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STUDYING EFFECT OF BOSWELLIA, BETA HYDROXY ACID (BBA) ON DOPAMINERGIC DIFFERENTIATION OF THE EMBRYONIC STEM CELLS EXPRESSING NURR1 AND GPX1 GENES

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ABSTRACT

Introduction: In the studies, it has been mentioned that the main cause of Parkinson's disease is specific loss of dopaminergic neurons in the Mesencephalon area. Pharmacotherapy has been the main treatment for Parkinson's disease up to now but side effects of these drugs and temporary treatment of disease's symptoms are of the main drawbacks of this therapeutic method. It has been recently shown that BBA increases length and number of neuron branches by affecting neural cells and it has been specified in the studies that one of the drugs with plant origin for neurodegenerative diseases in Iranian and Indian traditional medicine is Boswellia (Beta Hydroxy Acid (Bba) content). Considering the mentioned materials, the most important goal of the present research is to study effect of Boswellia (Beta Hydroxy Acid (Bba) content) on dopaminergic differentiation of the embryonic stem cells expressing nurr1 and gpx1 genes. **Method:** In the present research, cellular tests were used to differentiate embryonic stem cells of rats expressing nurr1 and gpx1 genes to dopaminergic neurons with function and EB construction protocol was used for differentiating between embryonic stem cells to dopaminergic neurons. Then, we evaluated expression of neural progenitor cells markers (Nestin) and markers of

dopaminergic neurons (neuron Tau, TH, Nurr1, Pitx3) with Real time PCR quantitative technique at the end of the third, fourth and fifth stages. In the third and fifth stages, specific markers expression was studied in protein level with Immunocytochemistry method. **Results:** It was shown that concurrent expression of Nurr1 and GPX1 along with BBA treatment with concentration of 30nM had the highest effect on increase of neural and neuron markers and finally expression of specific markers of dopaminergic neurons. **Discussion and conclusion:** Results show that BBA had positive effect on differentiation of stem cells into dopaminergic cells and dopaminergic neurons markers in the studied groups were expressed and the highest expression of these markers was observed in the group with external genes treated with BBA.

KEYWORDS: Boswellia, dopaminergic, stem cells, nurr1 ،gpx1.

INTRODUCTION

Parkinson's disease (PD) was first described by Dr. James Parkinson in 1817. He named this disease as Shaking palsy which is known as Parkinson's disease. The main cause of Parkinson's disease is the specific loss of dopaminergic neurons in the Mesencephalon area. In healthy people, movements of body are regulated by the dopaminergic neurons but dopaminergic neurons are lost in black body in the people with Parkinson's disease and the person will not have motor control due to phenomenon of dopamine depletion in neural arteries.[1]

Parkinson's disease is the most prevalent neurodegenerative disease after Alzheimer's disease.[2] Clinical aspects of the Parkinson's disease in clued secondary motor symptoms, dragging feet on the ground at time of walking and stammering and non-motor symptoms such as sensory and cognitive disorder.[3]

Brain is susceptible to oxidative stress damage due to high amounts of non-saturated fatty acids, low levels of antioxidants, high iron content in special areas such as Globus Pallidus and black body.[4] In addition, these cells permanently lose their performance due to indivisibility of neurons when they are damaged for one time.[5] For this reason, brain has more sensitivity to oxidative stress than other organs of the body.[6] For this reason, oxidative stress causes several chronic neurodegenerative diseases such as Parkinson's disease, Alzheimer, Huntington etc.[4]

In the past, it was mentioned that intensive reduction of glutathione, oxidation and nitration of proteins, lipids and DNA, increase of SOD and the increased level of free iron in black body of the Parkinson's patients were shown.[7]

Dopamine is one of the most important neurotransmitters which are produced in body. As a potential substrate, dopamine has been introduced in formation of synapses and memory mechanism. Many physiological activities have been attributed to this matter and these cases include mood, sleep, motivation, reward, Voluntary kinetic movement, attention and learning. One of the common damages of the main neural pathways in Mesencephalon area is the absence of dopamine or change in its secretion. Due to key role of these pathways in control of the body's system (such as movement control and keeping balance), defect in them cause many disorders. To clarify the subject, it can be mentioned that very high or very low dopamine rate can disrupt normal balance between dopamine system and system of other neurotransmitters and confront with the voluntary movement and this causes no control in kinetic movements in the living creature.[8] One of the important characteristics of the symptoms observed in Parkinson's disease is changes in population of dopaminergic neurons which are located in SNc part in Mesencephalon.[9,12]

In recent years, new attitudes have been mentioned for studying differentiation of dopaminergic neurons pathways in Mesencephalon area and several transcription factors have been identified for development and identification.[13] Nurr1 comes from intracellular nuclear receptors and mutation in this gene is accompanied by neuron disorders.[14] This gene is expressed in central nerves system particularly in SNpc ،VTA.[15] Lee refers to important effect of this gene in all life stages whether embryonic life period and after that and differentiation to dopaminergic cells and in all life stages of dopaminergic neurons.[15] GPX1 is considered as one of the main antioxidant enzymes of the mammals' cells which inactivates hydrogen peroxide and protects it against oxidative stress. GPX1 is expressed in Microglia in high level and in neurons in lower level.[16,18]

Since Parkinson's disease which is a neurodegenerative disease which affects neuron populations, attempts have been made to find the strategy which slows neurodegenerative processes.[19] Although patients lose about 50% of the *dopaminergic neurons of black body and 60-80% of the striatum dopamine when detecting disease, the remaining neurons can be a target for treatment through neural protection and repair.*[20,22] *On the other hand, a way of coping with oxidative stress is use of exogenous antioxidants.*[23] *For this reason, there is*

need for alternative strategies based on antioxidants for coping with harmful effects of oxidants and keeping balance of cell repair. Boswellia is collected from Burseraceae family which grows in dry regions of Africa and Asia. Therapeutic use of extract of this plant for neurodegenerative diseases relates to ancient medicine of Egypt, India and China.[24] Most studies on extract of this plant have been conducted on Triterpens which is generally called Boswellic *acid. These compounds and particularly 11-keto-beta-* Boswellic *acid with inhibition of 5-lipooxygenase pathway have anti-inflammatory effects*[25] *and also have anti-apoptotic effects in inhibition of tumor growth.*[26] Frankincense (containing BBA) was applied as a drug in prevention and treatment of amnesia in India several thousand years ago.[27] On the other hand, use of traditional medicine and herbs is increasing for improving diseases and effects of BBA on neural progenitor and stem cells extracted from hippocampus have been investigated in a study. This study has shown that BBA can increase growth and the number of collaterals and also stabilize polymerization of microtubules.[27] Considering the mentioned materials, goal of the present research is to study effect of synergism of Nurr1 transcription factor expression and GPX1 enzyme along with BBA matter in improvement of efficiency of differentiation of transgenic stem cells to *dopaminergic neurons.*

PROCEDURE

In the present research, cellular tests were used to differentiate embryonic stem cells of rats expressing nurr1 and gpx1 to dopaminergic neurons with performance.

Cellular methods include isolation and culture of mouse embryonic fibroblast (MEF) cells for preparation of MEF from pregnant mice, 12.5 day embryos and amnion and chorion layers were isolated. Then, the abdominal area and nerve cord and head were isolated from embryo. Then, the rest of body of embryo was disintegrated with scalpel blade and then it was incubated inside incubator along with trypsin for 10-20 min at 37°C. after isolation of larger tissues which have not been digested, the cells were isolated and isolated through centrifuge. MEF cells were cultured on petri dishes or flasks covered with gelatin in DMEM 01 % FBS culture medium. Flasks containing MEF with 90-100% of the coated cells on the flask for 3-4 hours were treated in concentration of mitomycin and then were prepared for use as feeder after 3-4 times of washing with PBS and then embryonic stem cells R1were cultured.[28] It is necessary to note that culture medium of embryonic stem cells is KO-DMEM which contains 10% of SR1 and 10% of FBS. This medium contains Pen/Strep with ultimate concentration of µg/ml01 for each one of them, unessential amino acids with ultimate concentration of

µM011 and l-glutamine with ultimate concentration of mM011 and beta- Mercaptoethanol with ultimate concentration of ‹011 µM and U/ml LIF0111. Culture medium of MEF and HEK-293T ‹high glucose DMEM cells has 10% of FBS and pen/strep. Culture medium of R1 stem cells was replaced every day and culture medium of MEF and HEK-293T cells was replaced every 2-3 days. Then, cells R1 were passed and counted.

Differentiation of R1-NG cells to dopaminergic neurons

In this research, we used EB construction protocol for differentiation of embryonic stem cells to dopaminergic neurons.[29] Embryonic stem cells were proliferated in the first stage, EB formation was done in the second stage, EB culture was done on Matrigel and PLL(Poly l-Lysine) in the third stage, positive Nestin cells proliferation toward mesencephalic differentiation was done in the fourth stage and differentiation to dopaminergic neurons was done in the fifth stage.

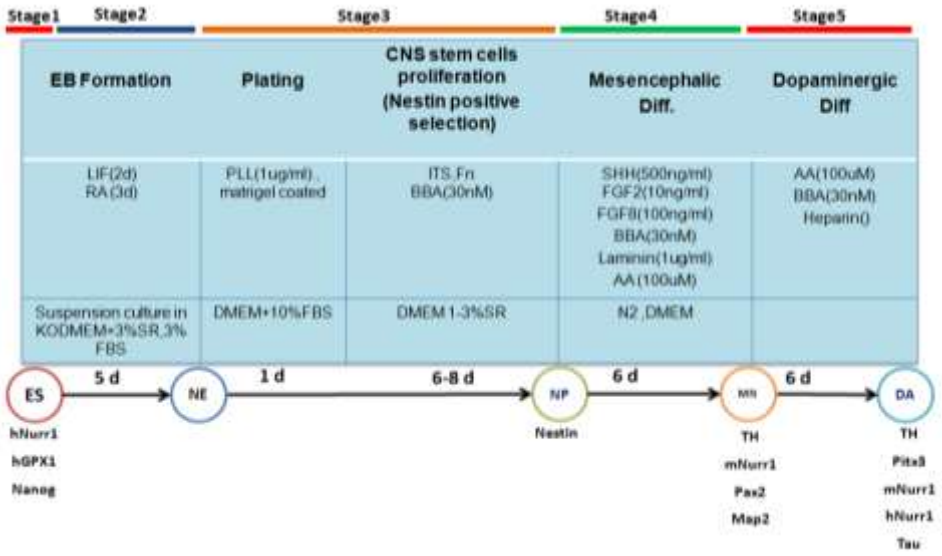

Figure 1- Five-staged protocol of ES cells differentiation to dopaminergic neurons by constructing EB

To study differentiation trend and identify condition of cells by studying gene expression in mRNA level, differentiation was done at the end of stages 3, 4 and 5 and then RNA extraction was done and cDNA was also prepared.

At the end of the third, fourth and fifth stages of differentiation, we evaluated expression of markers of neural progenitor cells (Nestin) and markers of dopaminergic neurons (neuron Tau, TH, Nurr1, Pitx3, gpx1) with Real time PCR technique. In the third and fifth stages, expression of specific markers was studied with Immunocytochemistry method.

Studying performance of dopaminergic neurons with HPLC

To perform this test, after cells reached end of the fifth stage of differentiation, they were studied for measurement of dopamine secretion rate.

The cells culture medium is replaced with 56mM KCl and HBSS and incubated for 15 min in cell culture incubator. Then, the supernatant was collected and kept in freezer at 70C until HPLC test was performed.

In all stages of this research, RNA extraction from cells was done with manual method and using Trizol.

For each one of the RNA samples, cDNA was made (ultimate concentration of ng/µl20) with Fermentase kit or Roche method.

RESULTS

Embryonic stem R1 cells which were cultured in specific culture medium and on MEF cells (Figure 1) were contaminated with viruses with Nurr1, GPX1 and eGFP genes as control in presence of polybrene (8µg/m1). Efficiency of contamination was between 40 and 50% of the cultured cells after being observed with fluorescent microscope. These cells survived due to expression of puromycin resistant protein in presence of 1µg/ml of puromycin and also GFP protein was expressed and clones were observed below green fluorescent microscope.

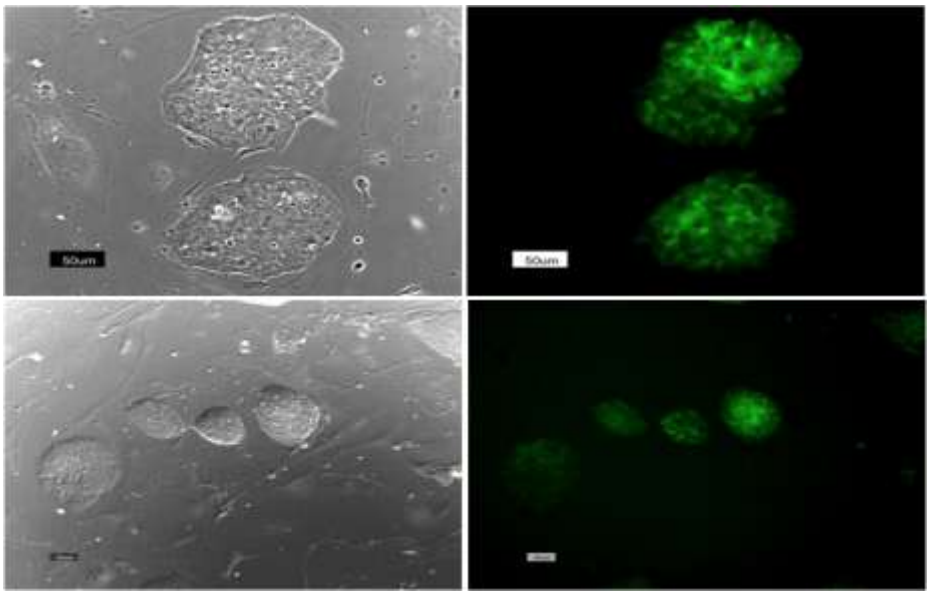

Figure 1: Clones of mouse's embryonic stem R1 cells in invert and fluorescent microscope. Green color indicates GFP protein expression (the size of strip is equal to 50 µm).

PRODUCTION OF EB FROM R1 AND R1-NG CELLS IN THE SECOND STAGE

In the second stage of differentiation, stages of forming embryoid bodies (ebs), eb structures were made from r1 and r1-ng cells. Ebs were treated for 3-4 days with retinoic acid. In these structures, spherical appearance and round margins are the desirable characteristics for differentiation of embryonic stem cells and eb structures in r1-ng samples and the control sample were similar to each other and had regular and spherical margins (figure 2).

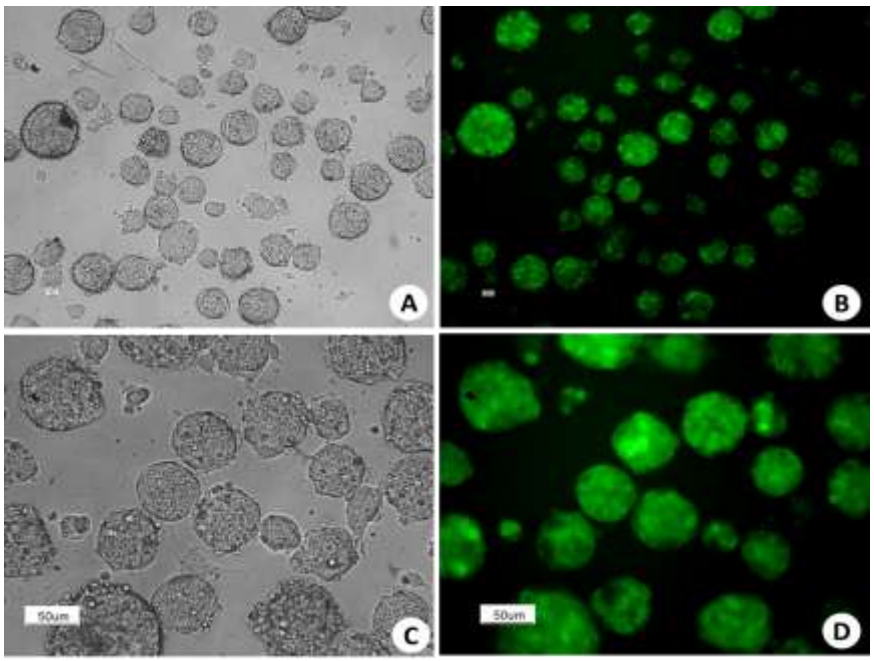

Figure 2: production of eb from r1 and r1-ng cells in the second stage of differentiation: a,b images relate to r1-gfp and c,d images relate to r1-ng. The above images relate to the third day of starting formation of eb.

Neuron progenitor cells in the third stage.

At The end of the third stage, Nestin gene expression was measured as a specific marker for neural progenitor cells quantitatively in the control groups, R1 cells with N/G gene and R1-NG cells treated with BBA. Results showed that population of the positive Nestin cells was significantly higher than that of the control group (4.5 times) in the group with gene and treated with BBA (Diagram 1).

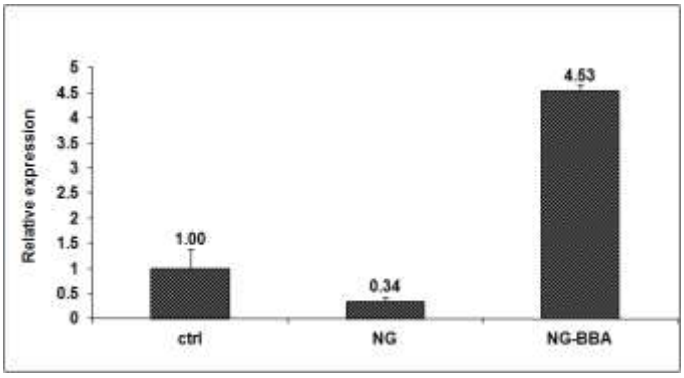

Diagram 1: Quantitative comparison of Nestin gene expression in the third stage of differentiation: ctrl ،NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes, respectively and R1 cells have Nurr1 and GPX1 genes treated with beta-Boswellic acid.

Neuron progenitor cells derived from stem cells: In this stage, it was observed with invert optical microscope and population of these cells was confirmed in the images (Figure 3). Nestin protein expression was also studied in this stage with Immunocytochemistry method and expression of this protein was also confirmed (Figure 4).

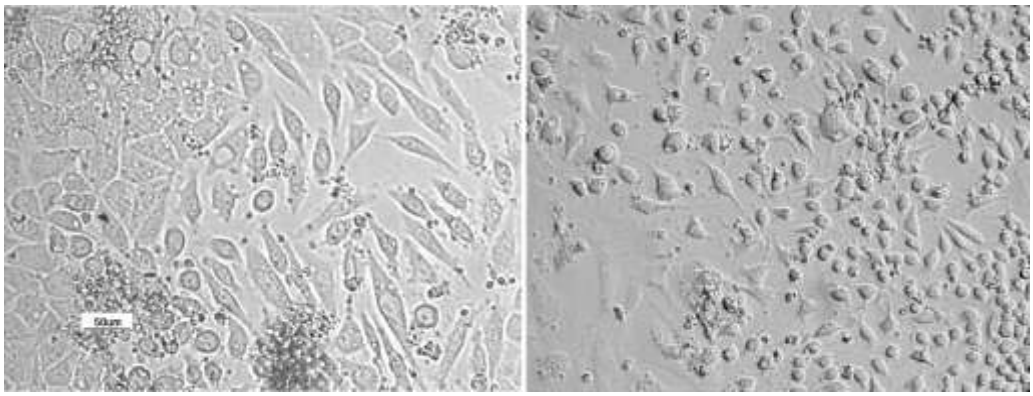

Figure 3: Optical microscopic images of the third stage of differentiation: right side: R1-NG+BBA and left side: control

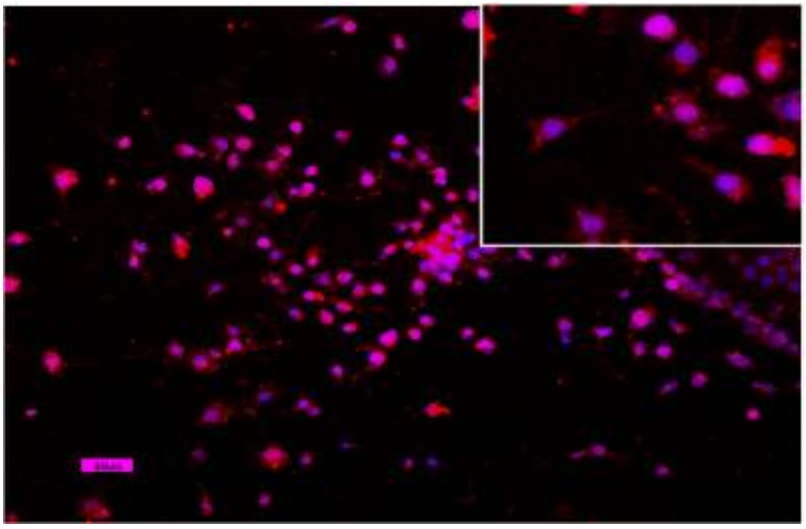

Figure 4: Confirmation of Nestin marker expression in the third stage of differentiation with Immunocytochemistry. In the Figure, nucleus of the c ells is blue (stained with DAPI) and marker of the NESTIN neural progenitor cells is also red (stained with Jred).

Mesencephalic neurons in the fourth stage of differentiation

In the fourth stage of differentiation, neural progenitor cells (positive Nestin) are differentiated to Mesencephalic neurons. In this stage, Mesencephalic neurons are differentiated to dopaminergic neurons in presence of factors such as SHH and heparin. In microscopic observations, neuron structures were observed in culture containers each with clear axon and dendrite processes (Figure 5).

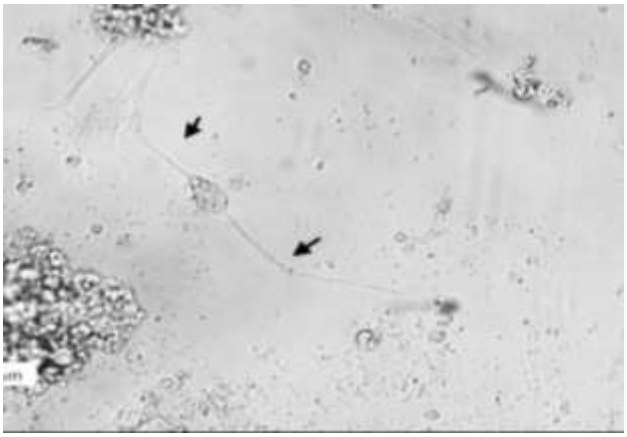

Figure 5: Mesencephalic neurons in the fourth stage of differentiation.

Dopaminergic neurons in the fifth stage of differentiation.

In the last stage of differentiation of stem cells to dopaminergic neurons, expression of specific markers of this stage was evaluated in protein level and mRNA.

Result of studying gene expression with real time PCR showed that specific markers of dopaminergic neurons are expressed in all samples (control and treatment) (Diagram 2) indicating differentiation of the control and treatment groups to dopaminergic neurons.

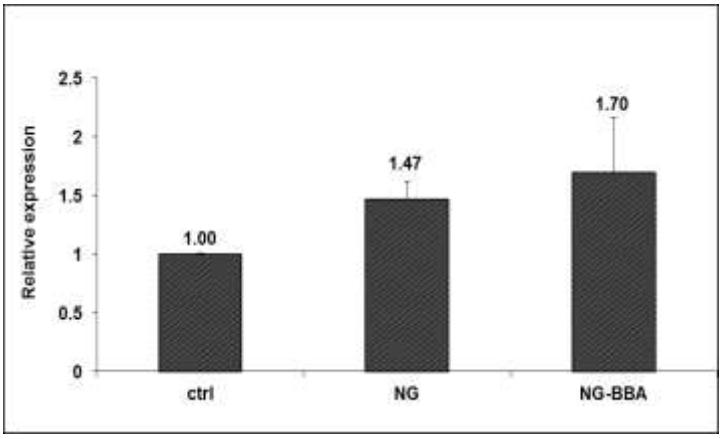

Diagram 2: Quantitative comparison of mNurr1 gene expression in the fifth stage of differentiation: ctrl ،NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes, respectively and R1 cells have Nurr1 and GPX1 genes treated with beta-Boswellic acid.

Expression of TH marker was studied as an enzyme interfering in dopamine synthesis process and real time PCR results showed that its expression in the treatment groups was higher than that in the control group (Diagram 4). Expression of two specific markers of dopaminergic neurons which are expressed in adult dopaminergic neurons, mouse Nurr1 and Pitx3 were studied. Results showed that the treatments with external gene and treatment with gene with BBA have more expression than the control (Diagrams 2 and 3).

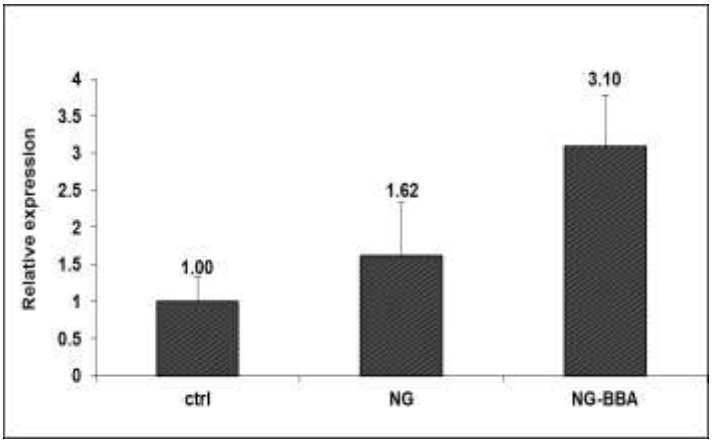

Diagram 3: Quantitative comparison of Pitx3 gene expression in the fifth stage of differentiation: ctrl ،NG and NG-BBA are R1 cells , R1 cells with Nurr1 and GPX1 genes , respectively and R1 cells have Nurr1 and GPX1 genes treated with beta-Boswellic acid.

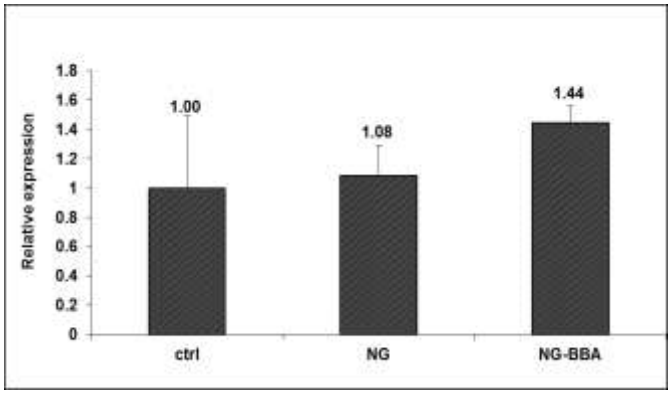

Diagram 4: Quantitative comparison of TH gene expression in the fifth stage of differentiation: ctrl ،NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes, respectively and R1 cells have Nurr1 and GPX1 genes treated with beta-Boswellic acid.

EVALUATING PERFORMANCE OF DOPAMINERGIC NEURONS WITH HPLC

After differentiation of cells to dopaminergic neurons, cells with KCl 56mM were treated in HBSS solution for 15 mi n in the last stage. By doing so, neurons are depolarized and release dopamine similarly to natural condition of cells. After collection, the above solution was stored and produced at appropriate time and dopamine secretion was confirmed in the samples with HPLC (diagram 5).

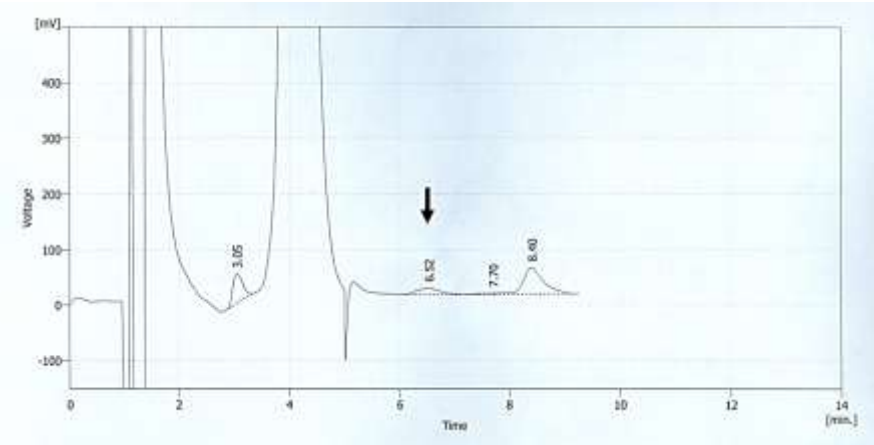

Diagram 5: studying dopamine secretion in the fifth stage of differentiation (peak of 6.52 indicates dopamine in the sample).

DISCUSSION

Parkinson's disease is the most prevalent neurodegenerative disease after Alzheimer. In this disease, dopaminergic neurons (DAn) of nigrostriatal area are destroyed selectively. At present, the main cause of Parkinson's disease is unknown but oxidative stresses play

important role in creation and progress of the disease. For this reason, protection of these neurons against oxidative stresses can lead to survival of the transplanted neurons and treatment of patients with Parkinson's disease.

Pharmacotherapy has been the main treatment of this disease but side effects of these d rugs and temporary treatment of symptoms of the disease are of the main shortcomings of this therapeutic method. One of the drugs with plant origin for neurodegenerative diseases in Iranian and Indian traditional medicine was the Beta Hydroxy Acid (Bba). It has been recently shown that BBA increases length and number of neuron branches by affecting neural cells. BBA was first studied as a treatment to study its role in differentiation of embryonic stem cells to dopaminergic neurons.

One of the strategies for treatment of Parkinson's disease is cellular transplantation and replacement of the lost neurons in these patients. Different cellular sources are available for provision of these cells and dopaminergic neurons derived from embryonic stem cells are one of the most accessible sources. Differentiation of these cells to dopaminergic neurons is possible according to a 26-28 day protocol as co-culture with stromal cells with protocol based on EB formation. EB formation protocol (used in this research) was first used by Dr. McKee for differentiation of mouse embryonic stem cells (without any external gene)[30] and completed more in 2002 and they could obtain dopaminergic neurons from mouse embryonic stem cells without coculture with stromal cells.[31] In the present research, possibility of differentiation of transgenic cells (with foreign gene eGFP and with Nurr1 and GPX1) to dopaminergic neurons was tested and we could obtain dopaminergic neurons with performance from both cellular ranks similarly to work of Dr. McKee (Diagram 5 and Diagram 4).

Nurr1 reanscirption factor is one of the important proteins in natural genetic evolutin process of dopaminergic neurons in embryonic period.[32] This protein is expressed as marker of dopaminergic neurons in the end stages of differentiation of dopaminergic neurons. Martinant et al. showed that concurrent expression of Nurr1 and Pitx3 in stages of differentiation of embryonic stem cells to dopaminergic neurons can increase efficiency of the differentiation.[33]

Boswellia extract is one of the prescribed drugs for the patients with neurodegenerative diseases. For this reason, potential role of this matter on neural system and particularly, its role in neurons and the effective pathways are questioned. In recent report, Karima and et al.[27], it was shown that BBA can increase length and the number of axon and dendrite processes by affecting neural progenitor cells extracted from brain tissue. This matter stabilizes microtubules in vitro by affecting microtubules. In the third stage of differentiation, expression of neural progenitor cells (Nestin) marker with real time PCR and Immunocytochemistry was quantitatively studied. Results showed that the treatment with the foreign genes treated with BBA expresses Nestin marker more than the control group.

Expression of all specific genes of the studied dopaminergic neurons in the fifth stage of differentiation was higher in the treatment group with foreign genes treated with BBA than the control group (Diagram 2, Diagram 3 and Diagram 4) indicating increase of efficiency of differentiation in BBA treatments along with foreign genes.

The main used protocol was the five-stage differentiation protocol based on EB.[30,31] RA and BBA and Matrigel from SHH and growth factors and heparin and ascorbic acid were applied in treatment.

CONCLUSION

Considering the mentioned facts, the results can be classified as follows:

BBA has positive effect on differentiation of stem cells to dopaminergic neurons.

Nurr1 and GPX1 expression can incease speed of difrentiation in the protocol in the third stage and increases Nestin expression.

Effect of BBA on R1-NG cells in the third stage of difrentiation increases population of beural progenitor cells expressing Nestin.

In the fifth stage of difrentiation, markers of dopaminergic neurons were expressed in all three groups and the group with foreign genes treated with BBA expressed more markers.

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