

**THE ADMINISTRATION
OF TRANS-
RESVERATROL AT
DOSES OF 1 AND 3 MG/
KG REVERSES THE
POSTURAL IMBALANCE
IN HEMIPARKINSONIAN
RATS WITH A
BENEFICIAL POST-
TREATMENT EFFECT**

José L. Bata-García

Universidad Autónoma de Yucatán. Centro de Investigaciones Regionales “Dr. Hideyo Noguchi”-Unidad Biomédica. Mérida, Yucatán, Mexico. ORCID iD: 0009-0002-6526-4931

Gilda C. Solís-Uicab

Augusta Soortswear de México. Mérida, Yucatán, Mexico. ORCID iD: 0009-0002-8113-0108

Jorge J. Alcocer-Sosa

IMSS-Bienestar; Main office; Barrio de Santiago Mérida, Yucatán, Mexico. ORCID iD: 0009-0006-1024-8140

Karen C. Hernández-Monsrreal

Hospital Psiquiátrico de Campeche. Kobén, Campeche, Mexico. ORCID iD: 0009-0007-0536-6799

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Carlos E. Pérez-Osorio

Universidad Autónoma de Yucatán. Medicine course.

Unidad Interinstitucional de Investigación Clínica y Epidemiológica.

Mérida, Yucatán, México.

ORCID iD: 0000-0003-0657-8615

Abstract: Parkinson's disease is chronic and causes a postural imbalance that affects the gait and balance of patients. There is progressive loss of nigro-striatal dopaminergic neurons. It generates a high economic cost and current therapies produce unwanted effects. The objective of the present work was to evaluate the beneficial effect on postural adjustment with the systemic administration of trans-resveratrol at doses of 1 and 3 mg/kg in rats with hemiparkinsonism induced with 6-OHDA. The 14 rats used showed a significant reduction in steps with the contralateral paw (PC) without involvement of the ipsilateral paw (PI) post-injury. The vehicle did not produce reversal of motor impairment, but there was deterioration in the ipsilateral paw during washing. The motor impairment produced by 6-OHDA was reversed, both in the CP and the IP with the administration for 3 weeks of trans-resveratrol at 1 and 3 mg/kg, significantly reducing the asymmetry between both legs, without presenting development of effects. collaterals. The motor improvement produced by trans-resveratrol was maintained up to 6 months after stopping the treatment of both doses of 1 and 3 mg/kg, although at the dose of 1 mg/kg the improvement had some fluctuations. The above suggests that trans-resveratrol is effective in reversing postural imbalance without causing side effects and that this effect can last for a long time after stopping treatment, suggesting that it could be a safe therapy with a prolonged effect in patients with PD.

Keywords: Step adjustment. Postural instability. Hemiparkinsonism. Neuroprotection. Trans-resveratrol.

INTRODUCTION

Parkinson's disease (PD) includes postural imbalance that makes patients have difficulty starting and maintaining walking and falls are frequent, which can cause mild to very serious

fractures (Bloem et al, 2004), considerably affecting quality of life of the patient and it is a chronic condition, against which to date there is no cure and conventional therapy only acts by reducing the severity of the symptoms, having to be applied for the rest of the patient's life once started.

The development of chronic preclinical models with injury to the nigrostriatal pathway with 6-hydroxydopamine (6-OHDA) and with greater predictive value, analogous to PD in humans, has allowed the evaluation of long-term effects with drugs with antiparkinsonian potential. The step adjustment test (AJP) evaluates the number of steps taken by each front leg and is analogous to the "push test" used by neurologists in the diagnosis of PD (Schallert & Tillerson, 2000). Due to the development of motor abnormalities and "on-off" periods with prolonged administration of L-DOPA, the preferred therapy given (LeWitt, 2009), Much of the current research is focusing on finding new pharmacological alternatives that are effective and that do not develop tolerance or long-term adverse effects and that, if present, are minimal. Some studies have suggested the antiparkinsonian potential of resveratrol, such as a study where a significant reduction in the number of contralateral turns (CG) was observed with apomorphine in hemiparkinsonian rats, administering grape juice extract over a long period of time, which has a high content of resveratrol (Eshraghi-Jazi et al., 2012) and in another study using mice treated with MPTP (acute model of PD) it was found that when resveratrol was administered at a high dose (50 mg/kg, PO) the mice showed significant motor improvement in the motor behavior in the open field (Anandhan et al., 2010). Based on the above, the objective of the present work was to evaluate whether daily administration for 3 weeks of trans-resveratrol at doses of 1 and 3 mg/kg to hemiparkinsonian rats produces

recovery in postural adjustment, without developing tolerance and/or dyskinesias and whether the improvement is prolonged after stopping treatment.

MATERIALS AND METHODS.

Animals. To evaluate the effect of the administration of trans-resveratrol on the improvement in AJP in animals with a chronic model of PD, 14 adult male rats of the Wistar variety, raised in, with an average weight of 421.1 ± 17.46 g (range between 348 to 540 g) at the beginning of the experiments. The rats remained throughout the entire time in a room with a constant temperature (23 ± 1 °C) and a 12/12 h light-dark cycle system (on at 07:00 a.m.). The contralateral turn (CG) and AJP tests were carried out between 08:00 and 16:00 h. In accordance with the recommendations of the Official Mexican Standard (1999) and The Guide for the Care and Use of Laboratory Animals (National Research Council, 2011), every effort was made to avoid any unnecessary suffering to the animals.

Drugs. The drugs were the following: Desipramine-HCl (Spectrum, Gardena, CA, USA); 6-OHDA-HBr (Sigma, St. Louis, MO, USA); Apomorphine-HCl (APO.HCL, Sigma, St. Louis, MO, USA); Trans-resveratrol (SIGMA). All drug solutions were prepared fresh and protected from light before administration. Desipramine (25 mg/ml) was dissolved in a mixture of distilled water plus dimethyl sulfoxide (12.5%). 6-OHDA (3 mg/ml) and apomorphine (0.25 mg/ml) were dissolved in distilled water with ascorbic acid (0.5 mg/ml). AND I resveratrol at doses of 1 and 3 mg/kg was administered orally. Based on the low solubility of resveratrol, it was suspended in a mixture (which from now on we will refer to as Vehicle) of 0.985 ml of Aspartame solution (Canderel®) + distilled water (67 mg/ml) + canola oil at 1.5%, sonicating for 3 min,

to facilitate the rats' ingestion of the trans-resveratrol without difficulty.

Injury Surgery. To protect noradrenergic neurons, desipramine (25 mg/kg, ip) was administered at least 30 min before the administration of 6-OHDA. The rats were anesthetized with pentobarbital sodium (45 mg/kg, ip) and placed in a stereotaxic apparatus (Stoelting) with the incisor bar 3.3 mm below the interaural line. A single dose of 6-hydroxydopamine (6-OHDA, 10.5 µg/3.5 µl) was manually injected, at a rate of approximately 0.2 µl/min, into the substantia nigra pars compacta (SNpc) of the right side of the rat brain. at the coordinates: AP, -5.3 mm from bregma; L, -1.8 mm in reference to the midline, and V, -7.6 mm with respect to the surface of the dura mater (Paxinos and Watson, 1986). The infusion was performed through a 30-gauge needle connected to a polyethylene catheter (PE10) and a 10 µl microsyringe (Hamilton™). At the end of the injection, the needle was held for an additional minute before being removed, for better diffusion of the toxin.

Apomorphine-induced GC test. In order to select animals with dopaminergic denervation greater than 90%, 14 days after injury all rats were subjected to the apomorphine-induced GC test. The rats were placed inside a hemisphere (41 cm diameter). Afterwards, a dose of the dopamine agonist apomorphine (0.25 mg/kg, sc) was administered and the rotating behavior induced by apomorphine was recorded for 90 min. The GC test was repeated 2 more times at one-week intervals. All tests were videorecorded to subsequently count the number of GCs and only those rats that performed a minimum of 250 GCs during the last test were selected to continue to the treatment phase with vehicle or with trans-resveratrol at the different doses. The previous selection criterion was managed based on previous studies where, with

immunostaining against tyrosine hydroxylase (TH), a dopaminergic denervation of more than 90% was confirmed in the SNpc in rats that performed the same number of GCs (Bata-García et al, 2007).

Treatments. Treatments were started from the 8th week after injury and were continued daily for 3 consecutive weeks from days 57 to 75 post-injury (PL), after which they were discontinued. The vehicle and both doses of trans-resveratrol were administered orally in a volume of 1 ml/kg through a stainless steel cannula connected to an insulin syringe. In all cases the animals voluntarily ingested the solution sweetened with Aspartame, making it unnecessary to introduce the cannula into the esophagus. The animals were distributed according to the following groups: A, Control Group (VEH) that received the vehicle; B, Group RESV-1 (Trans-resveratrol at 1 mg/kg); C) Group RESV-3 (Trans-resveratrol at 3 mg/kg).

Step Adjustment Test. It is a test to evaluate postural adjustment, in which the steps taken by each rat to maintain balance were counted while it was pushed sideways with the experimenter's extended hand and moved along a smooth stainless steel surface (1 m length) at a speed of approximately 20 cm/s, first to the left and then to the right (test), 4 times per side (4 trials/rat/day). The activity of the rats during the tests was videotaped for later analysis and the number of steps taken with the front paw of the side towards which the rat was moved was counted. To avoid bias in the response to the test, all experiments were carried out by the same experimenter (Bata-García et al., 2007; 2010).

The ipsilateral and contralateral terms were managed in relation to the side of the 6-OHDA lesion. In this study, the SNpc was injured on the right side and, therefore, the term ipsilateral was used when referring to the right front paw, while the contralateral was

the left paw. Previous studies in animals with unilateral lesion of the SNpc with 6-OHDA have reported an impairment in the use of the PC, but not in the PI (Bata-García et al., 2007; 2010). To evaluate the effect of daily administration for 3 consecutive weeks of 1 and 3 mg/kg of resveratrol, in this study the AJP test was used due to its clinical relevance since it is analogous to the “push test”, used by the neurologists for the diagnosis of PD (Schallert and Tillerson, 2000). The test was carried out on alternate days (Monday-Wednesday-Friday) for all stages of the study: A) Pre-injury Baseline (BPRE); B) Baseline Post-injury (BPOST); C) Treatment and D) Washing (after stopping treatment). To obtain BPRE values, the test was performed on days 6 to 2 prior to injury. To obtain BPOST values, the test was again used from days 50 to 54 after injury. After the 8th week PL, treatment was started with a 3-week duration (days 57 to 77 PL), receiving their dose between 8:00 and 11:00, the evaluations were from day 57 to 75 PL. On the evaluation days, administration was carried out 60 min before the AJP test, between 10:00 and 16:00 h. Washout continued for up to 6 months after the last administration of vehicle or trans-resveratrol for both doses. During the Washout phase, evaluations were carried out every 4th week (month) until completing 6 months of post-treatment evaluations.

Identification and evaluation of side effects during treatments. Although treatment with L-DOPA has been shown to be the most effective means of reducing the symptoms of Parkinson’s disease in humans, its long-term use is associated with the appearance of complications, such as oscillations in the efficacy of the drug or the phenomenon of “on-off” and abnormal involuntary movements or dyskinesias (AIMs) (Sheenhan, 2000). The development of dyskinesias has also been reported in

hemiparkinsonian rats, under the following criteria: a) induced by L-DOPA, b) affecting only the side contralateral to the lesion, and c) repetitive, but not attributable to any normal behavioral pattern (Rangel -Barajas et al., 2011). In the present study, we evaluated whether dyskinesias appeared during drug treatment, individually for 3 min every 20 min, for 2 hours, with a maximum of 6 observations per day.

Data processing. The results were expressed as mean \pm SEM. For the AJP test, for the 14 selected rats, after counting the number of steps, the percentage of deterioration (PD) in the execution of steps was calculated for each paw, subtracting the number of steps on day 54 PL with the number of steps on day 2 of the BPRE and the result was divided by the number of BPRE steps and subsequently multiplied by 100. The procedure was repeated for the opposite leg and the averages for each were calculated. To evaluate the effect of the treatments on the number of steps of the PI and PC of each group, the average number of steps per group was compared against BPOST. The latency to the maximum effect during treatment was defined as the number of days elapsed from the start of treatment, until the day the maximum step value was reached. To evaluate the permanence of the effect of the treatments on the steps of both paws, the averages obtained in the wash were compared against day 75 PL. To evaluate the effectiveness of treatment with trans-resveratrol on the asymmetry between the PI and PC produced by the injury, the averages of the %Asim obtained during the treatment were compared against the %Asim of day 54 PL and to evaluate if this effect was maintained, the averages of %Asim during washing were compared against the %Asim of day 75 PL. To calculate the %Asim, the product of PC/PI \times 100 was subtracted from 100, for each rat in each group.

Statistic analysis. To evaluate the percentage of impairment (PD) due to the lesion, the average steps with each paw of each group of the 14 rats on day 54 PL were compared and the values were compared against the PD of the groups for day 2 of BPRES, using a paired Student's t test. To evaluate whether the treatments caused motor recovery, the averages of the AJP of the PC were compared against the AJP of day 54 PL, using a repeated measures (RM) ANOVA test followed by a Dunnett's post-hoc test. To evaluate whether the effect of treatment on the deterioration in the number of CP steps was maintained after its interruption (washout), the AJP results from the last day of each month during washout (days 103, 131, 159) were compared, 187, 215 and 243 PL) against day 75 PL, using a repeated measures ANOVA test followed by a Dunnett's post-hoc test. To compare the latency to the maximum effect of resveratrol at both doses on the number of steps of the contralateral paw, a Student's t test for independent groups was used. To evaluate the effect of treatments on step asymmetry caused by injury, %Asim during treatments were compared against BPOST, using an MR ANOVA test followed by a Dunnett's post hoc test. To evaluate whether the effect of the treatments on step asymmetry was maintained in the wash, the %Asim of days 103, 131, 159, 187, 215 and 243 PL were compared against day 75 PL, using an ANOVA test. of MR followed by a Dunnett post-hoc test. The minimum significance level was established as $p < 0.05$. All statistical analyzes were performed using version 5 of GraphPad Prisma (La Jolla, CA, USA). In consideration of ethical principles and international policies regarding the use of animals in neuroscience research, the smallest number of animals necessary to obtain results with statistical significance was used.

RESULTS

Effect of unilateral lesion of the nigrostriatal dopaminergic pathway with 6-OHDA on contralateral turning behavior induced with apomorphine. For this study, during the third test with apomorphine (0.25 mg/kg, sc), 14 rats performed an average of 539.6 ± 62.10 contralateral turns (CG) (range: 269 to 1007 GC), in a 90-minute recording and were distributed in the following groups: A, Control Group (VEH), N = 4; B, Group RESV-1 (Trans-resveratrol at 1 mg/kg), N = 5; C) Group RESV-3 (Trans-resveratrol at 3 mg/kg), N = 5.

Effect of 6-OHDA lesion on AJP of the ipsilateral and contralateral forelimbs of rats. Injured rats showed a significant reduction ($p < 0.001$, paired Student's t test) in step execution (BPRES: vs. BPOST: 5.73 ± 0.54 steps, N = 14) for PC only, with an average PD of $53.37 \pm 4.70\%$ deterioration.

Effect of treatment on deterioration in AJP due to 6-OHDA lesion and its change during washout. Only for the Control group treated with vehicle, the AJP of the PI suffered a significant decrease in the third week of treatment (54 PL: 13.13 ± 0.16 steps vs. day 75 PL: 11.75 ± 0.27 steps, $p < 0.001$) (Figure 1A), which lasted until the 6th month of washout (Figure 1D).

The step decrement of PC after injury remained unchanged throughout the treatment period (Figure 1A) and throughout the washout period (Figure 1D). In the groups treated with the 1 and 3 mg/kg doses, PI remained unchanged during treatment (Figures 1B and 1C) and during washout (Figures 1E and 1F). The AJP of PC increased significantly with treatment at both doses (day 54 PL vs. day 75 PL; RESV-1, 6.85 ± 0.30 vs. 12.3 ± 0.14 steps, $p < 0.001$; RESV-3: 3.55 ± 0.34 vs. 12.20 ± 0.27 steps, $p < 0.001$) (Figures 1B and 1C) effect that lasted during the 6 months of washout, although

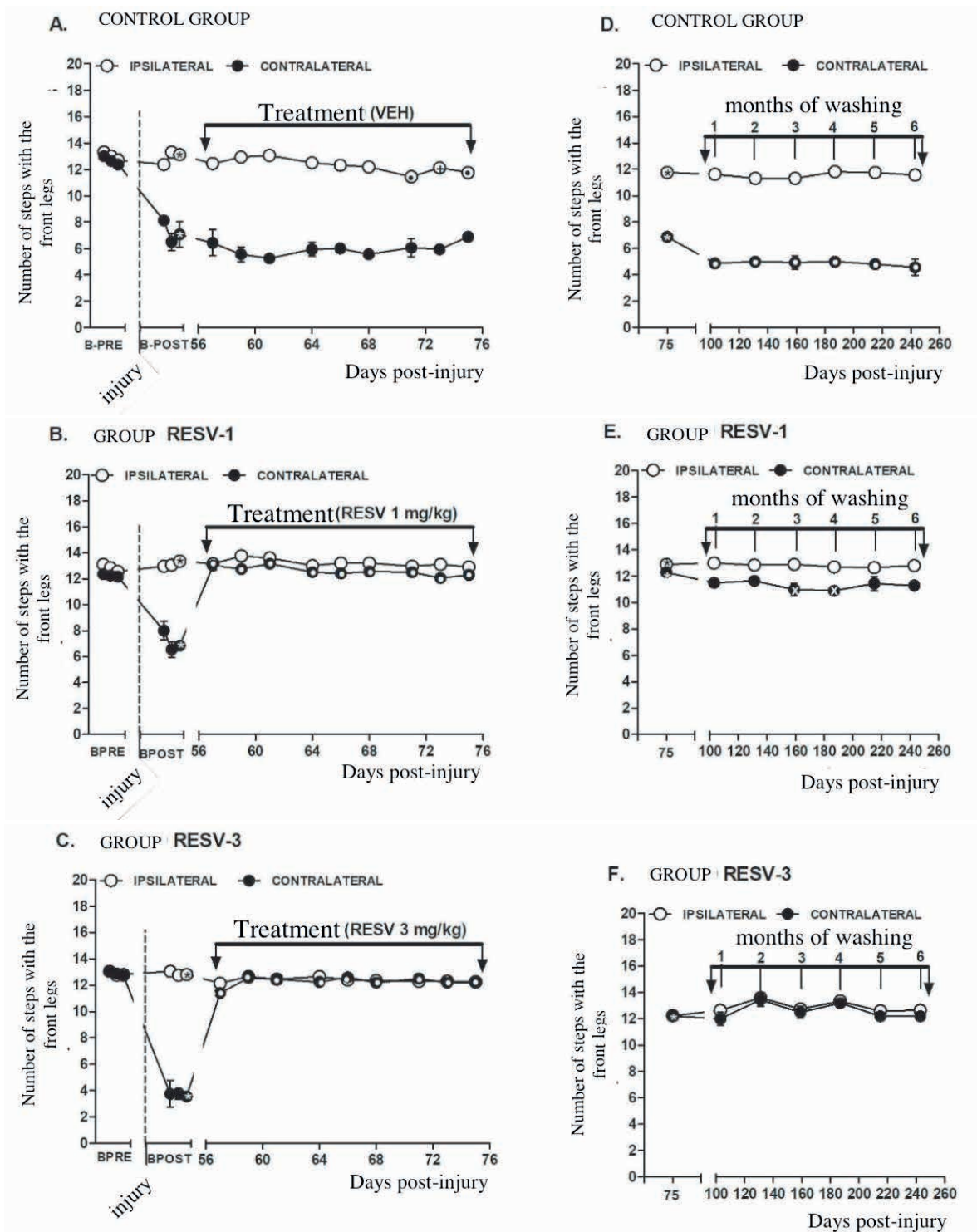


Figure 1. Effect of treatment on the number of steps of the IP and PC after injury with 6-OHDA. The use of the ipsilateral front paw (white circles) and the contralateral one (black circles) to execute steps throughout the Treatment (delimited by downward arrows, for each group) and during washing vs. on day 54 PL (asterisk inside the white circle for the ipsilateral paw and asterisk in the black circle for the contralateral one, respectively ($p < 0.01$; $p < 0.001$)).

with some oscillations with a significant reduction on days 159 and 187 PL for RESV-1 (day 75 PL: 12.20 ± 0.27 steps vs. day 187 PL: 12.20 ± 0.27 steps, $p < 0.01$), without returning to the values of day 54 PL (Figures 1E and 1F).

Effect of treatment on %Asim in AJP by 6-OHDA lesion and its change during washout. The injury increased the asymmetry between the IP and PC in all groups. For Control (day -2: 2.12 ± 3.08 vs. day 54 PL: $45.92 \pm 8.20\%$, $p < 0.01$); RESV-1 (day -2: 3.22 ± 1.37 vs. day 54 PL: $48.74 \pm 1.86\%$, $p < 0.0001$); RESV-3 (day -2: 0.73 ± 1.72 vs. day 54 PL: $72.34 \pm 2.58\%$, $p < 0.01$). Treatment with the vehicle did not reduce the asymmetry during treatment, which was maintained until day 243 PL of washout (Figures 2A and 2D).

Both doses of trans-resveratrol produced a reduction in %Asim from the first week of treatment (RESV-1: day 54 PL: $48.74 \pm 1.86\%$ vs. day 75 PL: $4.65 \pm 0.981\%$, $p < 0.0001$; RESV-3: day 54 PL: 72.34 ± 2.58 vs. day 75 PL: 0.39% , $p < 0.0001$) (Figures 2B and 2C) and the effect was maintained until day 243 PL of washing (Figures 2E and 2F). The %Asim treatment of the RESV-1 and RESV-3 groups had no significant difference with their respective values obtained in BPRE; that is, the symmetry observed before the injury was recovered.

DISCUSSION

In the present study, it was observed that rats that were unilaterally and stereotaxically injured in the SNpc with the neurotoxin 6-OHDA, showed a significant reduction in the number of steps of the foreleg contralateral to the injury, without affecting the use of the ipsilateral paw (hemiparkinsonism), in accordance with previous studies (Bata-García et al., 2007; 2010). This effect is associated with the loss of dopaminergic neurons of the SNpc that innervate the striatum, generating postural asymmetry. In patients with Parkinson's disease (PD), factors

such as successotoxic damage, caused by an excessive concentration of glutamate in the SNpc (Lipton & Rosenberg, 1994), variation in regional oxidative metabolism and the production at toxic levels of free radicals or species reactive oxygen (ROS) can cause deterioration in mitochondrial activity (Stanga et al., 2020) and this, in turn, contributes to the generation of more ROS and the death of dopaminergic neurons of the SNpc (Schapira, 1990). The use of the chronic model with injury to the nigrostriatal dopaminergic pathway with the neurotoxin 6-OHDA is a frequently used model due to its analogy with the mechanisms and symptomatology of PD in humans (Woodgate et al., 1999; Blum et al., 2001).

The unilateral deterioration in the AJP, after the 6-OHDA lesion, became bilateral in the long term in the rats that only received vehicle as treatment, which lasted during the 6 months of the washout period. It is important to note that the period in the AJP of the ipsilateral paw observed in the Control group was not observed in the groups treated with trans-resveratrol at both doses; That is, trans-resveratrol prevented the deterioration in AJP of PI in the long term, suggesting a long-term neuroprotective effect, in the cerebral hemisphere where 6-OHDA was not applied. Only the oral administration of trans-resveratrol at doses of 1 and 3 mg/kg caused a significant increase in the execution of steps with the PC during treatment, with recovery of around 100% of the symmetry in execution of steps between PC-PI, without significant difference with the symmetry before the injury and without causing the development of tolerance or the appearance of dyskinesias. It is important to note that, unlike other drugs, such as caffeine at doses of 1 and 3 mg/kg (Bata-García et al., 2007; 2010), the motor improvement achieved with treatment with trans-resveratrol at 3 mg /kg remained stable

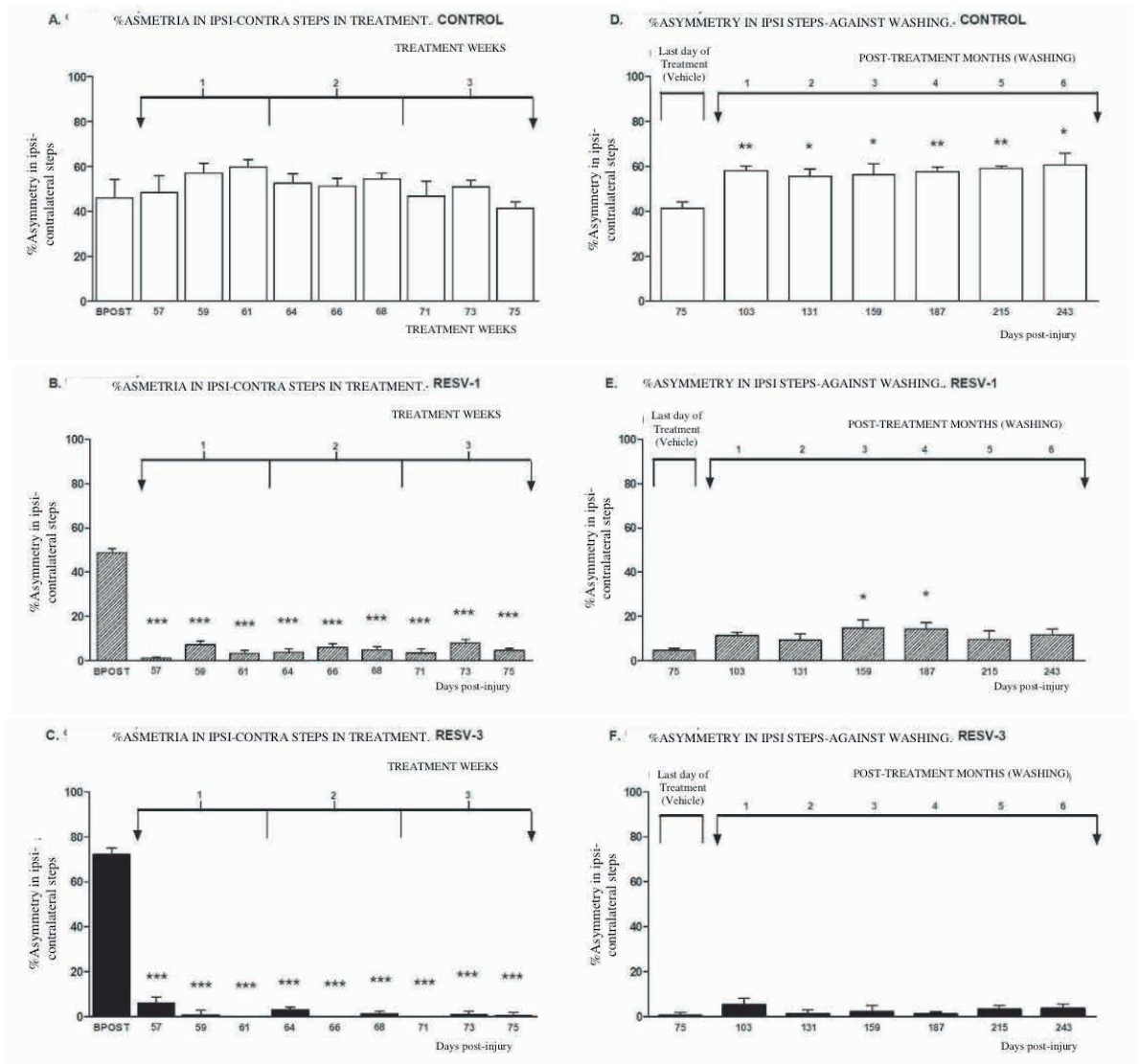


Figure 2. Evaluation of the Percentage of Asymmetry in the execution of steps with the ipsi- and contralateral paws during Treatment and Washing in hemiparkinsonian rats. B and D: Treatment. E and F: Wash ($p < 0.001$; $p < 0.01$; $***p < 0.001$).**

once treatment was stopped, at least during the 6 months that the washout lasted and with some oscillations in months 3 and 4, but without losing the motor improvement at the dose of 1 mg/kg. In rats, it is estimated that a time of 6 months is equivalent to 18 years in humans (Andreollo NA et al., 2012), which is relevant if it is taken into account that it is a time after stopping treatment.

The rapidity with which low-dose resveratrol produced the maximum recovery in the AJP of the contralateral paw and the maintenance of the prolonged effect after the interruption of treatment in the present study could not be explained solely by its pharmacokinetics, since although it has rapid absorption, reaching a peak concentration between 10 to 60 min, has low bioavailability and is rapidly eliminated. In pharmacokinetic studies it has been found that the amount of free resveratrol represents only a small fraction of the dose in plasma (1.7 – 1.9%), with glucuronide and sulfate conjugates predominating in both plasma and urine (Boocock et al., 2007). As suggested by Soleas and collaborators (1997), the levels of free resveratrol in the serum could be seriously underestimated due to the large amounts potentially contained in the cellular fraction, since more than 90% of the free trans-resveratrol is bound to lipoproteins in human plasma. in a non-covalent manner (Burkon and Somoza, 2008). The effects of resveratrol could then not be a result of the visible fraction in plasma but rather of the unassessed cellular fraction of resveratrol, since a large portion of the molecules could bind to cell membranes or lipophilic tissue. Resveratrol administration confers resistance against mitochondrial dysfunction (Zhou J et al, 2021; Zamanian MY et al., 2023) and neuronal death by increasing the activity of manganese-superoxide dismutase (Mn-SOD) (Chang et al., 2013), activates SIRT1, delaying the toxic effect induced by α -synuclein (Dillin

and Kelly, 2007) and facilitates cell survival in response to oxidative stress, catalyzing the deacetylation of p53 (Kume et al., 2006) reduces the release of ON and the increase in the production of free radicals (Dohi et al., 2010; Buljeta I, Pichler A, Simunovic, Kopjar. Beneficial effects of red wine polyphenols on human health: comprehensive review.).

The prolonged persistence of a significant recovery in the AJP of the contralateral paw in hemiparkinsonian rats treated with trans-resveratrol suggests the existence of prolonged plastic changes, which could involve not only changes in the number or sensitivity of receptors at the membrane level, but also other modifications in membrane morphology, such as membrane projections, forming new synapses and/or protein expression, as well as various neuroprotective mechanisms. Furthermore, it has been observed that resveratrol attenuates damage to dendrites and death of DAergic cells induced with rotenone in co-culture of DAergic cells with microglia cells (Chang et al., 2013), suggesting a neuroprotective effect at the intracellular level. and in the membrane structure of DAergic cells, which could explain the prolonged benefit in the AJP of the hemiparkinsonian rats in the present study.

CONCLUSIONS

Our results clearly showed that daily administration for 3 weeks of trans-resveratrol at doses of 1 and 3 mg/kg produced a beneficial effect on the execution of steps in both PI and CP of hemiparkinsonian rats, without the development of side effects and that Motor improvement is maintained for a long time after stopping treatment. These results, together with previous reports, confirm the antioxidant and neuroprotective capacity of trans-resveratrol and could be a better alternative to the use of conventional therapy drugs. The mechanisms of trans-resveratrol

involved in its acute and/or prolonged effect on recovery in the execution of steps with the contralateral paw in hemiparkinsonian rats are not well known and although there is some indirect evidence, it would be advisable to carry out studies to evaluate the changes.

neurochemical and genetic expression that could explain the beneficial effect on postural imbalance during the 3 weeks of treatment and the plastic changes involved in maintaining its long-term beneficial effect.

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