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Developmental effects of rotenone pesticide exposure on the rat nigro-striatal dopaminergic system

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Original article

SUMMARY

Rotenone is a pesticide used in Mexico, despite the experimental evidence showing dopaminergic neurons degeneration induced by this compound, which may lead to a psychomotor impairment. However, there are no studies about the possible effects of rotenone on the offspring when they are indirectly exposed through their mothers. In this study rotenone was administered to female rats during pregnancy and nursing, in order to assess its effects on the offspring's dopaminergic neurons as well as on motor coordination at 30 or 60 postnatal days.

In order to quantify the neurons that are immunoreactive to tyrosine hydroxilase of the substantia nigra six groups of pregnant Wistar rats were injected: intact (control), with rotenone solvent (vehicle), and four groups injected with the following doses of rotenone: 0.2, 0.4, 0.6 y 1.0 mg/kg/day. In a parallel experiment, the offspring of other groups of dams treated with rotenone 1 mg/kg/day, or controls were evaluated at the motor coordination test at 30 and 60 postnatal days.

Rotenone treated (1 mg/kg) dams showed a lower amount of dopaminergic neurons in the substantia nigra. This effect was also noticed in the offspring but at all doses of rotenone used, either at 30 or 60 postnatal days. Furthermore, the offspring of rotenone indirectly exposed dams significantly increased the time in which they accomplished the motor coordination test.

These data indicate that rotenone is able to damage the dopaminergic neurons of the offspring when exposed through their mothers. This effect in the offspring is noticed with lower rotenone doses than in adult rats. Therefore, those individuals indirectly exposed to rotenone could have less dopaminergic neurons at early stages of life, a fact that increases the risk of developing disorders related to the brains' dopaminergic system.

Key words: Rotenone, Parkinson's disease, attention-deficit hyperactivity disorder, tyrosine hydroxilase, substantia nigra, attention deficit hyperactivity disorder, motor coordination.

RESUMEN

La rotenona es un pesticida utilizado en México a pesar de que se ha demostrado experimentalmente que produce una degeneración de las neuronas dopaminérgicas, y puede derivar en deterioro psicomotor. Sin embargo, no existen estudios de la exposición indirecta a rotenona a través de las madres en el efecto que produzca sobre su descendencia. Nosotros administramos rotenona a ratas durante la gestación y la lactancia para evaluar las alteraciones producidas sobre las neuronas dopaminérgicas y la coordinación motora de sus crías, a los 30 o 60 días posnatales. Para cuantificar las neuronas inmunorreactivas a tirosina hidroxilasa de la sustancia nigra, se inyectaron subcutáneamente seis grupos de hembras Wistar: intactas (control), con solvente de rotenona (vehículo) y cuatro grupos con rotenona en dosis: 0.2, 0.4, 0.6 y 1.0 mg/kg/día. En un experimento paralelo, las crías de otros grupos de hembras tratadas con rotenona 1 mg/kg/día o controles fueron evaluados en la prueba de coordinación motora a los 30 y 60 días posnatales.

Las madres tratadas con 1 mg/kg de rotenona tuvieron menos neuronas dopaminérgicas en la sustancia nigra. Dicho efecto se observó también en las crías, pero con todas las dosis de rotenona utilizadas, tanto a los 30 como a los 60 días posnatales. Además, la exposición indirecta a rotenona aumentó significativamente el tiempo que requirieron las crías para ejecutar la prueba de coordinación motora.

Estos datos indican que la rotenona es capaz de inducir daño en las neuronas dopaminérgicas de las crías cuando son expuestas a través de sus madres. Este efecto en las crías se observa con dosis menores de rotenona que en ratas adultas. Por lo tanto, los individuos indirectamente expuestos a rotenona podrían tener menos neuronas dopaminérgicas desde etapas tempranas de la vida, lo que aumenta el riesgo de desarrollar trastornos relacionados con el sistema dopaminérgico.

Palabras clave: Rotenona, enfermedad de Parkinson, trastorno por déficit de atención con hiperactividad, sustancia nigra, tirosina hidroxilasa, coordinación motora.

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INTRODUCTION

Rotenone (ROT) is a compound extracted from the *Lonchocarpus* plants, which acts as a pesticide. Due to its natural origin, ROT is widely used as a plague-control insecticide in several crops, and as a piscicide for controlling certain fish species that may be undesirable in aquifers. Its high liposolubility enables it to easily cross the biological membranes. In the cells, ROT has a highly specific inhibitory effect upon Complex I of the mitochondrial respiratory chain.[1-3] In Mexico it is particularly used in the Pacific coast shrimp production, where ponds are 5.8%-ROT treated. Nevertheless, there are no studies of the impact that it may have in human health regarding occupational exposure or consumption. Dhillon et al.[3] analyzed the effect of ROT occupational exposure in a Texas town, finding an increase in the incidence of Parkinson's disease associated with this pesticide's use. However, the outlook in Mexico is still unknown.

Current evidence states that the exposure to pesticides such as ROT, maneb or paraquat causes degenerative injuries on brain dopaminergic neurons.[4-6] The reduction of this type of neurons is more likely to cause psychomotor effects on a Parkinson's disease type syndrome. Nonetheless, so far the effect of dopaminergic cells' degeneration on functional disturbances resulting in mental disorders has been scarcely studied. It has been found that ROT subcutaneous administration in adult rats results in the *nigrostriatal* dopaminergic system's degeneration, which leads to a psychomotor impairment, besides helping the aggregation of the α-synuclein protein in the synaptic terminals of such pathway.[1,7] The fact that the ROT induces these cytological markers of Parkinson's disease in rodents has made it an animal model of such disease. However, other brain dopaminergic nucleus could also be affected, although the functional study of the psychiatric disturbances they generate is particularly difficult to be assessed in animals.

In this article we study the effect of ROT exposure in very early stages of life on dopaminergic neurons in rats' substantia nigra (SN). The pesticide was given by the mother from the beginning of pregnancy through the end of nursing, which allowed studying its effect on the offspring, using as a reference the damage caused in mothers, since it has been presented in adult animals. Pregnant rats were assessed at the end of the 51-day treatment, while their offspring were assessed 30 and 60 days after birth (DAB). The assessment consisted in finding the number of neurons with positive immunoreactive for tyrosine hydroxilase (IR-TH) in the SN, which labels dopaminergic cells because the tyrosine hydroxylase (TH) is the limiting enzyme in the biosynthetic pathway of the dopamine. The reduction of IR-TH neurons is an indication of harm over this dopaminergic nucleus. Additionally, we assessed the effect on motor coordination through the Drucker's angle beam testing.[8]

MATERIALS AND METHODS

Subjects and procedures

Twenty-four 250g (8.81oz) female Wistar rats were divided into twelve boxes; two females were placed with a sexually expert male. After 48hrs it was verified —through the vaginal plug— that females were pregnant, a fact that determined the beginning of the ROT treatment. Pregnant female rats were placed in individual boxes and were kept in standard conditions with a 12/12-hour light-dark cycle reversal (turning lights off at 7am), at a constant (21 ± 1.0 °C) temperature and (55 ± 5%) humidity, with water and food *ad libitum*. Pregnant rats were divided into six groups (n=4 each): an intact control group that did not receive any treatment; a control group injected with 100 µl of a 1:1 solution of polyethylene glycol and dimethyl sulfoxide (PEG: DMSO) called "vehicle" group for only receiving the ROTs solvent (Cat. R8875, Sigma, San Luis, MO), and four experimental groups with different ROT doses: 0.2 mg/kg, 0.4 mg/kg, 0.6 mg/kg and 1.0 mg/kg, respectively.

Both vehicle and ROT were daily and subcutaneously administered to pregnant rats during the 21 days of pregnancy and the 30 days of nursing. All animals were treated in accordance with the (NOM-062-ZOO-1999) Official Mexican Standard or Norma Oficial Mexicana. At the end of the treatment the brains of the mothers and of the 30-day after birth (DAB) offspring were taken out. The offspring brains of 60 DAB were studied in order to have a group used to assess the 30-day ROT effect after the exposure is over. Brains were frozen at -20°C to make coronal resections of 40 µm. Sections were processed with antibodies against TH, with the purpose of immunohistochemically mark SN cells having this enzyme (dopaminergic neurons.

Immunohistochemical procedure for TH

Female rats and their offspring were anesthetized with a sodium pentobarbital overdose, 1 or 0.5 ml, respectively (Pfizer, Mexico City, Mexico) and a transcardial perfusion was performed with 200ml of a PBS (Phosphate-Buffered Saline solution: 0,1 M, pH 7.4, NaCl 130 mM, Na_2HPO_4 7 mM and NaH_2PO_4 3 mM) solution, and subsequently were fixed with 250 ml of paraformaldehyde at 4% dissolved in PBS solution. Brains were extracted and post-fixed in the same solution during 12hrs, and subsequently they were placed in a cryoprotector solution consisting in sucrose at 30% dissolved in PBS-4°C solution.

From the SN brain portion, according to the rat's brain stereotactic atlas,[9] 40 µm thick sections were obtained in a freezing microtome (Cryo-Cut American Optical).

As for the TH immunohistochemical, the brain slices were placed in free flotation in multi-well boxes and were washed up to three times with PBS during 10 minutes per

wash (PBS 3×10 min); they were incubated for one hour at 4°C in a PBS solution with 3% of Triton X-100 (Sigma) and 0.01% of bovine serum albumin (BSA; Sigma). Subsequently, the sections were incubated 12 hours in a PBS solution containing the rabbit polyclonal antibody against TH (1:1000; Biotecnología Santa Cruz); this incubation was kept under slight stirring conditions and 4ºC. The next day, after washed in PBS 3×10 min, the sections were incubated in PBS solution with a secondary biotynilated antibody (IgG of biotynilated rabbit diluted at 1:250; ABC Vector Burlingame, CA, USA) during 2 hrs at room temperature; after this time the sections were washed with PBS 3×10 min. In order to visualize the antigen-antibody union reaction, the sections were incubated 2 hrs in a single solution of avidin conjugated with horseradish peroxidase (ABC VectaStain Elite Kit Vector, Burlingame, CA, USA). After a 3×10 min wash with a Tris-HCl solution 0.1 M, pH 7.4, the sections were incubated 5 minutes with 0.1% of diaminobenzidine (Sigma), and then hydrogen peroxide (H_2O_2) was added at 0.24% in order to reveal the antibodies location. The reaction was monitored with a microscope, and when it was clearly visible it was stopped through PBS 3×5 min washes. Finally, the sections revealed were placed over a microscope slice. They were dehydrated with alcohols and xylol, and finally covered with Cytoseal XYL (Richard Allan – a scientist) and a microscope slice.

Immunohistochemical analysis

To quantify IR-TH neurons, SN limits were established according to the rat's brain stereotactic atlas,[9] starting in -5,20 and finishing at -6,30 mm of Bregma (approximately 800 im of the SN were assessed). Images were captured with a camera (Evolution VF Cooled Color Camera Medica Cybernetics) adapted to a microscope (Olympus IX-71, Japan) with a 4X and 20X magnification; once acquired they were analyzed with the Image ProPlus 6.0 program. IR-TH neurons were manually counted. The criterion to consider an IR-TH dopaminergic neuron was the finding of the completely marked cytoplasm, and the perfectly defined and not marked nucleus. Twenty brain sections of each animal were analyzed by two independent researchers. With these data the IR-TH neurons average was calculated for each one of the 10 animals; the results are the average of the IR-TH neurons of 10 subjects.

Motor coordination test

Thirty offspring subjects of 30 DABs divided into three groups: control, vehicle and ROT 1 mg/kg (n=10 each) were assessed at 30 and 60 DABs with the motor coordination test designed and validated by Drucker and García.[8] This device consists in a 2m [6.5ft]-long wooden beam, placed with a 15º-inclination, so that the rat can scale. Its home box is placed on the top section of the beam as a stimulus for the accomplishment of the test. Initially, the rats are trained to climb on a 24 mm [1 in]-wide beam during five days before doing the test; the test starts randomly changing the thickness of the beam in a 9, 6 and 3 mm wide. The rat is placed at the bottom of the beam and then allowed to walk until reaching the top end, recording the time in which it arrives. A maximum 120-secs limit is used, at the end of which if the rat has not reached its home box it is manually removed and placed in its box, receiving a 120-secs grade. The results were expressed as the average of the total time (secs) that the 10 subjects lasted out in each experimental condition to accomplish the test.

RESULTS

ROT effect in pregnant rats

Pregnant rats treated with ROT during 51 days had a less number of IR-TH neurons in the SN. Figure 1 (panels A, D, J, M and P) shows representative images of the areas corresponding to the SN, where a reduction of IR-TH neurons proportional to the increase of the ROT dose can be noticed. The mean of the number of IR-TH neurons in adult females without (control) treatment was 200.5, and in those treated with the vehicle was 160.5; the animals treated with ROT had values of 219.5, 171.5, 128.5 and 41.75 neurons by field, corresponding to the dose of 0.2, 0.4, 0.6, and 1.0 mg/kg of ROT, respectively (Figure 2).

The one-way analysis of variance (ANOVA) revealed a significant effect of the treatment ($F_{(5,18)}$=11.04; p<0.0001). Tukey's *post-hoc* test of multiple comparisons showed that the control, vehicle and 0.2 mg/kg of ROT groups had significant differences against the group that received the greatest dose of ROT (p<0.001).

ROT effect in the offspring

The number of IR-TH neurons in the SN of the offspring showed a pattern similar to the mothers treated with ROT: the IR-TH neurons diminished as the ROT dose increased; this effect was found in the 30-DAB offspring indirectly exposed *in utero* and during nursing at ROT. Figure 1 (panels B, E, H, K, N and Q) shows the SN of these animals where the reduction in the IR-TH neurons population is noticed. A similar ROT effect was found in the animals of 60 DAB, which had been 30 days without being exposed to the xenobiotic.

Figure 1 (panels C, F, I, L, O and R) shows the effect of ROT on the IR-TH neurons of such animals.In 30-DAB animals the average values of the number of neurons under control and vehicle conditions were 190.7 and 161.7, while in the groups treated with the different ROT doses were

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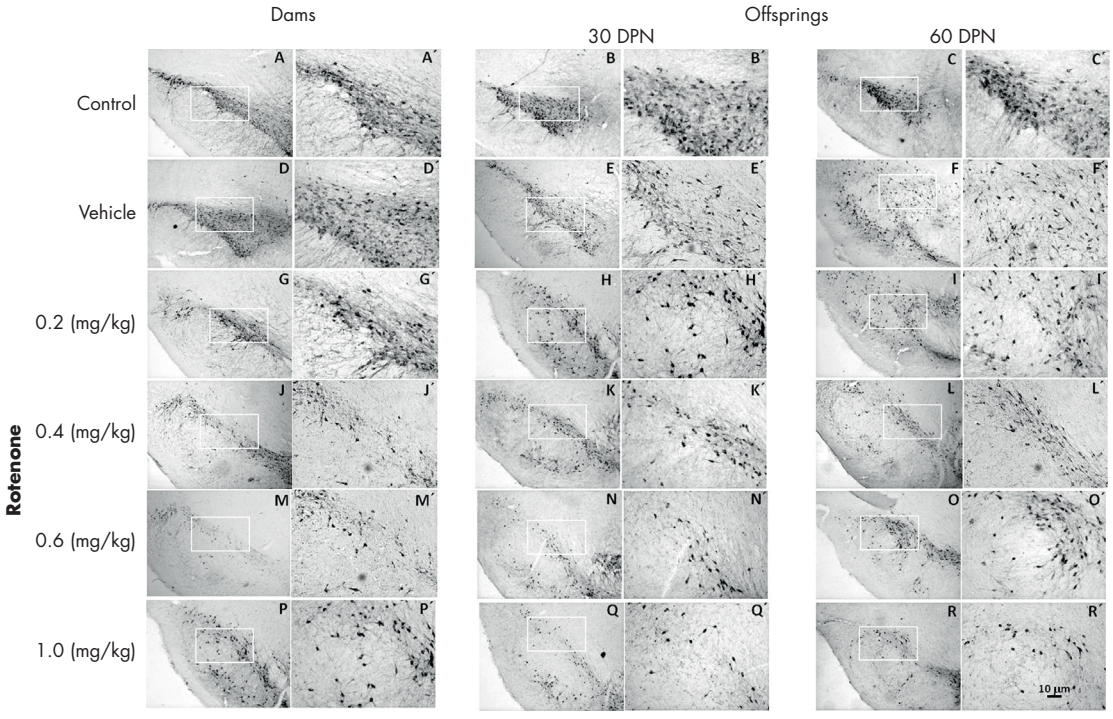

Figure 1. Representative photographs of the substantia nigra of mothers treated with different ROT doses and their offspring. The first two columns (letters A, D, G, J, M and P, and those marked with an apostrophe ') present the mother rats tissue at 4X (left) and 20X (right) magnifications, where a lower density of IR-TH neurons can be noticed as the ROT dose is increased. Panels B, E, H, K, M and P present substantia nigra of the 30-day after birth (DAB) offspring with the same magnification: (4X left, and 20X right). Panels C, F, I, L, N and Q present the substantia nigra of the 60-day after birth (DAB) offspring with the same magnification (ibid). The right-hand side photographs show the representative area of the substantia nigra in which the IR-TH neurons counting was carried out. The bar is shown in a 10 µm scale.

135, 102.9, 75.18 and 56.5, respectively (Figure 3A). The one-way ANOVA showed significant differences of the treatment ($F_{(5,54)}$=1268; p<0.0001). Tukey's *post-hoc* test of multiple comparisons revealed that the control and vehicle had differences with all groups treated with ROT (p<0.001). The density of IR-TH neurons diminishes in a similar way in 60-DAB offspring; the average values of the number of neurons under control and vehicle conditions were 191 and 165, respectively, while in the groups treated with the different ROT doses were 106.8, 65.35, 56.38 and 32.52, respectively (Figure 3B). The one-way ANOVA showed significant differences due to the treatment ($F_{(5,54)}$=344,1; p<0.0001), and Tukey's *post-hoc* test revealed differences between the control and vehicle groups, and all groups treated with ROT (p<0.001).

ROT effect on the motor coordination test

The results obtained when assessing the offspring at 30 and 60 DAB in the motor coordination test are shown in Figure 4. Offspring exposed to 1mg/kg of ROT took longer for the accomplishment of the motor coordination test. As shown in Figure

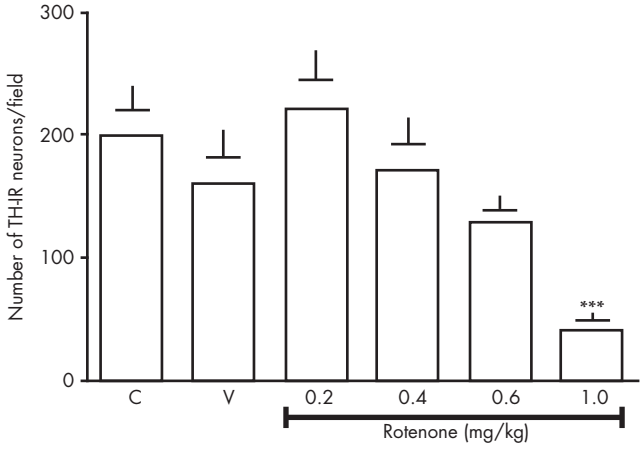

Figure 2. Number of SN IR-TH neurons of adult rats. The graph shows the 51-day ROT treatment effect on these rats. Each bar represents the average ± the standard error of the number of IR-TH neurons by field, obtained from 20 sections per subject of each group (n = 4). Asterisks (***) show the statistical significance group (p<0.0001). C = control group; V = vehicle; and the bar with the Rotenone sign represents the animals treated with different ROT doses.

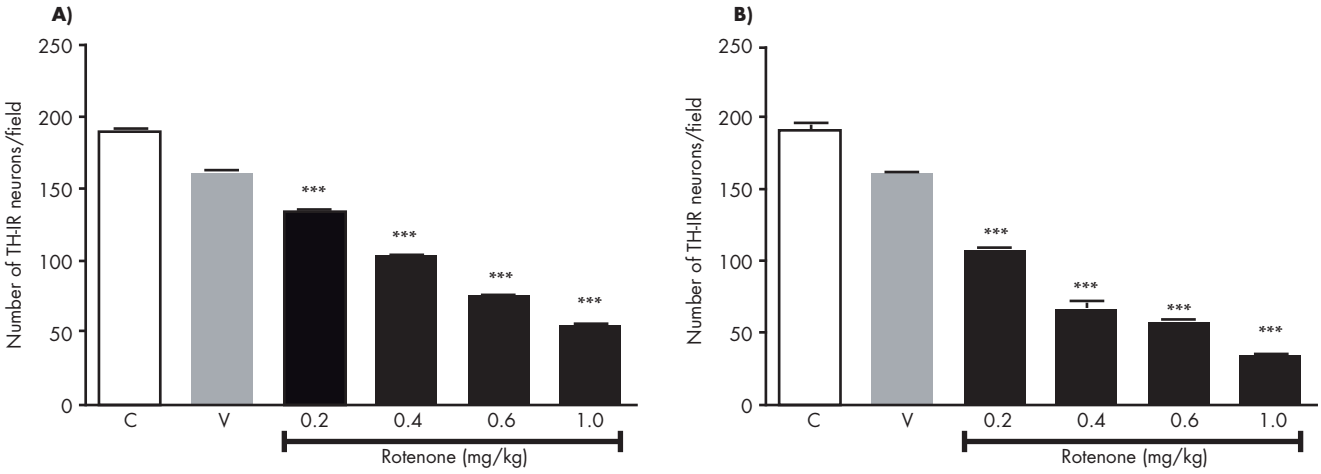

Figure 3. Number of SN IR-TH neurons. Effect of the ROT in utero and nursing exposure in 30-DAB (A) and in 60-DAB offspring (B). Each bar represents the average ± the standard error of the number of IR-TH neurons of the substantia nigra by field, obtained from 20 sections per subject of each group (n = 10). Asterisks (***) represent the significant differences (p<0.0001) obtained between the groups C = control, V = vehicle vs. the groups exposed to the different ROT doses.

4A, for 30-DAB offspring the total time (secs) increased for carrying out the test as the thickness of the bar diminished from 9 to 6 and 3 mm. The analysis of variance showed a significant effect of the ROT treatment ($F_{(8,81)}$=478; p<0.0001). Tukey's *post-hoc* test of multiple comparisons showed that the offspring exposed to 1mg/kg of ROT, significantly increased the time for accomplishing the test as compared to the control and vehicle offspring (p<0.001). Figure 4B shows that the deleterious effect continues on the motor coordination in these same offspring at 60 DAB ($F_{(8,81)}$= 344.1; p<0.0001). Tukey's *post-hoc* test showed significant differences (p<0.001) on the total time (secs) they take in accomplishing the test under the same experimental conditions.

DISCUSSION

There are multiple environmental factors that may cause a variety of toxic effects in the organism. Different studies show that exposure to xenobiotic such as metals,[10] pesticides including ROT,[1,4] fungicides and herbicides such as maneb and paraquat,[11-13] among many others, may generate damage to the nervous system.[14] Grandjean and Landrigan[10] found that human fetus exposure to industrial chemicals caused damages in the brain during the neurodevelopmental process that could predispose it to several neurological or psychiatric diseases in the long-term.

To our knowledge there are no previous studies dealing with the *in utero* effects of ROT, hence we assess in Wis-

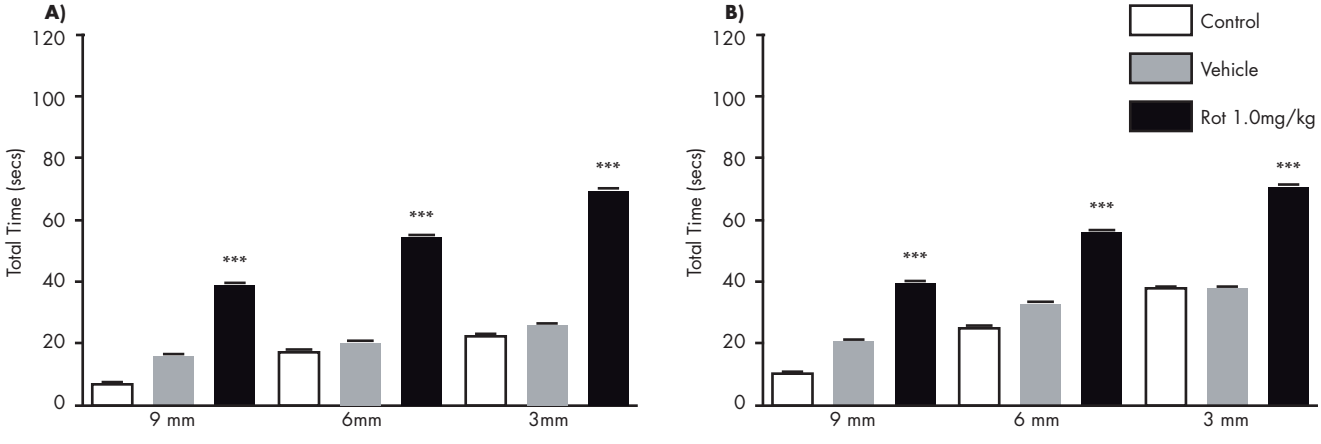

Figure 4. Time used to accomplish the motor coordination test. Panel A shows the animals assessed at 30 DAB and panel B the same offspring assessed at 60 DAB. The offspring exposed to ROT (1 mg/kg) had significant differences (*** p<0.05) compared to the control and vehicle groups. The abscissa axis indicates the thickness of each bar in which the animals were assessed.

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tar rats the exposure to this pesticide, during pregnancy and nursing, regarding the number of their offspring dopaminergic neurons and motor coordination. The experimental design contemplated two postnatal moments for investigating the changes occurred in the offspring brains: one at the end of indirect ROT exposure, and the other 30 days after the end of treatment, in order to determine whether there were changes related to the brain development.

ROT subcutaneously administration in the mothers was used as the damage control on dopaminergic cells, since the mothers and offspring were exposed to the same blood ROT concentrations. Our findings indicate that mother rats had a significant reduction in the number of immunoreactive neurons to TH (dopaminergic neurons) in the SN, but only with the 1.0 mg/kg dose. Our results replicate the findings previously reported by Betarbet et al.,[1] who treated adult male rats with 2mg/kg of ROT during two weeks and noticed a reduction in the number of IR-TH neurons. In all previous studies higher doses than 2 mg/kg of ROT have been used, and results similar to ours have been obtained.[1,7,15-18] In our experimental conditions we did not notice significant effects at lower doses, therefore, we propose that 1 mg/kg of ROT should be the minimum effective dose to cause a significant damage on dopaminergic neurons in the SN of adult rats.

A previous study had suggested that female rats are pesticide resistant such as maneb and paraquat;[19] however, our results do not indicate any female rat special resistance to ROT, maybe because the latter affects metabolic pathways different than the pathways affected by maneb and paraquat in their neurotoxic action.[20]

The primary concern of our work was exploring the damage that ROT could cause on SN dopaminergic neurons during the offspring development. Our results show that ROT doses administered through the mother caused a significant reduction in the number of dopaminergic neurons (IR-TH). This is in contrast to the effect noticed in the mothers, in which only the highest ROT dose caused a significant decrease. This difference could be because the neuronal phenotype is defined in the mothers, which confers resistance to the neurotoxin effects, while in the offspring the neuronal phenotype is not committed and the process of differentiation makes them vulnerable to damage provoked by external factors.[21] Furthermore, it seems that the offspring postnatal brain development is also an important factor for the ROT effect, since the 60-DAB animals, which were 30 days without exposure, showed a further reduction in the number of IR-TH neurons as compared to the 30-DAB animals, which had ended their exposure to the pesticide when assessed. This can be attributed to ROT accumulation in the adipose tissue, which could be subsequently released in a tonic way in the offspring.

Although both offspring groups were exposed to ROT during the same time (the difference between them lies in the offspring that remained without exposure during 30 days), the apparently higher damage provoked by ROT on the 60-DAB offspring neurons was not correlated with the results of the motor coordination, since there were no significant differences between the animals tested as of 30 days and those tested as of 60 days of age, regarding the accomplishing time to climb through the angle beam (Figure 4).

During fetal stage the brain is sensitive to those damages that could be caused by several external factors; during this period, the protection offered by the placenta is limited, especially against highly-liposoluble substances such as ROT. Likewise, the blood–brain barrier in fetuses is not completely developed since it finishes its development after birth,[22] and because the brain keeps developing during post-natal stage, the vulnerability period is extended. It has been reported that newborn mice exposed to lower doses of neurotoxic agents, such as paraquat insecticide, do not show immediate effects; nonetheless, in adulthood they present behavioral changes and learning deficiencies, which have been interpreted as a consequence of brain damage provoked by the early exposure to this substance.[11,23]

Barlow et al.[11] proved that the presence of pesticides during the prenatal and perinatal period causes the reduction in the number of dopaminergic neurons and increases these neurons' sensitivity to the degeneration due to subsequent damages occasioned by other environmental or age-related factors.[24-26] Our results seem to support their hypothesis.

It has been suggested that children accidentally exposed —during fetal development— to pesticides are at an increased risk to develop the Attention Deficit Hyperactivity Disorder (ADHD), especially in cases in which the exposure is made through food treated with pesticides.[27,28] ADHD is closely related to dopaminergic system disorders; Warton suggests that children who suffer from this may have the dopamine release altered at the nigrostriatal terminals.[29] Our study suggests that ROT exposure by maternal transmission could have a causal relationship with these observations.

Additionally, it has been documented that the exposure to pesticides —among which ROT is included— increases the incidence of Parkinson's disease on exposed individuals.[3,4,30] On the other hand, Whatley has proposed an indirect physiopathologic mechanism for the development of schizophrenia, since ROT may affect the expression of Mitochondrial genes in dopaminergic neurons and this, in turn, could make easier the development of such disease.[31] Actually, among the ROT effects is the reduction of serotonin in the hippocampus, which facilitates depression-related behaviors that have been explored in animal models.[32]

In summary, ROT is capable of inducing neuronal damage before birth; individuals exposed during their development could have a lower number of dopaminergic neurons from the early stages of life, which would make them more vulnerable to other harmful factors, thus increasing the risk to develop —in early years— disorders related to dysfunctions of the dopaminergic system such as ADHD, or late alterations like Parkinson's disease, without disregarding other types of functional or psychiatric disorders.

In Mexico, as far as we know, there is very few knowledge about the association between environmental xenobiotic and neurological disorders in individuals exposed during early stages of life. Supposedly in Mexico there are approximately 1.5 million of children under the age of eighteen who suffer from ADHD, and most of them have not been diagnosed.[33] It is likely that an important number of individuals are affected due to the *in utero* exposure to environmental toxins.[13]

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