Effects of Rosemary Oil (Rosmarinus officinalis) supplementation on the fate of the transplanted human olfactory bulb neural stem cells against ibotenic acid-induced neurotoxicity (Alzheimer model) in rat

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Abstract

Rosemary oil (ROO) is known to have multiple pharmacological effects: it is an antioxidant, anti-inflammatory, and cytoprotective. In the present study, we examined the effects of ROO on Human olfactory bulb neuronal stem cells (hOBNSCs) after their transplantation into rats, with the ibotenic (IBO) acid-induced cognitive deficit model. After 7 weeks, cognitive functions were assessed using the Morris water maze (MWM). After two months, blood and hippocampus samples were collected for biochemical, gene expression, and histomorphometric analyses. Learning ability and memory function were significantly enhanced ($P < 0.05$) after hOBNSCs transplantation and were nearly returned to normal in the treated group. The IBO acid injection was associated with a significant decline ($P < 0.05$) of total leukocyte count (TLC) and a significant increase ($P < 0.05$) in total and toxic neutrophils. As well, the level of IL-1β, TNF-α CRP in serum and levels of MDA and NO in hippocampus tissue were significantly elevated ($P < 0.05$), while antioxidant markers (CAT, GSH, and SOD) were reduced ($P < 0.05$) in treated tissue compared to controls. The administration of ROO before or with cell transplantation attenuated all these parameters. In particular, the level of NO nearly returned to normal when rosemary was administrated before cell transplantation. Gene expression analysis revealed the potential protective effect of ROO and hOBNSCs via down-expression of R-βAmyl and R-CAS 3 and R-GFAP genes. The improvement in the histological organization of the hippocampus was detected after the hOBNSCs transplantation especially in h/ROO/hOBNSCs group.

Keywords Alzheimer’s disease · Rosemary oil · Olfactory bulb neural stem cells · Gene expression · Immunocytochemistry

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AD           | Alzheimer’s disease |
| ROO          | Rosemary oil |
| IBO          | Ibottenic acid |
| hOBNSCs      | Human olfactory bulb neural stem cells |
| TLC          | Total leukocyte count |
| TNF-α        | Tumor necrosis factor alpha |
| IL-1β        | Interleukin-1β |
| CRP          | C-reactive proteins |
| CAT          | Catalase |
| GSH          | Reduced glutathione |
| SOD          | Superoxide dismutase |
| MDA          | Malondialdehdy |
| NO           | Nitric oxide |

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Introduction

Alzheimer’s disease (AD) is one of the major public health concerns associated with a progressive and irreversible cognitive deficit and memory loss in aged people (Gjoneska et al. 2015). Currently, the European Prevention of Alzheimer’s Dementia (EPAD) committee has classified AD as the most widespread neurodegenerative disease. Globally, around 29.8 million individuals were affected by Alzheimer’s disease in 2015, with the number expected to rise to 115 million by 2050 (Alipour et al. 2019).

Chronic neuroinflammation and neuronal loss due to the extracellular aggregation of an amyloid-β peptide (Aβ) as senile plaque and the intraneuronal deposition of neurofibrillary tangles and a wide range of metabolic dysfunctions are all hallmarks of neuropathological features of AD (Alipour et al. 2019). The hippocampus is most vulnerable to these pathological alterations and affected than other areas of the brain (Giraldo et al. 2014). Until now, all the drugs available for AD such as memantine, partial antagonist of N-methyl-D-aspartate receptor (Olivares et al. 2012), only relieve clinical symptoms but are unable to prevent progression or to replace the degenerated neurons (Cummings et al. 2014). Consequently, the development of novel therapies to alleviate AD pathologies, inhibit neuronal death, replace dead neurons, eliminate toxic deposits, and provide a better niche for remaining cells are very necessary.

Traditional medicine has become more highly regarded in the past few last decades. Several plant materials are rich in natural antioxidants (Abu-Al-Basal 2010). Rosemary (Rosmarinus officinalis) is one of the plants that are widely used around the world as a potent source of natural antioxidants. The efficacy of rosemary as a natural antioxidant is principally attributed to its chemical constituents, including carnosic acid, carnosol, 1,8-cineole, α-pinene, β-pinene, diterpenes, and myrcene-rich essential oils (Ngo et al. 2011). Several studies have demonstrated therapeutic and pharmacological effects of rosemary essential oil, as an anti-nociceptive, anti-inflammatory agent, and cognition-enhancing (Rasoolijazi et al. 2015).

Over the past few years, stem cell-based therapy has been tested in both animal models and clinical trials and has become a potential novel approach for several human diseases, such as autoimmune diseases, and neurodegenerative diseases (Bel et al. 2003; Ge et al. 2018; Hall and Shenoy 2019).

In AD, several reports have shown improvement in cognitive and memory performances in rats with an AD model after their transplantation with stem cells (McGinley et al. 2018). Among various stem cells, the human adult olfactory bulb neural stem cell (hOBNSCs) provides a potential source for autologous stem cells, while avoiding the ethical issues involved in the use of human embryos (Casalbore et al. 2009).

The therapeutic potential of the hOBNSCs for treating many neurodegenerative diseases such as AD (Marei et al. 2015a), Parkinson’s (Marei et al. 2015b), and spinal cord injury (Marei et al. 2016), as well as their ability to survive and differentiate into neuronal cell types after transplantation has been established in many of our previous studies (Marei et al. 2015a, b; Marei et al. 2016).

The ibotenic acid (IBO) induced AD model has been chosen for this study as intrahippocampal injection of IBO in rats produces nearly the same symptoms and pathological changes that are seen in humans with AD (Karthick et al. 2016). The present study was thus performed to determine whether rosemary oil (ROO) can alleviate effects on the hippocampus and enhance the differentiation of hOBNSCs into different neuronal linages that might replace damaged neurons resulting from the IBO injection, and to determine the best time for administration of ROO (before hOBNSCs transplantation or concurrently with it).

Materials and methods

Preparation of oil extract

Rosemary (R. officinalis L.) essential oil used in this work was kindly extracted by the Department of Botany, Faculty of Science, Mansoura University (Mansoura, Egypt). The oil extraction was performed using the hydro distillation process (Tigrine-Kordjani et al. 2012) and then kept at room temperature till be used. As described by manufacturer, the rosemary oil contained about 32.5% 1,8-cineole, 13.7% α-pinene, 11.3% β-pinene 15.2% p-cymene, 12.2% camphene, 8.6% camphor and 6% of other unidentified compounds.

Animals

Fifty adult male rats (Sprague Dawley strain, 3 months old and 200–250 g weight) were used in this study. They were housed in suitable cages (5 rats/cage) under standard hygienic environmental conditions (12 h light/12 h dark cycle, 25 ± 5 °C, and 65% humidity) and freely supplied with a balanced diet and clean water. All rats were fed conventional basal diet to meet the NRC requirements (1995). The protocol of this experiment was performed according to the ethical committee Guidelines of Faculty of Veterinary Medicine, Mansoura University (Approval number; R/4/307/2019).
Study design

The rats were randomly divided into five groups (10 rats/group) as follows: Control group; no manipulation was received. IBO acid model group; bilaterally intra-hippocampus injected with IBO acid (4 μL). IBO/hOBNSCs group; bilaterally intra-hippocampus injected with IBO acid (4 μL) followed by bilateral intra-hippocampus transplantation of hOBNSCs (4 μL) 22 days after IBO acid injection. IBO/ROO/hOBNSCs group; bilaterally intra-hippocampus injected with IBO acid (4 μL) followed by rosemary oil administration (30 mg/kg) by stomach tube at the 7th day and once a day thereafter for 2 weeks; this was then followed with hOBNSCs transplantation on the 22nd day. IBO/hOBNSCs + ROO group; bilaterally intra-hippocampus injected with IBO acid (4 μL) followed by concurrently hOBNSCs transplantation with rosemary (30 mg/kg) oil administration at 22nd day (once a day/2 weeks) after IBO injection. The study design was summarized in Fig. 1.

Human olfactory bulb neural stem cells (hOBNSCs) isolation and culture

The hOBNSC were isolated and cultured according to the previously described methods (Casalbore et al. 2009). This step was explained in detail in our previous studies (Marei et al. 2015a, b, 2016). The olfactory bulbs were collected from six adult patients (39–45 years) undergoing craniotomy at the Institute of Neurosurgery, Catholic University, Rome, Italy. Informed consent was received according to the Ethical Committee protocols of the Catholic University.

Immunocytochemical assessment

Immunostaining of the cells’ composition was performed with appropriate specific antibodies to evaluate the cells’ multipotentiality, using the method published by Pagano et al. (2000). The primary antibodies used (all from Sigma-Aldrich) were anti-Nestin (1:200, rabbit, for undifferentiated neural stem cells), anti-beta tubulin III (1:100, rabbit, for immature neurons), anti-MAP2 (1:200, rabbit, for mature neurons), anti-GFAP (1:400, mouse, for astrocytes) and anti NG2 (1:100, rabbit, for oligodendrocyte progenitors). The secondary antibodies (also from Sigma-Aldrich) were tetramethylrhodamine isothiocyanate ([TRITC], 1:200, anti-rabbit, anti-mouse, and Fluorescein iso-thiocyanate (FITIC), 1:200, anti-rabbit, anti-mouse. After that, the culture was washed and incubated for 15 min with 4, 6-diamidino2 phenyl indole dihydrochloride ([DAPI], nuclear stain, 1 mg/ml, and then examined with FluorsaveTM [Calbiochem; La Jolla, CA, USA].

Morris water maze test (MWMt)

After acclimatization to the environment for one week, the rats were subjected to the Morris water maze to assess spatial memory and learning. The methodology of this test was completely explained in a previous study (Vorhees and Williams 2006). The animal’s behavior, the escape latency time (time needed to reach to platform), and time in the target quadrant (quadrant time) were tracked and measured with a digital camera. The scores were recorded on the 6th day after training (before IBO injection) and at the end of the study (before sample collecting with one week).
IBO acid-induced memory deficient rat model

Memory deficient model was performed by bilateral IBO acid (Sigma, St. Louis, MO) injection into the rat’s hippocampus. IBO acid was dissolved in 10mM artificial cerebrospinal fluid (CSF) at a concentration of 8 mg/ml (Karthick et al. 2016). The surgery protocol was the same as that explained in our previous study (Marei et al. 2015a). To anesthetize the rats, a mixture of ketamine (80mg/kg body weight) and Xylazine (10mg/kg body weight) was intraperitoneally administered. Each rat was carefully placed in a stereotactic frame under complete aseptic conditions. Intra-hippocampus injection of 4 µL of IBO acid solution was made slowly over 5 minutes using a 10 µL Hamilton syringe (coordinate, ML: 2.5 mm, AP: 3.5 mm, and VD: 2.7 mm relative to the bregma) according to the brain atlas of Paxinos and Franklin (2001). Following the injection, the skin was sutured, and the rats were returned to their cages when fully recovered from anesthesia.

hOBNSCs transplantation

Animals in IBO/hOBNSCs, hOBNSCs/ROO/hOBNSCs, and IBO/hOBNSCs + ROO groups were subjected to hOBNSCs transplantation surgery on the 22nd day after IBO injection. A Trypan blue exclusion test was used immediately before cell transplantation to detect the cell viability (not less than 95% viability) under a phase-contrast microscope (Glas et al. 2012). The total cell number was calculated with a hemocytometer then the cells were suspended in artificial CSF (60,000 cells/µL). Animals were rearresthetized and 4 µL of cell suspension was transplanted (2 µL for each side) into each rat using a 10 µL Hamilton syringe at the same coordinates of IBO injection. The immune suppressor cyclosporine (Sandimmune®, Roche, Basel, Switzerland) was injected daily (10 mg/kg/S.C.) one day before cell grafting and throughout the experiment’s course. The treatment protocol was summarized in Fig. 1.

Sample collection

Samples were concurrently collected from each group 11 weeks after IBO acid injection (8 weeks after cell transplantation). Two blood samples were collected through cardiac puncture from each animal. One blood sample was collected with anticoagulant (EDTA) for blood counting. The other sample was collected into plain tubes, allowed to clot at room temperature for 15 min, and centrifuged at 3000 rpm (4 °C) for serum separation. The separated serum was frozen at –80 °C for further estimation of cytokine levels. After that, rats were euthanized, and their brains were dissected. The hippocampus was collected from each rat. Tissue samples were divided into three groups. The 1st group was fixed in neutral formalin 10% (48 h) for histological examination and morphometric analyses; the 2nd group was preserved in Trizol Reagent (Invitrogen, UK) for gene expression analyses and the 3rd group was homogenized and cold centrifuged; the supernatants were separated and carefully collected into clean sterile tubes to be used in the evaluation of antioxidants/oxidative stress parameters.

Total and differential leukocyte count

Total leukocytes (TLC) were counted manually after soluition in Turkey’s solution. Blood smears were also prepared and stained with Wright’s Giemsa stain for differential leukocyte counts. Later, neutrophil–lymphocyte ratio (NLR) and lymphocyte-monocyte ratio (LMR) were calculated (Borné et al. 2016).

Serum cytokines and C-reactive proteins (CRP)

Serum tumor necrosis factor-alpha (TNF-α) and interleukin (IL-) concentrations were measured in serum using ELISA, with ready-made commercial kits purchased from Quantikine Co. (USA). CRP was estimated according to the standard protocol of ELISA ready-made kits obtained from Cobas Co. (USA).

Antioxidants/ Oxidative stress parameters

The hippocampal levels of catalase (catalog No.; CA 25 17), glutathione (GSH, catalog No.; GR 25 11), superoxide dismutase (SOD, catalog No.; SD 25 21), and malondialdehyde (MDA, catalog No.; MD 25 29), were estimated using commercial test kits obtained from Biodiagnostics company (Cairo, Egypt). Additionally, hippocampal level of nitric oxide (NO) was determined according to the method of Miranda et al. (2001) with some modification. All tested parameters were determined spectrophotometrically (Lambda EZ201; Perkin Elmer).

Total RNA extraction and gene expression analysis

RNA was extracted from the hippocampus specimens with the use of Trizol Reagent (Invitrogen, UK) following the procedures of the manufacturer. The concentration of RNA and purity were checked with a “Q5000” Quawell nanodrop spectrophotometer (USA). The integrity of RNA was also assessed by gel electrophoresis. An equivalent of 2 µg of RNA was transferred to cDNA with a kit supplied from Introntbio, South Korea (Hisenscript™ cDNA synthesis kit) according to the instructional manual.

Quantitative real-time PCR (qRT-PCR) was performed on a Rotor-Gene Q cycler (Qiagen, Heidelberg, Germany), using Quantitec SYBR Green PCR kits (Qiagen, Heidelberg, Germany). The sequences of the primers used in
this study are shown in Table 1. The β-actin gene acted as an internal control for normalizing expression levels of the target genes (Livak and Schmittgen 2001). The total volume of the reaction is 20 µL, which contained 10 µL 2× SensiFast SYBR, 3 µL cDNA, 5.4 µL for H2 (distilled water), and finally 0.8 µL of each gene-specific primer. The amplification cycling conditions were: 95 °C for 10 min then 40 cycles of 94 °C for 40 s; 55 oC for 30 s is the optimum temperature for annealing of the chosen primers; then elongation for 30 s at a temperature of 72 oC and again at 72 °C for final elongation over 10 min. At the end of the amplification cycle, a melting curve was created after the completion of the amplification phase; the relative expression analysis of target genes was complete using the 2−ΔΔCt procedures (Pfaffl 2001).

**Statistical analysis**

Statistical analysis of the results was performed using SPSS PC (version 19, IBM Analytics, New York, New York, USA). All values were expressed as mean ± SME. The data were analyzed using a one-way analysis of variance (ANOVA) test followed by Tukey’s post hoc test for multiple comparisons. Differences were considered statistically significant at \( P < 0.05 \).

**Results**

**hOBNSCs immune cytochemical assessment**

The hOBNSCs remained undifferentiated when cultured in serum-free proliferation media. The cells only proliferated in clusters or aggregated cells known as neurospheres. These neurospheres were separated into single cells using enzymatic digestion accurate. More than 90% of the cells possessed intact cell membranes that excluded trypan blue dye, confirming cell viability for transplantation (Fig. 2a, b). During the growth phase, more than 90% of the cells remained undifferentiated as they give only positive immune staining of nestin (Fig. 2c). After one week of differentiation, most of the cells gave positive immune reactivity for GFAP and MAP2 (Fig. 2d, e) and a small number gave positive immune reactivity for NG2 and (Fig. 2f) b-Tubulin III (Fig. 2g) confirming the multipotency of the cells.

**Water maze test**

The mean time of latency to the platform was significantly increased (\( P < 0.05 \)) in the IBO acid lesioned group compared to the control group. The latency time was significantly decreased (\( P < 0.05 \)) after the transplantation of

| Gene            | Primer sequence                      | Accession no |
|-----------------|--------------------------------------|--------------|
| Human MAP2      | F’5-ATTTAACAGCGAAAGGACA-3’ R’5-TGGTGGTACCTCCTCC-3’ | NR_164696.1 |
| Rat GFAP        | F’5-CGTCCTGACGCTTATAC-3’ R’5-AAG AAC TGG ATC TCC TCC TC-3’ | NM_017009.2 |
| Human GFAP      | F’5-CATGACTTTGTCCCCATTTCT-3’ R’5-GTGTGTTGTTG-3’ | NM_002055.5 |
| Rat Amyloid beta| F’5-TACCCCTTAGTACACT-3’ R’5-CTA GAC AAC ACC GCC CAC-3’ | NM_000484.4 |
| Human CNP-1     | F’5-CGTCCTAGATGCAAGCGCTC-3’ R’5-CAGCAGGTCTCCTGAGCAG-3’ | NM_001330216.2 |
| Rat Caspase3    | F’5-AGTGGACCCACCTGAGTGCAG-3’ R’5-AGT CTG CAG CTC CTC CACAT-3’ | NM_012922.2 |
| Rat β-actin     | F’5-TCCCTGACGCGCAAGTACTC-3’ R’5-GTTCAGTAACAGTCCGCTAGA-3’ | XM_032887061.1 |

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**Histomorphometric analyses**

Formalin-fixed hippocampus samples were processed for paraffin wax embedding by dehydration in ascending grades of ethanol (50, 70, 80, 95, and 100 /1 h for each), then cleared in two changes of xylene (1 h/each); they were paraffin wax impregnated and embedded, sectioned (5 µm) with a rotatory microtome and mounted on coated glass slides. The mounted sections were stained with Hematoxylin & Eosin (H&E) stain or cresyl violet stain and examined under a light microscope. For morphometric analysis, morphometric measures were obtained from all groups. Five different samples from five different rats were taken. Three H&E-stained sections from each sample were examined under higher magnification (×400) (Gao et al. 2006). The mean thickness of the pyramidal layer at CA1 and CA3 and the granular layer of the dentate gyrus was measured with an image analyzer. At the same time, the mean number of their viable neurons was also counted.

**Table 1** Forward and reverse primers sequence and accession number of the studied genes

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hOBNSCs compared to the IBO group especially in the IBO/ROO/hOBNSCs group which nearly reached that of the control one (Fig. 3A).

The mean time that rats spent in the goal quarter (quadrant time) was significantly decreased ($P < 0.05$) in the IBO acid lesioned group compared to the control group. The transplantation of hOBNSCs significantly increased ($P < 0.05$) the quadrant time compared to the IBO acid lesioned group especially in IBO/ROO/hOBNSCs group as it nearly returned to normal. (Fig. 3B).

**Total and differential leukocyte count**

Total and differential leukocyte counts are depicted in Table 2. Significant decline of total leukocyte, lymphocyte, and monocyte count with an elevation of total and toxic neutrophil counts in the IBO-intoxicated group compared to control one ($P < 0.05$). Besides, IBO induced a significant increase in NLR compared to controls ($P < 0.05$). Whereas, hOBNSCs transplantation with or without ROO administration (IBO/hOBNSCs, IBO/ROO/hOBNSCs, and IBO/hOBNSCs + ROO groups) restored changes occur in NLR, total and differential leukocyte counts, especially, IBO/ROO/hOBNSCs group, unlike that of IBO-intoxicated group ($P < 0.05$). No significant difference in LMR among the different experimental groups.

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Fig. 2 (a and b) Phase contrast micrograph showing single hOBNSCs during counting on hemocytometer and viability test with trypan blue exclusion test, live cells (arrow), and dead cells (arrowhead). (c, d, e, f and g): Fluorescence image (20X) of hNGF-GFP-OBNSCs during proliferation phase on serum-free growth media showing: The hOBNSCs were stained for (a) Nestin (white arrowhead) and GFAP markers (white arrow), (d and e) MAP2 (vertical arrow) and GFAP (arrow) markers, NG2 (curved arrow) and GFAP (arrow), (g) β-Tubulin (arrow with interrupted tail) and GFAP (arrow). Note nuclei stained with DAPI (tailed arrow).
Serum cytokines and CRP

The levels of pro-inflammatory cytokine (IL-1β, TNF-α) and CRP were significantly higher in the IBO-intoxicated relative to the controls ($P < 0.05$) (Fig. 4). However, treating rats with ROO before or with transplantation of stem cells as in IBO/ROO/hOBNSCs and IBO/hOBNSCs + ROO groups significantly reestablished levels of serum cytokines when compared to the IBO group ($P < 0.05$) (Fig. 4).

Hippocampal level antioxidants/ oxidative stress markers

The antioxidative markers (CAT, GSH, and SOD) exhibited significantly lowered hippocampal activities in the IBO and stem cell treated groups (IBO/hOBNSCs group) when compared to the controls ($P < 0.05$). On the other hand, the hippocampal lipid peroxide (MDA), and NO levels showed a significant increase in the IBO group compared to the control rat ($P < 0.05$). Significantly higher activity of antioxidant insult (CAT, GSH, and SOD) in both IBO/ROO/hOBNSCs and IBO/hOBNSCs + ROO groups relative to that of the IBO group ($P < 0.05$). Moreover, injection of stem cells alone or with ROO induced a significant decline when compared to controls ($P < 0.05$), with the main lowest level in IBO/ROO/hOBNSCs group (Table 3).

Gene expression with real-time PCR

As shown in Fig. 5, a forty-seven-fold increase in the expression of R-βAmyl was observed in the IBO group in comparison with the control one ($P < 0.05$). This was significantly decreased in all studied groups that received stem cells alone (IBO/hOBNSCs) or in combination with rosemary (IBO/ROO/hOBNSCs and IBO/hOBNSCs + ROO groups), reaching the control level.
On the other hand, the apoptotic marker, R-caspase-3, showed an 11-fold increase of expression in the IBO group when compared with the control group which was the highest group \( (P < 0.05) \). Meanwhile, expression of R-caspase-3 was significantly decreased in IBO/hOBNSCs and IBO/ROO/hOBNSCs groups when compared with the control group \( (P < 0.05) \), followed by IBO/ROO/hOBNSCs+ROO group which was similar in expression to the control group with non-significant differences between both groups \( (P < 0.05) \).

Similarly, the expression of RGFAP showed a significant increase in expression by twenty-two-fold in the IBO group compared to the control group \( (P < 0.05) \). Conversely, the expression of RGFAP was significantly decreased in...
IBO/hOBNSCs, IBO/ROO/hOBNSCs, and IBO/hOBNSCs + ROO groups like the control group. Furthermore, the mean mRNA expression of the HGFAP was increased in IBO/hOBNSCs group by fivefold compared with the control group \( (P < 0.05) \), while the IBO/ROO/hOBNSCs group showed a significant increase in expression of the HGFAP gene by almost 2.5-fold compared with the control group \( (P < 0.05) \). Meanwhile, the IBO group showed the least expression of HGFAP compared with other groups \( (P < 0.05) \). The expression of HMAP2 in both IBO/ROO/hOBNSCs and IBO/hOBNSCs + ROO groups showed a non-significant difference between both groups \( (P < 0.05) \). Besides, the expression of HMAP2 showed the highest expression in IBO/ROO/hOBNSCs group with a 13-fold increase in expression when compared with the control group \( (P < 0.05) \). Both IBO/hOBNSCs and IBO/hOBNSCs + ROO groups showed nearly 15-fold expression of HCNP-1 in comparison with the control group \( (P < 0.05) \), while IBO/ROO/hOBNSCs and IBO showed nearly the same level of expression as the control group \( (P < 0.05) \).

**Histomorphometric results**

The light microscopic examination of the H&E-stained section of the control group exhibited the normal histoarchitecture of the hippocampus. It comprised two major interlocking c-shaped parts, which are the hippocampus proper (CA1, CA2, and CA3) and the dentate gyrus (DG) (Fig. 6 (1)). In the current study only CA1 (Fig. 6), CA3 (Fig. 7), and the dentate gyrus (Fig. 8) were subjected to examination as CA2 is poorly defined and small. Both CA1 and CA3 were arranged in three layers; polymorphic, pyramidal, and molecular. The pyramidal layer of CA1 (Fig. 6-(1a, 1b)) and CA3 (Fig. 7-(1a, 1b)) was the principal layer that contained...
pyramidal neurons with light vesicular nuclei, scattered astrocytes, and scattered oligodendrocytes. Cresyl violet stained sections through these pyramidal neurons revealed a rim of violet staining Nissl granules forming cap on neurons.

H&E-stained sections from the IBO group revealed that the pyramidal layer appeared loose with a significant decrease ($P < 0.05$) in thickness (Fig. 6 (6)) and numbers (Fig. 6, (7)) of their viable neurons compared to the control group; nearly all the pyramidal neurons in CA1 (Fig. 6-(2a, 2b)) and CA3 (Fig. 7-(2a, 2b)) appeared shrunken and deeply eosinophilic with pyknotic or completely lost nuclei. The density of the Nissl granules was markedly decreased or completely depleted in the pyramidal neurons using a cresyl violet stain. Massive gliosis and widely spread congested blood capillaries were detected in the molecular and polymorphic layers.

The pyramidal layer at CAI retained some of its normal histology in IBO/hOBNSCs group (Fig. 6-(3a, 3b))

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**Fig. 6** Photomicrograph of a section of rat's hippocampus at CA1. (1) stained with H&E showing: different fields of hippocampus, PO=polymporphic layer, PY=pyramidal layer in CA1 and CA3, MO=molecular layer, GR=granular DG=dentate gyrus. (1a, 2a,3a,4a,5a). stained with H&E and (1b,2b,3b,4b,5b) stained with cresyl violet showing: viable pyramidal neuron (arrow), astrocyte (arrowhead), oligodendrocyte (vertical arrow), axonal tract (thick arrow), massive gliosis (inside circle), dead neurons (corrugated arrow), edema and vacuoles (tailed arrow). (6) and (7) represent pyramidal layer at CA1 thickness and CA1 viable neuronal numbers respectively, data are expressed as Mean±SEM. The different letters indicate significant difference ($P < 0.05$) between experimental groups: 1 (control group), 2 (IBO group), 3 (IBO/hOBNSCs group), 4 (IBO/ROO/hOBNSCs group) and 5 (IBO/hOBNSCs+ROO group)
and returned to normal when the rosemary oil was given before (IBO/ROO/hOBNSCs group) (Fig. 6-(4a, 4b)) or concurrently with the cell transplantation (IBO/hOBNSCs + ROO) (Fig. 6-(5a, 5b)). The number of viable pyramidal neurons was significantly increased ($P < 0.05$) after hOBNSCs transplantation compared to the IBO group (Fig. 6-(7)), especially in IBO/ROO/hOBNSCs and IBO/hOBNSCs + ROO groups as there is no significant difference between them and the control group.

The histoarchitecture restoration in CA3 was more readily detected in IBO/ROO/hOBNSCs group (Fig. 7-(4a, 4b)) as the thickness (Fig. 7-(6)) and the number (Fig. 7-(7)) of intact pyramidal neurons nearly returned to the normal. On other hand, some of the dark shrunken neurons were still detected in IBO/ROO/hOBNSCs group (Fig. 7-(3a, 3b)) and IBO/hOBNSCs + ROO group (Fig. 7-(5a, 5b)), and the total number of their viable neurons (Fig. 7-(7)) was still significantly decreased ($P < 0.05$) compared to the control one; however, it was significantly increased ($P < 0.05$) compared to IBO group.

The dentate gyrus was formed of three layers; the molecular, granular, and polymorphic layers (Fig. 6-(1)).
granular layer is considered the principal layer of the dentate gyrus and is formed of densely packed granular cells. The superficial part of this layer included mature small rounded granular cells with light vesicular nuclei, while the subgranular zone contained immature neurons with oval dark nuclei (Fig. 8-(1a, 1b)). The Cresyl violet stain of the granular layer appeared as blue cells with light vesicular nuclei surrounded by violet Nissl granules. The granular layer in the IBO group appeared disorganized and with significant decreases ($P < 0.05$) in thickness and numbers of mature granular cells compared to the control group (Fig. 8-(2a, 2b)). Massive gliosis and widely spread congested blood capillaries were detected in the molecular and polymorphic layers. The number of mature granular cells was nearly returned to the numbers of the normal group in IBO/ROO/hOBNSCs group (Fig. 8-(4a, 4b)), since in the IBO/hOBNSCs group (Fig. 8-(3a, 3b)) and IBO/hOBNSCs + ROO group (Fig. 8-(5a, 5b)) the number of the mature granular cells still significantly decreased ($P < 0.05$) compared to control group; however, they were significantly increased compared to IBO group (Fig. 8-(7)).

**Fig. 8** Photomicrograph of a section of rat’s hippocampus at dentate gyrus (1a,2a,3a,4a,5a) stained with H&E and (1b,2b,3b,4b,5b) stained with cresyl violet showing: mature granular neuron (arrow), immature granular neuron (arrowhead), sever congestion and edema (thick arrow), disorganized granular layer (asterisk), astrocyte (corrugated arrow), microglia (tailed arrow). The granular layer at dentate gyrus thickness and viable granular neuronal numbers respectively (6, 7), data are expressed as Mean ± SEM. The different letters indicate a significant difference ($P < 0.05$) between experimental groups: 1 (control group), 2 (IBO group), 3 (IBO/hOBNSCs group), 4 (IBO/ROO/hOBNSCs group) and 5 (IBO/hOBNSCs + ROO group).
Discussion

The injection of IBO in the hippocampus was associated with up-regulation in the activity of caspase-3 resulting in oxidative stress, astrogliosis, and amyloid-beta accumulation (Vargas et al. 2010). The histological alteration following IBO administration is similar to the histopathological events seen in the brain of patients with AD (Marei et al. 2015a). We used hOBNSCs that have been reported in many previous studies for their ability to differentiate into different neuronal and glial elements in vitro (Marei et al. 2015a, b, 2016). Thus, combining complementary strategies might be required to enhance the proliferation and differentiation of hOBNSCs in this hostile environment. Based on these findings, our study was designed to investigate whether hOBNSCs alone can survive, differentiate and replace all damaged neurons or need further assistance by adding ROO as an anti-inflammatory and antioxidant substance (Rašković et al. 2014).

Routine peripheral blood parameters could be new inflammatory markers associated with the inception and prognosis of CNS diseases (Pikija et al. 2018). Our results showed that TLC, lymphocytes, and monocytes significantly declined while NLR, total and toxic neutrophils were significantly elevated in the IBO group. These findings agreed with those of (Dong et al. 2019) who speculated that a higher neutrophil count and NLR could be a favorable conducive diagnostic biomarker for AD.

Additionally, IL-1β and TNF-α are the pro-inflammatory cytokines, generated from microglia and astrocytes (Zuliani et al. 2007) and are involved in the phosphorylation process, the key pathogenic event of AD, as it can cross the blood–brain barrier causing neurodegenerative changes (Li et al. 2003). On the same line of our results (Swardfager et al. 2010) a significantly higher concentration of IL-6, TNF-α, IL-1β, TGF-β, IL-12, and IL-18 was observed in peripheral blood of AD subjects. The elevation of inflammatory mediators and CRP in the current study may be correlated to activation of astrocytes and microglia by potential damage in hippocampus tissue (amyloid plaques and neurofibrillary tangles) (Venegas and Heneka 2017). After activation, astrocytes enhance the production of ROS and NO, release TNF-α, IL-1 β, and IL-6, and increase GFAP expression (Palpagama et al. 2019). The up-regulation of GFAP expression is commonly considered a distinctive feature of neuroinflammation in many neurodegenerative conditions, including AD (Millington et al. 2014). Similar to our findings, Stoltenberg-Didinger et al. (1996) investigated that the expression of R-GFAP was increased after the exposure to IBO, a potent neurotoxic substance, which also accompanied an increase in the number of glial cells which express these signals. The histological image of the hippocampus of the IBO group in the current study also exhibited massive gliosis which was detected in the molecular and polymorphic layers of the hippocampus.

Rosmarinic acid from R. officinalis is recognized as a tool in the treatment of inflammation and oxidative stress (Rašković et al. 2014) in neurodegenerative diseases. In our study, we investigated the protective effects of ROO on inflammatory responses and oxidative stress in the case of AD. Our results showed that administration of ROO before or after cell transplantation significantly increases lymphocyte count and TLC, while reducing NLR, toxic, and total neutrophil counts. Similar to our findings, neutrophils and monocytes were dramatically increased; but the levels of lymphocytes and eosinophils were increased in rabbits treated with R. officinalis L. extract after prolonged exposure to lead (Mohamed et al. 2016).

Our experiment also demonstrated an anti-inflammatory activity of ROO, even when administrated before or with transplantation of stem cells, compared to the IBO group. Similarly, cineole administration significantly inhibited cytokine release from blood cells (IL-1β, TNFα, IL-4, IL-5, IL-6, and IL-8) with minor effects on chemotactic cytokines (Ansari and Scheff 2010). Similar to previous studies for their ability to differentiate into different neuronal and glial elements in vitro (Marei et al. 2015a, b, 2016).

In the current study, a forty-seven-fold increase in the expression of RAmyl was detected in the IBO acid group in comparison with the control group. As mentioned above, the accumulation of Aβ resulted in the activation of astrocytes. The activated astrocytes enhance the production of ROS. Therefore, Aβ is linked with the formation of ROS and the induction of oxidative stress that occurs in the case of AD (Tillement et al. 2010).

The significant reduction in antioxidant levels (GSH, SOD, CAT) corroborates the idea that oxidative stress is the early event that has a crucial role in the disease progression (Ansari and Scheff 2010). Similar to our findings, IBO has been reported to exhibit toxicity through the increasing activity of NO and the production of nitric oxide levels (Karthick et al. 2016). Additionally, the reduction in GSH, SOD, and CAT levels may be linked to the loss of neurons in AD (Bains and Shaw 1997) and increasing the free radical load, which triggers oxidative stress (Bains and Shaw 1997). Moreover, in our study, the hippocampal activities of antioxidant molecules were significantly restored with ROO administration, compared to the IBO or cell-treated groups. This was attributed to the active component of rosemary oil (1,8-cineole, α-pinene, and β-pinene and myrcene-rich essential oils) exhibiting greater free radical scavenging activity (Ngo et al. 2011).

Furthermore, clinical data demonstrate that a relationship exists between apoptosis, Aβ, and ROS. Aβ could serve as an extracellular signal molecule for caspase-3 activation (Choi et al. 1988), meanwhile, ROS can act as a potent intrinsic stimulus for caspase-3 (Redza-Dutordoir and Averill-Bates
and caspase-3 expression were still elevated after hOBNSCs transplantation without ROO compared to the control group resulting in the highest expression of hGFAP was detected in IBO/hOBNSCc group.

The amelioration of astrocytosis, beta-amyloid accumulation, and TNFα elevation in IBO/hOBNSCs group are not fully understood but may be attributed to the release of some diffusible bioactive trophic factors from the hOBNSCs itself that may decrease many of these neurotoxic substances and so enhance survival and differentiation; or it may be attributed to the increase in the expression of IGF-1 in this group, as normal astrocytes have a high capacity for the production and storage of GSH compared to neurons. Subsequently, they can protect the newly formed neurons from oxidative stress by releasing GSH into the surrounding environment (Baxter and Harding 2016). Furthermore, astrocytes also play a role in the degradation and clearance of Aβ through the expression of different types of proteases that are involved in the cleaving of Aβ (Redza-Dutordoir and Averill-Bates 2016).

Loss of neurons at CA1, CA3, and dentate gyrus after Intra hippocampus injection with IBO was associated with a progressive decline of cognitive function (Terry and Davies 1980). The behavioral deficit aspect of this model was our main interest. In the current study, the main latency time and quadrant time in the IBO group were significantly increased and decreased respectively compared to the control group; that confirmed that IBO administration is associated with impairment of spatial memory and cognitive functions. The enhancement in these times was detected after cell transplantation but nearly reached normal when ROO was given before cell transplantation. These results confirm the definitive objective of hOBNSCs transplantation, which is the achievement of cognitive functional recovery. The improvement in the learning and memory function in the current experiment was mostly detected in IBO/ROO/hOBNSCs group as this group possesses the highest antioxidant capacity, the lowest inflammatory cytokines, and the lowest expression of caspase and amyloid-beta aggregation.

Conclusion

Taken together the histological, biochemical and gene expression and behavioral findings are strongly suggestive of the administration of ROO before cell transplantation enhances these cells to fast differentiation and integration with the neighboring cells than when given concurrently with the cells and further that administration of ROO before cell transplantation is a promising candidate for cell-based therapy for AD.
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Data Availability All data are included in the submitted manuscript.

Conclusions We report the first isolation and characterization of human olfactory bulb neural stem cells (HOB-NSCs) from different anatomical sites of the olfactory bulb of aborted human fetuses. These cells have a high proliferation rate and can be induced to differentiate into neurons and astrocytes, suggesting that they may have potential for use in regenerative medicine.

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