The neuroprotective and anti-inflammatory effects of *Myracrodruon urundeuva* are related to inhibitions of brain inflammatory enzymes, cytokines and HDAC

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**ABSTRACT**

*Myracrodruon urundeuva* (Anacardiaceae) is a Northeast Brazil species popularly used for its anti-inflammatory properties. Considering the involvement of neuroinflammation in neurodegenerative diseases, the objectives were to evaluate the neuroprotective actions of a standardized extract from *M. urundeuva* (SEMU) on a Parkinson’s disease (PD) model, focusing on brain inflammatory targets. Male Wistar rats were anesthetized subjected to stereotoxic surgery and a unilateral striatal 6-OHDA lesion. The animals were divided into sham-operated (SO), untreated 6-OHDA-lesioned and SEMU (20 mg/kg and 40 mg/kg, p.o., 14 days) treated groups. The SO group (Control) was subjected to the same procedures, but injected with saline. Afterwards the animals were sacrificed and their brains processed for dopamine (DA) determination (striata) and immunohistochemical assays (striata and hippocampal) for COX-2, iNOS, TNF-α, NF-kB and HDAC. The data were analyzed by one-way ANOVA and Tukey as the post hoc test. The results showed a reversion of striatal DA contents in the SEMU treated, relatively to the untreated 6-OHDA group. SEMU decreased the immunostaining for COX-2, iNOS, TNF-α and NF-kB in striata and hippocampal areas. In addition, SEMU decreased hippocampal HDAC immunostaining, shown to be affected in PD. The results point out to the potential neuroprotective actions of SEMU, and its chalcone contents may be responsible for the observed effects.

**Key Words:** *Myracrodruon urundeuva*; Cyclooxygenase; Nitric oxide synthase; TNF-α; NF-kB; Histone deacetylase; neuroprotection; Parkinson’s disease

**BACKGROUND**

Parkinson’s disease (PD) is the second most common neurodegenerative disorder and the first on movement disorders worldwide, afflicting about 5% of people over 85 years of age. The pathology of PD is characterized by a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), associated instead of to the degeneration of projecting nerve, leading to the cardinal features of disease as tremors, muscular rigidity, bradykinesia, and postural and gait abnormalities (1).

Despite having been implemented in the 60’s, levodopa remains the “gold standard” anti-Parkinson therapy. However, its use leads to complications, such as highly disabling fluctuations of motor activity and dyskinesias (2,3).

All other options of treatment are merely palliative and neuroprotective therapies, but the cure of PD remains an unrealized goal. This, in part, may reflect a misunderstanding of the cause of neuronal death in PD. Among several reputed factors that contribute to PD pathology, inflammatory mechanisms may play an important role (4,5). It has been clearly demonstrated, in cerebral spinal fluid and blood of PD patients, high concentrations of pro-inflammatory cytokines, as well as enzymes associated with inflammation, such as inducible isoform of nitric oxide synthase and cyclooxygenase 2 (6). This abnormal production of pro-inflammatory mediators by activated microglia and astrocytes may act as an environmental stressor to promote progressive degeneration of dopaminergic neurons (7).

The activated microglia may trigger an active self-perpetuating cycle of oxidative stress and chronic neuroinflammation (8). Reactive oxygen species (ROS) act as secondary messengers capable of modifying gene expression in microglia-associated neurodegenerative diseases by activating mitogen activated protein kinases (MAPKs) and transcription factors, as nuclear factor-kB (NF-kB) (9-11). Considering neuroinflammation and oxidative stress as some of the active or main driving forces in the neurodegenerative process of PD, the search of effective therapeutic strategies for its treatment is of paramount importance. *Myracrodruon urundeuva* Fr. Allemão, known as ‘aroceira do sertão’ in the Brazilian Northeastern region, belongs to the Anacardiaceae family and is widely used in popular medicine for treatments of pain and infections, especially in the genitourinary tract. Recent progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) in the basal ganglia leads to the clinical manifestations of PD, such as highly disabling fluctuations of motor activity and dyskinesias (2,3). However, despite the availability of treatments for PD, the disease remains progressive, and complications such as motor fluctuations and dyskinesias may develop over time.

**MATERIALS AND METHODS**

**Drugs and reagents**

Ketamine (5% Vetanarcol) and xylazine (2% Kense) were purchased from König (Santana de Parnabul, São Paulo, Brazil). 6-hydroxydopamine was from Sigma Aldrich (MO, USA). The antibodies were for immunohistochemistry assays, attempting to elucidate the SEMU’s mechanism of action.

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The loss of dopaminergic neurons and neuroinflammation shown in PD is a chronic process, associated with a glial response composed of activated glial cells, including astrocytes and microglia (22,23). These assays were performed, as previously described (16), except for the primary antibodies. Thus, in the present study, TNF-α, COX-2, iNOS and NF-κB monoclonal antibodies were from Santa Cruz Biotecnology (CA, USA). The HDAC antibody (rabbit polyclonal IgG) was from Merck KGaA (Darmstadt, Germany). All antibodies were diluted according the manufacturers’ instructions. The data from 3 animals were analyzed by the Image J software (NIH, USA) in the Nikon Eclipse Ni microscope coupled to the DS Ri2 camera (for the capture of the entire image area). The results were expressed as optical density per field.

Statistical analysis

The results are expressed as means ± SEM. For multiple comparisons, the data were analyzed by one-way ANOVA, followed by Tukey as the post hoc test and significant results presented as q (the studentized range statistics) and p values. The results from the immunohistochemical assays were quantified by the Image J software (NIH, USA) and also analyzed by oneway ANOVA, followed by Tukey as the post hoc test.

RESULTS

DA contents in 6-OHDA lesioned striata in the untreated 6-OHDA groups and in SEMU treated groups (6-OHDA+SEMU20 and 6-OHDA+SEMU40), (Figure 2) shows that the 6-OHDA lesion (right striatum) causes a 70% reduction in DA contents, in relation to the SO group. Smaller decreases were demonstrated in lesioned right striata of the 6-OHDA groups, after SEMU treatments with the doses of 20 (45% decreases) and 40 mg/kg (23% decreases). These results point out to the neuroprotection afforded by SEMU treatments, in this PD model.

Immunohistochemistry for COX-2, iNOS, TNF-α and NF-κB in striata from 6-OHDA-lesioned groups, before (untreated 6-OHDA groups) and after SEMU treatments. Immunostaining for COX-2 revealed a 21-times increase after SEMU treatments, in this PD model.

Immunohistochemistry for COX-2, iNOS and TNF-α in hippocampi from 6-OHDA-lesioned groups, before (untreated 6-OHDA groups) and after SEMU treatments. Immunostaining for COX-2 revealed a 21-times increase after SEMU treatments, in this PD model.
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CA3 subfield; in untreated 6-OHDA groups compared with the SO group. Five- and only 2-times increases were observed in this region, after SEMU treatments with the doses of 20 and 40 mg/kg, respectively (Figure 4A). The untreated 6-OHDA group showed a high immunostaining for iNOS in the CA3 area, compared with the SO group (almost 300%). This value went down to 206% and to only 35% after SEMU treatments with the doses of 20 and 40 mg/kg, respectively (Figure 4B). The neurotoxin 6-OHDA also increased by 28-times TNF-alpha levels in the CA3 hippocampal subfield, compared with the SO group. This increase was of only 10 times in the 6-OHDA group after SEMU treatment with the dose of 40 mg/kg (Figure 4C).

Immunohistochemistry for histone deacetylase (HDAC) in hippocampi and temporal cortices from 6-OHDA-lesioned rats, before (untreated 6-OHDA) and after SEMU treatments. HDACs are known to reduce gene expression and regulate protein clearance. Inhibitors of HDACs have been reported to be potentially efficacious in neurodegenerative pathologies, including Parkinson’s disease (24-26). We showed a 94-times increase in immunostaining for HDAC in the CA1 subfield, in 6-OHDA lesioned animals, related to the SO groups. This value decreased to 7- and 5-times in the 6-OHDA lesioned group, after the treatments with SEMU, 20 and 40 mg/kg (Figure 5A). Similar, although higher values were observed in the CA3 subfield, with increases of 197-times in untreated 6-OHDA-lesioned groups. These values dropped to 60- and 20-times increases after treatments with SEMU at the doses of 20 and 40 mg/kg, respectively (Figure 5B). The untreated 6-OHDA lesioned group presented increases in HDAC immunostaining (14-times increase) in the dentate gyrus, compared to the SO groups, while SEMU treatments, at the doses of 20 and 40 mg/kg, showed only 8- and 2-fold increases, respectively (Figure 5C).

In addition, a 23-times increase in HDAC immunostaining was shown in TC, in the untreated 6-OHDA lesioned groups, compared with the SO group. This value decreased to 17- and 7-times after treatments with SEMU, at the doses of 20 and 40 mg/kg (Figure 6).
Figure 4) Representative photomicrographs showing reduced immunostaining for COX-2, iNOS and TNF-alpha (×400, scale bar=50 µm), in CA3 areas from 6-OHDA-lesioned rats after SEMU treatments, compared to the untreated 6-OHDA groups (3 animals per group). The data were quantified, using the entire image area, by the Image J software (NIH, USA). COX-2: a. vs. SO, q=15.21, p<0.001; b. vs. 6-OHDA+SEMU20, q=5.924, p<0.01; c. vs. 6-OHDA+SEMU40, q=12.97, p<0.001; d. vs. SO, q=8.162, p<0.001; e. vs. 6-OHDA+SEMU20, q=5.924, p<0.01; f. vs. 6-OHDA+SEMU40, q=12.97, p<0.001. iNOS: a. vs. SO, q=62.57, p<0.001; b. vs. 6-OHDA+SEMU20, q=17.79, p<0.001; c. vs. 6-OHDA+SEMU40, q=55.42, p<0.001; d. vs. SO, q=40.14, p<0.001; e. vs. 6-OHDA+SEMU40, q=33.52, p<0.001; f. vs. SO, q=7.155, p<0.01. TNF-alpha: a. vs. SO, q=95.89, p<0.001; b. vs. 6-OHDA+SEMU40, q=65.10, p<0.001; c. vs. SO, q=30.74, p<0.001 (one-way ANOVA and Tukey as the post hoc test).

**DISCUSSION**

In this study, we investigated potential anti-neuroinflammatory effects of a standardized extract from *Myracrodruon urundeuva* in hemi-parkinsonian rats. Neurotoxin-based animal models have largely been used to induce selective neuronal death, in both in vitro and in vivo studies. 6-Hydroxydopamine (6-OHDA) is the most reproduced model of Parkinson’s disease, inducing a rapid and pronounced dopaminergic neurodegeneration with the associated motor dysfunction (27). The toxin initiates dopaminergic neurons (DA) degeneration through a combination of oxidative stress and mitochondrial dysfunction, making the tool suitable for an effective model (28). Oxidative stress activates microglia and leads to neurotoxicity of DA neurons (29).

As the treatment of PD focuses on symptomatic relief, rather than the improvement of pathogenesis progression, the discovery of triggering pathways is of great importance in the search for effective therapeutic strategies. It is well established the relationship between severity of motor dysfunction and the selective loss of dopamine neurons, what is at least in part associated with severe oxidative stress and inflammation (30,31). In this context, the development of disease-modifying agents based on the use of drugs with antioxidant and anti-inflammatory actions, along with other pharmacological properties, would be useful for retarding the development and progression of PD (32,33).

Thus, oxidative injury and neuroinflammation contribute significantly to PD pathogenesis. In order to investigate alternative and early intervention approaches to prevent or halt the progressive nature of PD, we evaluated the potential of SEMU in 6-OHDA-induced neurodegeneration. With central nervous system activities yet poorly studied, *M. urundeuva* has well-defined anti-inflammatory and antioxidant actions, mainly attributed to its content of phenolic compounds (13,34,35). Based on these findings, we demonstrated that chalcones from *M. urundeuva* exerted neuroprotection, on rat mesencephalic cells, by reducing the oxidative stress and apoptotic injury caused by 6-OHDA. Subsequently, the neuroprotective activity of the
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standardized extract of M. urundeuva was confirmed, in vitro, by inhibitions of microglia and astrocyte activation (15,16).

PET analysis showed that PD patients, with or without dementia, present significant microglial activation in cortical brain regions, suggesting that neuroinflammation could occur early in PD, persisting as the disease progresses (36). Insults to the central nervous system (CNS) triggers microglial activation, leading to the recruitment of peripheral leukocytes. Furthermore, inflammatory mediators not only modulate immune cells, but also contribute to neurodegeneration, resulting in a vicious cycle of inflammation and neuronal death (7). Thus, targeting microglial activation appears as a valid therapeutic strategy for PD treatment (37).

Our results show that 6-OHDA caused in the striatum an increase in immunohistochemical labeling of inflammatory mediators, such as TNF-alpha, COX-2, iNOS, as well as the transcription factor NF-kB, whereas its immunostaining was restored to the normal range after the SEMU treatment. The effects of 6-OHDA are in agreement with other studies that show the activation of microglia and subsequent increase of the overexcited mediators to be a major cause of the neurotoxic effects (38,39). Activation of NF-kB has been linked to oxidative stress-induced apoptosis in PD and seems to be involved in regulating inflammatory responses in microglial cells (40).

The TNF-alpha signaling pathway induces upregulation of inflammatory mediators involved in apoptotic cells, including caspase-1, caspase-3, and TNF-alpha receptor R1. This pro-inflammatory cytokine has already been identified in the substantia nigra (SN) from Parkinsonian patients, indicating the occurrence of a proapoptotic environment in PD (41). Prolonged TNF-alpha expression induced in the SN of adult rats leads to dopaminergic neuronal death, motor symptoms and microglia activation (42). Furthermore, inhibition of NF-kB attenuates production of TNF-alpha and other glial derived inflammatory mediators, thus reducing the neurotoxicity of activated microglial cultures on dopaminergic neuron-like cells (43).

Accordingly, the effects observed with SEMU may be exerted through inhibition of the NF-kB activation in DA neurons, inducing down-regulation of COX2 in the brain that could subsequently decrease the release of TNF-alpha. Inhibition of COX-mediated DA oxidation (44), as well as microglia-derived toxic mediator production are likely to be among the mechanisms that contribute to decreased incidence of PD in chronic NSAID users (45). A non-selective COX2 inhibitor has shown to present a neuroprotective activity in the 6-OHDA rat model of PD (46), what is consistent with the neuroinflammatory hypothesis for PD pathogenesis. The activation of NF-kB also promotes the induction of iNOS from activated microglia (6). This, in turn, increases the production of NO that reacts with superoxide anion and generates peroxynitrite, a highly reactive molecule, causing striatal neurodegeneration in the 6-OHDA model of PD (47). In our study, evidence for the anti-inflammatory activity of SEMU is further supported by the attenuation of iNOS striatal immunostaining, what can be explained by its NF-kB inhibitory activity.

Figure 5) Representative photomicrographs showing reduced immunostaining for HDAC (×400, scale bar=200 µm) in hippocampal, CA1 and CA3 and DG areas, from 6-OHDA lesioned rats after SEMU treatments, compared to the untreated 6-OHDA groups (3 animals per group). CA1: a. vs. SO, q=59.76, p<0.001; b. vs. 6-OHDA+SEMU20, q=15.64, p<0.001; c. vs. 6-OHDA+SEMU40, q=57.06, p<0.001; d. vs. SO, q=44.07, p<0.001; e. vs. 6-OHDA+SEMU40, q=41.37, p<0.001. CA3: a. vs. SO, q=45.32, p<0.001; b. vs. 6-OHDA+SEMU20, q=8.419, p<0.001; c. vs. 6-OHDA+SEMU40, q=40.99, p<0.001; d. vs. SO, q=36.90, p<0.001; e. vs. 6-OHDA+SEMU40, q=15.12, p<0.001 (one-way ANOVA and Tukey as the post hoc test).
In conclusion, for the first time, this study demonstrated that a standardized extract from \textit{M. urundeuva} is able to reduce the inflammatory environmental consequence of microglial activation induced by 6-OHDA dopaminergic neurodegeneration, through inhibition of inflammatory enzymes, NF-\(\kappa\)B as well as HDAC. Most importantly, taken together, the present findings and those from previous reports indicate that SEMU appears to be a promising agent of natural origin for protection against PD and neurodegeneration.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

REFERENCES


