecylsulfate. Then, 100 µM 5,5'-dithiobis(2-nitrobenzoic acid) in a buffer 100 mM Na_3PO_4 (pH 8.0) were added and the mixture incubated at room temperature for 20 min with continuous mixing. After centrifugation at 13,000 rpm for 5 min, the absorbance of the resulting solution was measured at 412 nm and the thiol content calculated from this data using as absorption coefficient for 2-nitro-5-mercaptobenzoic acid e = 13,600 M^{-1} cm^{-1} and expressing the corresponding parameter as nmol thiols/mg protein.

Statistical analysis

Results were expressed as the mean ± SD from five animals. Statistical differences were tested using one-way ANOVA followed by LSD for multiple

comparisons ($p < 0.05$). All statistical analyses were performed using Sigmastat 3.0 from Jandel Scientific (San Diego, CA, USA).

Results

Effects of 6-OHDA administration on TBARS concentration

As shown in Fig. 1, the intrastriatal administration of 6 µg of 6-OHDA in the right striatum caused a significant and progressive increase of TBARS con centration in both right striatum ($F(7,24) = 51.73$, $p < 0.001$) and right ventral midbrain ($F(7,24) = 72.81$, $p < 0.001$), obtaining a peak-effect 48 h after the injection. Although, the increase in the right striatum was significant at 5 min versus 1 h for ventral midbrain, the augmentation observed after 48 h was higher in the right ventral midbrain (+83%) than in the right striatum (+26%). At 7-day post-injection, the values returned to those of the controls. A significant and progressive increase in the TBARS concentration was also observed in the contralateral side, affecting again to both left striatum ($F(7,24) = 10.39$, $p < 0.001$) and left ventral midbrain ($F(7,24) = 6.30$, $p < 0.001$). The peak-effect was also observed at 48 h, but the increase was lower (+23% in the left striatum and +26% in the left ventral midbrain). At 7-day postinjection the values returned to the concentrations found in the corresponding controls.

Effects of 6-OHDA administration on protein carbonyl content

The protein carbonyl content also exhibited a significant and progressive time course increase (Fig. 2), which affected both right striatum ($F(7,24) = 57.55$, $p < 0.001$) and right ventral midbrain ($F(7,24) = 54.76$, $p < 0.001$). In this case, the increase observed in the protein carbonyl content was not significant till 1-h post-injection. However, the peak-effect was observed once again 48 h after injection,

and higher in the right ventral midbrain (+42%) than in the right striatum (+38%). A significant and progressive increase in the protein carbonyl content was also observed in the contralateral side, affecting to both left striatum ($F(7,24) = 5.95$, $p < 0.001$) and left ventral midbrain ($F(7,24) = 3.48$, $p < 0.05$). However, in this case, although the peak-effect was also observed at 48-h post-injection, the increase found was lower (+12% in the left striatum and +11% in the left ventral midbrain). The corresponding concentrations achieved values close to those found in the controls at 7-day post-injection.

Effects of 6-OHDA administration on protein free thiol content

Figure 3 shows the time-course of the changes observed for free thiol content in proteins following 6-OHDA administration. As can be seen, a significant and progressive reduction in protein thiol content was found in both right striatum ($F(7,24) = 5.06$, $p < 0.05$) and right ventral midbrain ($F(7,24) = 25.87$, $p < 0.001$). The maximal decrease for both right striatum (–7%) and right ventral midbrain (–20%) was found at 24 h. Although the peak-effect did not happen at the same time for both TBARS concentration and protein carbonyl content, the value obtained for protein thiol content at 24 h was not significantly different when compared with that observed 48-h post-injection. The protein thiol content in the contralateral side only exhibited a significant reduction in the left striatum ($F(7,24) = 5.06$, $p < 0.05$), because the changes observed in the right ventral midbrain did not reach statistical significance ($F(7,24) = 0.87$, $p > 0.05$). Once again, the minimum value found in the left striatum for protein thiol content was observed 24-h post-injection and the decrease was of –8%, a value lower than that found in the ipsilateral side. No significant differences were found when the value obtained at 24 h was compared with that obtained at 48 h.

Discussion

As shown by the data here reported, the intrastriatal injection of 6-OHDA causes a continuous increase in the indices of oxidative stress in the ipsilateral side (striatum and ventral midbrain), which expands over 48 h for both lipid peroxidation and protein carbonyl content, and over 24 h for protein free-thiol content. This quick evolution of the indices of oxidative stress agrees with both morphological observations showing that dopaminergic neurons start to die within the first 24 h [11, 20, 21] and that rapid autoxidation of 6OHDA takes place under physiological conditions [13, 14, 26]. Once peak-values are reached, each of the indices begins a slow decline to values very close to those found in controls after 7-day post-injection. Taking into account the fact that the degeneration of the nigrostriatal system can last for 1–2 weeks [27, 28], our data appears to show that the here reported oxidative damage is the cause of the dopaminergic lesion and not a consequence of this process, but this is still open to question [29]. Furthermore, the fact that the neurodegenerative process continues when the indices of oxidative stress returned to the initial values (control values) appears to prove that the oxidative stress generated by 6-OHDA autoxidation causes irreversible damage in dopaminergic neurons and endangers their survival. Similar findings of early oxidative stress have been observed after MPTP application [30] as well as after chronic treatment of rats with rotenone [31]. However, the fact that in our study the indices of oxidative stress increase simultaneously in both striatum and ventral midbrain seems to discard previous suggestions involving a chemical axotomic action of 6-OHDA in the delay and gradual degeneration of dopaminergic neurons found in this model of dopaminergic neurodegeneration [28]. At this point, it is interesting to note that the maximum increase in the indices of oxidative stress is higher in the ventral midbrain than in the striatum and occurs simultaneously. Assuming the accepted involvement of oxidative stress in apoptosis [32], the apoptotic-like features observed 1–3 weeks after lesion [12], and the ability shown by caspase

inhibitors to protect against degeneration [33], our results corroborate the involvement of the oxidative stress caused by 6-OHDA autoxidation as a triggering factor in the development of neurodegeneration. In view of both the recently reported ability of oxidative stress to act as a primary event for *a*-synuclein polimerization and the involvement of these aggregates in neurodegenerative processes [34, 35], the fibrillization of *a*-synuclein could also be responsible for the slow rate of neurodegeneration observed after unilateral, intrastriatal administration of 6-OHDA. Evidently, both our results and the suggested hypothesis do not discard the suggested involvement of microglia in the corresponding neuronal death [36, 37], because the formation of certain uncharged ROS during the 6-OHDA autoxidation, with a relatively non short half-life, makes these substances highly diffusible through biological membranes and suitable for cellular signaling [38, 39].

It is also important to emphasize that the unilateral injection of 6-OHDA also caused a significant increase in the indices of oxidative stress of the contralateral side, affecting both the striatum and the ventral midbrain. Our results show that the magnitude of these increases was lower than that found in the ipsilateral side, which agree with the reported inability of intrastriatal and unilateral injections of 6-OHDA to cause loss of cell bodies in the contralateral side [40].

However, these data clearly preclude the use of the contralateral side as a control to assess neurochemical changes induced by 6-OHDA in this experimental model of Parkinson's disease. This may be explained by the above-mentioned ability of certain ROS generated by 6-OHDA autoxidation to diffuse through biological membranes.

In summary, our data confirm that intrastriatal and unilateral injections of 6-OHDA cause oxidative stress (lipid peroxidation and protein oxidation), which

increases during the first 2-day post-injection and returns to approximate control levels at the 17-day post-injection. This appears to be the triggering factor for the neurodegenerative process, and the retrograde neurodegeneration following the first week post-injection seems to be a consequence of the cell damage caused within the first days post-injection. Finally, when this model is used to assess new neuroprotective strategies or to study the oxidative potential of a specific factor, the measurement of brain indices of oxidative stress (TBARS and the content of oxidized groups in proteins) should be performed at 48-h post-injection, which is when maximum values are obtained.

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Fig. 1 Changes in TBARS levels in the ipsilateral and contralateral side of both striatum (*St*) and ventral midbrain (*VM*) after stereotaxic, unilateral (*right*), intrastriatal injection of 6-OHDA (6 µg in 5 µl of sterile saline containing 0.2% ascorbic acid) to rats. Each point represents the mean ± SD from five animals ($n = 5$). *Asterisks* denote values significantly different from the corresponding control (LSD test; $p < 0.05$)

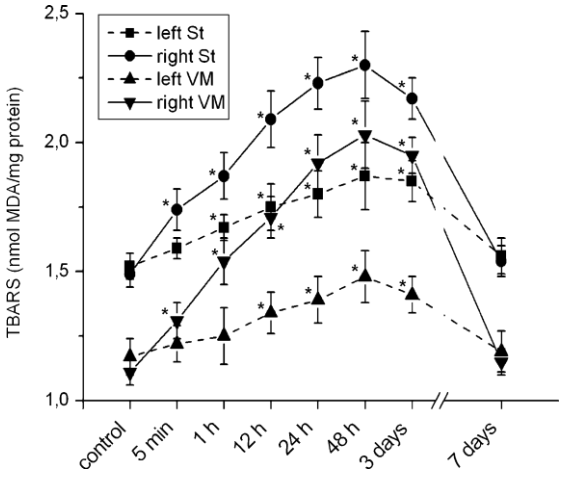


Fig. 2 Changes in protein carbonyl content in the ipsilateral and contralateral side of both striatum (*St*) and ventral midbrain (*VM*) after stereotaxic, unilateral (*right*), intrastriatal injection of 6-OHDA (6 µg in 5 µl of sterile saline containing 0.2% ascorbic acid) to rats. Each point represents the mean ± SD from five animals ($n = 5$). *Asterisks* denote values significantly different from the corresponding control (LSD test; $p < 0.05$)

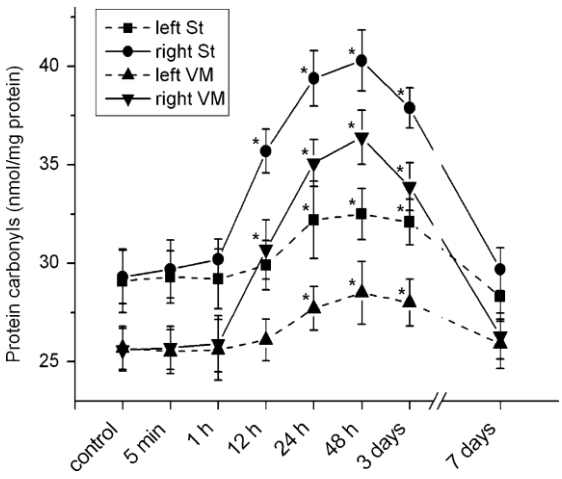
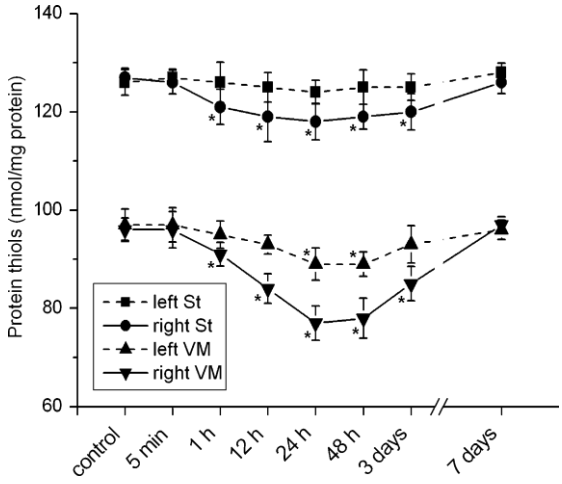


Fig. 3 Changes in protein free thiol content in the ipsilateral and contralateral side of both striatum (*St*) and ventral midbrain (*VM*) after stereotaxic, unilateral (*right*), intrastriatal injection of 6-OHDA (6 µg in 5 µl of sterile saline containing 0.2% ascorbic acid) to rats. Each point represents the mean ± SD from five animals ($n = 5$). *Asterisks* denote values significantly different from the corresponding control (LSD test; $p < 0.05$)



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