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Neuroprotective effects of caffeine in the model of 6-hydroxydopamine lesion in rats

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Abstract

The work shows the effects of caffeine after the intrastriatal injection of 6-OHDA in rats, considered as a model of Parkinson disease (PD). Two weeks after the 6-OHDA lesion, rats exhibit a characteristic rotation behavior as a response to the apomorphine challenge. Our results showed significant increases in the number of apomorphine-induced rotations in 6-OHDA-lesioned rats, as compared to sham-operated animals. A partial recovery was observed in 6-OHDA-lesioned rats, after caffeine (10 and 20 mg/kg, i.p., daily for 14 days) treatment. The stereotaxic injection of 6-OHDA produced loss of striatal neurons, as indicated by the decrease in monoamines levels, in the ipsilateral side (75–85%) when compared to the contralateral side. Significant decreases in noradrenaline levels were seen in the ipsilateral side of 6-OHDA group (62%), and this effect was not significantly reversed in caffeine-treated groups. While significant decreases in dopamine levels were seen in the ipsilateral side of 6-OHDA group (78%), in the caffeine-treated group (10 and 20 mg/kg, i.p.) the decreases were only 53 and 18%, indicating significant recoveries. In conclusion, our data demonstrated beneficial effects of caffeine in this model of PD, suggesting the potential use of A2A antagonists as a novel treatment for this neurodegenerative disease.

Keywords: Caffeine; 6-Hydroxydopamine; Parkinson's disease; Rat striatum

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the *substantia nigra*, with severe dopamine depletion in the striatum. Current therapies with antiparkinsonian agents partially alleviate the symptoms of the disease, but have not been found to avoid the progression of dopaminergic neurons degeneration. Another major limitation of PD medications is their sometimes disabling side effects. The most irreversible adverse effect of the chronic dopaminergic therapy is dyskinesia. This side effect limits symptomatic therapy, thus increasing the search for new nondopaminergic alternative or adjunctive treatments for PD.

Adenosine, an endogenous modulator of biological functions, interacts with at least four receptors classified as A_1, A_{2A}, A_{2B} and A_3. Several data showing the co-localization of A_{2A} and D_2 receptors suggest that the blockade of A_{2A} receptors produces direct effects on D_2 receptors (Fredholm and Svenningsson, 2003). Evidence that adenosine A_{2A} antagonists could constitute a potential therapeutic tool for the therapy of PD, is provided by results obtained in rodent and primate models of PD, which report that A_{2A} antagonists exert antiparkinsonian activity (Morelli, 2003). Adenosine A_{2A} receptors are abundant in the caudate-putamen, and involved in the motor control in several species.

Caffeine is an alkaloid widely consumed for its CNS stimulating properties (Moo-Puc et al., 2003). Several studies revealed an inverse association between risk of PD and caffeine intake (Ross et al., 2000; Ross and Petrovitch, 2001; Schwarzschild et al., 2002; Baraldi et al., 2003). Experimental evidence also suggests that caffeine has potential antiparkinsonian properties, as demonstrated by its protective effects (Joghataie et al., 2004) and the blockade of striatal adenosine receptors. The CNS effects of caffeine appear to be mediated primarily by its

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antagonistic actions at the A_1 and A_{2A} subtypes of adenosine receptors (Fredholm et al., 1999). A_{2A} receptors are particularly relevant to PD because their expression in the brain is largely restricted to the striatum (Fisone et al., 2004), the main target of dopaminergic neurons that degenerate in PD. The presence of A_{2A} receptors was also reported in the human brain (caudate nucleus and putamen) by Ishiwata and collaborators (Ishiwata et al., 2005).

The unilateral 6-hydroxydopamine nigrostriatal lesion has been widely used as a rodent model of Parkinson's disease. It has been reported that reactive oxygen radicals are involved in the toxicity of 6-OHDA-induced nigrostriatal lesions. Increased free radicals levels occur on aging and are proposed to be a contributing factor for Parkinson's disease (Alfavaro et al., 2004). Methylxanthines, including caffeine and their metabolites, have proven to inhibit oxidative damage induced by these reactive species (Lee, 2000). Joghataie et al. (2004) reported that 6-OHDA-induced loss of nigral neurons and associated alterations in behavioral responses to dopaminergic stimulation can be attenuated by caffeine. Together, the protective effects of caffeine and its metabolites in rodent models of PD support a causal basis for the inverse relationship between human caffeine consumption and the subsequent risk of PD development (Xu et al., 2005). The present study was designed to investigate the beneficial effect of caffeine in a model of PD, using unilateral intrastriatal 6-hydroxydopamine (6-OHDA)-lesioned rats.

2. Materials and methods

2.1. Drugs

Caffeine, 6-hydroxydopamine, ascorbic acid, apomorphine, sodium octanesulfonic acid, acetonitrile and tetrahydrofuran were purchased from Sigma Chemical Co. USA. All other drugs were of analytical grade.

2.2. Animals and experimental procedures

Adult male Wistar rats (from the Animal House of the Federal University of Ceará), weighing 200–250 g at the start of the experiment, were housed five to six per cage, maintained in a 12 h light/dark cycle with free access to water and standard food. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals from the US Health and Human Services Department. Animals were anesthetized with a combination of Ketamine (100 mg/kg, i.p.) and Xylasine (5 mg/kg, i.p.) and given a unilateral stereotaxic injection of 12 μg 6-OHDA (12 μg/μl) with 0.2 mg/ml L-ascorbic acid (Sigma), into the right striatum (AP 0.9/1.4, ML 3.0, DV 3.3, from Bregma), according to the Atlas of Paxinos and Watson (1986), using a 10 μl Hamilton syringe. Sham-operated animals received vehicle and were used as controls. Caffeine (Sigma, 10 and 20 mg/kg, i.p.) was administered 1 h after 6-OHDA lesions and once a day for the next 13 days. Fifteen days after the 6-OHDA injection (24 h after the last injection of caffeine or vehicle), the behavior was assessed by monitoring body rotations induced by apomorphine (3 mg/kg, i.p.). The number of net rotations (the number of 360° contralateral turns) was recorded for 60 min and, at the next day, animals were sacrificed, and the striatal tissue was collected and stored at −70 °C until use.

2.3. Monoamine levels

For the measurement of monoamine levels and their metabolites noradrenaline (NE), dopamine (DA), 3,4 dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), the brain tissue was used to prepare 10% homogenates. Homogenates were sonicated in 0.1 M $HClO_4$, for 30 s, centrifuged at 4 °C for 15 min at 15,000 rpm, and the supernatant was filtered (0.2 μm, Millipore). A 20 μl sample was then injected into a high-performance liquid chromatograph (HPLC) column. The mobile phase was 0.163 M citric acid, pH 3.0, containing 0.02 mM EDTA, with 0.69 mM sodium octanesulfonic acid (SOS), as ion pairing reagent, 4% v/v acetonitrile and 1.7% v/v tetrahydrofuran. NE, DA, 5-HT and their metabolites were electrochemically detected, using an amperometric detector (Shimadzu, Japan), by oxidation on a glassy carbon electrode at 0.85 V relative to the Ag–AgCl reference electrode. The amount of monoamines was determined by comparison with standards injected into the HPLC column at the day of experiment, and their concentrations were expressed as ng/mg tissue.

2.4. Cell culture and cell viability studies

Mesencephalic cells were isolated from embryo brains obtained from female Wistar rats, at their 16–18 days of gestation, as described elsewhere (Ahmadi et al., 2003). Briefly, tissue blocks from coronal sections of occipital cortical areas of embryos were submitted to a median horizontal cutting. The anterior region was discarded and the posterior one, containing predominantly mesencephalic cells, was used. After treatment with trypsin, the cells were suspended in the Neurobasal Medium without L-glutamine, supplemented with B-27 Supplement Minus from Gibco, USA, containing streptomycin, penicillin, and actinomycin. The cell suspension was platted on 96 well plates coated with poly-L-lysine, at a density of 5×10^4 cells/well. Cultures were maintained at 37 °C under a 5% CO^2 atmosphere. After 4 days in culture, caffeine (CAF, 5 and 10 μg/ml) was added to the cells, 3 h before 6-OHDA (40 μl). The neurotoxicity was evaluated using the MTT [3-(4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium assay. After 24 h of incubation with 6-OHDA, the medium was replaced and MTT was added to the cell culture, at a final concentration of 200 μM, and incubated again for 3 h. Then, after cell washing with PBS, 150 μl DMSO were added and, after 5 min stirring, the absorbance was measured with a microplate reader at 595 nm. The inhibition of MTT reduction indicates the degree of 6-OHDA-induced toxicity. Experiments were performed in triplicates at three different days.

2.5. Statistical analysis

All results were presented as means±S.E.M. One-way ANOVA was used, followed by the Student–Newman–Keuls test, except for the MTT assay where the Tukey's test for multiple comparisons was used. Results were considered significant at $p<0.05$.

3. Results

Two weeks after the intrastriatal injection of 6-OHDA, rats exhibited rotational behavior in the direction opposite to the lesion (contralateral rotation), following the apomorphine administration. Significant increases in the number of apomorphine-induced rotations were seen in 6-OHDA-lesioned controls, as compared to the sham-operated group (178.7±7.8 vs. 7.0±0.5 turns/h; F (3, 34)=172.8; $p<0.001$). A partial motor recovery was observed in 6-OHDA-lesioned rats treated with caffeine, that reduced profoundly the number of apomorphine-induced rotations by 47 and 69% (caffeine 10 and 20 mg/kg, i.p., respectively), when compared with the 6-OHDA-lesioned group (controls) (Fig. 1).

The 6-OHDA stereotaxic injection produced oxidative damage that resulted in the loss of neurons, as indicated by the decrease in monoamines and their metabolite levels in the ipsilateral side (75–85%), when compared with the contralateral side of controls. No differences were seen on contralateral sides among sham-operated, 6-OHDA-lesioned controls and 6-OHDA-lesioned rats after caffeine treatment.

Significant decreases in dopamine (DA) levels were seen in the ipsilateral side of controls (78%). However, in the caffeine-treated group, at the doses of 10 and 20 mg/kg, the decreases were only 53 and 18%, respectively, indicating significant recoveries which were almost complete at the higher dose of caffeine (F (5, 34)=36.61; $p<0.001$). The same occurred with its metabolite DOPAC that decreased by 75% in controls, and by 41 and 33% in the caffeine-treated group, at the doses of 10 and 20 mg/kg, respectively (F (5, 34)=19.66; $p<0.001$). Furthermore, HVA levels also decreased by 51% in controls, and by 10 and 5%, respectively, in the lesioned groups treated with the same doses of caffeine (10 and 20 mg/kg; F (5, 34)=16.32); $p<0.001$) (Fig. 2).

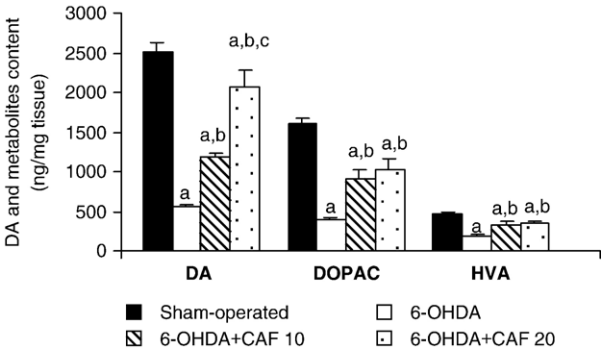

Fig. 2. Effects of caffeine (CAF) on levels of DA, DOPAC and HVA, in the rat striatum (ng/mg tissue). Animals received CAF (10 and 20 mg/kg, i.p., daily, for 14 days), starting 1 h after 6-OHDA lesion. Data are reported as means±SEM, from 6 to 8 animals. a, vs. sham-operated; b, vs. controls (6-OHDA alone); c, vs. CAF10, respectively; at $p<0.05$ (ANOVA and the Student–Newman–Keuls test).

Significant decreases in noradrenaline (NE) levels were also seen in the ipsilateral side of controls (62%) and in the caffeine-treated group, at the doses of 10 and 20 mg/kg (53 and 56% decreases, respectively; F (5, 34)=22.69; $p<0.001$), as shown in Fig. 3. A decrease of 61% was observed in 5-HT levels, in the 6-OHDA-lesioned group (controls), in the ipsilateral side as related to the contralateral side (F (5, 34) =29.03; $p<0.001$). On the other hand, in the caffeine-treated group, at the doses of 10 and 20 mg/kg, this decrease was 58 and 42%, respectively. No significant differences were seen in the levels of 5-HIAA among the groups (Fig. 3) (F (5, 34) =3.66; $p<0.001$). Furthermore, no difference was seen between the sham-operated (contralateral and ipsilateral sides) and the contralateral side of the 6-OHDA-lesioned rats (controls).

Results of the MTT test showed that the exposure of mesencephalic dopaminergic cells to 40 μM 6-OHDA caused a 34% reduction in cell viability, as compared to controls

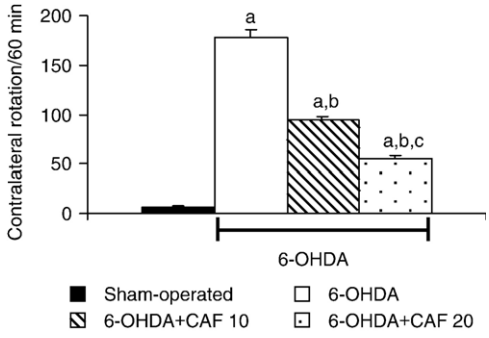

Fig. 1. Effects of caffeine treatment (CAF 10 and 20 mg/kg, i.p., for 14 days, starting 1 h after 6-OHDA lesion) on apomorphine-induced (3 mg/kg, i.p.) rotational behavior. Two weeks after 6-OHDA striatal injection, the number of net contralateral rotations was counted for 60 min. Data are reported as means±SEM, from 6 to 8 animals. (ANOVA and the Student–Newman–Keuls test). a, vs. sham-operated; b, vs. controls (6-OHDA alone); c, vs. CAF10, respectively; at $p<0.05$.

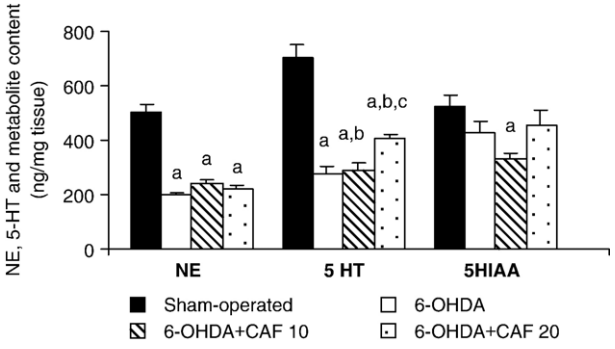

Fig. 3. Effects of caffeine (CAF) on levels of NE, 5-HT and 5-HIAA, in the rat striatum (ng/mg tissue). Animals received CAF (10 and 20 mg/kg, i.p., daily, for 14 days), starting 1 h after 6-OHDA lesion. Data are reported as means±SEM, from 6 to 8 animals. a, vs. sham-operated; b, vs. controls (6-OHDA alone); c, vs. CAF 10, respectively; at $p<0.05$ (ANOVA and the Student–Newman–Keuls test).

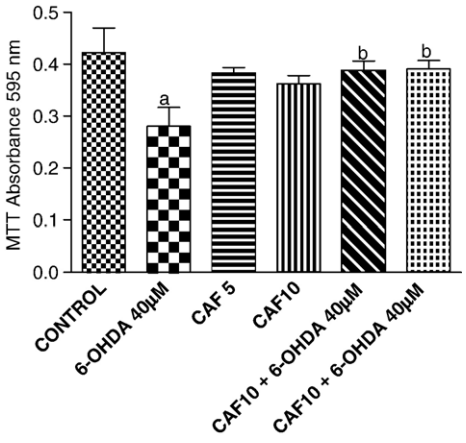

Fig. 4. Effects of caffeine (CAF) on the 6-OHDA-induced toxicity, in rat mesencephalic dopaminergic cells. After 4 days in culture, CAF was added to the cells, 3 h before 6-OHDA (40 μM). The neurotoxicity was evaluated by the MTT assay. a and b vs. control and 6-OHDA, respectively (one way ANOVA and Tukey tests, at $p<0.05$).

(MTT absorbance at 595 nm, control = 0.4223 ± 0.0476; 6-OHDA = 0.2808 ± 0.0360). While caffeine at 5 and 10 μg/ml concentrations did not alter cell viability, it blocked the 6-OHDA-induced cytotoxicity, and values of MTT reduction were closed to those of controls (in the absence of 6-OHDA) (Fig. 4).

4. Discussion

Adenosine A_{2A} receptor antagonists, including the non-specific adenosine antagonist caffeine, are a novel non-dopaminergic therapeutic strategy for PD treatment (Yu et al., 2006). For instance, istradefylline is the first of several A_{2A} receptor antagonists in development for PD and is now under clinical trials. Results indicate that the drug reduces motor fluctuation induced by L-Dopa.

Epidemiological studies have linked the consumption of coffee and other caffeinated beverages to a reduced risk of the subsequent development of PD. The popularity of caffeine as a psychoactive drug is due to its stimulant properties, which depend on its ability to reduce adenosine transmission in the brain. Adenosine A_1 and A_{2A} receptors are expressed in the basal ganglia, a group of structures involved in various aspects of motor control, and caffeine acts as an antagonist to both types of receptors (Fisone et al., 2004). Psychomotor-stimulant and other CNS effects of caffeine appear to occur, at least in part, through its antagonism of the adenosine A_{2A} receptors (Fredholm et al., 1999).

Caffeine as well as more specific antagonists of the adenosine A_{2A} receptor have been found to attenuate neurotoxicity in mouse models of PD. Furthermore, in addition to possessing a neuroprotective potential, caffeine and other A_{2A} antagonists have been known to reverse motor deficits in PD models and to be neuroprotective in the MPTP and 6-OHDA models of PD (Chen et al., 2002; Ikeda et al., 2002). Thus, A_{2A} antagonists may offer neuroprotective benefits as well as symptomatic relief (movement enhancing). This benefit of blocking A_{2A} receptors, coupled with their remarkably restricted expression in the basal ganglia, have made A_{2A} antagonists attractive targets for drug development (Schwarzschild et al., 2002). Thus, caffeine has recently been included in the list of the twelve most promising neuroprotective agents for clinical trials in PD (Ravina et al., 2003).

In the present study, behavioral and biochemical effects of caffeine were investigated in a model of PD. For this purpose, apomorphine-induced rotations and monoamine levels of the SNC were quantified. Previous studies have demonstrated that the most important biochemical changes in the 6-OHDA-lesioned striatum are reductions in dopamine (DA) and its metabolite levels. This effect causes a prominent functional and motor asymmetry that can be evaluated by dopamine receptor agonists, such as apomorphine, resulting in rotations in a direction that is contralateral to the lesion, which are considered as reliable indicators of nigrostriatal dopamine depletion (Hudson et al., 1993). This is consistent with the present results, that showed apomorphine-induced rotations following a unilateral injection of 6-OHDA into the striatum. This effect would involve dopamine receptor supersensitivity caused by the loss of DA terminals, resulting in a significant reduction of DA levels in the 6-OHDA-lesioned striatum.

Our results demonstrated a reduction in apomorphine-induced rotations following caffeine administration, as compared to the untreated lesioned group (controls). This reduction could be attributed to beneficial effects of caffeine, attenuating striatal degeneration, and may be due to its ability to block adenosine A_{2A} receptors that are concentrated in dopaminergic areas of the brain (Joghataie et al., 2004).

We also showed that 6-OHDA induced significant decreases in NE, DA, DOPAC and HVA levels, measured in the ipsilateral side of controls when compared with the sham-operated group. We also demonstrated that 5-HT levels decreased, but levels of its metabolite, 5-HIAA, were unchanged. These findings are consistent with other studies describing biochemical changes in the 6-OHDA-lesioned striatum (Ichitani et al., 1994).

The caffeine treatment partially restored the content of monoamines and their metabolites in 6-OHDA-lesioned rats. In the present study, the finding that the caffeine treatment can attenuate apomorphine-induced motor deficits suggests increases in DA levels in the lesioned striatum and subsequent reduction of the receptor supersensitivity. These results were supported by measurements of DA and its metabolite levels which were partially recovered in the ipsilateral side of the caffeine treated group.

The influence of caffeine on the dopaminergic innervation of the striatum can be attributed to its ability to prevent the death of dopaminergic neurons originating in the substantia nigra. Oztas et al. (2002) demonstrated that the MPTP-induced loss of nigral dopaminergic neurons (TH-immunoreactive) can be prevented by caffeine. Recently, Joghataie et al. (2004) reported that nigrostriatal neurons within the SNC were mainly preserved against neurodegenerative effects induced by 6-OHDA, in caffeine-treated lesioned group. Another work (Pierri et al., 2005) demonstrated that after MPTP treatment in mice, an A_{2A}

antagonist (KW-6002) was neuroprotective and had anti-inflammatory effects at the level of the substantia nigra.

In conclusion, the data of the present study demonstrated that caffeine has beneficial effects in the model of 6-OHDA-induced striatal lesions in rats, attenuating motor dysfunctions and increasing dopamine levels. Alternatively, caffeine could act as a neuromodulator, thus functionally compensating for the loss of dopaminergic neurons. Recently (Cauli et al., 2005), it was demonstrated that repeated caffeine exposure enhanced the motor stimulant effects elicited by dopamine agonists, through sensitization of D1 receptors in 6-OHDA-lesioned rats. Since the effects of caffeine appear to be mediated by its antagonistic action at A_{2A} receptors, more specific antagonists of these receptors could be helpful as a novel therapeutic strategy for the treatment of PD.

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