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PRODUCTION OF MOUSE EMBRYONIC STEM CELLS EXPRESSING THE GPX1 AND NURR1 GENES BY USING LENTI VIRUS VECTORS; NEW CELLS SOURCES FOR CELL THERAPY IN PARKINSON'S DISEASE

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ABSTRACT

Background: Parkinson's disease is the second most common neurological developmental disease which has involved the high population of elderly people in our society. In this disease, the dopaminergic neurons (Dan) of the Nigrostriatal are selectively lost. Now the main cause of Parkinson's disease is unknown, but oxidative stress play an important role in the development and progression of disease. Cell therapy as a treatment for the replacement of lost neurons can be a permanent and effective treatment for this disease. Methods: in the present study, by using the techniques of gene transfer based on lenti virus vectors, the Nurr1 genes (a transcription factor involved in the development of Dan) and GPX1 (an antioxidant enzyme) was transferred to the R1 mouse embryonic stem cells. And the permanent expression of these genes was confirmed in these cells. The above transgenic stem cells were differentiated according to a five-step protocol and by treatment of growth factors and molecules on Matrigel with Poly-L-Lysine. and at the each of differentiation stage, expressing specific markers of each stage were studied by using

the real time PCR techniques and immunocytochemistry techniques. Results: the results show that the infected stem cells with lenti virus, after receiving GPX1 and Nurr1 genes, still have maintained their pluripotency characteristics. Also, in infected cells, the transgenes are expressed at the mRNA level and the ICC results showed that the Nurr1 protein is expressed in the nucleus of infected cells and the GPX1 protein is expressed in the cytoplasm of these cells. Investigating the pluripotency markers expressing, Nanog protein in these cells as the RT-PCR shows for this market that the expression of pluripotent stem cell markers in these cells are at the acceptable level. Conclusions: the results of this study can be used in pre-clinical cell therapy of Parkinson's disease and transplantation to the animal model. Also, cell therapy as a treatment for the replacement of lost neurons can be a permanent and effective treatment for this disease.

KEYWORDS: mouse embryonic stem cells differentiation, Nurr1, GPX1, lenti virus vectors, Parkinson.

INTRODUCTION

After Alzheimer disease, Parkinson disease is the most common neurodegenerative disease. the prevalence of this disease in people over 50 years old is 1-2 percent. Tremor at rest, Bradykinesia and Rigidity and difficulty in movements starting are the major symptoms of Parkinson disease.[1,2] Parkinson's disease occurs under the situations of environmental factors and only 5% of patients inherited this disease and multiple genes participate in the recessive or dominant inheritance of Parkinson and all of them include alpha synuclein, Parkin, Dj-1 and LRRK2 and PINK1. The cause of dopaminergic cell death in Parkinson's disease is unknown but in the last two decades, the underlying genetic factors of this disease, oxidative stress and mitochondrial dysfunction are known as the major factors in Neurodegeneration of these neurons.[4] in cell level, the characteristics of Parkinson include selective loss of dopaminergic neurons in the substantia Nigra Pars Compacta and Lewy Bodies containing the Sinoculein alpha in cytoplasm of neurons.[5,6] Understanding the production mechanism and loss of neural cells caused by neuronal death such as genetic problems are results of changes and their adjustments needs an accurate understanding of transcription factors.[7] Research teams in studies that have done on the effects of transcription factors and production and survival of dopaminergic neurons, detect some important transcription factors including Pitx3, Nurrl, FGF8 (Fibroblast Growth Factor 8), Lmz1a, Lmxib, GDNF and SHH (Sonic Hedgehog) to produce the dopaminergic neurons.[8]

Another effective factor in development of dopaminergic neurons are Lmx1b and Lmx1a that the gene Lmx1a expression is dependent on the SHH gene expression.[11,9] The transcription factors which are used for cellular differentiate mainly is from natural developmental pathways. Two genes Pitx3 and Nurrl are the most important transcription factors that many researches are done on their effects on neuronal survival and production. According to the done studies, the researchers found that Pitx3 gene is the most important transcription factors that play an important role in the maturation of dopaminergic neurons and neurons protection.[12] According to the done studies on the context of transcription factor roles in generation of neuronal cells and the treatment of Parkinson's disease such as pitx3, Nurrl and GDNF, researchers are trying to investigate the treatment conditions of this disease by using these genes induction to the cells in vivo and in vitro conditions. Researchers have used some of these factors to differentiate the embryonic cells to differentiate of dopaminergic neurons.[13] Nurr1 is a transcription factor which is expressed in over 95% of the SNpc and VTA neurons.[14] in mice, at the 10, 5 days, the fetal life appears. This day is the last day of fetal life The Nurr1 expression is maximized during the fetal life and its expression during life time is in the high level.[15] This issue points to that Nurr1 has an important role in the differentiation and survival of dopaminergic neurons.[16] this transcription factor as an auto receptors has the self-regulatory activities. This gene, itself in the neuronal cells, stem cells and precursor of dopamine materials and stem cells, helps as a promoter for the expression of marker genes in dopaminergic neurons in vitro conditions.[12] Some researchers stated that in experiments on mice that the Nurr1 gene lost on them, mice die one day after birth and after their studies, it was concluded that the cause of their death was the destruction of VTA and SNpc neurons. Also other research groups in the experiments, the Nurr1 gene silencing is affected after Pitx3 gene expression and decreases.by comparing the Nurr1 +/+ mice with the Nurr1 -/- mice as well as Nurr1 +/- mice, they observed that in Hetero genes Nurr1-/-, the dopamine level is too low. And in Nurr1 +/- mice, the dopamine level in corpus striatum is normal, but after a while, the Nurr1 protein level and subsequent of dopamine level compared to the dopamine level in Nurr1 +/+ mice reduces.[17] Nurr1 gene and transcription gene expression are one the effective factor in evolution and formation of dopaminergic neurons as well as it strengths the Adrenal aldosterone hormone. In addition to the regulating the TH gene expression, this gene regulate the DAT, AADC, VMAT2 and P75kip2 gene expression that all of these genes are essential in evolution if phenotype neuronal dopaminergic.[17] Some researchers introduced the measure changes in gene expression as a diagnostic marker for the induced diseases of neurons degeneration such as Parkinson's disease. GPX1 acts as a

Homo Tetramer[19] it is found in cytosol, nucleus and mitochondria.[18] Due to the widespread tissue distribution of glutathione peroxidase 1, this enzyme has been considered as one of the major antioxidant enzymes in the detoxification of hydrogen peroxide.[20] later it is shown that GPX1 shows a spatial distribution with lewy Bodies in neurons and it seems that this enzyme is involved in the degradation steps of objects and hence it is said that neurons are able to conduct the Antioxidant enzymes to the production area as of oxidative stress.[21] Since the recovery of dopamine amount effects on the early stages of Parkinson's diseases, but due to its side effects and worsen the patient's condition, it is not considered as a good treatment, to replace the lost dopaminergic neurons, the researches focused on the neural transplantation.[22] Although the early attempts at transplantation of cell lines such as fibroblast, Schwann, Miblast, giloma and … even were manipulated by different genes, due to the tumor formation or be detecting by the immune system, it did not show success in the treatment of Parkinson's disease. the efforts were continued to find a more suitable cell sources.[23] So, due to the given information and the role of murine embryonic stem cells by the Nurr1 gene expression and Gpx1 gene expression in the treatment of Parkinson's disease is the aim of this research and the purpose of this study is the production of transgenic mouse embryonic stem genes with the Gpx1 and Nurr1 gene expression by using the lenti virus vectors.

METHODS

In the presented research, in order to difference the mouse embryonic stem cell of gpx1 and nurr1 gene expression to the dopaminergic neurons with the performance and it was used by using the lentivirus vectors in Parkinson's disease from the bacterial and cellular experiments. The Molecular methods including competenting of the E.coli STBL4 bacteria and transforming the E.coli STBL4 competent bacteria to determine the accuracy of plasmid colonies they were exploited in low scale and manual and appropriate enzyme tests are done on these plasmids and after the plasmid extraction, these tests have been done (Maxi prep) and then digestion was done with BamHI enzyme and the product was electrophoresis on agarose gel and then the sample was observed and photographed by using the Gel Doc instrument. The cellular biology methods includes respectively isolation and implantation of the mouse embryonic fibroblasts (MEF) was used for preparing MEF from pregnant mice the 12.5 day embryos and the chorionic layers were separated then the abdomen and spinal cord of the fetus was separated. In the continuing, the remains body of the fetus was fragmented with scalpel blade and then treated and digested with trypsin, it was incubated for 10-20 min

at 37 Celsius degrees. After removal of undigested tissues which are larger, the cells were separated by centrifugation. Then, the MEF cells were implanted on Petri dishes or flasks coated with gelatin in the implant environment DMEM10% FBS. In the required time, the flasks contained MEF with 90-100 percent of covered flask floor for the 3-4 hour is treated in the mitomycin concentrations and after 3-4 washing with PBS, and they are ready to be used as a feeder and then after that, the implantation of embryonic stem cells R1[24] was done. It should be noted that the implantation environment is a stem cell KO-DMEM which consist 10 percent of SR and 10 percent FBS. This environment contain Pen/Strep with a final concentration 50 µg/ml for each of them and the unnecessary amino acids are with the final concentration 100µM and with L-glutamine with a final concentration 100 mM and beta-mercaptoethanol with final concentration 100 µM, LIF1000 U/ml. The implantation environment of MEF cells and high glucose DMEM, HEK-293T culture medium have 10 percent FBS and pen/strep. The implantation environment of R1 is exchanged every day and the implantation environment of MEF and HEK-293T are exchanged every 2 or 3 days. And then R1 cells were passaged and counted. In the later steps, transfection and construct the lent viral particles contains Nurr1 and GPX 1 were done and finally the viruses containing the Nurr1 and GPX1 gene were stored at -70. In this study, our selective marker for transgenic cells is the resistance gene to puromycin and GEP gene. So, before cells infecting with viruses, to obtain a lethal dose of Puromycin resistance gene for the R1 cells which did not have resistance gene, experiments were performed and the lethal puromycin dose was decreased.

Infecting the R1 cells with Nurr1 and GPX1 genes

The R1 ES cells were cultured on the mouse embryonic fibroblast cells and by helping trypsin they become as an isolated from. Single cells with polybrene (8µg/ml) with the ratio of 1:1 with lentivirus Nurr1 and GPX1 cells were infected in 6 homes on MEF cells, 12 hours after incubation, the cells environment were switched to a new environment. After infection, the implantation environment of stem cells was added first two or three days after infection to the implantation environment without antibiotics of Puromycin. Due to the presence of the resistance gene to Puromycin in the virus vector lenti is as a selective marker and it is excepted that the R1 cells which are given to them a virus and express the gene and they should survive in this concentration of Puromycin and other cells should die. Environment exchange with the implantation environment containing Puromycin was done every two days. After 10 days, it was seen that the well which was infected with 1 µg/ml, has approximately

70 percent cell and the well without virus was empty. During this time, the cells that have not received Nurr1 genes, were deleted by using Puromycin the cells are selected for reproduction and they were passaged on the new feeder layer and RNA extraction was performed from the uninfected and infected cells. Furthermore, the Nurr1 and GPX1 gene expression at the mRNA level were confirmed by using RT-PCR and in the protein level by using immunocytochemistry. At all stages of this study, the RNA extraction was done with cells and using manual method. For all RNA samples, the cDNA construction (final concentration) was done by using Fermentas kit or the Roche method.

RESULTS

Constructing the lent viral viruses contain Nurr1/GPX1/eGFP. The results show that after transfection of lenti virus vectors Nurr1/GPX1/eGFP and constructing the lent virus particles in HEK-293T cells were observed by fluorescent microscope 24 hours after cells transfection. In the cell implication dishes, GPX1-GFP and eGFP gene transfection were observed in green due to the reporter gene which represents the transfection level and producing the lenti virus particles with high titers (about 90-100 percent of cells) (figure 1 and figure 2).

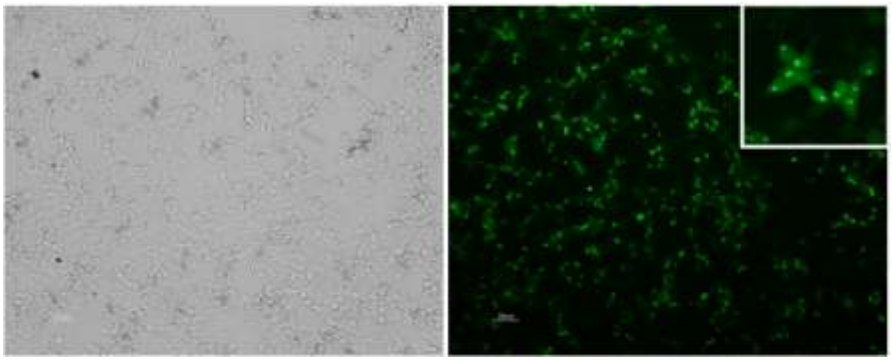

Figure 1: transfection and production of lenti viral particles contain hGPX1 in HEK-293T cells. (eGFP expression as the reporter and the hGPX1 expression)

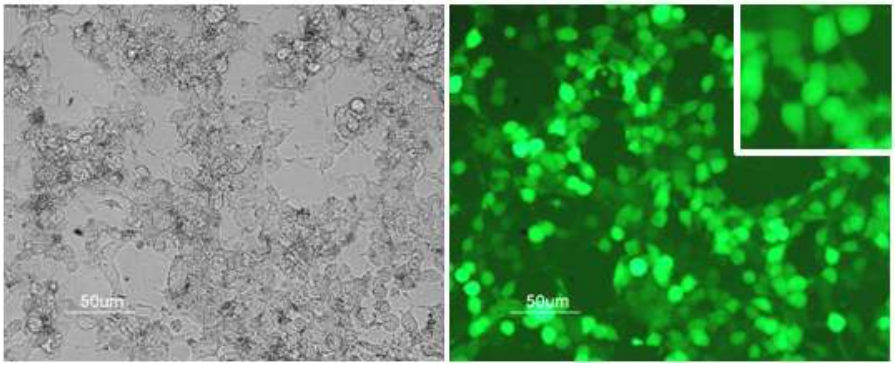

Figure 2: Lv-eGEP transfection in HEK-293T cells.

Implantation and infection of R1 cells and selection of R1-NG clones

The R1 ES cells in the implantation environment were cultured on MEF cells (Figure 3). In polybrene (8µ g / ml), they were infected with viruses contain NURR1 and GPX1 and eGFP genes as a control. Infection performance was observed after observing a fluorescent microscope between 40-50 percent of implanted cells. Sue to the protein expression to Puromycin, these cells were survived in the presence of 1µg/ml Puromycin and they express the GEP protein and the clones can be seen under the fluorescent microscope in green, (Figure 4).

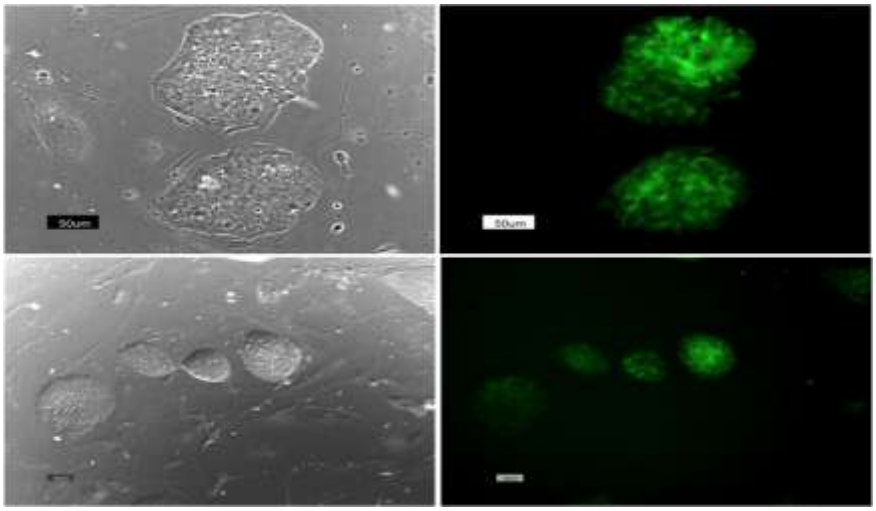

Figure 3: the clones of R1 mouse embryonic stem cell in inverted fluorescent microscope, the green color indicates the GFP protein (the bar size is 50µm).

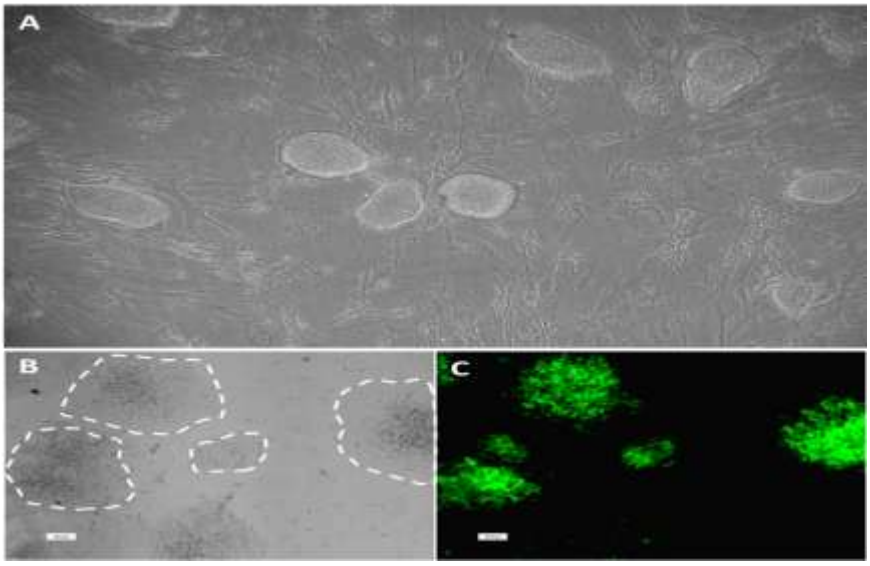

Figure 4: embryonic stem cell. R1 cells before infection, B and C: infected cells with Nurr1 and GPX1 viruses on the tenth day of Puromycin treatment.

Confirming ES cells expressing Nurr1/GPX1

After infecting R1 cells, the selection of expressing clone gene for resistance against Puromycin was done from the RNA extraction and cDNA construction. And they were evaluated with RT-PCR and hNurr1 and hGPX1 foreign genes and it was observed that these cells express the desired genes. Then in the protein level these two gene expression was confirmed by the ICC techniques. The Pluripotency marker Nanog expressing in these infected cells was evaluated and it was observed that the stem infected cells with lenti virus, have maintained their pluripotency characteristics after transfection by GPX1 and Nurr1 transgenes.

Expression confirming in the mRNA level by using RT-PCR

After infecting R1 stem cells and selection the Puromycin resistance gene expressing cells, from infected cells, the RNA extraction was done and for the hNurr1 and hGPX1 foreign genes, the RT-PCR action was done. PCR products were electrophoresis on agarose gel. The results showed that in infected cells, the foreign genes are expressed at the mRNA level.

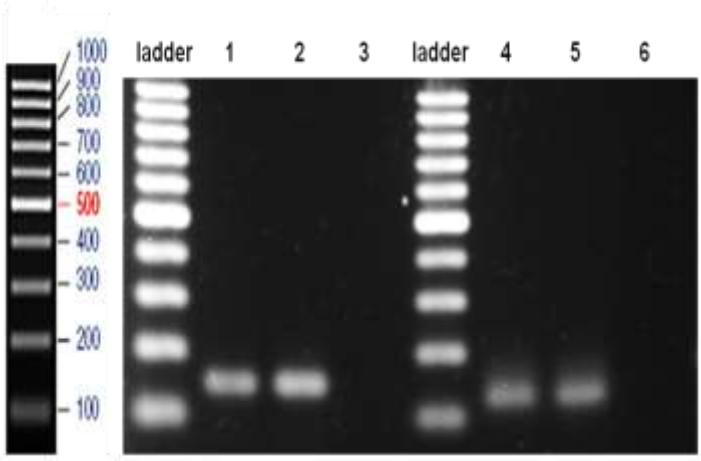

Figure 5: the expression confirming of hGPX1 and hNurr1 genes in infected cells.
1: hGPX1 gene for the infected instance with viruses. 2: positive control gene hGPX. 3: hGPX1 gene for the uninfected gene with virus and 4: the hNurr1 gene for the infected instance with viruses and 5: positive control for hNurr1 gene and 6: the hNurr1 gene for the uninfected gene with any gene.

(the fragments PCR size for hNurr1 and hGPX1 are equal to 145 bp and 127 bp).

The expression confirming of GPX1 and Nurr1 at the protein level with Immunocytochemistry (ICC)

After the gene expression hNurr1 and hGPX1 was confirmed at the R1 stem cells at the mRNA level. The R1-NG cells were cultured on MEF and these cells were fixed with Paraformaldehyde and immunocytochemistry (ICC) for the above cells.

The ICC results showed that Nurr1 protein is expressed in the nucleus of infected cells and the GPX1 protein is expressed at the cytoplasm of these cells (figure 6).

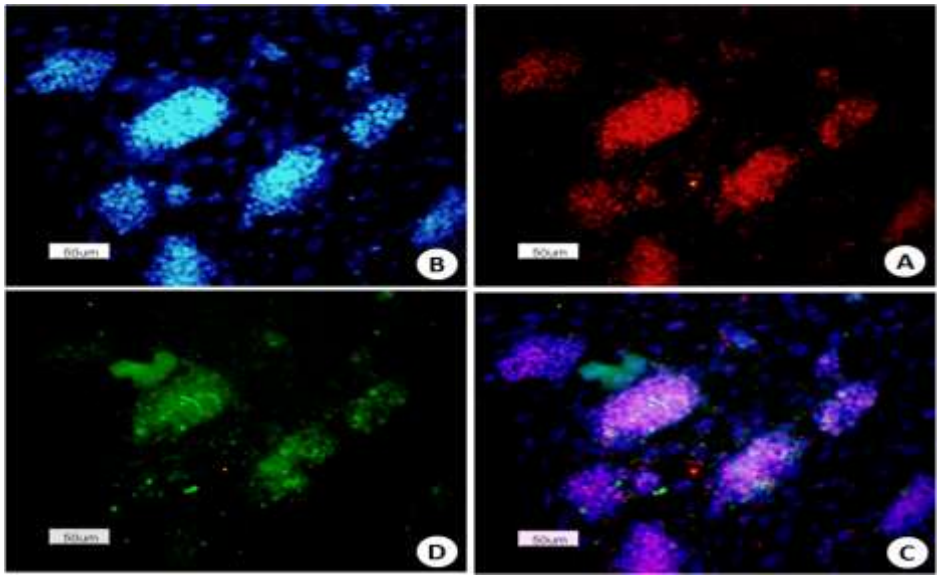

Figure 6: expression confirming of hNurr1 and hGPX1 genes at the protein level by immunocytochemistry techniques (ICC). A and B and C and D images shows the anit-mouse antibody of hNurr1 painted with Jred and the nuclei colored cells with DAPI and the combined picture, it is the GFP protein in selected clones.

Investigating the Pluripotent stem cells of R1-NG

After threating the stem cells of infected R1 with Puromycin, and with hNurr1 and hGPX1 viruses (R1-NG cells) and obtaining a homogeneous population of the Nurr1 expression cells and the expression confirming of these two foreign genes, this process was done at the mRNA and protein level. In this experiment, for evaluating, the presence of these two foreign genes is effective in the pluripotent characteristics of these cells, the marker expression evaluating and Nanog protein were done in these cells. The result of RT-PCR for this marker shows that the marker expression of pluripotent stem cells is created in an acceptable level.

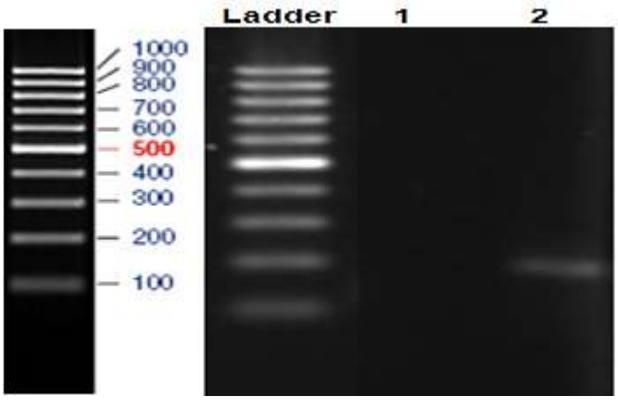

Figure 7: Nanog gene expression in NG-R1 cells as a pluripotent embryonic stem cells. 1 and 2, respectively are the instances of mouse embryonic fibroblast cells and the R1 infected cells with Nurr1 and GPX1 viruses

(the fragment size of PCR is 175 bp).

DISCUSSION

After Alzheimer's disease, Parkinson disease is the most common neurodegenerative disease. in this disease, the dopaminergic neurons (Dan) of the Astragal field are degenerated, selectively. In the present, the main cause of Parkinson disease is unknown but oxidative stress has an important role in disease creation and development so protecting these neurons against the oxidative stress can help to the survival of grafted neurons and treatment of Parkinson's disease. One of the forward ways in the treatment of Parkinson's disease is the cell transplantation to replace the lost neurons in these patients. There are several different cell sources to supply these cells and the dopaminergic neurons derived from embryonic stem cells is one of the most accessible sources, differentiation of these cells into the dopaminergic neurons, according to a 26-28 day protocol is possible as Co-culture with stromal cells or the protocol based on EB. The protocol of EB construction (used in this study) was used by Mckay doctor for murine embryonic stem cell differentiation (without any foreign gene).[25] and in the 2002 year it was completed and they could to obtain dopaminergic neurons from the mouse embryonic stem cells with stromal sells.[26] in this study, for the first time, by using a Lenti viral gene delivery, the combination of Nurr1 and GPX1 transferred into the mouse embryonic stem cells R1, and we obtained the embryonic stem cels expressing of Nurr1 and GPX1 gene (figure 5 and 6). Also, the possibility of transgenic cells (including the eGFP foreign gene or Nurr1 and GPX1) to the dopaminergic neurons was investigated and we were able obtain dopaminergic neurons from both cell lines similar to the Mckay doctors' sorks. (figure 7). The Nurr1 transcription factor is one of the major proteins in the normal

development of dopaminergic neurons in the embryonic stage.[30,27] This protein is expressed as a marker of dopaminergic neurons I later stages of dopaminergic differentiation. Martinant et al showed that the simultaneous expression of Nurr1 and Pitx1 in all cell differentiation process of embryonic stem can increase the efficiency of differentiation.[12] One of the goals of this research was improving the differentiation efficiency of dopaminergic neurons in the presence of foreign Nurr1 and GPX1 foreign genes. As it was shown in the results, the presence of transcription Nurr1 factor in treated cell groups (R1-NG) compared to the control group (R1), the markers of dopaminergic neurons (mNurr1,Pitx3,TH) express higher amount (figures 5,6,7). So, similar to the martinant 's works and at al, the cells containing the Nurr1 foreign genes with GPX1 in compared with control group, have a higher efficiency to the dopaminergic neurons.[12]

CONCLUSIONS

This research's results can be used for the purposes of pre-clinical of cellular treatment of Parkinson's diseases and transplantation to the animal model and it can followed the similar studies for the induced stem cells (iPS cells).

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