ROLE OF STEM CELLS AND PRAMIPEXOLE IN COUNTERACTING ROTENONE NEUROTOXICITY

BY

Seham Gad ElHak, Abdel Aziz Ghanem, Hasan Abd Elghaffar*, Sahar Abd ElAziz ElDakroory, Dina ElTantawy and Mohamed Salama**

Departments of of Forensic Medicine and Clinical Toxicology, Clinical Pathology, Pathology**, Faculty of Medicine, Mansoura University, Egypt*

ABSTRACT

Rotenone is one of the pesticides which thought to have neurotoxic effect that could potentially play a role in the development of Parkinson's disease (PD). Environmental exposure to this pesticide is supposed to be contributed to the increased incidence of PD. This study was done to evaluate rotenone neurotoxicity and the curative role of pramipexole and stem cells therapy in mice. Forty male BALB/c mice were used and divided into 4 equal groups. The control group (G.1) received only carboxymethyl cellulose orally once daily at a volume of 10 ml/kg. The second group was given a daily rotenone oral dose of 30mg/kg for 28 days. The third group was given oral rotenone (30mg/kg/d for 28 days) then Pramipexole was started from the15th day in a dose of 1 mg/kg/d orally for 14 days with continuing the rotenone course. The fourth group received rotenone (30mg/kg/d orally for 28 days) and in the 15th day 1X10^{5} of Wharton jelly derived mesenchymal stem cells (WJCs) were given intrathecally and then they completed the rotenone course. At the 23rd day all the animals were subjected to behavioral test for evaluating the degree of PD development. At the end of the 28 days all animals were sacrificed by overdose of phenobarbital and their brain were subjected to immunohistochemical analysis for dopaminergic neurons staining for anti TH antibodies. Behavioural test showed improvement of mice activity in the pramipexole group (18 seconds). Also intrathecal stem cells administration to the mice improved their test performance to reach about 12 seconds. Immunohistochemistry results revealed that the rotenone-induced loss of TH-immunopositive neurons in the SNpc was inhibited by the pramipexole treatment . Intrathecal stem cells administration had also improved the neuronal loss. In conclusion ,the results of this study revealed the neuroprotectant and regenerative capacities of pramipexole and stem cell therapy in improving the rotenone intoxicated mice. So, they could be potential therapeutic approaches in rotenone neurotoxicity specifically toxic parkinsonism.

INTRODUCTION

Environmental toxins have been shown to contribute to the increasing incidence of Parkinson's disease (PD). Pesticides, which represent one of the primary classes of environmental agents associated with PD, share the common feature of being in-

tentionally released into the environment to control or eliminate pests. Pesticides consist of multiple classes and subclasses of insecticides, herbicides, rodenticides, fungicides, fumigants and exhibit vast array of chemically diverse structures (Hatcher et al., 2008).

Humans have used rotenone-containing plants as pesticides for centuries (Cabras et al., 2002). As rotenone is plant-derived, it has been considered an "organic" pesticide, and was commonly used as a household insecticide, (in home & gardening), agriculture, and to kill fish. The ubiquitous use of rotenone in both work and home settings that occurred until recently suggests that many people may have been exposed to this environmental contaminant (Caroline et al., 2011).

Parkinson's disease is a common neurodegenerative disorder, characterized by relatively selective degeneration of dopaminergic neurons in the substantia nigra. Epidemiological studies indicate that pesticides are the leading candidates of environmental toxins that may contribute to the pathogenesis of PD (Tanner et al., 1989; Jimenez-Jimenez et al., 1992; Semchuk et al., 1992; Gorell et al., 1998; Betarbet et al., 2000; Di Monte et al., 2002; Baldi et al., 2003; Di Monte, 2003).

Pramipexole is a selective dopamine D2 receptor agonist, approved since 1997 in the US and most European countries. Pramipexole is indicated for the symptomatic treatment of idiopathic Parkinson's disease (PD), either alone or in combination with levodopa (Antonini and Calandrella, 2011).

Stem cells have been the subject of increasing scientific interest because of their utility in numerous biomedical applications. Stem cells are capable of renewing themselves; that is, they can be continuously cultured in an undifferentiated state, giving rise to more specialized cells of the human body such as heart, liver, bone marrow, blood vessel, pancreatic islet, and nerve cells. Therefore, stem cells are an important new tool for developing unique, in vitro model systems to test drugs and chemicals and a potential to predict or anticipate toxicity in humans (Davila et al., 2004).

Stem cells can be classified into two major categories, according to their developmental status: embryonic and non-embryonic, or adult, stem cells. Embryonic stem (ES) cells are pluripotent cells, isolated from the inner cell mass of the blastocyst-stage mammalian embryo (Nagy et al., 1990). Pluripotent cells are capable of giving rise to most tissues of the organism, including the germ line during development.

Adult stem cells (ASCs), also known as

mesenchymal stem cells (MSCs) or multipotent adult progenitor cells (MAPCs), are specialized cells found within many tissues of the body where they function in tissue homeostasis and repair. Multipotent cells are precursor cells capable of differentiation into several different cell types but not all cell types in the organism like pluripotent cells (Davila et al, 2004).

AIM OF THE WORK

The aim of this study is to assess the neurotoxicity of rotenone and study the role of pramipexole and stem cells in counteracting this toxicity.

MATERIAL AND METHODS

Material:

a) Chemicals:

1- Rotenone (white fine powder) was purchased from Sigma (St. Louis, MO- USA).

2- Carboxymethyl cellulose (CMC) [white granules] was obtained from El Gomhourya Company (Mansoura, Egypt).

3- Mouse monoclonal antibodies against tyrosine hydroxylase (TH) were purchased from Sigma (St. Louis, MO- USA).

4- Biotinylated secondary antibodies for TH staining, avidinbiotin-peroxidase complex (ABC) solutions, diaminobenzidine (DAB) were obtained from Pathology Department-Mansoura Faculty of Medicine.

5- Phenobarbital (anaesthesia), phosphate buffered solution (PBS), paraformaldehyde (PFA) Low glucose Dulbecco's modified eagle medium (LG-DMEM), Trypan blue: were obtained from Medical Experimental Research Center (MERC) of Faculty of Medicne- Mansoura University.

b) Equipment:

The vertical grid apparatus: was made according to its specified dimensions (Kim et al., 2009).

Flowcytometry (Coulter Epics XL). Stereoinvestigator system and optical density measurements (Leica Q-win system).

c) Biological samples:

The umbilical cords were obtained from Elsherbiny Obstetrics Hospital, Damietta after taking consent of the mothers before delivery.

d)Animals:

Eight-month-old male BALB/c mice of average weight 20-25 g were purchased from vacsera animal house (Cairo, Egypt). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of

Laboratory Animals.The protocols of the research was approved by the Ethical Committee for Research at Mansoura University.

Study groups:

The mice were divided into 4 groups (10 mice each):

Group (1): control group, 10 mice received 0.5%carboxymethyl cellulose orally once daily at a volume of 10 ml/kg body weight.

Group (2): rotenone group, 10 mice received 30mg/kg rotenone by oral gavage daily for 28 days (Inden et al., 2009). Rotenone was suspended in 0.5% carboxymethyl cellulose sodium salt.

Group (3) : rotenone plus pramipexole group,10 mice received the rotenone for 28days, from 15th day on the animals received oral pramipexole in a dose of 1 mg/kg orally/ day for 14 days with continuing the toxin course (Alvarez-Fischer et al., 2007).

Group (4) : rotenone plus Intrathecal stem cells group, 10 mice received the rotenone dose (30mg/kg through oral gavage daily for 28 days). In the 15th day the animals received $1X10^5$ of Wharton jelly derived mesenchymal stem cells (WJCs) which were isolated according to Seshareddy et al. (2008) through intrathecal route (De Lacalle and Paino, 2002) , and then they completed the toxin course.

Methods:

Evaluation of rotenone neurotoxicity:

a) Behavioural test "Vertical grid test" (Kim et al., 2009):

At the 23rd day all the animals were subjected to behavioural test for evaluating the degree of toxic PD as follows:

The vertical grid apparatus is an open box of 8cm x 55cm x 5cm, set vertically. The back side of the vertically standing box is made of a wire mesh of 0.8cm x 0.8 cm, the front side is open, and the other four sides are made of black plexiglass. For stability, the bottom of the apparatus has a 5cm extension to the front. In the experiment, each mouse was carefully placed inside the apparatus at 3cm from the top, facing upward, and was left free to turn around and climb down. The trials were videotaped. The videos were replayed for recording the total time taken for the mouse to make a turn, climb down, and reach the floor by its forepaw (Figure 1).

b) Immunohistochemistry:

At the end of the 28 days, the mice were perfused through the aorta with 50 mL of 10 mM phosphate-buffered saline (PBS), followed by 150 mL of a cold fixative consisting of 4% paraformaldehyde, 0.35% glutaraldehyde and 0.2% picric acid in 100 mM phosphate buffer (PB), under deep

anesthesia with phenobarbital (100 mg/ kg, i.p.). After perfusion, the brain was quickly removed and postfixed for 2 days with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4°C. The brain pieces were cut using a cryostat and collected in 100 mM PBS containing 0.3% Triton X-100 (PBS-T). After several washes, the sections were stored until use in a free-floating state at 4°C for immunohistochemical analysis.

Brain slices were incubated with primary mouse monoclonal anti-TH antibody (diluted 1:10,000; Sigma) for 3 days at 4°C. After several washes, sections were incubated with biotinylated anti-mouse IgG antibody (1:2000), as appropriate, for 2 h at room temperature. The sections were then incubated with avidin peroxidase for 1 h at room temperature. All of the sections were washed several times with PBS-T between each incubation, and labeling was then revealed by 3,3'- diaminobenzidine (DAB). Slides were counterstained with Meyer`s hematoxylin, dehydrated and cover slipped. The resulting slides were examined under microscope to evaluate the degree of neurodegeneration. The same slides were exposed to stereological analysis as described below.

c) Stereological analysis of DA neurons in the ventral midbrain (Höglinger et al., 2007):

TH-immunopositive neurons in the substantia nigra pars compacta (SNpc) were estimated using stereological counts of cell numbers, on a Stereo-investigator system and optical density measurements on a Leica Q-win system). Six sections (30 μm-thick), from the anterior to the posterior midbrain, were used for counting in each case. The total number of TH-immunopositive neurons was estimated using the optical fractionator method.

Statistical Methods:

All data were given as the mean ± standard error of the mean (SEM). Two groups of data were analyzed by the Student's t-test. Three groups of data were analyzed by ANOVA with a Tukey post hoc test. For all tests, $p \leq 0.05$ was deemed significant.

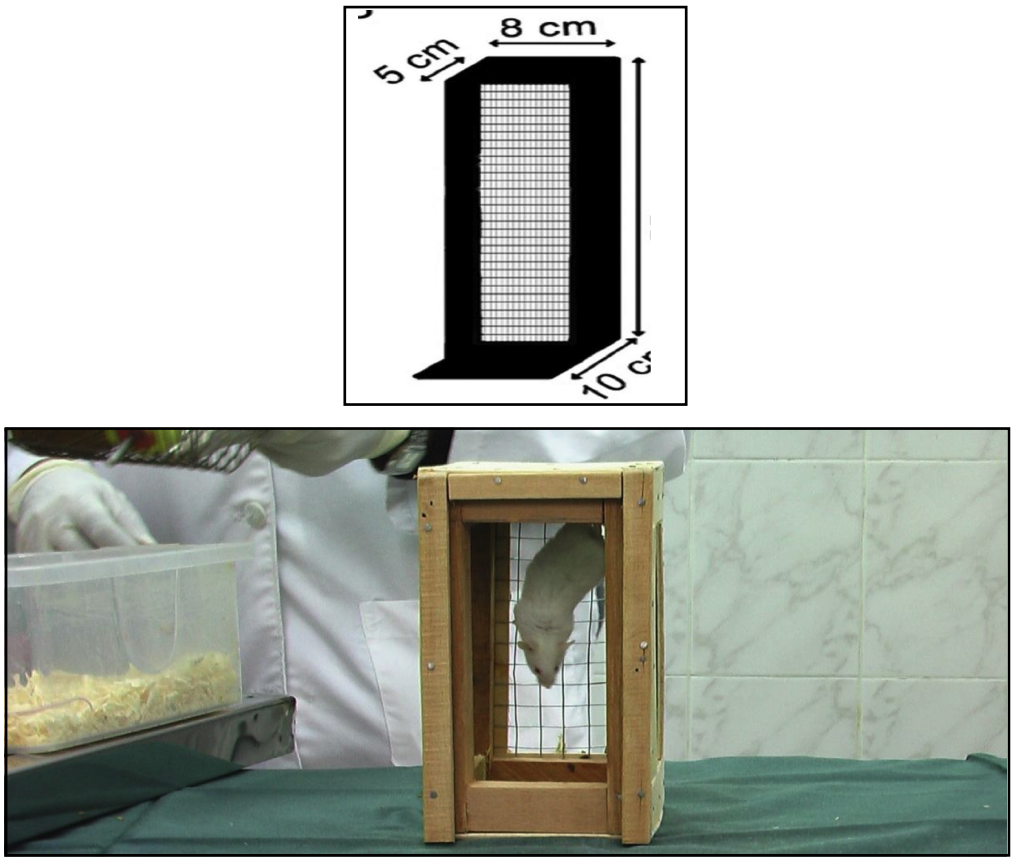


Figure (1) : The vertical grid apparatus; above as specified by Kim et al. (2009); below in Medical Experimental Research Center.

RESULTS

Effect of rotenone on nigrostriatal DA neurons in mice

As shown in (Figure 2B), the oral administration of rotenone at 30 mg/kg for 28 days obviously reduced the number of TH-immunopositive neurons in the SNpc. Stereological analysis of nigral TH-immunopositive neurons showed that rotenone caused a significant loss of DA neurons (Table 1).

Effect of rotenone on locomotor coordination in mice.

The control mice usually take about 10 seconds to complete the vertical grid test. On the other hand the mice of the rotenone group has taken more than 80 seconds to complete the test (Table 2).

Effect of pramipexole and stem cells on nigrostriatal DA neurons in rotenone group mice

On investigating whether treatment with pramipexole (oral 1 mg/kg/ day for 14 days) can protect DA neurons from damage caused by the chronic oral administration of rotenone. The rotenone-induced loss of TH-immunopositive neurons in the SNpc was significantly inhibit-

ed by the pramipexole treatment ($p<0.001$) (Figures 2B and 2C; Table 1).

Intrathecal stem cell administration has also improved the condition as the rotenone induced loss of TH+ve cells was nullified by coadministration of stem cells ($p<0.001$) (Figure 2D and Table 1).

Effect of pramipexole and intrathecal stem cells on locomotor coordination in rotenone mice.

In this study the control group usually take about 8 seconds to complete the vertical grid test. On the other hand, mice of the rotenone group has taken more than 80 seconds to complete the test. On studying the effect of treatment, it can be seen that mice of the pramipexole group take about 18 seconds to complete the test which represents a significant improvement in their activity($p<0.001$). Also stem cells administered intrathecally to the mice improved their performance in the test to reach about 12 seconds ($p<0.001$) (Table 2).

DISCUSSION

There are several subclasses of insecticides, many of these compounds are designed to be neurotoxic. Similarities between the insect and human nervous systems can lead to cross-toxicity of these compounds (Hatcher et al., 2008).

Rotenone is a naturally occurring pesticide derived from the roots of Derris elliptica and it is known to be a high-affinity specific inhibitor of mitochondrial complex I (Monti et al., 2009).

The possibility of a role of rotenone in PD due to its ability of inhibiting mitochondrial complex I (NADH dehydrogenase), has been raised (Hatcher et al., 2008). Betarbet et al. (2006) added its possible effect on α-synuclein and proteasomes system.

In our study, the brain of the rotenone exposed mice revealed degeneration of dopaminergic neurons by the immunohistochemical analysis. This is in accordance to the studies of Inden et al. (2009) and Takeuchi et al. (2009) who used the same course of treatment inspite of the difference regarding the mice strain.

On exposure to the vertical grid test, the intoxicated animals showed a significant longer time to return and go down through the grid which would be a success to this new assessment method especially in the case of rotenone where mild to moderate damage is expected in the dopaminergic neurons, so, we need a very sensitive test. This sensitivity appeared in the vertical grid test on accordance with the findings of Kim et al. (2009).

Earlier studies with rotenone exposure

found minimal nigrostriatal damage (Thiffault et al., 2000) or found damage to striatal dopamine fibers but not to nigral dopamine neuronal bodies (Ferrante et al., 1997). Other studies showed that chronic and subcutaneous administration of rotenone could result in a parkinsonian syndrome with selective dopamine neuron degeneration, oxidative damage and cytoplasmic inclusions reminiscent of early Lewy bodies (Betarbet et al., 2000). The damage reported in these studies was seen in the striatum first, followed by the SNpc (this is similar to the 'dying-back' phenomenon in PD); however, these changes were seen only in a subset of exposed animals. Additionally, increased oxidative stress, ubiquitin accumulation, proteasomal inhibition and inflammation all have been observed in response to rotenone exposure (Sherer et al., 2002; Liu, et al., 2003; Wang et al., 2006).

Concerning stem cell therapy in this study,transplantation of WJCs was associated with marked reduction of rotenone-induced neurodegeneration, as reflected by the increase in number of TH-positive nigral cell bodies in lesioned animals that received the WJCs graft. This finding confirms other data, showing that transplantation of mesenchymal stem cells (MSCs) (WJCs or bone marrow derived) - of either human or rodent origin - exerts protective and/or regenerative effects on nigrostriatal neurons (Bouchez et al., 2008; Levy et al., 2008).

Similar results, although in a different experimental context, have been reported by Park et al. (2008). In this case, the authors administered hMSCs, i.v., to rats treated, several weeks before, with a proteasome inhibitor. Proteasomal inhibition was associated with a substantial loss of TH-positive (dopaminergic) neurons in the substantia nigra pars compacta (SNpc), which was markedly reduced in rats infused with hMSCs.

In line with previous results (Bjiorklund et al., 2002; Ben-Hur et al., 2004; Blandini et al., 2010), stem cells graft also induced significant behavioral effects. The time taken by the mouse to turn around and go down in the vertical grid apparatus, was dramatically reduced in lesioned animals transplanted with WJCs. It can be concluded that transplantation of WJCs counteracts the progressive degeneration of the nigrostriatal pathway caused by specific neurotoxins, and associated motor abnormalities, even when the neurodegenerative process has already been set in motion and has reached a medium/advanced stage.This supports the protective potential of MSCs against neurodegeneration (Torrente and Polli, 2008).

As regard pramipexole therapy, the simultaneous daily administration of oral

rotenone and pramipexole prevented the decrease in number of dopaminergic neurons In the substantia nigra (SN). Stereological assessment of the number of TH positive cells in the bilateral SN confirmed the histological findings with statistical significance.

The use of stereology enriched the work through quantifying the number of dopaminergic neurons. Moreover, the stereology helped eliminating the subjectiveness in decision made by different pathologists through depending on the optical fractionator computerized system hence the name "unbiased cell count".

Another sign of pramipexole neuroprotective effect was observed through preventing the motor impairment elicited by rotenone as can be seen from grid test results.

These findings of neuroprotectant effect of pramipexole were in accordance to the work of Inden et al. (2009), and could be explained by the works of Riaz and Bradford (2005) and Winner et al. (2009) regarding the potency of pramipexole to induce neurogenesis and dopaminergic differentiation of NSCs. Also, Inden et al. (2009) found another suggested roles of pramipexole in neuroprotection such as: scavenging of -OH- and induction of B-cell lymphoma 2 (Bcl-2) protein.

It can be concluded that the exogenous administration of WJCs proved to have neuroprotective and regenerative effects as can be seen in the histopathological studies. So, stem cells transplantation could be a successful promising therapy in rotenone neurotoxicity specifically toxic parkinsonism which can serve in regeneration of damaged neurons and improving the patients' clinical condition. Also, pramipexole was tried aiming at stimulation of dopaminergic differentiation of NSCs. This approach proved to have neuroprotective effect as can be seen in rotenone toxicity.

Table (1): Stereological cell counts in substantia nigra of mice of the studied groups.

Control group	Rotenone group	Rotenone + pramipexole group	Rotenone + IT stem cells group
19700 ± 120	10000 ± 56*	19300 ± 88**	20000 ± 230***

IT: Intrathecal

*p ≤0.05 compared to the control group.

** p < 0.001compared to the rotenone group.

*** p < 0.001compared to the rotenone group.

Table (2): Vertical grid test results of the studied groups.

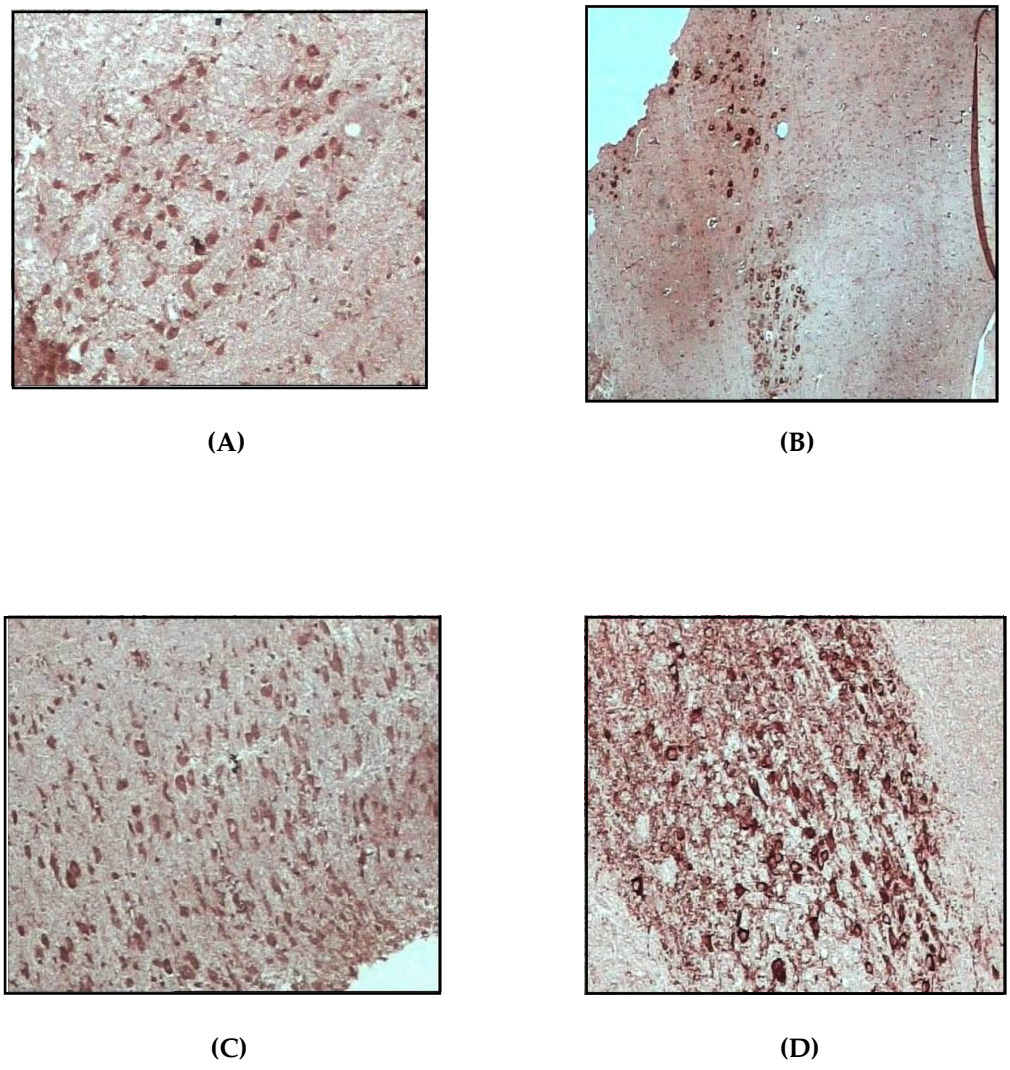
Groups	Control group	Rotenone group	Rotenone + pramipexole group	Rotenone + IT Stem Cells group
Total time to climb down (seconds)	10.9 ± 2.4	83.7± 6.8*	18.7± 3.4**	12.4± 3.5***

IT: Intrathecal

*p ≤ 0.05 compared to the control group.

** p < 0.001compared to the rotenone group.

*** p < 0.001compared to the rotenone group.



(A) **(B)**

(C) **(D)**

Figure (2) : Tyrosine hydroxylase immunohistochemistry of the control mice(2-A), the rotenone group (2-B), the rotenone+ pramipexole group (2-C) and the rotenone+ IT stem cells group (2-D). Medium magnification(*20) images in dorsolateral region of nigra show cell loss in this particularly vulnerable area in the rotenone group (2-B) with regeneration in the rotenone+pramipexole group (2-C) and the stem cells treated group (2-D).

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دور الخلايا الجذعية وعقار البراميبكسول في إبطال مفعول التسمم العصبي للروتينون

المشتركون فى البحث

أ.د.سهام جاد الحق — أ.د.عبد العزيز غانم
أ.د.حسن عبد الغفار* — أ. م. د.سحر عبد العزيز الدكروري
أ. م. د. دينا الطنطاوي — **د. محمد سلامة****

من أقسام الطب الشرعي والسموم الاكلينيكية، الباثولوجيا الإكلينيكية*، الباثولوجيا**

كلية الطب – جامعة المنصورة

يعتبرالروتينون من أحد المبيدات الحشرية المعروفة بتأثيرها السمي العصبي التي تلعب دوراً هاماً في تطور مرض الشلل الرعاش (داء باركينسون).حيث أن التعرض البيئي لهذا المبيد يُعد من العوامل المشاركة في زيادة معدل حدوث هذا المرض.وقد أجريت هذه الدراسة لتقييم التسمم العصبي للروتينون والدور الشفائى لعقار البراميبكسول والخلايا الجذعية في الفئران.أُستخدم فى هذا البحث أربعون فأرا تم تقسيمهم إلى أربع مجموعات متساوية كالتالي؛ المجموعة الأولى: مجموعة ضابطة تم إعطائها الكاربوكسي ميثيل سيليلوز(١٠مجم/كجم يومياً)عن طريق الفم لمدة ٢٨ يوماً. المجموعة الثانية: تم إعطائها الروتينون (٣٠ مجم/كجم يوميا) عن طريق الفم لمدة ٢٨ يوما. المجموعة الثالثة: تم إعطائها الروتينون (٣٠ مجم/كجم يوميا) عن طريق الفم لمدة ٢٨ يوما و أيضا تم اعطائها عقار البراميبيكسول(١مجم/كجم يوميا) بداية من اليوم الخامس عشر عن طريق الفم لمدة أربعة عشر يوماً.المجموعة الرابعة: تم اعطائها الروتينون (٣٠ مجم/كجم يوميا) عن طريق الفم لمدة ٢٨ يوما، وفى اليوم الخامس عشر تم إعطائها ٥١٠X١ من الخلايا الجذعية المستخرجة من الحبل السرى(عن طريق الحقن داخل القراب) مع الروتينون. وفى اليوم ٢٣ تم عمل اختبار سلوكي للحيوانات لمعرفة تأثير الروتينون على تطور أعراض داء باركينسون .وفي نهاية الثماني والعشرين يوماً تم حقن الفئران بمادة الفينوباربيتال وتشريحهم واستخراج عينات المخ وحفظها ثم التحليل الهستوباثولوجى المناعى لها ضد الأجسام المضادة للخلايا الدوباميينية. وقد أظهر الإختبار السلوكي تحسن في نشاط الفئران لمجموعتي البراميبكسول (١٨ ثانية) والخلايا الجذعية (١٢ ثانية).وأوضحت أيضاً نتائج تحليل الهستوباثولوجي المناعي أن عقار البراميبكسول قد ساعد في منع فقد الخلايا العصبية الناتج عن تأثير الروتينون ، وظهر هذا التحسن أيضاً في مجموعة الفئران التي تم علاجها بالخلايا الجذعية. وتتلخص نتائج هذا البحث في قدرة هذه التقنية العلاجية بإستخدام كُلاً من الخلايا الجذعية وعقار البراميبكسول علي تجدد و حماية الخلايا العصبية في الفئران من التأثيرالسمى للروتينون.ولذلك يُعدا من الطرق العلاجية الممكنة في التسمم العصبي بالروتينون وخاصة داء الباركينسون السمى.