ORIGINAL ARTICLE

Simultaneous treatment with cells and rosemary extract ameliorates 6-OHDA-induced toxicity in the hippocampus of mice

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ABSTRACT

In this study, we delved into the hippocampal region to understand the effects of adipose stem cells (ADSCs) and rosemary extract (RE). Our main objective was to explore how these substances influence spatial memory, neurotrophins, and changes in antioxidant enzymes. Moreover, we meticulously investigated the impact of dopamine deficiency, a notable characteristic linked with Parkinson's disease (PD), on memory impairment. This study comprised five groups of Wistar rats – all male, all selected randomly. We labeled two of these gatherings "lesion" (L) and "sham" (SH). Each got injections in the bilateral form with 6 μg – one group getting saline, while another got 6-OHDA. From couple weeks before the neurotoxin injection to 8 weeks later on, our lesion cohort was treated with rosemary at a dosage rate of 50 mg/kg body weight – let's call it RE for simplicity sake. Moreover, there is also this other lot, designated as cell-transplanted lesion group or catchy exercise (CE) as we prefer to interpret them; they had cell transplants conducted exactly 7 days after receiving their respective injections. Bringing up the rear, we got a group treated with both cell transplant and rosemary (CE+R). We performed spatial memory tests at 4 weeks, then again at 8. At the end of eighth week, the brains were extracted for q-PCR, enzymatic and immunohistochemical studies. Turning our gaze toward a comparison between the CE+R and CE groups versus the L group, we spot an intriguing drop in escape latency time. There is also more time spent in quadrants. Digging deeper into this matter, the CE+R bunch unveiled a clear surge when it comes to the expression of four genes, namely NGF, BDNF, NT3, and NT4! This was notable especially while comparing with both R and even other fellows from its very own broader group – CE. In a bit complex bit related to enzyme activity now, there is some good news as well for those in favor of potent antioxidants such as GPx or SOD. CE + R group, showed a significant increase of GPX and SOD enzymes, compared to the SH and L groups, and a significant decrease of MDA activity as compared to other treated groups. A significant decrease of escape latency and increase of time in quadrant were observed

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INTRODUCTION

During the early stages of Parkinson's disease (PD), there is a reduction in dopamine levels that happens because of the death of certain neurons in a specific part of the brain called

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in the CE+R and CE groups compared to L group. What's more, the levels of MDA in the CE+R group plummeted significantly when set up against the SH group. Wrapping things up, a definite downscale was observed in the density of GFAP-positive cells throughout different regions located within the hippocampus; this decline presented itself not solely in treatment groups but gripped onto those falling under SH as well, especially when compared to its comrade – the L group. Using ADSCs and taking RE orally have shown promising results in improving memory issues linked with PD.

Key words: Adipose stem cells, neurotrophins, oxidative stress, Parkinson, rosemary extract

the substantia nigra pars compacta (SNc). This decrease is connected to issues with memory and thinking abilities. The demise of these neurons occurs as a result of various factors such as oxidative stress, problems with mitochondria, and external toxins (1–4).[1-4]

People with PD who are in its advanced stages frequently suffer from a variety of cognitive deficits. According to Shao *et al*., the most noticeable deficits were in memory, which were followed by issues with attention, executive functioning, and visual-spatial skills. This is a common problem for people who have PD.[5]

Polyphenolic compounds, including carnosol, carnosic acid, ursolic acid, and rosemary acid, make up the main components of rosemary extract (RE). Carnosic acid, specifically, is the primary constituent of phenol-terpenes derived from rosemary and possesses various beneficial properties such as antioxidant, antimicrobial, anti-obesity, anti-tumor, neuroprotective, anticancer, and antidepressant effects. It also inhibits lipid peroxidation, biological membrane aberrations, and the release of oxygen and hydroxyl.[6-8] In a study conducted by Lee in 2008, it was found that rosmarinic acid exhibited neuroprotective properties by reducing oxidative stress and the apoptotic process in dopaminergic neurons.[9] Additionally, Du *et al.* demonstrated in 2010 that rosemary has therapeutic effects against MPTP and 6-OHDA neurotoxins through its antioxidant and anti-apoptotic activities.[10]

Previous studies have revealed that stem cells derived from adipose tissue, known as adipose tissue derived stem cells (ADSCs), have shown promising results in treating symptoms associated with cognitive impairment. These positive outcomes can be achieved in two ways: either by directly replacing the lost cells or indirectly through the release of neurotrophic factors.[11,12] Neurotrophic factors, which are a category of growth factors including NT3, NT4/5, NGF, and BDNF, play a vital role in protecting adult neurons from various types of harm such as ischemic, toxic, and mechanical damage, as well as apoptotic or necrotic neuronal death.[13]

It has been found by researchers that when they introduced stem cells from umbilical cord blood into the brains of mice who had Alzheimer's disease, they observed a noticeable reduction in oxidative stress and apoptosis. This groundbreaking treatment has also displayed encouraging outcomes in terms of improving memory and boosting learning capabilities.[14]

This work aimed to investigate changes in the expression of NT3, NT4, BDNF, and NGF genes, along with reactive markers, in the hippocampus of rats that were concurrently transplanted with ADSCs and RE after 6-OHDA was administered.

MATERIALS AND METHODS

The Pasteur Institute of Iran provided the adult male Wistar rats used in this study. The mice lived in an environment with a controlled temperature of 20°C–24°C and a continuous 12-h light/dark cycle. Drinks and food are easily available. When keeping animals, please follow the regulations of the Iranian Animal Welfare Committee. This study was approved by the Ethics Committee of Islamic Azad University (IR.IAU.DAMGHAN.REC.1400.028). He classified rats into five categories. SNc 6-OHDA was purchased from Sigma-Aldrich, USA, and administered bidirectionally to the lesion group (L) at a concentration of 6 µg/2 µL saline. In the sham-operated group, no toxin was used, and 2 µL of physiological saline was injected. Cell population: 3×10^6 ADSCs were injected intravenously after 6 days. Rosemary group (R): Gavaged daily with 50 mg/kg RE, from 2 weeks before to 8 weeks after lesion. Cells and Rosemary Collective (catchy exercise [CE]+R): Feed cells with RE. For example, the B.RE group and the CE group [Figure 1].

Modeling of memory impairment of Parkinson's disease in rats

The animals were given anesthesia to put them into a temporary sleep-like state. They were securely fixed using a special device that restricted their movements, making sure they could not move. After that, a specific area called the SNc was pinpointed on both sides to receive an injection of 6-OHDA. The injection was made by mixing 6 µg of 6-OHDA with 2 µl of saline solution. It was done with great precision, following specific coordinates: −8.0 mm from the skull in the up-and-down direction, ±2.1 mm from the midline in the left-right direction, and −5.0 mm from the bregma in the front-back direction.[15]

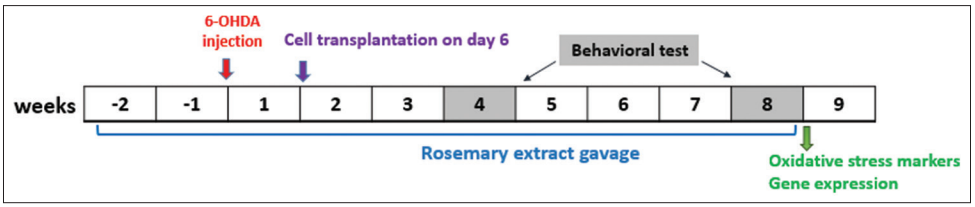

Figure 1: Experimental design: Timeline showing the behavioral and histological studies along with treatments

Isolation and culture of ADSCs

The process of extracting fat tissue from the space between the shoulder blades in rats was carried out. To ensure cleanliness, procedures that prevent infection were strictly adhered to. The enzymatic digestion of the tissue was done using a special type of collagenase called type I (provided by Sigma-Aldrich) at a temperature of 37°C for 1 h. After this, the cells were separated and placed in a culture medium known as α-MEM, which was enriched with 10% fetal bovine serum. The cells that were used for transplantation, which were from the fourth round of cultivation, were treated with BrdU (5′-bromo-2′-deoxyuridine, provided by Sigma) 48 h before the transplantation took place. This treatment involved adding 10 µl of BrdU to the culture medium. I injected a mixture of cells, containing 3×10^6 cells in a small volume of 50 µl, into the tail vein using a Hamilton syringe. This was done after treating the cells with trypsin and spinning them down in a centrifuge. It had been 6 days since the neurotoxin was given before this injection took place.[16]

Preparation of rosemary extract

RE, which included 40% carnosic acid, came from Hunan Geneham Biomedical Technology in China. It was then reduced with distilled water. Based on past research, the adequate amount of RE was 50 mg/kg.[17,18]

Behavioral test

In accordance with a previously published article, we conducted the Morris water maze test.[19]

Immunohistochemical study

The animals were perfused after 8 weeks, and their brains were removed and preserved for 24 h in a 4% paraformaldehyde solution. After that, coronal sections were cut to a thickness of 7 microns at a position of +2.8 mm with respect to the bregma, with a focus on the CA3, DG, and CA1 regions of the hippocampal tissue. Following this, the cells were incubated overnight at a temperature of 4°C with the primary antibodies anti-BrdU, anti-TH, and anti-GFAP. Subsequently, the sections were exposed to a substrate solution containing 0.1% DAB (Sigma-Aldrich, D7304) for a period of 15 min in the case of TH and BrdU. The secondary antibodies conjugated with HRP were then added. To capture an image of the object at a magnification of ×400, a Nikon DXM 1200 digital camera (USA) and a Nikon E600-Eclipse fluorescent microscope (Japan) were utilized.

Enzymatic activity assays

Ohkawa *et al.* used a specific measurement technique to determine if there were any substances that had interacted with thiobarbituric acid.[20] First, they divided the sample into small portions of 500 µL each and transferred them into tiny tubes. Then, they added a solution made up of 20% acetic acid (v/v) with a pH of 3.5, along with 50 µL of 8.1% sodium lauryl sulfate. They also introduced 700 µL of distilled water and 1500 µL of 0.8% thiobarbituric acid to this mixture. After vigorously mixing the ingredients together, they allowed the mixture to react in a hot water bath for 1 h. I constructed a calibration curve by using 1,1,3,3-tetramethoxypropane as a standard. Later on, I measured the absorbance of the resulting pink TBARS at a wavelength of 532 nm.

The activity of GPX was calculated using the Wendel method, which is described in reference.[21] In this analysis, tert-butyl hydroperoxide was used as the substrate. The disappearance of NADPH was observed by using a spectrophotometer at a wavelength of 340 nm. To start the reaction, a mixture was prepared that contained 2 mM glutathione (GSH), 0.15 U/ml GSH reductase, 0.1 mM NADPH, 0.4 mM azide, and 0.5 mM tert-butyl hydroperoxide. By evaluating the change in absorption per milligram of protein per minute, one can determine the enzyme activity.

Giannopolitis and Ries' findings indicate that the mechanism of action of SOD was the suppression of the photochemical reduction of NBT. A fluorescent lamp was used to illuminate the tubes holding the different mixtures so that the effects could be seen. The response began as soon as the light source was turned on, and the A560 readings were collected for 10 min in order to gauge the drop in NBT levels. The amount of enzyme needed to prevent 50% of NBT breakdown was determined to be one unit of SOD under the given experimental parameters.

Both the Lowry method and BSA were used to quantify the protein content in order to determine the precise amount that was present.[22]

Semi-quantitative reverse transcription polymerase chain reaction analysis

To obtain RNA from the hippocampal region, we utilized Gibco's TriZol reagent. To create cDNA, we adhered to

the guidelines provided by Takara, the manufacturer of the PrimeScript TM 1st Strand cDNA Synthesis Kit (Japan). For the quantitative polymerase chain reaction (Q-PCR) analysis [Table 1], we employed a q-PCR kit from Clontech (Cambridge, UK) and attained both forward and reverse sequences from GenBank.

We utilized electrophoresis to separate the PCR products on a 1.5% agarose gel, enabling us to observe the gene bands. This visualization was achieved by capturing images of the gel using a Uvidoc camera. In our analysis, we discovered gene band lengths of 405 bp for BDNF, 164 bp for NGF, 181 bp for NT3, 213 bp for NT4/5, and 203 bp for GAPDH in the resulting reverse transcription q-PCR products. To evaluate the intensity of these gene bands, we employed Image J software (National Institutes of Health, USA).

Statistical analysis

Utilizing GraphPad Prism 9 software [Information Technology Support Services (ITSS), California], we conducted one-way analysis of variance (ANOVA), two-way and paired-sample *t*-tests, and other data analysis techniques. Tukey's *post hoc* test was applied to assess the outcomes for every group. To present the results, the mean and standard error of the mean were used. A statistically significant *P* value was one that was <0.05.

RESULTS

Immunohistochemical studies

As shown in Figure 2, the density of TH-positive neurons in the L group [Figure 2a], decreased compared with the SH group [Figure 2b]. Furthermore, BrdU-positive cells were seen in the CE and CE+R groups [Figure 2c and d]. The anti-GFAP labeling showed a decrease in astrocyte density as compared to the lesion group [Figure 3a-d].

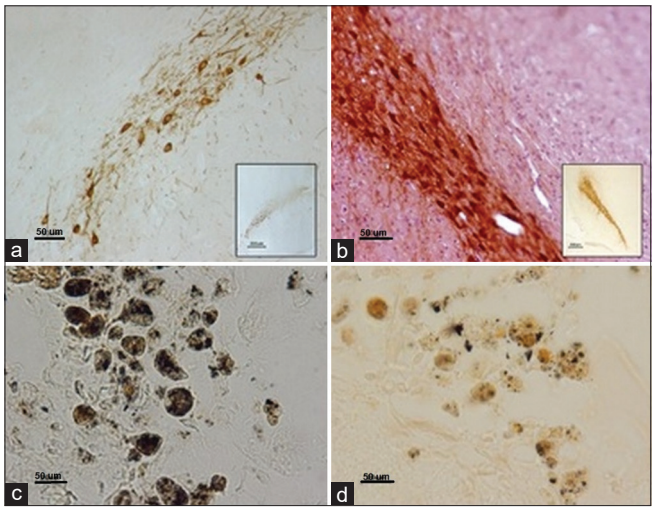

Figure 2: Positive immunostained cells in experimental groups: TH-positive cells in L group (a), and SH group (b); BrdU positive cells in CE+R group (c), and CE group (d)

Morris water maze test

During the probe test, we assessed two aspects: the amount of time spent in the target area and the time it took to reach the escape spot. This evaluation occurred at 4 and 8 weeks after a 4-day training session, as well as 24 h later. The results from week 4 showed a significant difference ($P < 0.01$) in the duration spent by the CE and R groups in the target quadrant compared to the SH group. As we reached the 8th week, there was a clear decline in this specific aspect for both the CE and R groups as compared to the SH group, and this decline was statistically significant ($P < 0.05$). Interestingly, unlike the CE and R groups, the CE+R group showed a significant increase in the amount of time spent, which was even more remarkable ($P < 0.01$) [Figure 4a].

By the 4th week after the neurotoxin injection, it was observed that the escape location latency differed significantly between the SH group and both the CE and R groups ($P < 0.01$). Additionally, during this same time period, the CE+R group exhibited a significant decrease in latency ($P < 0.001$) compared to both the CE and R groups. As we reached week 8, there was a noticeable distinction ($P < 0.01$) between the SH and R groups. On a contrasting note, Figure 4b reveals that the latency of the CE+R group was considerably lower than that of the R group ($P < 0.05$).

Expression of genes NT3, NT4/5, GDNF, NGF, and BDNF

Eight weeks after injury, one-way ANOVA showed significant differences between groups ($F_{4,15} = 25.77$, $P < 0.000$). Compared with the L group, the BDNF gene expression in the CE group and SH group was significantly increased ($P < 0.01$ and $P < 0.001$, respectively). Furthermore, there were no significant differences between the CE+R and SH groups. BDNF expression was significantly increased ($P < 0.001$) when comparing rosemary treatment alone to the combination of rosemary and cell therapy [Figure 5a]. The NGF gene in the SH, R, and CE groups showed a significant increase in significance ($P < 0.001$ and $P < 0.05$) in one-way ANOVA ($F_{4,15} = 17.81$, $P = 0.000$) compared to the L group [Figure 5b]. CE+R expression was higher than that in the

Table 1: The gene sequences of both the forward and reverse primers are being analyzed

Gene	Forward primer	Reverse primer
GAPDH	5-TGACATCAAGAA GGTGGTG AAGC-3	5-CCCTGTTGCTGTAGCCGTATTC-3
BDNF	5-GCCCAACGAAGA AAACCATA-3	5-GATTGGGTAGTTCGGCATTG-3
NGF	5-CCTCTTCGGACAC TCTGGA-3	5-CGTGGCTGTGGTCTTATCT-3
NT3	5-AGGTCAGAATTCC AGCCGAT-3	5-GTTTCCTCCGTGATGTT-3
NT4/5	5-TATGTGCGGCGTT GACTGC-3	5-CACAGTCAGAAGGCACGGTA-3

rosemary group ($P < 0.01$). Data analysis results showed that there was a significant difference in NT3 mRNA expression between groups (F4,15 = 23.2, $P < 0.000$). The expression of NT3 gene in the treatment group was significantly higher than that in the lesion area ($P < 0.01$). Compared with the CE and R groups, the NT3 gene expression in the CE+R group was significantly increased ($P < 0.05$) [Figure 5c]. There were significant differences in the NT5 gene between the five categories (F4,15 = 17.81, $P < 0.000$). Compared with group L, group CE and group SH were significantly larger ($P < 0.01$, $P < 0.001$). The gene expression in the CE+R group was significantly higher than that in the R group ($P < 0.01$) [Figure 5d].

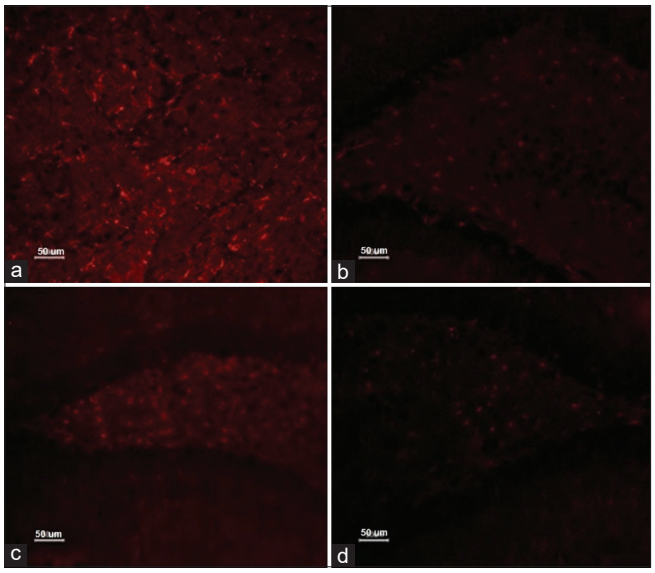

Figure 3: Anti-GFAP staining: Positive cells in L group (a); CE+R group (b); R group (c); CE group (d)

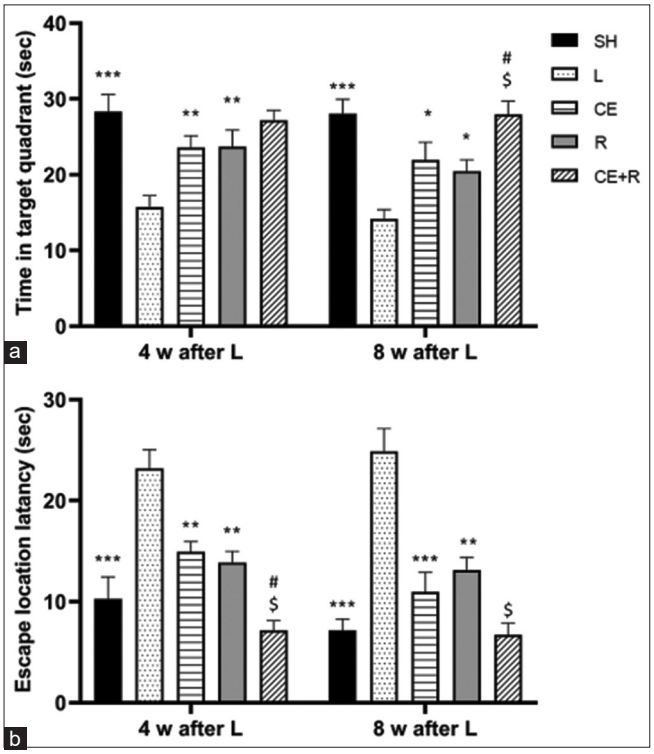

Figure 4: Memory evaluation of experimental groups at 4 and 8 weeks after neurotoxin injection. (a) Time in target quadrant (b) Escape location latency. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus L group. #$P < 0.05$ versus catchy exercise group. $$P < 0.05$ versus R group

Enzyme activity result evaluation

Upon analyzing the data, it became evident that there was a noteworthy alteration in the MDA level within the groups (F4,15 = 47.86, $P < 0.000$).

The levels of MDA in the SH, CE, and R groups were significantly lower compared to the L group. It was found that the MDA levels in the CE+R group were markedly lower as compared to both the R and CE groups [Figure 6a]. Through a one-way ANOVA test, it was observed that there exists a substantial disparity in the activities of GPX and SOD enzymes among the different groups. I would like to point out that upon comparing the affected area to the SH and R groups, we observe a substantial rise in the enzyme activity of GPX ($P < 0.05$, $P < 0.01$) and SOD ($P < 0.05$, $P < 0.001$), respectively. Additionally, it is worth noting that the CE+R group exhibited significantly heightened GPX and SOD enzyme activities in comparison to both the CE ($P < 0.001$) and R ($P < 0.05$) groups [Figure 6b and c].

DISCUSSION

Cognitive dysfunction is another feature of many patients with PD, and recently, it has become a severe challenge to many psychologists. Up to now, the study of stem cells and their therapeutic application has been advancing. Considering their neurotrophic effects and neuroprotection, ADSCs seem to be a good option for cell therapy. Rosemary, a green shrub, has recently been used in treating numerous neurological diseases due to its anti-inflammatory, anti-apoptotic, and neuroprotective features. Our previous report only focused on separate cells and RE pretreatment on memory deficits of PD rats.[16] In this study, cell therapy was used simultaneously with extract therapy to evaluate the protective role of combined cell and extract treatment on induced cognitive dysfunction and neurotoxicity in the PD model of the rat.

According to research findings, cognitive impairment – which often manifests as concentration and memory problems – occurs often in PD patients. The validity of the PD model employed in this study was verified by immunohistochemistry. The results of the investigation indicated a considerable drop in the TH-neutron density of the model.[4,23-25]

In weeks 4 and 8 following the lesion, the probe test evaluation was conducted 1 day after the training period

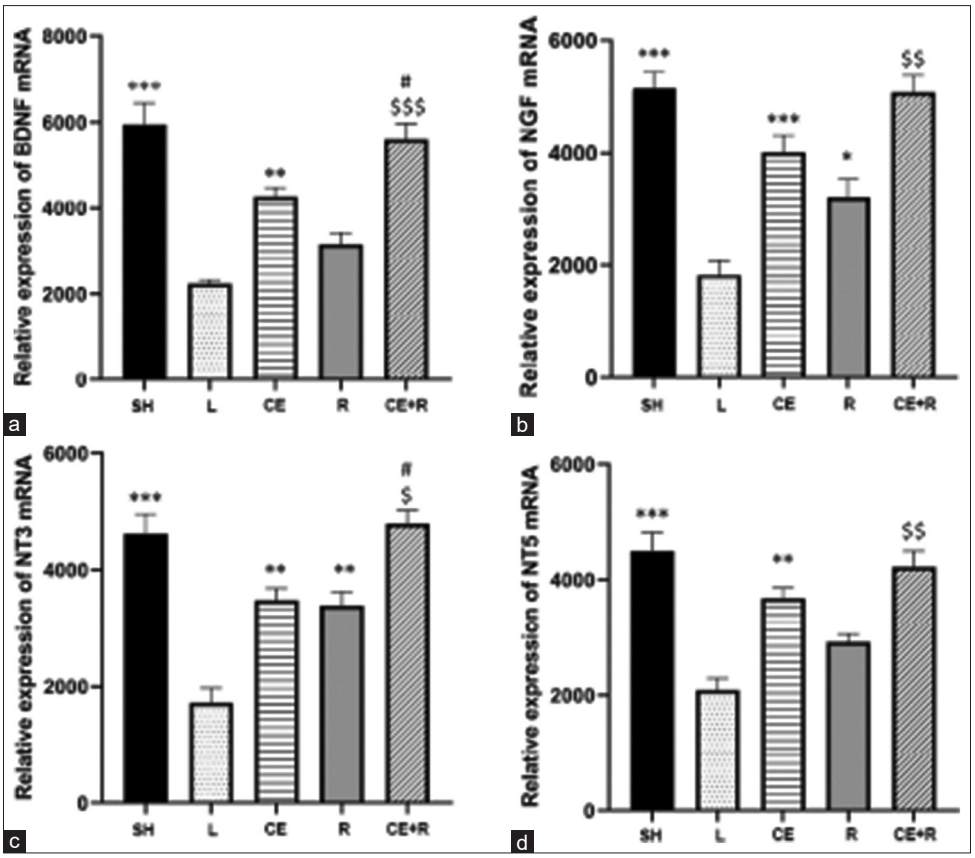

Figure 5: Relative expression of BDNF, GDNF, NGF, NT3, and NT4/5 genes in the experimental groups. (a) The expression of BDNF mRNA (b) The expression of NGF mRNA (c) The expression of NT3 mRNA (d) The expression of NT5 mRNA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus L group #*P* < 0.05 versus catchy exercise group. $*P* < 0.05, $$*P* < 0.01, $$$*P* < 0.001 versus R group

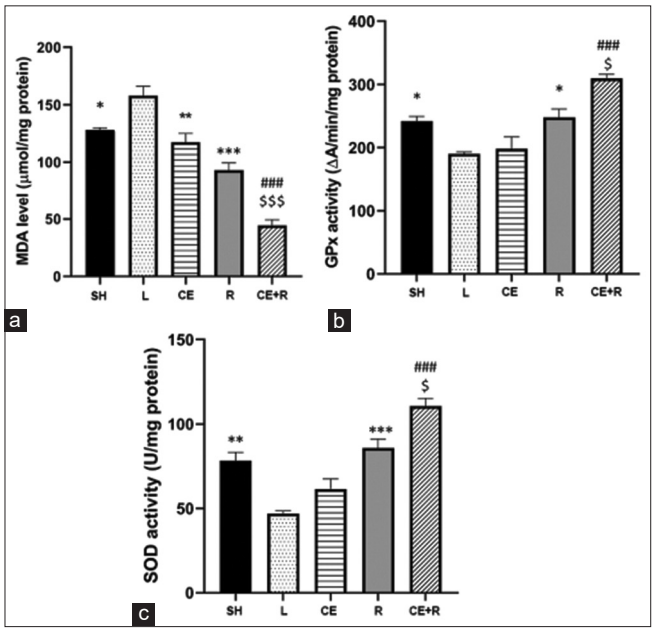

Figure 6: Enzyme assay results in all the experimental groups.(a) The activity of MDA enzyme. (b) The activity of GPX enzyme. (c) The activity of SOD enzyme. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus L group ###*P* < 0.01 versus catchy exercise group. $*P* < 0.05, $$$*P* < 0.001 versus RE group

concluded. The results showed that the treated and sham groups' escape latency was significantly lower than that of the lesion group. This indicates that the model's spatial memory was hampered by the neurotoxin injection. The CE+R group demonstrated a noteworthy reduction in escape latency in the 4th and 8th weeks when compared to the R group. In addition, compared to the R group, the CE+R group showed a significant increase in time spent in the target quadrant. These findings imply that the concurrent use of rosemary and cell therapy significantly increased the memory impairment linked to PD.

Consequently, this specific treatment not only improves memory but also successfully mitigates the cognitive deficits brought on by the injection of 6-OHDA.

In comparison to the sham group, the lesion group's NT3, NT4/5, BDNF, and NGF gene expression was significantly lower. The time of administration of rosemary extract is a very important factor in its effectiveness. Administration of the extract before damage prevents irreversible reduction of neurons. Furthermore, preoperative treatment has been demonstrated in earlier research to lower reactive oxygen species (ROS) levels and postpone neuronal deterioration.[26]

In this research, a noteworthy rise in BDNF and NT3 levels was noted in the PD rat model that received the extract orally prior to damage and later received cell injections, as compared to rats treated with only cells or rosemary and rats with lesions.

The transplantation of ADSCs into mice with Parkinson's models was found to result in a local increase of neurotrophic factors, including BDNF and NT3, according to Li *et al*.'s study in 2021.[27] Additionally, the intravenous administration of ADSCs has the potential to prevent neuronal death by either secreting neurotrophic factors or stimulating neurons to secrete neurotrophic factors, thereby promoting an antioxidant mechanism at the site of damage.[12,28]

In order to evaluate the enhanced performance of PD rats, our study measured the levels of oxidative stress markers, thereby assessing the anti-oxidative effects of cell transplantation and RE.

GPX and SOD enzyme levels were significantly higher in the hippocampal tissue of lesioned rats treated with rosemary and cell in combination than with cell treatment alone. In comparison to the other treated groups, the MDA levels in the CE+R group also significantly decreased. This indicates that the combination of rosemary and cell treatment effectively reduces oxidative stress by enhancing antioxidant enzyme activity and reducing MDA levels. According to Serafini *et al*., carnosic acid found in rosemary leaves acts as a protective agent for biological membranes, preventing lipid peroxidation by neutralizing harmful oxygen-free radicals.[29] Consistent with our findings, previous studies have shown that treatment with RE improves cognitive function by reducing neural degeneration and ROS levels while increasing catalase, GPx, and SOD activity in the injured hippocampus of rats.[30]

Through immunohistochemical analysis, the location of BrdU-labeled ADSCs in the hippocampus was verified. The results showed that these cells could move to the rat brain's affected areas and cross the blood–brain barrier. In this investigation, the GFAP immunohistochemistry results showed that the group with lesions had a higher concentration of astrocytes than the other experimental groups.

According to the report, individuals with PD showed a higher quantity of GFAP-positive astrocytes in their human samples.[31] Moreover, there has been a suggestion that glial cells may contribute to the demise of dopaminergic neurons by initiating apoptosis through cytokines such as interferon-gamma, IL-6, and IL-7, as well as producing TNF and nitric oxide. Hence, it is plausible to potentially diminish nerve damage in PD by suppressing the activity of glial cells during inflammatory processes.[32] This study illustrated a noteworthy reduction in the density of GFAP-positive cells across all treatment groups.

The fact that astrocytes can differentiate into neurons may be the cause of the decline in astrocyte counts in the therapy groups. Astrocytes have effects on the nervous system that are both harmful and protective. They generate vital compounds that are essential for protecting neurons impacted by PD, including GSH, SOD, NGF, and bFGF. On the other hand, hazardous ROS and reactive nitrogen species might arise as a result of a reduction in the antioxidant GSH. This then sets off the process of death in dopaminergic neurons by activating cytochrome C and caspases 3 and 8.[33,34]

CONCLUSION

It was possible to study how people with PD lose their memories with this model. By injecting OHDA-6 into both sides of the SNc and killing neurons in the hippocampus, this model lost its ability to remember where things were.

Thus, combining cell therapy and herbal medicine may improve PD's memory disorder by stimulating neurotrophic factors' expression in transplanted cells and maintaining their survival at the site of damage.

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Conflicts of interest

There are no conflicts of interest.

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