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Beneficial Effects of Rosmarinus Officinalis Leaves Extract on Memory Deficit of 6-Hydroxydopamine Lesioned Rats

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ABSTRACT

Background and Aims: Loss of dopamine in the hippocampus, causes memory deficiency in Parkinson's disease. Rosemary leaves extract (RE) has protective effects on neurons. It was evaluated whether RE restore defective memory of 6-OHDA injured rats.
Methods: Male Wistar rats were injected by bilateral intra-nigral of 6-OHDA (6μg) and divided into three treated and one lesioned group which were orally given RE (25, 50 and 100 mg/kg) once daily for 28 days and distilled water respectively. Fifth group as control received bilateral intra-nigral saline instead of neurotoxin. The escape latency, traveled distance and swimming speed parameters were measured 24h after the last training session (probe) and analyzed by two-way repeated measures ANOVA. BrdU-positive cells in the CA1, CA3 and dentate gyrus regions of the hippocampus were counted.
Results: Lesioned rats showed significant increase of escape latency and decrease of swimming time in the original platform quadrant, as compared with control group. RE at all doses, significantly decreased the escape latency ($P<0.05$) compared to lesioned group. There was a significant increase of swimming time in the original platform of 50, and 100mg/kg- treated groups as compared to lesioned rats.
Conclusion: RE improved spatial memory retention at doses of 50 and 100mg/kg and may serve as candidate for herbal therapeutic to improve spatial memory of PD. BrdU-positive cells of the CA1, CA3 and dentate gyrus increased significantly in the RE-100 treated group, as compared with the lesion group. Hippocampus was protected against neurodegenerative effects induced by 6-OHDA in the presence of RE.

Introduction

The dopaminergic mesencephalic neurons projecting to the hippocampus (HPC) are distributed in three cell groups: A8 region in the retrorubral field, A9 region in the substantia nigra pars compacta (SNc) and A10 region in the ventral tegmental area (VTA) of the medial part of SNc. The functional significance of the mesohippocampal dopaminergic system is the modulation of memory processes (1). The majority of dopaminergic (DA) innervations to HPC arise from the VTA, it was demonstrated that DA afferents from the VTA can facilitate long-term synaptic plasticity in the HPC (2-4). VTA has been shown to regulate HPC neural activity during spatial learning (5, 6) and encoding of HPC-dependent memories (7). Therefore, VTA inactivation disrupts HPC-dependent learning and

memory (5, 6). Because of this connection between VTA and HPC, depletion of hippocampal dopamine, resulting in memory deficits. PD is often complicated by a variety of cognitive symptoms that range from isolated memory and thinking problems to severe dementia. While the motor symptoms of PD are well known (tremor, rigidity, slowness of movement, imbalance), the commonly seen deficits in memory, attention, problem-solving and language are less understood. About 20% have more substantial cognitive impairment. Memory problems of PD are typically milder than of Alzheimer's disease (8-10).

6-hydroxydopamine (6-OHDA) is an oxidative neurotoxin to injure DA neurons in vivo and in vitro. It could induce catecholaminergic cell death by three main mechanisms: reactive oxygen species, hydrogen peroxide formation, or direct inhibition of the mitochondrial respiratory chain (11). 6-OHDA is also known to induce apoptosis (12, 13). Recently, it was found that 6-OHDA was recognized as a good model for early stage of PD, especially in terms of emotional and cognitive deficits (14).

Rosmarinus officinalis leaves extract (RE) shows very strong antioxidant activity (15, 16). RE possess a variety of bioactivities, including antioxidant, antitumor, anti-inflammatory and anti-HIV (17). Carnosic acid, like rosmarinic acid, has been shown to be neuroprotective in both in vitro models of neuronal death and in vivo models of neurodegenerative disease (18). It is composed of a vast number of polyphenolics such as carnosic acid, carnosol, rosemarinic acid, ursolic acid (17). Recently, it has been reported that pretreatment with carnosic acid can reduce cellular death in the cornu ammonis 1 (CA1) region of the HPC of an experimental model of Alzheimer's disease in rats (16). Rosmarinic acid (RA) is a naturally occurring hydroxylated polyphenolic compound in various plant families such as Lamiaceae herbs, Boraginaceae, sea grass family Zosteraceae, and fern family Blechnaceae (19). It has been known that RA has multiple biological activities such as anti-oxidative, anti-inflammatory and antiviral activities. RA displayed a strong antiviral and anti-inflammatory effect against Japanese encephalitis (20, 21). Lee HJ (2008) found that RA could modulate H2O2-induced cell death in human DA neurons (22). Sharmila R, 2012 revealed that oral administration of RA had potent anti-cancer, anti-lipid peroxidative and apoptotic effect in skin carcinogenesis (23). Lee et al has reported that RA could protect human DA neuronal cells against H2O2 toxicity (22). It was demonstrated that RA could exert its neuroprotective effects against 6-OHDA and 1-methyl-4-phenylpyridinium-induced neurotoxicities through its anti-oxidation and anti-apoptosis properties in vitro (24, 25).

Based on the role of 6-OHDA on the memory defect of PD, the cognitive enhancing effects of RE has gained much attention. The present study was designed to determine whether RE could improve memory impairment in 6-OHDA injured rats. Therefore, in the present study, 6-OHDA was employed to reduce neuronal density in HPC which caused memory impairment.

Materials and Methods

Animals

Male Wistar rats, weighing 200–250 g, (4-6 weeks-old) were purchased from Razi Institute, Karaj, Iran. Animals were housed in a room with a 12:12-hr light– dark cycle, and food and water were provided ad libitum. All procedures were conducted in agreement with the National Institutes of Health Guide for care and use of laboratory animals.

Surgery

The 6μg of 6-OHDA, which dissolved in 2μl of saline, was perfused bilaterally into SNc through a 30-gauge stainless needle using stereotaxy (26). The coordinates for the bilateral injections were −5.0 mm anteroposterior from bregma, ±2.1 mm mediolateral from midline, and −7.7 mm dorsoventral from the skull according to the Paxinos and Watson atlas (27).

Experimental protocol

The animals were randomly divided into five groups (n = 8 animals/group) as described below. Lesioned group: rats were injected by bilateral intra-nigral of 6μg of 6-OHDA and orally given distilled water once daily for a period of 28 days (14 days before and 14 days after surgery). Treated groups: Rats were treated orally with RE at various doses ranging from 25, 50, and 100 mg/kg once daily for 14 days before and after the neurotoxin injection. Control group which received bilateral intra-nigral saline instead of neurotoxin. RE containing 40% of antioxidant carnosic acid (carnosic acid powder CAP25-110401) was purchased from" HUNAN GENEHAM BIOMEDICAL TECHNOLOGY LTD "dissolved in distilled water.

Morris Water Maze Test

Nine days after surgery, all animals were evaluated for their spatial memory performance by MWM test. The swimming pool used for the test was 190 cm in diameter and 60 cm deep. The escape platform (100 cm^2) was fixed in a permanent position 2 cm under the water surface during the course of the MWM training procedure. The quadrant housing the escape platform was defined as the target zone. Spatial reference cues (arrow, star, circle, and rectangle) around the pool were remained constant during the test. For spatial learning acquisition test, the rats were trained in MWM for 4 consecutive days using 4-trial-per-day. The rats were placed into the pool facing the wall randomly from one of the three starting points located in the three quadrants except the quadrant with the platform. If the animals failed to find the platform by the maximum period of 60 seconds, they would be gently placed on the platform. At the end of each trial, the rats were allowed to rest on the platform for 20 s. A video camera that was mounted directly above the water maze pool linked to a computer recorded the animals' movement. For this purpose, the versatile tracking system of EthoVision (Noldus) was employed and the spatial memory was tested by measuring escape latency, traveled distance and swimming speed parameters. The time of escape latency and swimming distance to reach the platform were recorded by a video camera and analyzed using the computer software (Noldus). To assess spatial memory retention, a probe trial was performed 1 day after the last training trial, during which the platform was removed from the pool, while all other factors remained unchanged. Rats were allowed to swim for 90s. The time to reach the hidden platform (escape latency), the length of swim path (distance traveled), the time spent in target zone and swimming speed for each rat were recorded and used to assess the performance of the animal in this memory test (28, 29). One day after the last training session, the rats were tested during a 60-s probe trial (free-swim trial) in which the platform was removed from the pool. In this trial we recorded: (1) latency to cross the target platform location (time in seconds to cross the original platform position) (2) Total traveled distance (3) swimming speed and (4) percent of time spent in target zone (29).

BrdU administration

Bromodeoxyuridine (Sigma-Aldrich, St Louis, MO,USA) was dissolved in 0.9% normal saline and administered intraperitoneally during the last three days of treatments (i.e. days 12, 13 and 14). All rats received six injections of BrdU (50 mg/kg) twice daily, in 12 h intervals prior to transcardial perfusion (30).

Immunohistochemistry

After the perfusion with paraformaldehyde, the brains were quickly removed and post fixed overnight with 4% paraformaldehyde. The samples were embedded in paraffin and cut into approximately 10-µm-thick coronal sections with a microtome. For immunohistochemical detection of BrdU-labeled nuclei, coronal sections placed on poly-L-lysine-coated slides were incubated for 30 minutes with 2N HCl at 37°C for DNA denaturation. After blocking, sections were incubated overnight with mouse monoclonal anti-BrdU antibody (1:500; B2531, Sigma-Aldrich) at 4°C, and then incubated with anti-mouse IgG-Peroxidase conjugated antibody produced in goat (goat anti-mouse IgG conjugated with Peroxidase) (1:200, A2304, Sigma-Aldrich) for 2 hours. The peroxidase reaction was carried out by incubating with diaminobenzidine and hydrogen peroxide (Vector Laboratories) (31).

Cell counting

BrdU-positive cells located in the hippocampal dentate gyrus, CA1 and CA3 were counted in five to seven sections that were spaced 200µ apart and corresponded to coronal coordinates from bregma −2.80 to −4.52 mm, under ×400magnification (Olympus BX-51). Immunopositive cells were counted in 5 adjacent fields (each field was 400 $\mu m^{2)}$. Results were expressed as the average number of cells per section (31).

Statistical Analyses

Data were presented as mean ± SEM. Statistical analysis was performed using ANOVAs followed by a post hoc Tukey test using the statistical software SPSS (Windows version 11.5). Differences were considered significant at P <0 .05.

RESULTS

Behavioral analysis

Training

Animals were subjected for four days to the training set in the MWM. The differences in escape latency, swimming distance and speed parameters of training days among the five groups were analyzed. All groups of animals learned to achieve the hidden platform, as indicated by a

reduction in escape latencies and distance traveled. On the first and second days of training, no difference was found in escape latency between groups. The escape latency gradually declined over the training period for all groups indicating a gradual spatial memory acquisition in experimental animals. Paired-samples t-test showed that there was a significant difference between the fourth and the first day of training for the task of finding the hidden platform in terms of escape latency (P<0.05) in all groups. Two-way ANOVA with repeated measurements between groups in training days revealed a significant effect of groups [F (4, 30) =7.236; P = .000], a significant effect of days [F (3, 90) =21.239; P = .000] and no significant interaction [F (12, 90) =0.579; P = .854]. Intergroup comparison showed no significant difference on first and second days, but escape latency parameter was higher in lesioned group, as compare to control and RE100-treared groups, on third and fourth days (Fig. 1A).

Paired-samples t-test showed that there was a significant difference between the fourth and the first day of training for distance traveled (P<0.05) in all groups. Two-way ANOVA with repeated measurements between groups in training days revealed a significant effect of groups [F (4, 30) =7.236; P = .000], a significant effect of days [F (3, 90) =21.239; P = .000] and no significant interaction (F 12, 90 =0.579; P = .854). Intergroup comparison showed significant difference on first and second days between lesioned and control groups, also distance traveled parameter was higher in lesioned group, as compare to other groups, on third and fourth days (Fig. 1B).

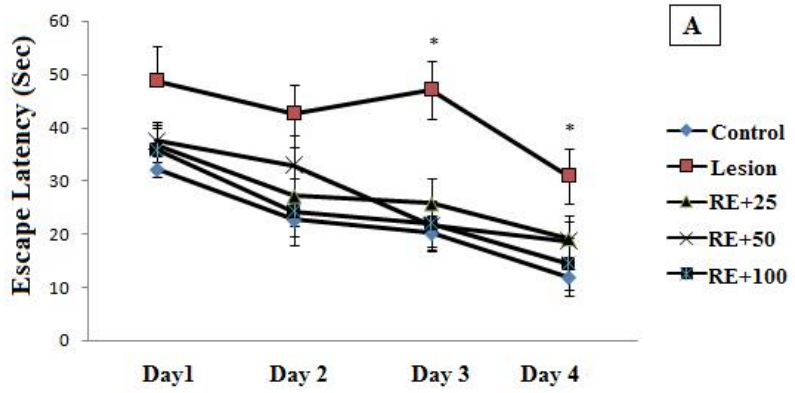

Fig. 1(A): The differences in escape latency of training days among the five groups. On the first and second days of training, no difference was found in escape latency between groups. There was a significant difference between the fourth and the first day of training for the escape latency in all groups. Data are expressed as mean ± SD. * (P<0.05) as compare to control and treated groups.

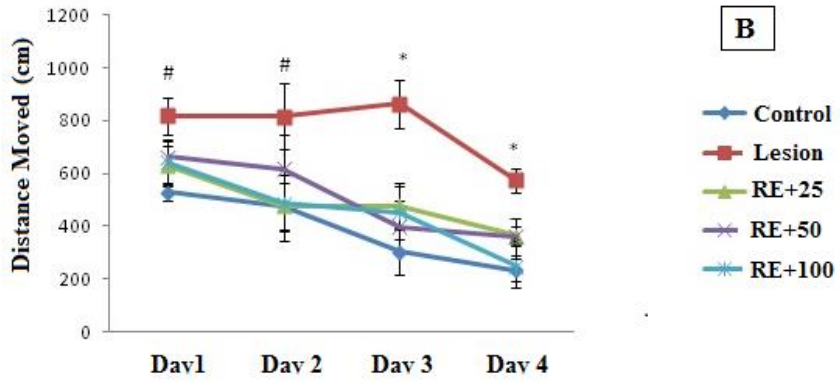

Fig. 1(B): The differences between days of training for distance traveled. There was a significant difference between the fourth and the first day of training for distance traveled (P<0.05) in all groups. Intergroup comparison showed significant difference on first and second days between lesioned and control groups (P<0.05), also distance traveled parameter was higher in lesioned group, as compare to other groups, on third and fourth days (P<0.01).

Paired-samples t-test showed that there was no significant difference between the fourth and the first day of training for the speed of swimming in all groups. Two-way ANOVA with repeated measurements between groups in training days revealed no significant effect of groups [F (4, 30) = 0. 315; P = .866], no significant effect of days [F (3, 90) = 0.403; P = .571] and no significant interaction [F (12, 90) =0.31; P = .986] (Fig. 1C).

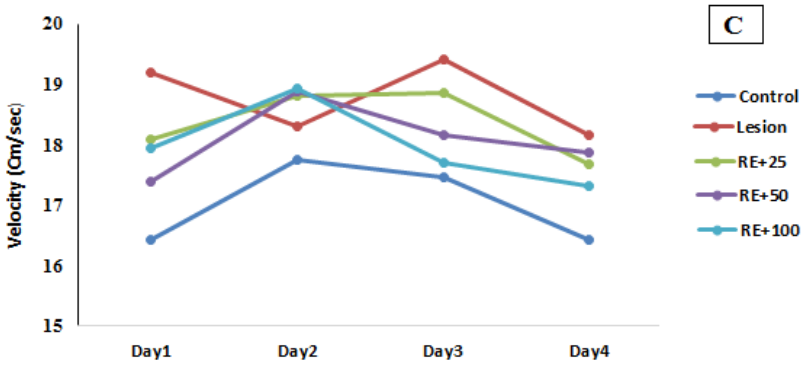

Fig. 1(C): The differences in speed of swimming of training days among the five groups. Between the fourth and the first day of training for the speed of swimming in all groups. There was no significant difference between the days in all groups.

Probe

Four parameters: escape latency, total traveled distance, swimming speed and percent of time spent in target zone were measured 24h after the last training session (probe) and analyzed by one-way ANOVA.

There was no significant difference in total traveled distance between groups on probe day [F (4, 30) =1.004, P=0.421], It means that our procedure had no effect on locomotion and motivation for finding platform (Fig. 2A). Also there was no significant difference in swimming speed between groups on probe day [F (4, 30) =1.189, P=0.33], so animals had no problem in movement (Fig. 2B). There was a significant difference of escape latency between different groups [F (4, 30) =25.065, P=0.000]. At the 14th day after lesion, RE at a dose of all doses significantly decreased the escape latency compared to lesioned group. There was also a significant increase in the escape latency in lesioned group compared to the control group (P<0.000) (Fig. 2C). Figure 2D demonstrated that there was a significant difference of percent of time spent in target zone different groups [F (4, 30) = 5.179, p = 0.003]. This parameter was significantly decreased in lesioned group as compared to the control (P<0.003). The time spent in target zone of rats received 50 and 100 mg/kg doses of RE was significantly longer than that of the lesioned.

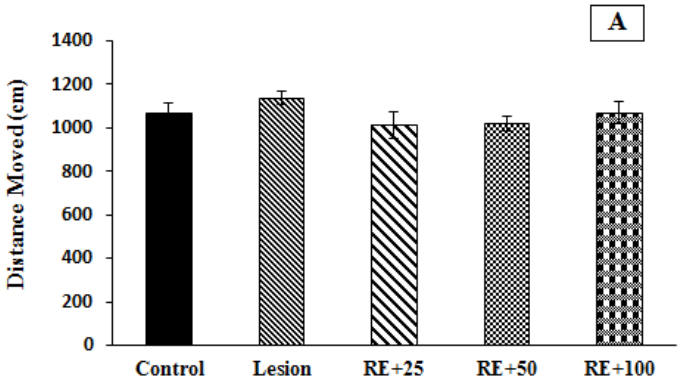

Fig. 2(A): The difference in total traveled distance between groups on probe day. There was no significant difference in total traveled distance between groups.

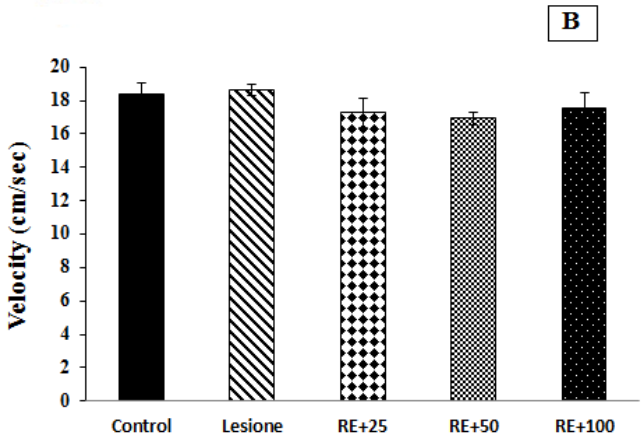

Fig. 2(B): The difference in swimming speed between groups on probe day. There was no significant difference in swimming speed between groups on probe day.

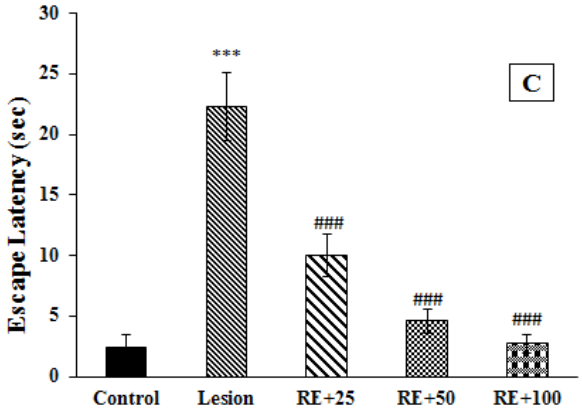

Fig. 2(C): The difference in escape latency between groups on probe day. At the 14th day after lesion, RE at a dose of all doses significantly decreased the escape latency compared to lesioned group. There was also a significant increase in the escape latency in lesioned group compared to the control group (P<0.000).

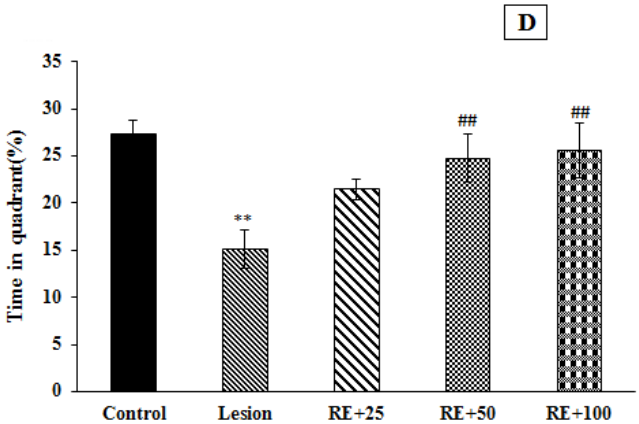

Fig. 2(D): The difference in percent of time spent in target zone (quadrant zone) between groups on probe day. Percent of time spent in target zone was significantly decreased in lesioned group as compared to the control (P<0.003). The time spent in target zone of rats received 50 and 100 mg/kg doses of RE was significantly longer than that of the lesioned.

Immunohistochemical analysis

The number of BrdU-labeled cells in the hippocampus of lesioned animals (n= 5 at each time point) were as follow, DG: 296.6±28.8, CA1: 85.33±4.3, and CA3: 52±5.81 count/400µm^2 and in RE100-treated group were, DG: 546±19.34, CA1: 125.33±4.8, and CA3: 82.66±7.3. It was indicated a significant increase of BrdU positive cells in DG, CA1 and CA3 of RE100-treated rats as compared to that of lesioned group (Fig 3-6).

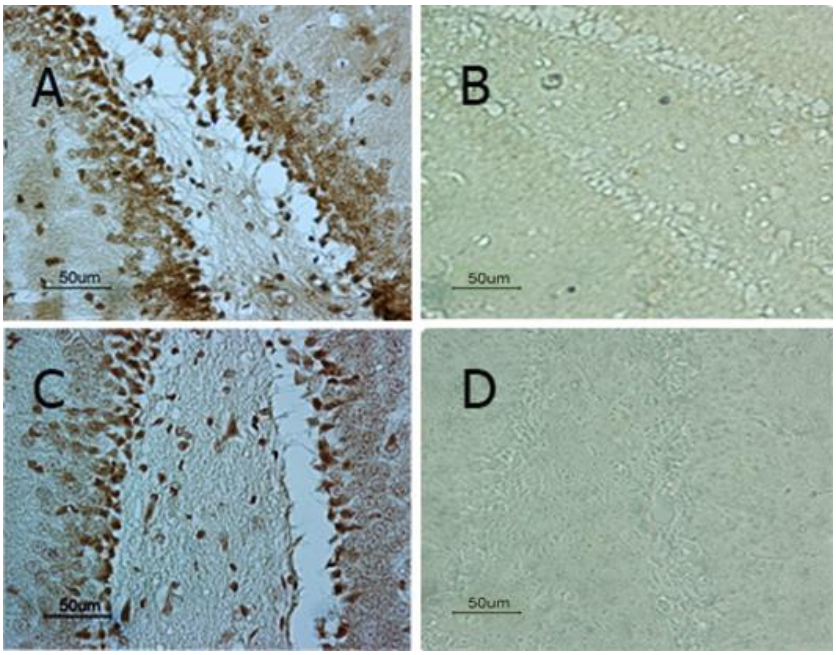

Fig.3 (A-D): Representative photographs illustrating BrdU-immunopositive cells in DG.
A: RE100-treated group, B: negative control of (A), C: 6-OHDA treated group, D: negative control of (C). RE100 treatment increased cell proliferation while 6-OHDA treatment decreased cell proliferation in various subregions of the hippocampus.

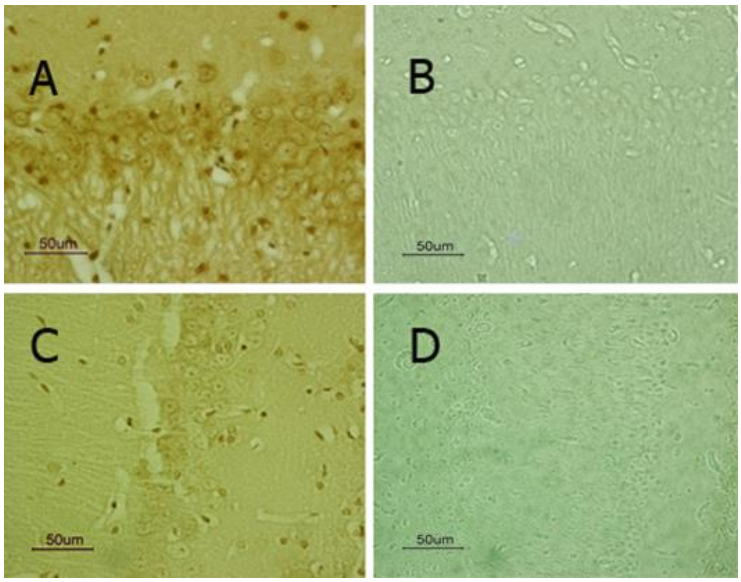

Fig.4 (A-D): Representative photographs illustrating BrdU-immunopositive cells in CA1.
A: RE100-treated group, B: negative control of (A), C: 6-OHDA treated group, D: negative control of (C). RE100 treatment increased cell proliferation while 6-OHDA treatment decreased cell proliferation in various subregions of the hippocampus.

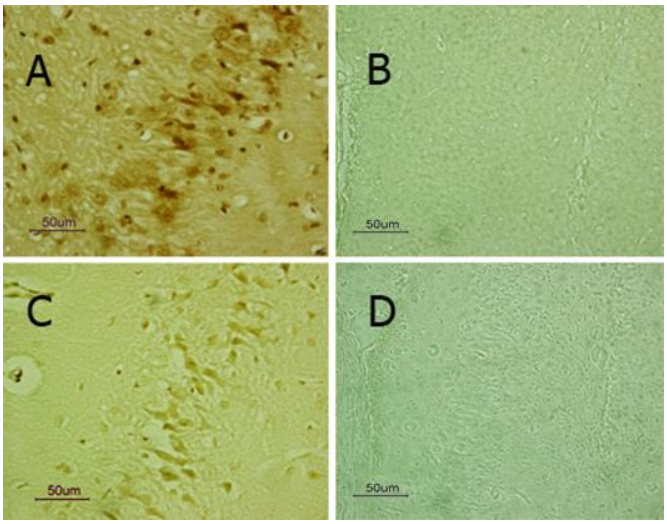

Fig.5 (A-D): Representative photographs illustrating BrdU-immunopositive cells in CA3.
A: RE100-treated group, B: negative control of (A), C: 6-OHDA treated group, D: negative control of (C). RE100 treatment increased cell proliferation while 6-OHDA treatment decreased cell proliferation in various subregions of the hippocampus.

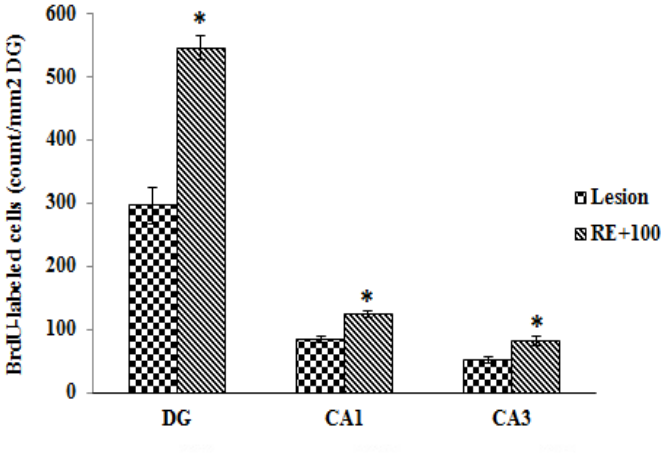

Fig. 6: Total number of BrdU-positive cells in dentate gyrus (DG), CA1 and CA3 of 100RE-treated rats were significantly increased as compared to lesion

d rats.

DISCUSSION

Because of the hippocampus and midbrain connection (dopaminergic loop), the releasing dopamine affect hippocampal-dependent memory (4, 32-34). Dopamine, a neuromodulator, which is known to regulate several forms of learning and memory. The contribution of cortical DA to the working memory processes altered in the late stage of PD has been emphasized (35). Gasbarri (1996) reported that the lesion of mesohippocampal dopaminergic system induced spatial memory impairment (36). Moreover, it was shown in previous studies that rats with SNc lesion induced by 6-OHDA present deficits in spatial memory tasks without gross motor alterations like in PD (14, 17). In the present study, the rats were subjected to 6-OHDA injection into the SNc, which is an appropriate model for memory impairment of PD. The motor symptoms of PD are more known than cognitive disabilities. Patients who go on to develop PD may have detectable non-motor feature including memory dysfunction, before they have motor symptoms, because nigral neuronal damage occurs in PD, before clinical symptoms are detectable (37). This symptom accompanying the early phase of PD affects quality of patients' live. The hippocampus is the brain area that plays an

important role in spatial memory (38), thus we determined the cognitive performance of the rats using the MWM test which is the best tool to determine spatial memory and learning in rodents (28, 39). This task is based upon the premise that animals have evolved an optimal strategy to explore their environment and escape from the water with a minimum amount of effort, i.e., swimming the shortest distance possible. For spatial learning acquisition test, the time (escape latency) and swimming distance to reach the platform were recorded for each rat. The involvement of RE on hippocampal memory related to DA systems has not been investigated much, compared to other DA systems. To evaluate the effect of RE on memory function, the spatial learning acquisition and memory retention of rats were tested using the MWM (28, 40, 41).

Although following 4 days of training in the MWM in our experiments, all groups of animals learned well as indicated by decrease in time and distance for finding the hidden platform, but lesioned group was weak in learning. At the end of training there was a significant increase in escape latency and swimming distance in lesioned group compared to the control group. Reduction in escape latency and swimming distance on third and fourth days of training in RE treated groups demonstrated improvement of cognitive impairment and elevation of learning ability.

To assess spatial memory retention, a probe trial was performed, during which the platform was removed from the pool, and four parameters were measured. No significant difference, in the traveled distance and swimming time tests between the all experimental groups emerged, so 6-OHDA-induced bilateral destruction of dopaminergic neurons produced a significant decrease in short-term memory without significantly affecting locomotion. There was a significant difference of escape latency between lesioned and control animals, which indicated an impairment in spatial memory at two weeks after injury and also they spent less time in the target quadrant than control rats.

It was further showed that injured rats received the RE treatment had stronger spatial bias in the probe test than the controls, this group spent significantly shorter time to find the hidden platform (escape latency) compared to lesioned group. RE treated group spent more time in the target quadrant than lesioned rats. There was no difference of escape latency and time spent in target quadrant between control animals and rats fed on the diet supplemented with 50 and 100 mg/kg RE. These groups also showed significant decrease and increase of escape latency and time spent in target quadrant, respectively as compared to lesion group. These findings suggest that dietary supplementation of RE improved memory acquisition and retention in 6-OHDA injected rats. Singhal (2012) reported some medicinal herbs such as RA in the treatment of memory loss of Alzheimer disease (42). Meizoso (43, 44) and Alkam et al (45) demonstrated that RA, in addition to decreasing the stress in Alzheimer rats, could improve spatial memory in Y maze.

We determined the effect of RE on the neuron density in the various subregions (CA1, CA3, and dentate gyrus) of the hippocampus. Because the dose of 100 mg/kg RE was the most effective dose, we evaluated the histological effects of RE in this group.

Our finding suggested that the number of BrdU-positive cells of RE100-treated rats significantly increased in all subregions of the hippocampus as compared to injured rats and it was linking to memory improvement in treated rats, by oral administration of RE.

Further studies are needed to determine whether BrdU-positive cells increased in RE100-treated after injury. One possible mechanism is that RE effects as an NGF inducer. RE would promote synthesis of NGF by glioblastoma human cells in vitro (46). NGF administered by injection via peripheral routes is unable to reach the brain through the BBB. Therefore low molecular weight NGF inducers that pass through the BBB would be good candidates for anti-dementia drugs (46). ITO et al, reported that the up regulatory action of RA induced cell proliferation may be one of the mechanism of the antidepressant-like effect of RA (47). In recent years much effort has been dedicated to the development of herbal therapy. We found that RE significantly improved the memory impairment induced by 6-OHDA injection, indicated by the decrease of time spent to find out platform (escape latency) and the increase of retention time. It hopes that these data may be beneficial for the future herbal medicine for treatment of PD. This herbal therapy would delay or prevent clinical symptoms of PD.

CONCLUSION

The present study demonstrated that RE, improved spatial learning and memory in injured rats. The 100 mg/kg dose showing significant improvement in the maximum parameters of memory

performance could be considered as the most effective dose. It was concluded that RE exerts the memory enhancing effect in this PD model and it was related to the number of neurons in hippocampus. Consequently, the use of RE as an adjuvant therapeutic agent for the treatment of cognitive impairment in PD should be considered. However, further investigations are still required.

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