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ANALYZING THE EFFECT OF BETA-BOSWELLIC ACID (BBA) TO DOPAMINERGIC NEURON DIFFERENTIATION OF TRANSGENE EXPRESSING MOUSE EMBRYONIC STEM CELLS

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ABSTRACT

Introduction: Parkinson's disease is a degenerative neurological disorder associated with progressive loss of dopamine-secreting neurons in the direction of the Substantia nigra and the basal ganglia. In the past, drug treatment was the only known solution for the disease. But now it has been tries to use modern medical methods and herbal extracts to reduce the effects of chemical drugs. Given the above, the main aim of the present study was to investigate the effect of beta-boswellic acid (BBA) for dopaminergic production of produce embryonic stem cells expressing the foreign genes using lentivirus vectors in Parkinson's disease. **Method:** In this study, using the gene transfer techniques based on lentivirus vectors, Nurr1 genes (A transcription factor involved in the development of DAn and GPX1 (An antioxidant enzyme) are transferred to the R1 murine embryonic stem cells and the permanent expression of these genes in these cells is confirmed. For the differentiation of murine embryonic stem cells expressing the nurr1 and gpx1 genes to Dopaminergic neurons with the function the cell experiments are used and the making EB protocol was used to distinguish the embryonic stem cell to dopaminergic neurons. Then at the end of the third,

fourth and fifth phases of differentiation we analyzed the expression of markers of neural precursor cells (Nestin) and the markers of dopaminergic neurons (Tau neuron TH, Nurr1, Pitx3) by Real time PCR quantitative techniques. At the third and fifth phases the expression of specific markers was studied. **Results:** The results showed that the stem cells infected with lentivirus maintained their pluripotency characteristics after receiving Nurr1 and GPX1 genes. Also in infected cells, foreign genes are expressed at the level of mRNA and also the results indicated that the simultaneous expression of Nurr1 and GPX1 along with BBA treatment had the greatest effect in increasing the neural and neuronal markers and eventually in the expression of specific markers of dopaminergic neurons. **Conclusion:** The results suggest that the BBA has a positive impact on the differentiation of stem cells from dopaminergic cells and the markers of dopaminergic neurons in the groups under study was expressed and in the group of foreign genes treated with BBA the highest amount of these markers was observed. Also the therapy cell as a treatment to replace lost neurons can be a permanent and effective treatment for the disease.

KEYWORDS: beta-boswellic acid, differentiating murine embryonic stem cells, dopaminergic, lentivirus vectors, Parkinson.

INTRODUCTION

Parkinson's disease is a degenerative neurological disorder associated with progressive loss of dopamine-secreting neurons in the direction of the Substantia nigra and the basal ganglia. The disease occurs in all races and in the United States and Western Europe it has the prevalence of 1-2 people per thousand populations which is approximately the same between both genders. The condition becomes more common with age. The most common cause is idiopathic or also seen in using drugs, toxins or other neurological diseases.[1] In Parkinson's disease, the normal balance between dopamine and acetylcholine existing in the striatum is scrambled. Clinical major expressions include: resting tremor, major clinical expressions include.

Resting tremor, which rises in times of stress and it gets better with voluntary work. Rigidity or increased gear type resistance against passive movement which are the most observed characteristic of the disease. Tone is impaired due to the curved position of the patients.[2 - 6] Slow motion including slow movements, the difficulty in starting movements and loss of automatic movements.[1] It has been stated that Parkinson's disease is not a fatal disease, but since it reduces the ability of the individual, it causes the lifetime reduction of the patient.

Other complications resulting from the disease itself or due to dopaminergic therapy, include complications of unstable body movement and posture, abnormal gait and jerky movements of the hands and feet.[3] As mentioned before the main cause of the Parkinson's disease is the decline and loss of brain dopaminergic (caused by the death of dopaminergic cells secreting in Substantia nigra), but this is not the only cause but it is also attributed to the loss of nigrostriatal dopaminergic rout neurons.[7] This rout consists of the dopaminergic neurons the cell body of which is in the Substantia nigra and their axons and the neuronal processes reach the striatum.[7] However, the neuropathology of Parkinson's disease is restricted to nigrostriatal path and also the cellular tissue disorders have been found in other dopaminergic and non- dopaminergic cell groups.[7] To date, little information has obtained about how and why Parkinson's disease begins the process of neuronal degradation and continues. Currently, there are various methods of treatment for these patients the most important of which are the medical treatments. Based on pathogenic mechanisms which is the destruction of dopamine-secreting neurons in the Substantia nigra and lack of dopamine in the basal ganglia of the brain, the dopamine medicines and anticholinergic drugs were used for treatment. But despite the treatments available, firstly, this treatment does not result in complete resolution of the inability of the patient and the patient is exposed to risks of falls due to mobility problems such as trauma resulting from hitting the ground, nutritional problems and etc and secondly, the drugs have problems of early and late complications that cause compounded disease.[8,9] In recent years, new approaches to analyze the differentiation of the paths of the midbrain dopamine neurons have been proposed and in order to determine and identify the neurons several important transcription factors have been identified.[10] Nurr1 is a family member of nuclear intracellular receptors and the mutations in this gene is associated with neuronal disorders.[11] This gene is expressed in in the central nervous system, especially in SNpc , VTA.[12] Lee in all his research refers to the important effects of this gene in all stages of life, during fetal life and afterwards, differentiated into dopaminergic cells and all stages of life Dopaminergic Neurons.[12] GPX1 is considered as one of the major antioxidant enzyme of the mammals that inactivates hydrogen peroxide and protects against oxidative stress. GPX1 is expressed in microglia in the upper level and in neurons in lower level.[15-13] Recently it has been shown that GPX1 has a spatially co-distribution with lewy bodies in neurons and it seems that the enzyme is involved in the degradation of these material hence it is said that neurons are able to direct the antioxidant enzyme to the areas of producing oxidative stress.[16]

On the other hand it has been specified that oxygen free radicals and oxidative stress cause neurodegenerative disorders in the nervous system and even death. Studies have shown that the brain and peripheral nervous system due to high amounts of unsaturated fatty acid side chains are exposed to ROS (Reactive Oxygen Species) which ultimately reduces the weight of the brain.[17] Hence, One way to cope with oxidative stress is to use exogenous antioxidant.[18] Alternative strategies based on antioxidants to counteract the harmful effects of oxygen radicals, reduce cellular balance and maintain the cell rehabilitation balance is required. Boswellia from Burseraceae species growing in the arid areas of Africa and Asia are collected. Therapeutic use of the herb extract for the neurodegenerative disease goes back to the medicine of ancient Egypt, India and China.[19] Frankincense (containing BBA) has been used in India as a medicine in the prevention and treatment of dementia over thousands of years.[20] On the other hand, the use of traditional medicine and herbal remedies to cure the disease is increasing. The early attempts at transplantation of cell lines such as fibroblasts, Schwann, myoblast, glioma, and…that have been manipulation with different genes were not successful in the treatment of Parkinson due to the formation of tumors or detection by the immune system and the attempts to find a suitable cell source continued.[21] Thus, according to the above and the role of transgenic murine embryonic stem cells expressing Nurr1 and Gpx1 genes in treating Parkinson's disease the purpose of this study was to investigate the effects of beta Boswellia acid (BBA) on the production of dopamine in embryonic stem cells expressing the foreign genes using lentivirus vectors.

METHOD

In the present study in order to differentiate murine embryonic stem cells expressing nurr1 and gpx1 genes to dopaminergic neurons with performance using lentivirus vectors in Parkinson's disease the molecular, bacteria and cell experiments were devised. The methods of molecular method include: making competent E.coli *STBL4* cells, transforming competent bacteria *E.coli STBL4,* in order to confirm their accuracy of the colony their plasmids were extracted in small quantities and restriction digestion tests carried out on the plasmid for confirming right size and then Plasmid extraction in large quantities (Maxi prep) was performed. The enzyme digestion was performed by *Bam* HI enzyme and the product was electrophoresised on agarose gel. Then using the Gel Doc it was observed and photographed.

Cellular biology methods respectively include: Isolation and culture of murine embryonic fibroblasts (MEF) as feeder cells. To provide MEF from pregnant mice, 12.5 days embryos extracted and the and the amniotic and chorionic layers were separated. Then the abdomen

and head and spinal cord were removed from embryos. Then the rest of the body was dismembered with a scalpel blade and then with trypsin incubated in an incubator for 10-20 min at 37 ° C. After removal of larger undigested tissues, single cells were separated by centrifugation. Then the MEF cells were cultured on Petri or flasks coated with gelatin in DMEM 10% FBS. The time required for flasks containing MEF with 90-100% of the cells covered at the bottom of the flask were treated in mitomycin concentration for 3-4 hours and then after 3-4 rinses with PBS were ready to be used as a feeder and then the culture of embryonic stem cells R1 (16) was performed. It should be noted that the embryonic stem cell culture medium is KO-DMEM.

R1 stem cell culture medium was changed every day and the MEF and HEK-293T culture medium was changed every 2-3 days. Then the R1 cells passageing and counting was performed each 4-5 days. In the later stages of transfection and generating lentiviral viruses containing Nurr1 and GPX1 genes was performed and finally the viruses containing Nurr1 and GPX1 genes Were stored at -70.

Infecting R1 Cells with Nurr1 and GPX1 Viruses

R1 stem cells cultured on embryonic fibroblast cells, were isolated by trypsin to form Single cells. Single cells in polybrene (8µg / ml) with a 1: 1 ratio were infected with lentivirus particles Nurr1 and GPX1in containers of 6 wells on MEF cells.

After 10 days it was observed that the cells that infected with 1 µg / ml had about 70% of cells but the well without virus (control) was almost devoid of cells. During this time, the cells that have not received Nurr1 gene were excluded by Puromycin. The selected cells to proliferate were passaged on a new feeder and the RNA extraction was performed on the infected and uninfected cells. Next, the expression level of Nurr1 and GPX1 at mRNA, were confirmed by RT-PCR.

R1-NG Cell Differentiation into Dopaminergic Neurons

In this study the EB building protocol was used for embryonic stem cells differentiation into dopaminergic neurons.[22] in the first stage embryonic stem cells proliferation, in the second stage building the embryoid bodies (EB formation), in the third stage EB culture on matrigel and PLL, at the fourth stage nestin-positive cells profiliation toward mesencephalic cells and in the fifth stage differentiated into dopaminergic neurons were performed.

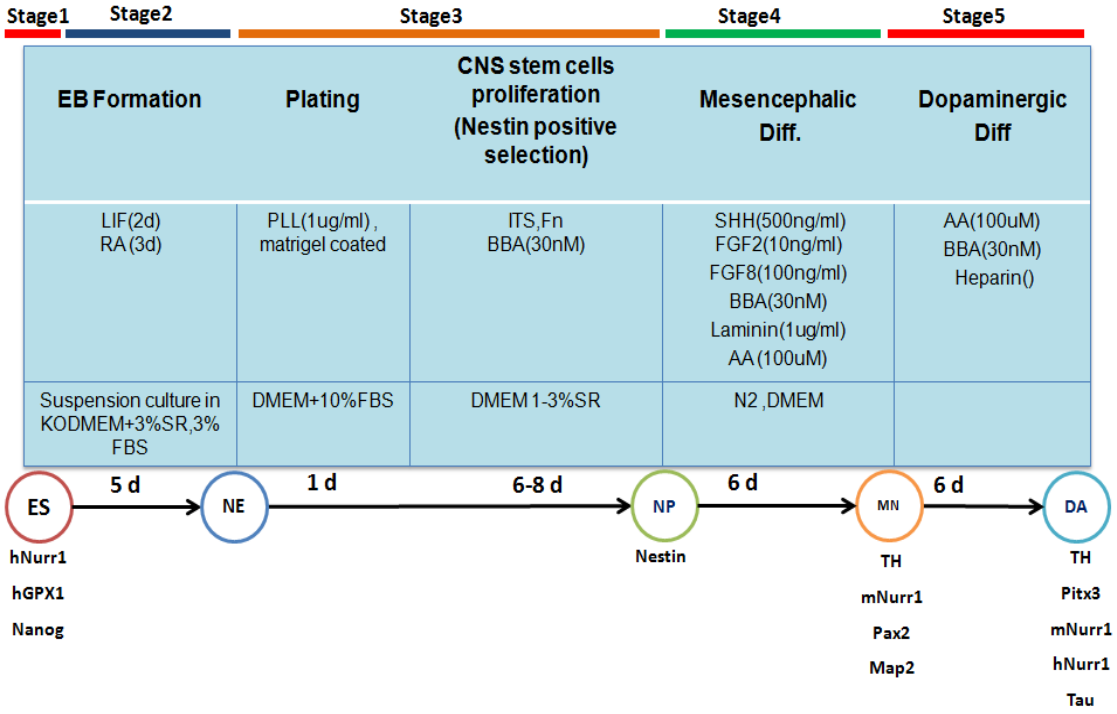

Figure 1: The the five stage Protocol of ES cell differentiation into the dopaminergic neurons by EB formation.

To study the process of cell differentiation and identification of the cells through the gene expression at the level of mRNA, at the end of the stages 3, 4 and 5 of differentiation was performed, RNA was extracted and then cDNA was prepared.

At the end of the third, fourth and fifth stages of differentiation we examined the expression of neural precursor cell markers (Nestin) and neuronal dopaminergic markers (Tau neuronal, TH, Nurr1, Pitx3, gpx1) by quantitative Real time PCR techniques. In the third and fifth stages, the expression of specific markers at the protein level was analyzed by immunocytochemistry method.

Evaluation of Dopaminergic Neurons with HPLC

To perform this test, after the cells reached the end of stage 5 of differentiation they were analyzed to measure the release of dopamine. Cell culture medium is replaced with 56mMKCl and HBSS and cells were incubated for 15 min in the incubator. Then the supernatant was collected and were stored at -70 quickly until HPLC test. In all stages of the research, RNA extraction of cells was performed manually using Qiazol. For each of the RNA samples, building cDNA (The final concentration 20 ng / µ l) was done using the Fermentas kits or Roche procedure.

RESULTS

The results of this study can be divided into two categories: 1. The production of stem cells expressing Nurr1 / GPX1 genes using lentivirus vectors, 2. Studying the effect of BBA on dopamine producing cells expressing Nurr1 / GPX1 genes.

Building Lentiviruses Containing Nurr1 / GPX1 / eGFP Genes

The results show that after trabsfection of lentivirus vectors Nurr1 / GPX1 / eGFP and building lentivirus particles in HEK-293T cells after 24 hours the cells transfection were observed by fluorescence microscopy. In the containers of cell culture the transfection of eGFP gene and GPX1-GFP was green due to having the reporter gene indicating the accuracy of transfection and production lentivirus particles of high titer(about 90-100% of the cells) (Fig 1& 2).

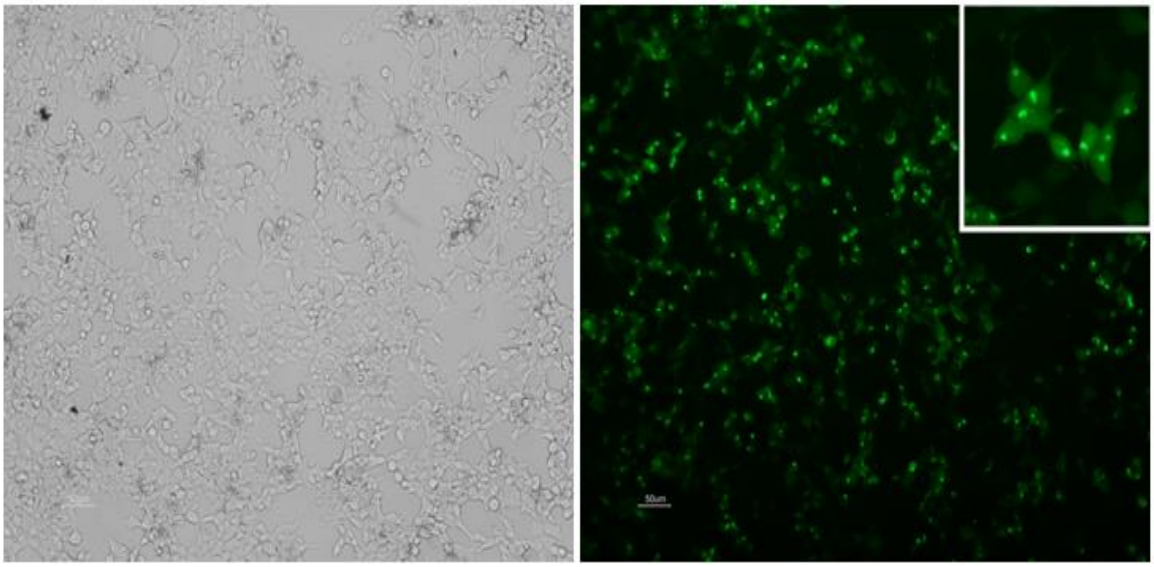

Figure 1: Transfection and production of lentivirus particles cells containing hGPX1 in HEK-293T cells. (EGFP expression as the reporter and expression hGPX1gene)

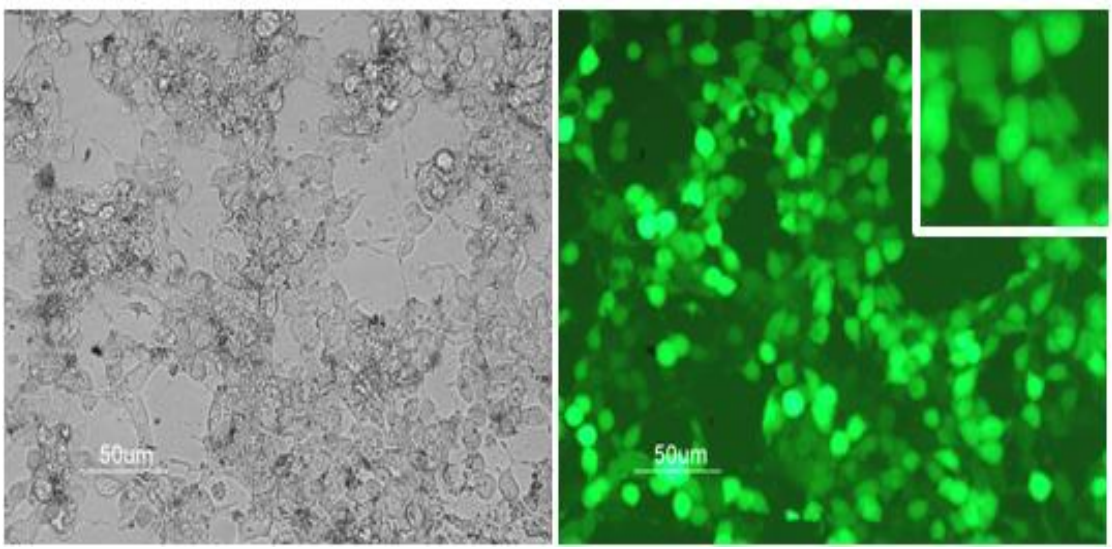

Figure 2: Transfection Lv-eGFP in HEK-293T cells.

Culture and Infection of R1 Cells and the Selection of Clones R1-NG

The R1 embryonic stem cells cultured in a specific medium and on the MEF cells (Fig. 3), were infected in the presence of the polybrene (8µg / ml), with viruses containing Nurr1, GPX1 and eGFP genes. Infecting performance was about 40-50% of the cultured cells after observing by a fluorescent microscope. These cells due to the expression of Puromycin resistant protein survived in the presence of 1µg / ml Puromycin and also expressed GFP protein and the clones can be seen under fluorescent microscope as green (Figure 4).

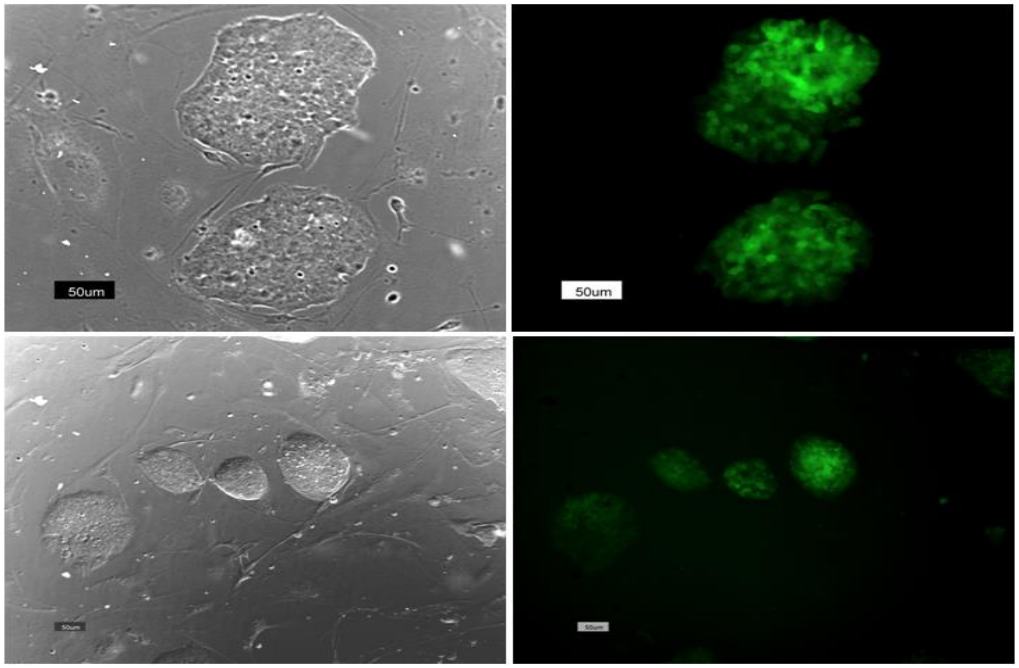

Figure 3: R1 murine embryonic stem cell clones in inverted fluorescent microscope. The green color indicates the protein expression GFP (Measuring tape is 50 µm).

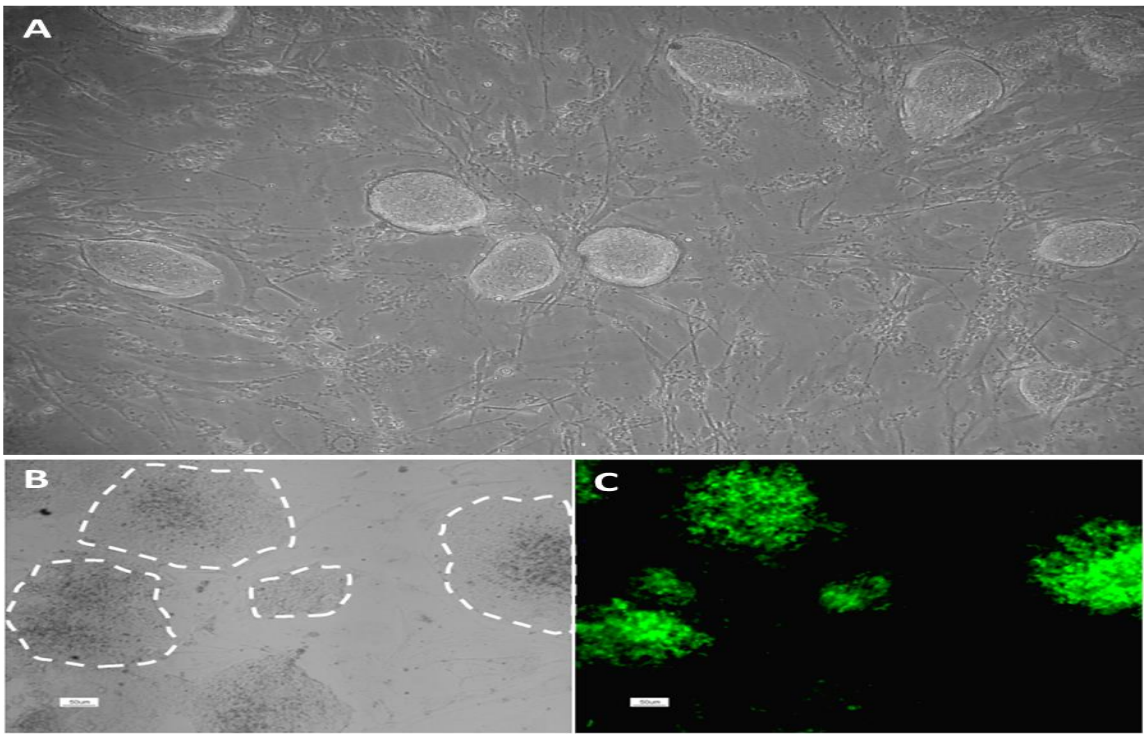

Figure 4: R1embryonic stem cells. A cultured cells prior to infection, B and C cells infected by Nurr1 and GPX1 viruses on the day 10 of Puromycin treatment.

Confirmation of ES Cells Expressing Nurr1 / GPX1

After infecting R1 cells the clones expressing Puromycin resistance were selected among the clones obtained from extracting RNA and building cDNA was accomplished and measured RT-PCR of the expression of the hNurr1 hGPX1 foreign genes and it was observed that these cells express the mentioned genes. The expression of Nanog Pluripotency markers in these infected cells was evaluated and observed that stem cells infected with lentivirus retained their pluripotency characteristic after receiving the Nurr1 and GPX1 genes.

Confirming the Expression at the Level of mRNA using RT-PCR

After infecting R1 stem cells and selecting cells expressing the Puromycin resistance gene from the infected cells RNA was extracted and reaction was performed for foreign hNurr1 and hGPX1 genes. The results of PCR on an agarose gel were electrophoresed. The results showed that in infected cells foreign genes are expressed at the mRNA level.

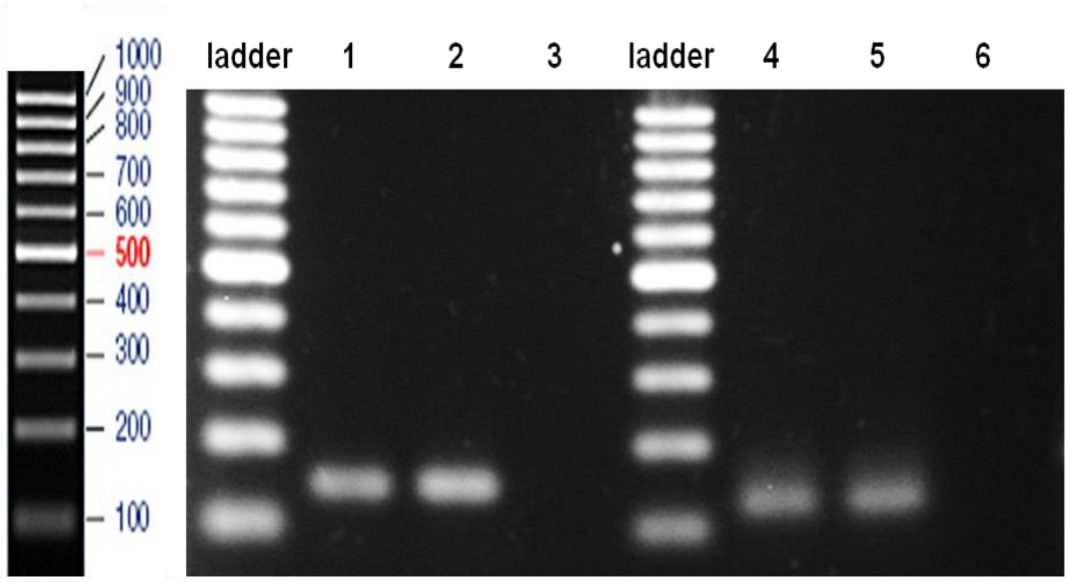

Figure 5: Confirming the expression of hNurr1 and hGPX1 genes in infected cells. 1: hGPX1 Gene For samples infected with viruses, 2: positive control hGPX1 gene 3: hGPX1 Gene For samples not infected with the virus, 4: Gene hNurr1 For samples Infected with viruses, 5: positive control for hNurr1 gene 6: hNurr1 Genes For samples, not infected with any virus. (In order of size of PCR pieces for hNurr1 And hGPX1 Equal 145 bp and 127 bp).

Producing EB from R1 and R1-NG Cells in the second stage

In the second stage of differentiation, the stage of embryoid bodies formation, the EB structures were made of R1 and R1-NG cells. The EBs was treated with retinoic acid EB for 3-4 days. In these structures the spherical and regular rounded margins are among the optimal features of for embryonic stem cell differentiation and the EB structures in R1-NG and control samples are similar with regular spherical margins (Fig. 6).

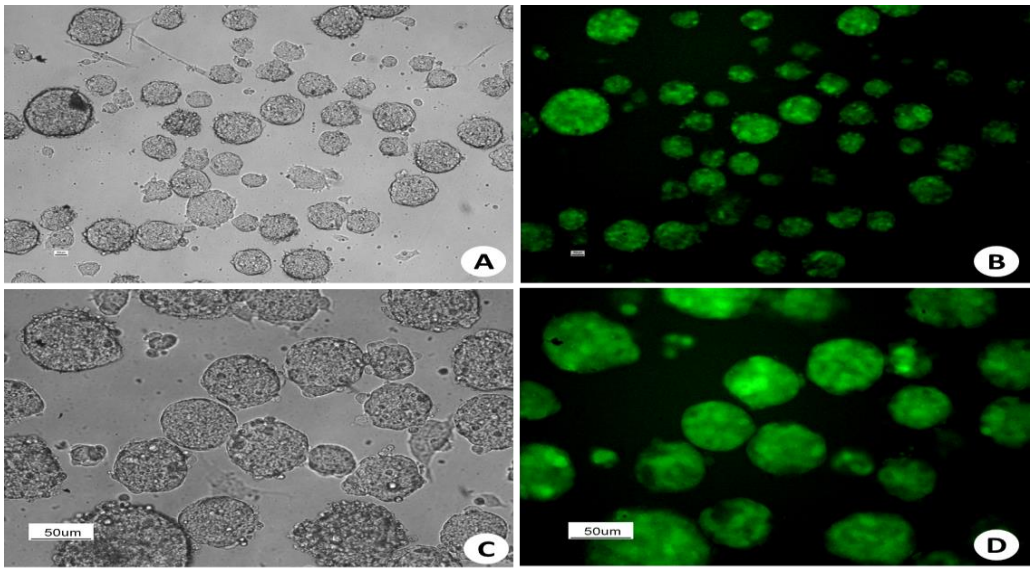

Figure 6: EB Production from R1-NG and R1 cells at the second stage of differentiation. Images A, B images present R1-GFP cells and C, D images represent R1-NG. The above images refer to the third day of the building EB.

Neuronal Precursor Cells in Stage 3

At the end of the third stage of differentiation Nestin gene expression as a specific marker for neural precursor cells was quantitatively measured in control, R1 cells with N / G gene and R1-NG treated with BBA cells. The results showed that the BBA treated with gene group the nestin-positive population of cells was significantly (4. 5times) higher than the control group (Chart 1).

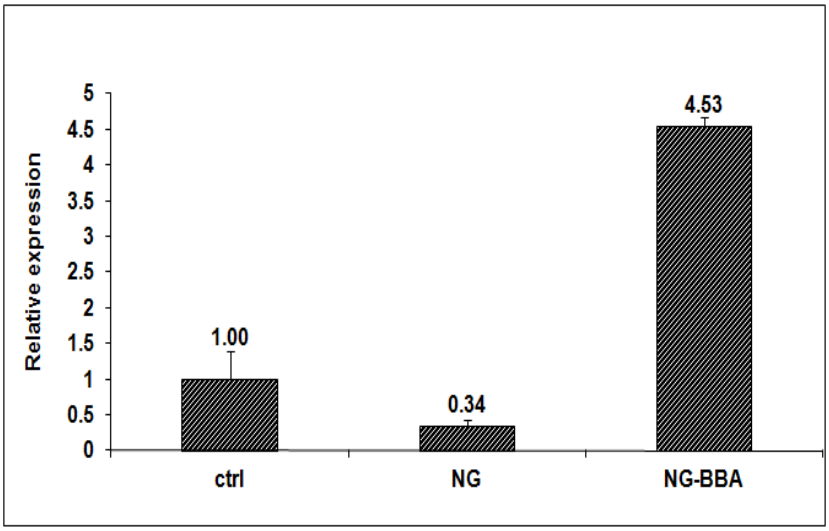

Chart 1: Quantitative comparison of Nestin gene expression in the third stage of differentiation. Ctrl, NG and NG-BBA are, R1 cells, R1 cells with Nurr1 and GPX1 genes and R1 cells with Nurr1 and GPX1 gene treated with BBA respectively.

Neural precursor cells derived from the stem cells at this stage were observed with inverted optical microscope and the population of these cell was confirmed at images (Figure 7). Analyzing the expression of the Nestin protein at this stage was studied by immunocytochemistry technique which confirmed the expression of the protein (Figure 8).

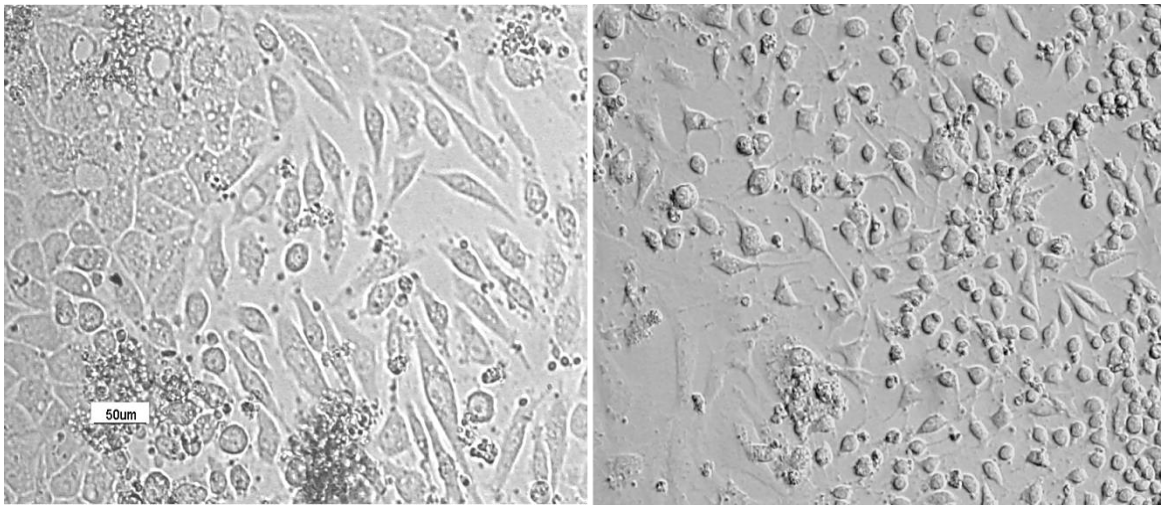

Figure 7: Optical microscope images of the third stage of differentiation. Right figure: R1-NG + BBA and left figure: control.

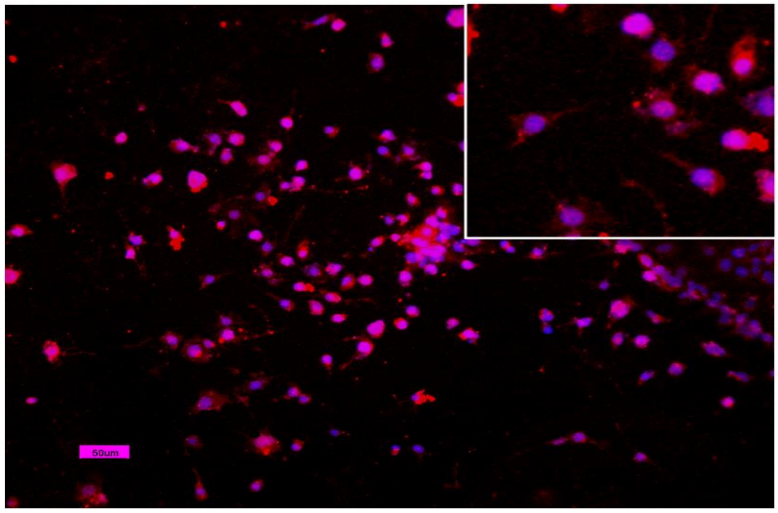

Figure 8: Confirmation of the expression of Nestin markers in the third round stage of differentiation using immunocytochemistry. In the image the nuclei is blue (stained with DAPI) and NESTIN markers of neural precursor cells stained with Jred is red.

Mesencephalic Neurons in the Fourth Stage of Differentiation

In the fourth stage of differentiation, neural precursor cells (nestin-positive) differentiated into mesencephalic neurons. At this stage mesencephalic neurons differentiated into dopaminergic neurons in the presence of factors such as SHH and heparin. In microscopic

observations neuronal structures were observed in culture containers each with axonal and dendritic frills (Figure 9).

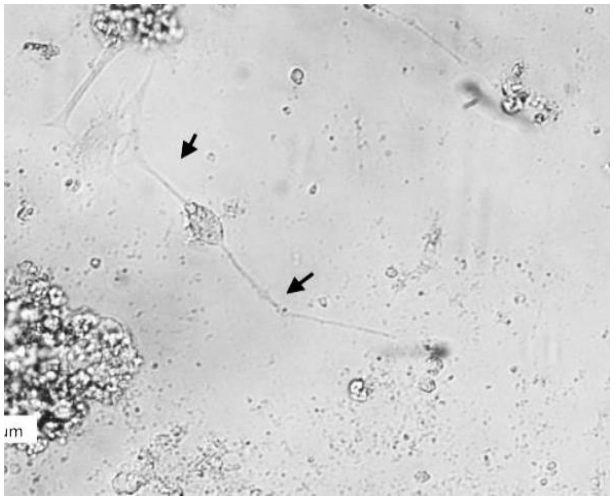

Figure 9: mesencephalic neurons in the fourth stage of differentiation.

Dopaminergic Neurons in the Fifth Stage of Differentiation

In the final stage of differentiation of stem cells into dopaminergic neurons, the expression of specific markers of this stage was measured in mRNA and protein levels.

The results of gene expression analysis by real time PCR showed that in all the samples (control and treatment) markers of dopaminergic neurons are expressed (Chart 2) which indicates the differentiation of dopaminergic neurons in the treatment and control group.

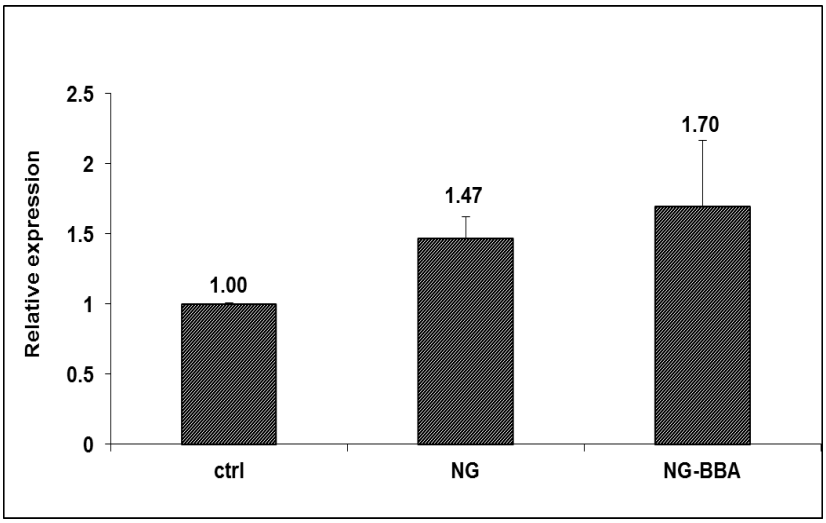

Chart 2: Quantitative comparison of mNurr1 gene expression in the fifth stage of differentiation. Ctrl, NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes and R1 cells with Nurr1 and GPX1 gene treated with BBA respectively.

The expression of TH as an enzyme involved in dopamine synthesis was studied, the results of real time PCR showed that its expression in the treatment groups was higher in the control group (Figure 4). Also the analysis of the expression of the two specific markers of dopaminergic neurons expressed in mature dopaminergic neurons, murine Nurr1 and Pitx3 were studied. The results showed that in the treatments with foreign gene and treatments with gene with BBA have higher expression than the control (Chart 2 and 3).

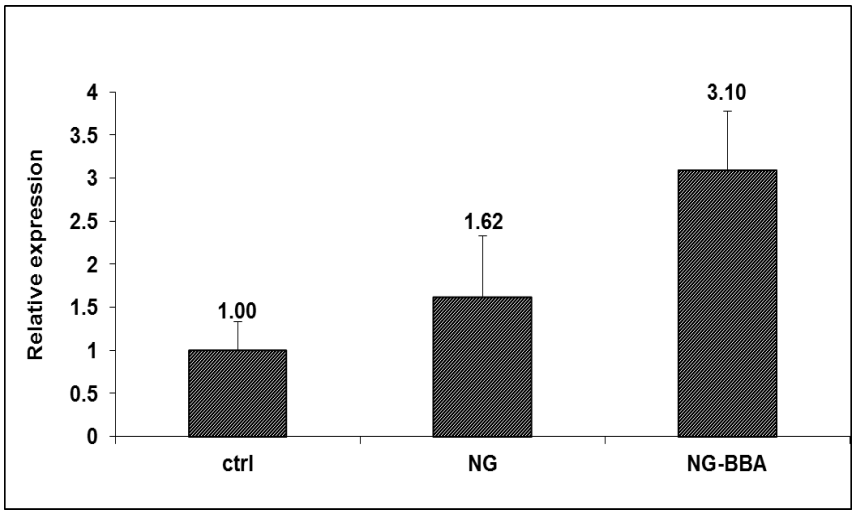

Chart 3: Quantitative comparison of Pitx3 gene expression in the fifth stage of differentiation. Ctrl, NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes and R1 cells with Nurr1 and GPX1 gene treated with BBA respectively.

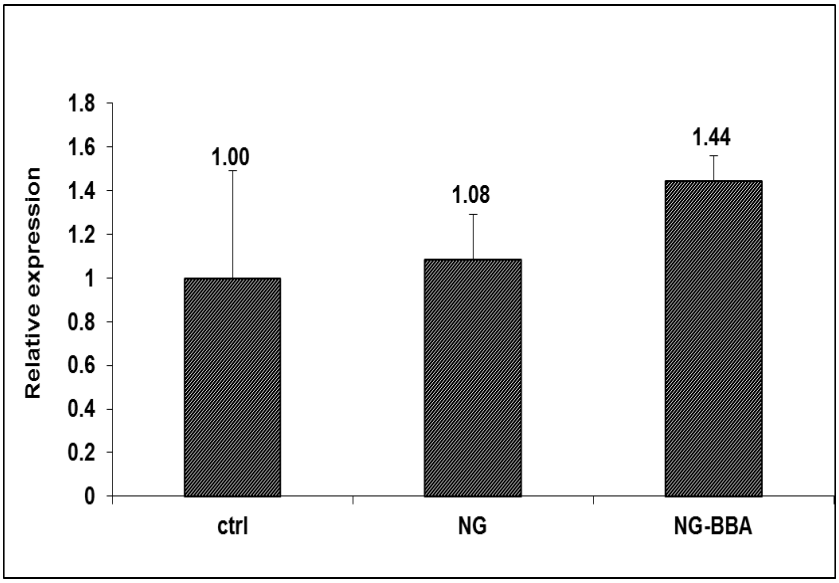

Chart 4: Quantitative comparison of TH gene expression in the fifth stage of differentiation. Ctrl, NG and NG-BBA are R1 cells, R1 cells with Nurr1 and GPX1 genes and R1 cells with Nurr1 and GPX1 gene treated with BBA respectively.

Performance Evaluation of Dopaminergic Neurons by HPLC

After differentiation of dopaminergic neuronal cells in the final step the cells were treated with KCl 56mM in HBSS solution for 15 minutes. Thereby the membrane of neurons was depolarized and the cells release dopamine as normal conditions. After collecting the solution it is stored and the production, dopamine release in the samples was approved with HPCL (Chart 5).

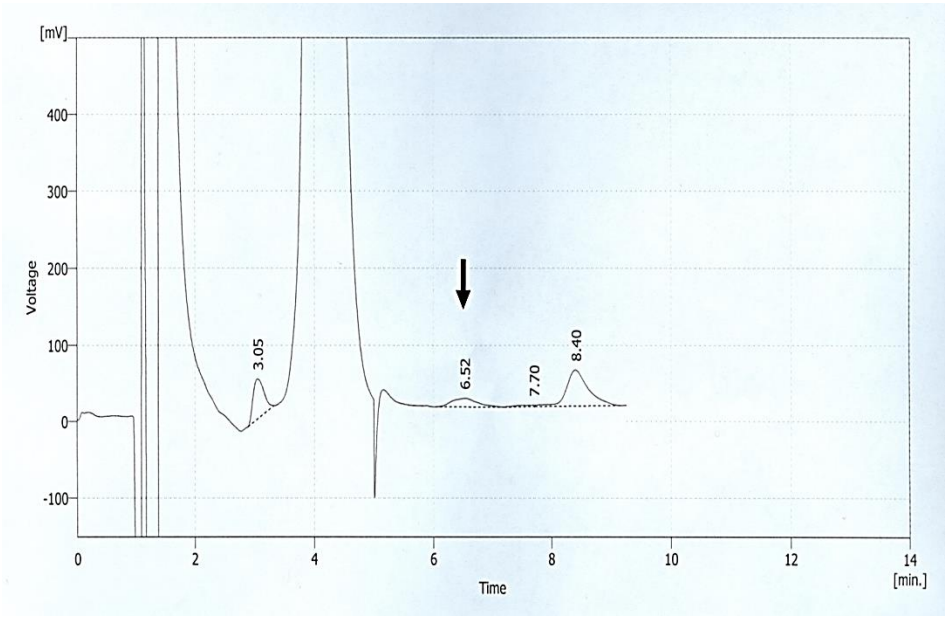

Chart 5: Evaluation of dopamine release in the fifth stage of differentiation (6.52 peak is indicative of dopamine in the sample).

DISCUSSION

So far treatment of Parkinson's disease has been done by chemical medicines. But today the science focusing on herbal therapies have led to the improvement or the treatment of many diseases of unknown origin. Since the side effects of the chemical medicines are much higher than the herbal therapies, this paper attempted to use the Boswellia as a memory booster plant in order to produce dopaminergic cells an the other hand it has been attempted to treat the Parkinson's disease through culturing the embryonic stem cells expressing Nurr1 / GPX1 the result of which is discussed as follows.

Discussing the Results of the Building EB Protocol

The protocol of building EB (used in this study) was first used by Dr. McKay to differenciate R1 murine embryonic stem cells (no foreign gene)[22] and in 2002 was completed and they

could obtain dopaminergic neurons from the murine embryonic stem cells without being cultured with stromal cells.[23]

In this study for the first time using a lentivirus vector-based gene transfer we transferred the combination of Nurr1 and GPX1 genes into R1murine embryonic stem cells and obtained the transgenic embryonic stem cells permanently expressing the Nurr1 and GPX1 genes (Fig 5). Also we measured the possibility of differentiating transgenic cells (containing eGFP foreign gene or Nurr1 and GPX1) into dopaminergic neurons and we could obtain the performing dopaminergic neurons similar to doctor McKay from both cell categories.

The transcription factor Nurr1 is one of the important proteins in the normal developmental process of dopaminergic neurons in the embryonic period.[23-25] This protein as a marker of dopaminergic neurons is expressed in the final stages of dopaminergic neurons differentiation. Martinant et al demonstrated that the simultaneous expression of Nurr1 and Pitx3 in the embryonic stem cell differentiation stages into dopaminergic neurons could increase the efficiency of differentiation.[26]

One of the main goals of this research is to improve the efficiency of the differentiation of embryonic stem cells into dopaminergic neurons in the presence of Nurr1 and GPX1 foreign genes. As shown in the results the existence of Nurr1transcription factor in the cells of the treatment group (R1-NG) was expressed higher than the control group (R1) markers of dopaminergic neurons(mNurr1, Pitx3, TH) (Figure 5). So similar to Martinant et al the cells with Nurr1 foreign genes with GPX1 had higher differentiation efficiency toward dopaminergic neurons compared with the control group.[26]

The Effect of Bba on Differentiation of Dopaminergic Stem Cells

In Parkinson's disease dopaminergic neurons (DAn) of the nigrostriatal are removed selectively. Right now the main cause of Parkinson's disease is unknown but oxidative stress plays an important role in the development and progression of the disease so keeping these neurons against oxidative stress can be linked to neurons survival and help to treat Parkinson's disease.

Recently it was shown that BBA can increase the length and number of the neuronal branches through affecting the nerve cells. Hence, in the present study for the first time BBA is studies

as a treatment to analyze its role on the differentiation of embryonic stem cells into dopaminergic neurons.

One of the ways forward in the treatment of Parkinson's disease is the cell transplantation and replacing the lost neurons in these patients. There are several different cell sources to provide these cells that the derived dopaminergic neurons from embryonic stem cells is one of the most accessible sources. Differentiation of these cells into dopaminergic neurons is possible in a 26-28 day protocol as co-culture with stromal cells or a protocol based on building EB.
EB Building protocol (Used in this study) was first used by Dr. McKay to differentiate R1 murine embryonic stem cells (No foreign gene).[22] And it was completed in 2002 and they could obtain dopaminergic neurons from murine embryonic stem cells without stromal co-culture.[23]

In this study, the possibility of differentiating transgenic cells (with eGFP foreign gene or Nurr1 and GPX1) into dopaminergic neurons was measured and we could obtain the dopaminergic neurons from both categories of cells like Dr McKee (Chart 4 and 5).

The Nurr1 transcription factor is one of the major proteins in the normal development of dopaminergic neurons in the embryonic stage.[25] This protein as a dopaminergic neuron marker is expressed in the final stages of dopaminergic neurons differentiation. Martinant et al demonstrated that the simultaneous expression of Nurr1 and Pitx3 in the embryonic stem cell differentiation stages into dopaminergic neurons could increase the efficiency of differentiation.[26]

Boswellia extract has been one of the prescriptions for the patients with neurological diseases. Hence its possible role in nervous system and especially on neurons and its related pathways is under question. A recent report by Karima et al.[20] It was shown that BBA through affecting the the nerve cells derived from brain tissue can increase axonal and dendritic frills length and the number. Also this element through affecting the microtubules can stabilize microtubules in vitro conditions. In the third stage of differentiation the quantitative analysis of the markers of the neural precursor cells (Nestin) were examined by real time PCR and immunocytochemistry. The results showed that treatment containing treated foreign genes with BBA expresses more nestin than the control group.

Expressing all the specific genes of Dopaminergic neurons under study was higher in treatment containing treated foreign genes with BBA than the control group at the fifth stage (Chart 2, 3 and 4) which represents an increase of differentiation efficiency in treatment of foreign genes with BBA.

The main protocol used is a 5 stage protocol based on building EB.[23,24] That RA, BBA, Matrigel, growth factors, heparin and ascorbic acid were used.

CONCLUSIONS

According to what is mentioned so far the results can be categorized as follows

The results of this study can be used in the pre-clinical objectives of cell therapy for Parkinson's disease and linked to animal model and the similar studies may follow for the induced stem cells (iPS cells). BBA has a positive effect in the differentiation of stem cells into dopaminergic cells. The combined expression of Nurr1 and GPX1 can increase the speed of the differentiation protocol in the 3rd stage and increase the expression of Nestin. The effect of BBA on R1-NG cells in the 3rd stage of differentiation increased the population of neural precursor cells expressing Nestin. In the fifth stage of differentiation in all three groups the dopaminergic neurons markers were expressed and these the highest expression of these markers were observed in the group with foreign genes treated with BBA.

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