

Evaluation of neural gene expression in serum treated embryonic stem cells in Alzheimer's patients

Leila Dehghani¹, Batool Hashemi-Beni², Elahe Poorazizi¹, Fariborz Khorvash³, Vahid Shaygannejad³, Maryam Sedghi⁴, Sahar Vesal³, Rokhsareh Meamar^{1,3}

¹Department of Medical Sciences, Islamic Azad University, Najafabad Branch, ²Department of Anatomical Sciences, Isfahan University of Medical Sciences, ³Isfahan Neurosciences Research Centre, Isfahan University of Medical Sciences, ⁴Genetics Laboratory, Alzahra University Hospital, Isfahan University of Medical Sciences, Isfahan, Iran

Background: Previous studies confirmed that neural gene expression in embryonic stem cells (ESC) could influence by chemical compounds through stimulating apoptotic pathway. We aimed to use ESCs-derived neural cells by embryoid body formation as an *in vitro* model for determination of neural gene expression changes in groups that treated by sera from Alzheimer's patients and compare with healthy individuals. **Materials and Methods:** ESC line which was derived from the C57BL/6 mouse strain was used throughout this study. ESC-derived neural cells were treated with serum from Alzheimer's patient and healthy individual. Neural gene expression was assessed in both groups by quantitative real-time polymerase chain reaction analysis. The data was analyzed by SPSS Software (version 18). **Results:** Morphologically, the reducing in neurite out-growth was observed in neural cells in group, which treated by serum from Alzheimer's patient, while neurite growth was natural in appearance in control group. Microtubule-associated protein 2 and glial fibrillary acidic protein expression significantly reduced in the Alzheimer's patient group compared with the control group. *Nestin* expression did not significantly differ among the groups. **Conclusion:** Neural gene expression could be reduced in serum treated ESC in Alzheimer's patients.

Key words: Neural cell, neural gene expression, neurotoxicity

INTRODUCTION

Embryonic stem cells (ESCs) which derived from the inner cell mass of the blastocyst are pluripotent cells that able to differentiate into different cell lineages *in vitro* such as neural cell, cardiac cell, and etc.^[1,2] This differentiation ability have introduced them as a valuable model for diagnosing the mechanisms of cell survival, differentiation^[3] and especially, to create the neural cells for evaluating neurodegenerative disorders process, which are one of the main cause of human disabilities.^[4] It has been confirmed that neural gene expression in ESCs could influence by chemical compounds as drug, may be through stimulating apoptotic pathway.^[5,1] In some neurodegenerative disorders like as Alzheimer's and Parkinson diseases, there is an overall shrinkage of brain tissue.^[6] As the disease progresses, more nerve cells lost, leading to changes in behavior, such as wandering and agitation.^[7-10] One of the main causes of neurodegeneration is accumulation of b-amyloid plaques in the brains in Alzheimer's disease.^[7] It was implicated that disruption of normal cellular processes and high assembly of reactive oxygen and nitrogen species resulted apoptosis.^[8,9] Here, we used ESCs-derived neural cells by embryoid body (EB)

formation as an *in vitro* model for determination of neural gene expression changes in groups that treated by sera from Alzheimer's patients and compare with healthy individuals.

MATERIALS AND METHODS

Mouse embryonic stem cells (mESCs) culture and differentiation mESCs, Royan B1 cell lines derived from the C57BL/6 strain,^[11] were kept in an undifferentiated state in Knock out Dulbecco's Modified Eagle Medium (KDMEM) (Invitrogen) supplemented with 15% ESC-qualified fetal calf serum (ES-FCS) (Invitrogen) as previously reported. Neural differentiation of cells (800 cells per 20 µl hanging drops) was induced by culturing the cells in hanging drops to form EBs for 2 days as previously reported.^[12] EBs was collected and went under suspension culture in the presence 1 mM retinoic acid (RA) (Sigma-Aldrich) for 4 days.^[12] On day six, the EBs were placed on gelatin coated 12 well plates, for further 8 days in the presence of neurobasal medium (Invitrogen), supplemented with 5% sera from healthy individuals (as control group) and sera from Alzheimer's patients (as experimental group), 0.1 mM non-essential amino acids (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM

Address for correspondence: Dr. Rokhsareh Meamar, Isfahan Neurosciences Research Centre, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: Meamar@Pharm.mui.ac.ir

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Mbeta-mercaptoethanol, 1% penicillin–streptomycin (Invitrogen) and B-27 supplement (Gibco). For assessment of neural gene expression (microtubule-associated protein 2 [*Map2*] as a mature neuron marker), *Nestin* (as an immature neuron marker) and (Glial fibrillary acidic protein [*Gfap*] as mature astrocyte marker), we studied the following sera added to mESCs-derived neural cells culture medium: (1) Sera from healthy individuals (2) sera from Alzheimer's patients and gene expression was evaluated by real-time Polymerase Chain Reaction (RT-PCR).

Quantitative real-time polymerase chain reaction analysis

Cells were collected by centrifugation and total RNA was extracted by RNeasy mini kit (Qiagen) and then treated with DNase I (Fermentas) to avoid any DNA contamination (cDNA) and was synthesized using Moloney Murine Leukemia Virus (MMLV) Reverse Transcriptase according to the manufacture protocol (Fermentas). cDNA synthesis was performed using MMLV Reverse Transcriptase and random hexamer primer according to the manufacturer protocol (Fermentas). Real-time PCR was carried out in a thermal cycler Rotor gene 6000 (Corbett). The PCR mixture contained 10 μ l Rotor-Gene SYBR Green PCR Master Mix (TaKaRa), 5 pM of each primer, and 50 ng cDNA for each reaction in final volume of 20 μ l. All samples were assessed according to the level of β -tubulin expression, as internal control. All measurements were done in triplicates. Primer sequence were *Map2*; (forward: AAGTCACTGATGGAATAAGC, reverse: CTCTGCGAATTGGTTCTG), *Nestin*; (forward: CACACCTCAAGATGTCCC, reverse: GAAAGCCAAGAGAAGCCT) and *Gfap*; (CTCCAAGATGAAACCAAC, reverse: GCAAACCTAGACCGATACC).

Statistical analysis

The data were stated as mean \pm SD (standard deviation). One way ANOVA followed by the Turkey's *post hoc* test multiple group comparison to find the means that were significantly different from one another for data collected quantitative real-time-PCR. All experiments were replicated at least 3 times. A difference between groups was considered as statistically significant, if the $P < 0.05$.

RESULTS

In order to evaluate effect of Alzheimer's patient serum on neural gene expression in mESC-derived neural cells, plated EBs were treated in two groups; in the first group, plated EBs cultured in medium containing 5% serum from healthy individual as a control group or 5% serum from Alzheimer's patient. After 7 days post-plantation, neural gene expression was assessed and compared in both groups. Morphologically, the reducing in neurite outgrowth was observed in neural cells in group which

treated by serum from Alzheimer's patient [Figure 1a], while neurite growth was natural in appearance in control group [Figure 1b]. The expression pattern of *Map2*, *Nestin*, and *Gfap* were quantified by real-time PCR. *Map2* and *Gfap* expression significantly reduced in the Alzheimer's patient group compared with the control group [Figure 1c]. *Nestin* expression did not significantly differ among the groups [Figure 1c].

DISCUSSION

In our study, we applied ESCs-derived neuron as an *in vitro* model for evaluation of neural gene expression that caused by sera from Alzheimer patient when compared with control. Our results presented that mature neuron and astrocyte markers (*Map2* and *Gfap*) significantly decreased in patients group.

Previous studies have shown that the amyloid- β precursor protein cleaved, its products are placed in amyloid plaques in neurodegenerative conditions such as Alzheimer disease^[7] that affected neural gene expression^[6,12,13] and resulted in activating apoptosis pathway, as well.^[6,14] Wicklund's study has demonstrated that because of the existence of amyloid- β the number of functional neurons decreased and it provoked both neurogenesis and apoptotic pathway, separately.^[6,14]

Some studies have shown when ESC exposed to amyloid- β , *Gfap* expression was increased in neural differentiation process,^[6] but increment of *Gfap* expression was seen in present research. Compared with Wicklund's study, it seem this change has been made due to change in cell type or existence of other factor such as Nitric Oxide (NO).^[14-16] Furthermore, like as other study *Map2* expression reduced following treatment of ESC by Alzheimer's patient serum.^[6]

In previous literature, too much production of NO in serum of Alzheimer's disease has been associated with cytotoxicity in brain,^[17] which regulated apoptosis in these cells which were treated by serum from Alzheimer's patients. Not only was stress oxidative induced apoptosis, also neural gene expression was affected in ESC derived neural cells treated by Alzheimer's disease (AD) serum.^[7,17] Our previous study showed that *Map2* and *Nestin* expression decreased in neural cells treated by Methamphetamine.^[5]

In the current study, we supposed that neural gene expression has been reduced due to AD serum induced apoptosis and also NO production. Dildar *et al.*^[17] indicated serum levels of NO are elevated in probable AD patients and suppress neural gene expression.^[7]

Overall, the results of this study and its comparison with literature studies confirmed that firstly these cells

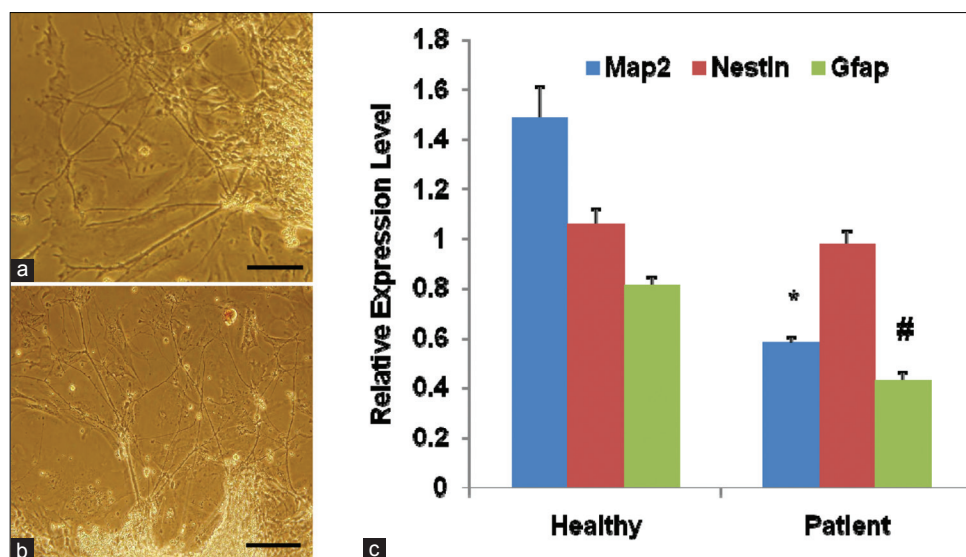


Figure 1: Status of neural cell with dendritic processes (a) in Alzheimer's patient group, (b) in control group, Scale bar: 20 μm, (c) Neural gene expression (*Nestin*, microtubule-associated protein 2 and Glial fibrillary acidic protein) in treated groups with serum from Healthy individual and Alzheimer's patient. Superscript letter indicates significant difference ($P \leq 0.05$) from means labeled with different letters as determined by one-way ANOVA analysis of triplicate samples

could be used as an efficient model for neurotoxic studies like as other studies, secondly neural gene expression decreased in Alzheimer's patients that need to be studied more and it can be concluded that Neural and cardiac differentiation protocol of mESCs has been applied to assess the developmental neurotoxicity and cardiotoxicity.^[2,5,18,19] Different studies have been tried to improve the mechanisms of activity or reduction of neurotoxicity, in addition to identifying new strategies that have higher therapeutic efficiency in neurodegeneration.

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