

RESEARCH ARTICLE

Effect of Human Umbilical Cord Blood Stem Cells on a Rodent Model of Parkinson's Disease

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Article History: Received: 15/4/2018 Received in revised form: 2/5/2018 Accepted: 7/5/2018

Abstract

Parkinson's disease (PD) is a neurodegenerative disease, which mainly affects the motor system. It is characterized by degeneration of dopaminergic neurons of the nigrostriatal pathway and formation of Lewy bodies. So far, there is no an effective treatment for PD, and the hope is to find neuroprotective strategies to stop or mitigate the disease progression. Therefore, this study aimed to find a treatment for PD with the help of regenerative medicine. Rotenone was administered subcutaneously in thirty adult female rats at a dose of 2 mg/kg BW for 35 days to induce PD. Afterwards; the animals were treated by either levocar or human umbilical cord blood mesenchymal stem cells (hUCB-MSCs). Body weight and behavior were recorded weekly around one month, then estimation of dopamine level in brain tissues and histopathological examination were also performed after one month. hUCB-MSCs-treated group showed an improvement ($P < 0.05$) of the body weight, behavioral assessment, dopamine level and the histopathological findings when compared with the untreated group. Levocar-treated group showed an improvement ($P < 0.05$) of body weight and behavioral assessment, whereas non-significant differences in dopamine level and the histopathological findings were noticed when compared with the untreated group. In conclusion, hUCB-MSCs significantly improved rotenone-induced PD, which evident by improving behavioral patterns, body weight, dopamine level and brain lesions.

Keywords: Parkinson's disease, Rotenone, Levocar, hUCB-MSCs, Rat.

Introduction

Parkinson's and Alzheimer's diseases are common neurodegenerative conditions [1]. The major pathognomonic lesion of PD is the formation of Lewy bodies [2]. Motor symptoms of PD such as tremors, rigidity, and bradykinesia are originated from the death of dopaminergic neurons [3]. All the current medications are aimed to controll the symptoms and improving the quality of life for PD patients [4]. Development of sporadic PD is based mainly on genetic mutation [5, 6] especially of alpha-synuclein gene [7]. Misfolding and aggregation in presynaptic phosphoprotein [8], leading to form esinophilic tangles, which known as Lewy bodies [9]. Neuronal cell death is mainly due to aggregation to these toxic molecules [10].

Exposure to pesticides is one of the environmental risk factors for the development of PD [11-14]. Rotenone is one of the most common pesticides, which can induce motor and non-motor deficits that are similar to human PD [15-20]. Over 30 years Levodopa is the most widely used treatment for PD, which is converted inside the dopaminergic neurons to dopa by dopa-decarboxylase. Lack of dopamine in the substantia nigra is responsible for the appearance of motor symptoms; few amount of L-DOPA is able to cross the blood-brain barrier (almost 5-10%) and the remaining amount causes side effects (vomiting, movement slowness and joint stiffness [21]. Mesenchymal stem cells are now appreciated as essential cells that govern

tissue homeostasis by regulating niches. This property along with their capacity for multipotential differentiation has greatly facilitated their role as a cell-based therapeutic agent [22].

Materials and Methods

Forty adult 8-week-old/ female albino rats weighing 200-250 grams were purchased from the Experimental Animal House at the Faculty of Veterinary Medicine, Zagazig University and housed in specific pathogen free rooms of animal house. Rats were fed *ad-libitum* commercial pelleted diet. These animals were randomly assigned into four groups as follows: the first control group was daily injected with sunflower oil (1 mL/kg BW S/C) throughout the experimental period [23]. The second group was subcutaneously injected daily with 2 mg/kg BW rotenone (Sigma-Aldrich, ST. Louis, MO, USA) for 5 weeks [23]. The third group was subcutaneously injected daily with 2 mg rotenone/kg BW for 5 weeks followed by oral administration of Levocar® (Alpha Cure Pharmaceuticals) until the disappearance of the symptoms according to National Parkinson Foundation, ½ tab/3 times for 7 days then 1 tab/3 times and re-evaluate the symptoms. If symptoms need further treatment then titrate up to 1.5 tab/3 times for 7 days, then 2 tab/3 times. The rat dose is 4.5 mg/200 gram of Levodopa [24]. The fourth group was subcutaneously injected daily with 2 mg rotenone/kg BW for 5 weeks followed by intravenous administration of 1.1 M cold mannitol ten minutes before hUCB-MSCs ($2 \times 10^7/1$ mL PBS) [25]. This work was carried out in the research unit located in the Faculty of Veterinary Medicine Zagazig University after the approval of the director responsible for the unit.

Human male umbilical cord blood mesenchymal stem cells (hUCB-MSCs)

Collection of human Umbilical cord blood

Male umbilical cord blood was obtained under complete aseptic conditions from the umbilical vein of nine full term delivery women at Zagazig University Hospital after getting informed consent from the mothers and their husbands. The samples were directly collected in a sterile 50 mL Falcon tubes

containing 2 mL Heparin and 5 mL of PBS at pH 7.2. Then the samples were transported at a temperature of 4°C in ice box to the Stem Cell Research Unit for isolation of stem cells. Then the isolation of umbilical cord blood MSCs was performed and the viability of cell was tested (for more details see Kern *et al.* [26] and Vassilios *et al.* [27], respectively).

Immunophenotypic characterization of hUCB-MSCs

Based on surface marker expressed on stem cells, we used monoclonal antibodies against human CD105 (a mesenchymal stem cell surface markers) and human CD45 (a hematopoietic stem cell surface marker), following the manufacturer's instructions. Briefly, the detached cells were washed twice with PBS and incubated with anti-human CD105 and human CD45 obtained from BD Bioscience for 30 minutes at 4°C in the dark. Cells were analyzed by FAC Scan flow cytometer (Becton Dickinson, Heidelberg, Germany) and Cell Quest software (Becton Dickinson), at Zagazig University Hospital Laboratories [28].

Behavioral assessment

Changes in body weight for animal's health evaluation were weekly recorded and they were monitored for any signs of motor symptoms. After 5 weeks of injection, behavioral patterns (postural reflex and forepaw grip time) were investigated. The postural reflex test was conducted to assess the sensorimotor function [29] and the rat's forepaw strength was assessed using a 5-mm-diameter wood dowel that was held horizontally and raised so that the animal supported its body weight [30,31].

Brain tissue sampling and preparation

After one month of treatment, five rats from each group were scarified. A part of brain tissue was collected in containers containing 10% neutral buffered formalin solution for studying histopathological changes. The other part of the brain was taken directly after slaughtering, thoroughly washed with distilled water, and then frozen at -20°C until used 1:5 (weight /volume) of brain tissue in cooled distilled water. Tissue specimens were homogenized for 5 minutes using electrical

homogenizer and centrifuged at 3000 rpm for 15 minutes to remove the cell debris. Supernatants were collected (whole homogenate) for RT-PCR of sex determining region on the Y chromosome (Sry) and other specimens were used for estimating dopamine level.

Homing of the hUCB-MSCs

The incorporation of the transplanted MSCs that were extracted from male donor into female brain tissue in the experimental groups were examined through RNA extraction from brain tissues of the slaughtered female rats at the end of the study, followed by RT-PCR. This method was used to detect the expression of sex determination region on the Y-chromosome male gene on brain tissue in the recipient female rat. To compare the results with the Sry gene of male rats, RNA extraction from adult male albino rat tissue sample followed by RT-PCR amplification of Sry gene was done according to previously published study [32]. The following primer pairs were designed using NCBI-Primer BLAST: human sex determining region primer (GenBank accession number (NM_003140.2); forward 5'-GAA TAT TCC CGC TCT CCG GAG-3', and reverse 5'- CCT GTT GTC CAG TTG CAC T-3'), rat sex determining region primer (Gene bank accession number (XM_017593858.1); forward 5'-AGA GAT CAG CAA GCA GCT GG-3' and reverse 5'-TCT TGC CTG TAT GTG ATG GC-3'), and rat β -actin as internal control gene (iNtRON Biotechnology, GenBank accession number

(V0127); forward: 5'- ATC TGG CAC CAC ACC TTC-3', reverse: 5'- AGC CAG GTC CAG ACG CA-3', product length= 302 bp).

The dopamine level in all animals was performed according to Manikoth *et al.* [33]. Histopathological examination of substantia nigra pars compacta (SNc) in brain was done according to [34].

Statistical analysis :

Statistical analyses were performed using Statistical Package for Social Science (SPSS, version 14). Collected data were presented as mean \pm S.E (stander error) for numerical variables and as percentages for qualitative variables. A one-way ANOVA was performed to assess the statistical differences between experimental groups. The comparison of means among the groups was performed with Duncan's multiple range tests. The significance level was set at $P < 0.05$.

Results

Viability test (trypan blue exclusion test) and flow cytometrical analysis

The result of trypan blue exclusion test indicated that the percentage of viable cell after the isolation procedure ranged from 94-96%. Viable cells appeared light in color, while dead cells appeared dark in color (Figure 1A). The cultivated hUCB-MSCs were positive for mesenchymal marker CD105 and negative for hematopoietic marker CD45 (Figure 1B).

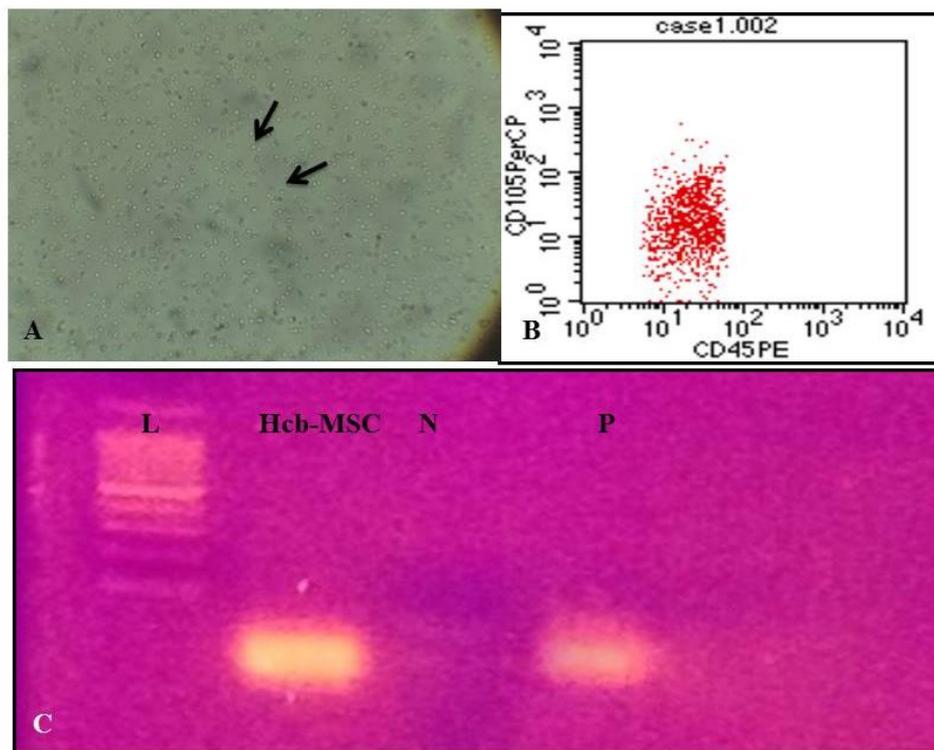


Figure 1: A) A light microscopic picture of trypan blue exclusion test showed the viable UCB-MSCs (light cells as pointed with arrows) X 20. B) Flow cytometrical analysis showed that hUCB-MSCs were positive for CD105 and negative for CD45. C) To address the homing of injected human-derived MSCs into injured brain tissues, the expression of human male gene-SRY was evaluated by RT-PCR. Brain tissue of animals that injected with male hUCB-MSCs showed expression of human male gene-SRY (lane-1), whereas those without MSCs showed no expression (N; lane-2). Positive control (P) is also shown (lane-3).

Body weight, behavioral assessment

Subcutaneous administration of rotenone induced significant decrease of body weight ($P \leq 0.05$) in all treated groups when compared with the control group (Figure 2A). Oral administration of Levocar and intravenous administration of hUCB-MSCs provoked a significant increase of body weight ($P \leq 0.05$) when compared with the untreated group (Figure 2B). The diseased group showed a

significant increase of posture reflex ($P \leq 0.05$) with a significant decrease in forepaw grip time ($P \leq 0.05$) when compared with the control group. Levocar- and hUCB-MSCs- treated groups induced a significant decrease in posture reflex ($P \leq 0.05$) with a significant increase in forepaw grip time ($P \leq 0.05$) when compared with the untreated animals (Figure 3).

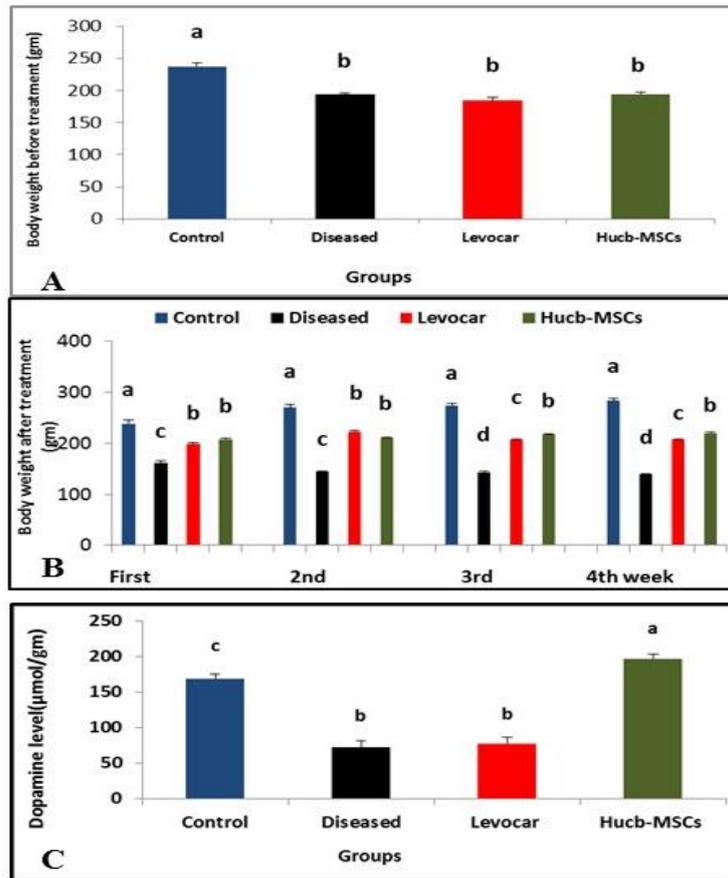


Figure 2: A) Effect of subcutaneous administration of rotenone (2 mg/kg BW daily for 5 weeks) on body weight before treatment. Effects of oral administration of Levocar and intravenous administration of hUCB-MSCs (2×10^7 in 1 mL PBS) on body weight after treatment (B) and on dopamine level ($\mu\text{moles/g}$) (C).

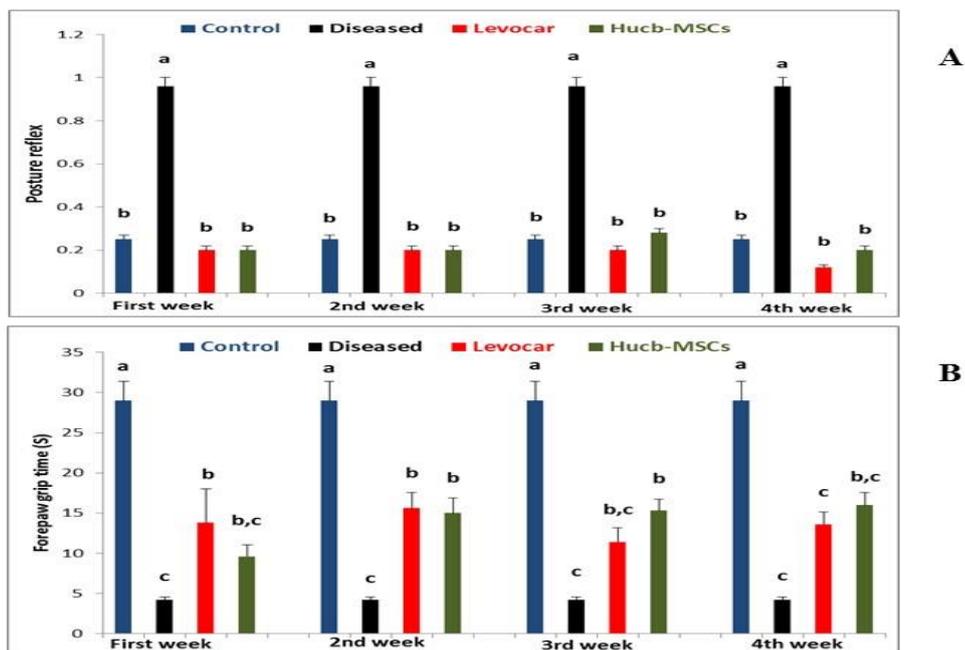


Figure 3: Effect of oral administration of Levocar and intravenous administration of hUCB-MSCs (2×10^6 in 1 mL PBS) on posture reflex test (A) and on forepaw grip time (B).

Homing of the transplanted hucb-MSCs and Dopamine level

Sry gene was detected in hUCB-MSCs-treated group as indicated by RT-PCR. The infused MSCs were migrated and homed into the injured brain tissue (Figure 1C). Diseased group evoked a significant decrease in dopamine level ($P \leq 0.05$) when compared with the control group. Levocar-treated group showed no significant difference in dopamine level when compared with the diseased group. hUCB-MSCs-treated group provoked a significant increase in dopamine level

($P \leq 0.05$) when compared with the untreated group (Figure 2C).

Histopathological results

Brain tissue especially at SNc in mid brain of the control rats showed normal neuroglial cells and blood vessels. While, in diseased and levocar treated rats showed esinophilic globose shaped neurofibrillary tangles (Lewy bodies) and degeneration of neurons. However, in hUCB-MSC-treated rats showed moderate degeneration of astrocytes (Figures 4 and 5).

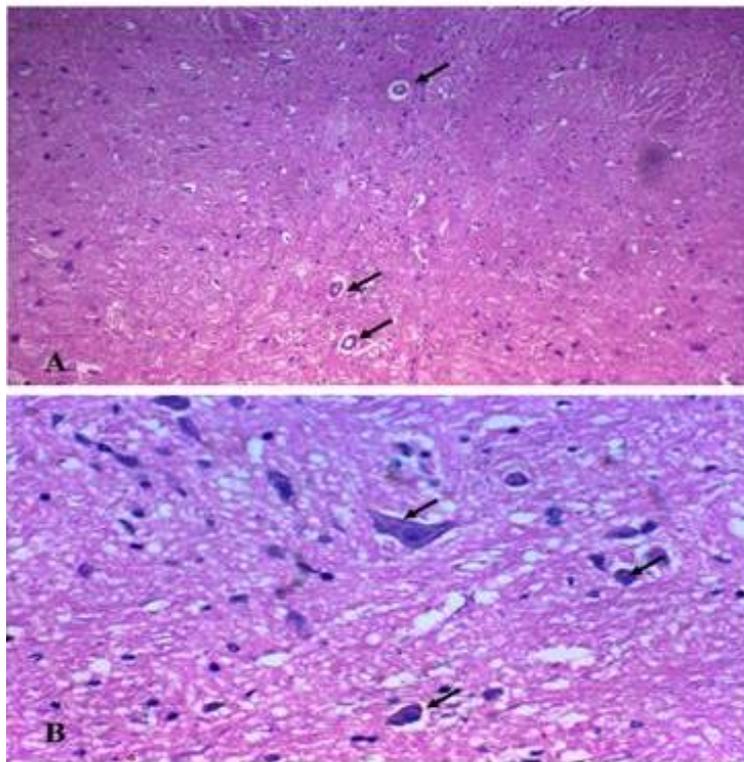


Figure 4: A) Photomicrograph of normal rat subcortical white matter showed normal neuroglial cells and blood vessels (black arrows) H&E x200. B) Photomicrograph of brain of diseased rat characterized by degenerated neurons, gliosis and esinophilic globose shaped neurofibrillary tangles Lewy bodies (black arrows) H&E x400.

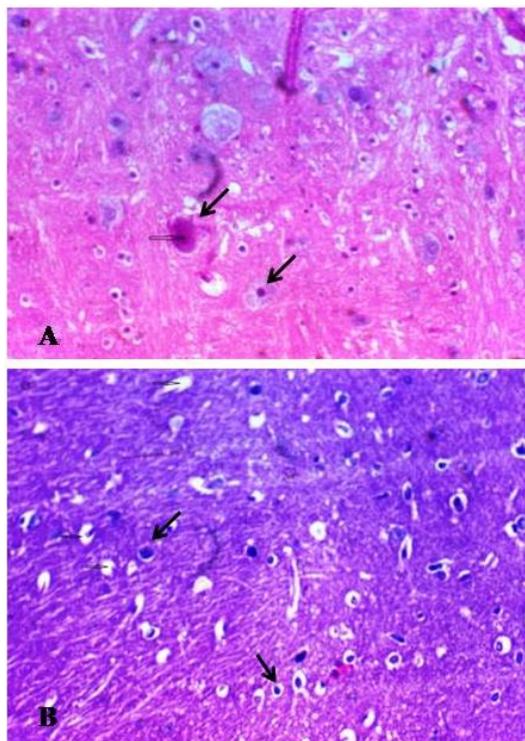


Figure 5: A) Photomicrograph of rats' brain (levocar treated) characterized by degenerated neurons and eosinophilic globose shaped neurofibrillary tangles (black arrows) H&E x400. B) Photomicrograph of rats' brain (hUCB-MSCs treated) characterized by moderate degeneration of astrocytes (black arrows) H&E x400.

Discussion

Parkinson's disease is one of the fastest growing neurological disorders in the developed world that mainly affects the motor system. Tremor, rigidity, bradykinesia and postural instability are the main features of PD [35]. The current data showed severe change in behavioral patterns of PD rats when compared with the control healthy rats, which are in agreement with Tonya *et al.* [36], who demonstrated that PD in mouse models reflect a basic motor symptoms that were confirmed with decreased locomotor activity in open field behavioral chambers and forepaw stride length

The results of the present study showed a significant decrease in general health and loss of body weight in diseased rats due to dyskinesia and other motor symptoms, which were comparable with the findings of Pfeiffer [37], who reported that PD causes constipation

and gastric dysmotility, which endanger comfort and general health]. In addition, lewy bodies affect neurons in the enteric nervous system that control gut functions even before affecting the functioning of substantia nigra neurons [38].

Parkinson's disease has no cure, but medications can mitigate the symptoms. Meanwhile, Levodopa is the drug of choice for treating motor symptoms [39]. Our results revealed that daily oral administration of levocar to PD rats (according to National Parkinson Foundation) ameliorated motor symptoms dyskinesia and tremors. These findings were in agreement with Chandran *et al.* [40], who found that rat model of PD challenged with L-DOPA displayed an increase of locomotor activity and enhanced the forepaw stride length. On the other hand, usage of levodopa for a long term leads to development of motor complications that

characterized by involuntary movements called dyskinesias.

With absence of the effective treatment for PD, the main objective is to replace the dopamine-destroyed neurons with the aid of regenerative medicine. Mesenchymal stem cells is considered one of the multipotent cells that has the ability to self-renewal, adhere to culture vessels. Moreover, it has a fibroblast-like shape and positively express stro-1, CD133, CD29, CD44, CD90, CD105, CD73, C-Kit, CD71 and CD106, while negatively express CD34, CD45, CD14, CD79 α and/ or CD19.

The current results were in consistent with the previous findings [41] that the isolated MSCs cells were positive for CD105, CD90 and CD73, while negative for CD45. In the present study, behavioral pattern was significantly improved in hUCB-MSCs treated rats, which reflected on general health and body weight.

Dopamine is a neurotransmitter released by neurons sending signals to other nerve cells. There are several distinct dopamine pathways in the brain, which play a major role in reward-motivated behavior. Losing the dopamine-secreting neurons at substantia nigra led to PD [42]. Our results showed a decrease in dopamine level in diseased and levocar treated groups, while there was an increase in its level in hUCB-MSC treated group. Similarly Obeso *et al.* [43] reported that, PD motor symptoms resulted from greatly reduced activity of dopamine-secreting cells caused by cell death in substantia nigra pars compacta. On the same ground, Alam and Schmidt, [15] stated that there was markedly lower level of striatal dopamine versus saline group of rats treated with rotenone.

The embryonic stem cells can modify the course of the disease. They can proliferate and generate dopamine neurons [44]. The dopamine neurons generated by stem cells showed an improvement in dopamine, serotonin levels and behavioral properties [45]. Microscopic findings of diseased and levocar treated groups are characterized by the abnormal deposition of esinophilic globose neurofibrillary tangles shaped (Lewy body) inclusions and degenerated neurons, which

were comparable with the findings of others [1]. In hUCB-MSCs treated group, microscopic and histopathological findings showed moderate destructed astrocytes and disappearance of esinophilic inclusions, Lewy bodies inside neurons at SNc. On the same ground, Johnson *et al.* [46] demonstrated that, transplantation of MSCs in PD model provided strong neuroprotective effect by transdifferentiation into neural cells and secret various neurotrophic and anti-inflammatory factors.

Conclusion

Based on results of current study, it could be concluded that hUCB-MSCs has a promising curing impact in treating rotenone induced PD, which was reflected by improving posture reflex, fore paw grip time test, body weight, dopamine level and brain lesions. Further studies are required to clarify the exact mechanisms by which MSCs could potentially help for recovery of PD patients.

Conflict of interest

The authors have no conflict of interest to declare.

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الملخص العربي

تأثير الخلايا الجذعية الوسيطة المعزولة من دم الحبل السري علي قوارض المصابة بالشلل الرعاش تجريبيا

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الشلل الرعاش هو مرض ناتج عن تلف في الخلايا العصبية والذي يؤثر بشكل رئيسي على نظام الحركة في الجسم و يتميز هذا المرض بفقدان الدوبامين في الخلايا العصبية الموجودة في مسار نيغروسترياتال ، وتشكيل أجسام ليوي. حتى الآن لا يوجد علاج فعال لهذا المرض، لذلك فالبحث مستمر عن استراتيجيات لوقف أو إبطاء تطور هذا المرض. هدفت هذه الدراسة إلى معرفة علاج المرض بمساعدة الطب التجديدي حيث تم حقن الروتينون لثلاثين فأر من الاناث تحت الجلد بجرعة 2ملغ / كجم من وزن الجسم لمدة 35 يوم لاحداث المرض، ثم علاج الحيوانات بالليفوكار اوبالخلايا الجذعية الوسيطة المعزولة من دم الحبل السري و تسجيل وزن الجسم و متابعة السلوك على مدار شهر واحد، ثم قياس مستوى الدوبامين في أنسجة المخ وكذلك الفحص النسيجي المرضي للدماغ بعد شهر واحد. اظهرت المجموعة المعالجة بالليفوكار تحسنا معنويا في وزن الجسم والتقييم الحركي بينما لم تظهر اي اختلاف معنوي في مستوي الدوبامين والنتائج النسيجية مقارنة بالمجموعة المصابة بالمرض , وأظهرت النتائج أن المجموعة المعالجة بالخلايا الجذعية أظهرت تحسنا معنويا في وزن الجسم، والتقييم الحركي، ومستوى الدوبامين، و كذلك في النتائج النسيجية مقارنة بالمجموعة المصابة بالمرض. والخلاصة ان الخلايا الجذعية الوسيطة المعزولة من دم الحبل السري ادت الي تحسن المرض الرعاش المستحث بمادة حقن الروتينون عن طريق تحسن في وزن الجسم والتقييم الحركي ومستوي الدوبامين والنتائج النسيجية للمخ .