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Over expression of Nrf2 in Umbilical Cord-derived Mesenchymal Stem Cells Up regulates Cytoprotective Genes, TXNRD1 and GCLC

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Abstract

Background: Mesenchymal stem cells (MSCs) are ideal cells for cell and gene therapy. However, the low survival of MSCs after transplantation has limited their applications. We aimed to evaluate the expression of cytoprotective genes including NQO1, TXNRD1, HO-1, GCLC following the over expression of Nrf2 in umbilical cord-derived MSCs (UC-MSCs).

Methods: 3-5 passages of UC-MSCs were cultured. Recombinant vector containing Nrf2 (pcDNA3.1-Nrf2) and empty vector were transfected into MSCs using FuGENE HD. After exposing the cells to UV light stress, RNA extraction and cDNA generation were performed. Using Primer3, software specific primers were designed for Nrf2, NQO1, TXNRD1, HO-1, and GCLC genes and the expression of these genes was evaluated by RT-PCR. The results were semi-quantified and analyzed statistically utilizing Image J2x software and ANOVA test.

Results: The expression of Nrf2 was up-regulated in UC-MSCs after transfection with pcDNA3.1-Nrf2 ($P<0.01$). Over expression of TXNRD1 and GCLC were also confirmed in these transfected cells ($P<0.05$ and $P<0.01$, respectively). However, expression of NQO1 and HO-1 did not alter in the transfected cells with pcDNA3.1-Nrf2 compared with those transfected with empty vector ($P>0.05$).

Conclusion: Over expression of Nrf2 resulted in the over expression of TXNRD1 and GCLC in MSCs and might be explained by the fact that a known part of Nrf2 cytoprotective mechanisms is controlled by the expression of these genes.

Keywords: Mesenchymal Stem Cells, Over-expression, Nrf2, TXNRD1, GCLC**Keywords:** Educational games, Computer games, Self-Efficacy, Hemophilia.

Introduction

Adult stem cells have attracted the attention of researchers in different scientific fields due to some limitations of embryonic stem cells [1]. Mesenchyme stem cells (MSCs) are the most important kinds of adult stem cells [1].

MSCs were first identified in 1966 by Perakova and Friedenstein during the separation of bone cells from mouse bone marrow [2]. These cells are a population of multipotential stem cells which are separated from various tissues including bone marrow, muscle, brain, fat tissue, umbilical cord blood, umbilical cord tissue and peripheral blood[3]. Because of several differences, these cells have some exclusive features such as cell proliferation and self-renewing ability, differentiation to mesodermal or even non-mesodermal lineages, and exhibition of fibroblast-like morphology [4]. Moreover, MSCs are ideal cells for cell therapy because of their immunomodulatory effects and rapid proliferation *in vitro*. Recently, MSCs have been very effective in the treatment of injured tissues, myocardial infarction, acute kidney injury and other diseases[5-7]. However, during the collection and culturing processes MSCs are damaged which could decrease their survival and function. Interestingly, there are some reports showing that less than 1% of transplanted MSCs are alive in the first one day after transplantation [8,9].

Hence using suitable approaches to increase MSCs resistance against stresses can improve the rate of successful MSCs transplantation. It seems that knowing and reinforcing the protective mechanisms of these cells will lead to increased MSCs viability. Genetic manipulation of MSCs using known cytoprotective genes is one of the most effective strategies to promote MSCs survival [9].

Nrf2, nuclear factor (erythroid-derived)-like 2 transcription factor is one of the most important cytoprotective genes. This transcription factor leads to cell survival against different stresses. Nrf2 is expressed naturally and attaches to the cytoplasmic protein and enters the cell nucleus under various stressful conditions, and induces the expression of several genes involved in oxido/reduction and detoxification. Nfr2 and its resulting proteins have an important role in protection against oxidative stresses, cell damages resulting from chemical materials, cancer prevention, and wound healing [10-13]. The expression of Nfr2 increases under stressful conditions and causes the transcription of some genes which increase the cell resistance against different stresses. Some of these genes are TXNRD1, GCLC, GSTs, UGT, HO-1, NQO1 that have detoxification and antioxidant abilities and eventually improve cellular defense mechanisms [14-18].

NQO1, NAD(P)H dehydrogenase quinine1, is the expressing gene of one oxidoreductase enzyme which has an important role in detoxification of quinine and its metabolites [19]. This enzyme also leads to the stability and endurance of P53, and protects cells against oxidative damages [20].

HO-1, hemeoxygenase-1, gene induces the expression of specific enzyme in stressful cellular condition that decomposes heme to Fe^{2+}, bilirubin, and Co [21]. Some of the most important functions of HO-1 are anti-apoptotic, anti-inflammatory and antioxidant activities [22,23].

In addition to being responsible for the vital biological response to oxidative stress, TXNDR1 gene regulates proliferation, apoptosis and survival of the cells through controlling thioredoxin activities[24]. GCLC, glutamate-cystene ligase catalytic, codes glutamate cytein ligas enzyme which is known as the first enzyme to inhibit glutamine production and participates in cellular stress response [25].

The described genes are Nrf2 downstream genes and well-known factors for cellular protection. In other studies, the effects of the Nrf2 over expression on cellular resistance against stressful microenvironment has been investigated [26-28], but alteration in the expressions of these mentioned genes which probably helps better understand the protective mechanisms of Nfr2, has been studied less.

Regarding the existing information and the importance of these genes in inducing cellular resistance, we aimed to evaluate the expression of some cytoprotective genes including NQO1, TXNRD1, HO-1, GCLC, after Nfr2 over-expression in UC-MSCs. In other words, by considering the expression of Nfr2 downstream genes, some of the probable protective mechanisms of Nfr2 could be determined.

Materials and Methods

Cell culture

UC-MSCs were previously separated from umbilical cord samples with informed consent and characterized in Iran Blood Transfusion Organization (IBTO) [29]. 3-5 passages of UC-MSCs were cultured in DMEM-low glucose with 10% FBS, 1% penicillin and streptomycin at 37°C and 5% CO_2. 5 days later, the morphological character of UC-MSCs was observed under invert microscope.

Transfection of the UC-MSCs

Recombinant plasmid containing Nrf2 gene (pcDNA3.1-Nrf2) was used which was produced and verified in the research center of IBTO [26]. To over express Nrf2 level, about 300000 UC-MSCs were cultured in 6-well plates. Through several experiments different ratio of DNA plasmid concentration and transfection reagent FuGENE HD (Roche, Germany) were mixed and added to the culture medium for 4 hrs according to the manufacturer's instructions. Transfected UC-MSCs with empty plasmid (non-containing Nrf2 plasmid) was considered as the control. Transfected cells were exposed to *UV* light for 1 hr to induce more Nrf2 expression. Then, the expression of Nrf2 was evaluated at three intervals (24, 48, 72 hrs) after transfection using the RT-PCR technique. The expression of NQO1, GCLC, HO01, TXNRD genes was also assayed using RT-PCR method.

RT-PCR

After UC-MSCs transfection and exposure

to *UV* light at the right time, RNA of transfected cells with recombinant plasmid (MSC-Nrf2) and empty plasmid (MSC-V) were extracted using RNA extraction kit (Invitrogen, Germany) according to the suggested instruction. cDNA was generated using the cDNA synthesis kit (BIONEER, USA). Specific primer sets were designed and blasted utilizing Primer3 or NCBI website. The PCR reaction was performed in suitable thermal conditions to evaluate the expression of Nrf2, TXNRD1, NQO1, GCLC, HO-1 genes. The expression of β–actin is also considered as normalizer.

Statistic analysis and quantifying the results

To optimize transfection and PCR reaction condition, several experiments were performed. The PCR images were semi-quantified using Image J2x software. Each test run was performed in triplicate. Statistic comparison was done using ANOVA and significant difference was reported with *P* value less than 0.05.

Results

Isolated UC-MSCs exhibited fibroblast-like morphology

UC-MSCs were cultured in DMEM medium with appropriate supplements. Microscopic observations showed that these cell were spindle shaped and fibroblastoid (figure 1 1). The differentiation potentials of these cells to osteocytes, chondrocytes and adipocytes as well as the expression of the MSCs surface markers (CD105, CD90, CD29) and no expression of the hematopoietic stem cells (HSCs) surface markers (CD34 and CD45) had been previously confirmed [29].

Nrf2 expression up regulated in transfected UC-MSCs with recombinant plasmid containing Nfr2

Recombinant plasmid containing Nrf2 gene (pcDNA3.1-Nrf2) and empty plasmid were transfected into the UC-MSCs using FuGENE HD. After several experiments and evaluation of Nfr2 expression with RT-PCR method, the best transfection duration which yielded increased Nrf2 expression was determined as 72 hrs. As shown in figure 2A, Nrf2 expression was up regulated in MSC-Nrf2 group transfected with recombinant pcDNA3.1-Nrf2. The quantifying of PCR image with Image J2x software was confirmed more expression level of Nrf2 in MSC-Nrf2 in comparison to MSC-V group that transfected with empty plasmid ($P<0.01$). The expression of β-actin was evaluated to ensure the validity of processes, and the result showed that both groups equally expressed this gene and there was no significant difference between the expression of β-actin in MSC-Nrf2 and MSC-V ($P>0.05$, figure 2B).

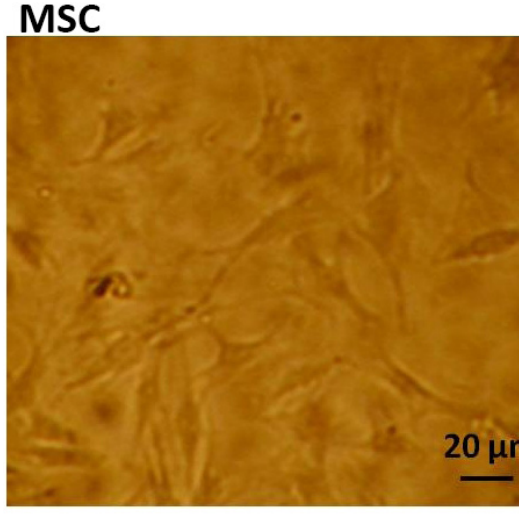

Figure 1: Fibroblast-like morphology of UC-MSC after 5 days of in vitro culturing. (Magnification × 200, Scale bar: 20 µm).

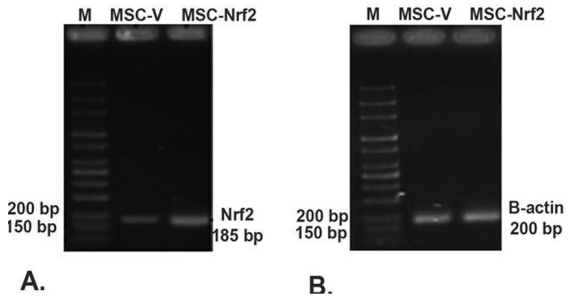

Figure 2: Evaluation of Nrf2 and β-actin expression by RT-PCR. A: Nrf2 expression was evaluated 72 hrs after transfection. MSC-Nrf2 expressed Nrf2 more than MSC-V (P< 0.01). B: Expression of β-actin was also evaluated. There was no significant difference between the expression of this gene in both groups (P> 0.05). M: DNA marker (100 bp), MSC-V: MSCs transfected with empty plasmid, MSC-Nrf2: MSCs transfected with recombinant pcDNA3.1-Nrf2 plasmid.

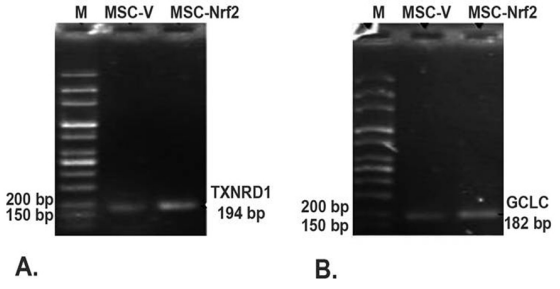

Figure 3: Evaluation of TXNRD1 and GCLC expression by RT-PCR. A: TXNRD1 expression was evaluated following by over expression of Nrf2. Expression of TXNRD1 was up-regulated in MSC-Nrf2 in comparison to MSC-V (P< 0.05). B: RT-PCR analysis indicated that there was significant difference between the expression of GCLC in MSC-Nrf2 and MSC-V groups (P< 0.01). M: DNA marker (100 bp), MSC-V: MSCs transfected with empty plasmid, MSC-Nrf2: MSCs transfected with recombinant pcDNA3.1-Nrf2 plasmid.

Expression of GCLC and TXNRD1 increased in transfected UC-MSCs with recombinant pcDNA3.1-Nrf2 plasmid

The expression of some Nrf2 downstream genes including HO-1, TXNRD1, NQO1, GCLC in transfected cells was also investigated. After electrophoresis of PCR products and quantifying the results, it was specified that TXNRD1 gene in MSC-Nrf2 group (transfected with pcDNA 3.1-Nrf2 plasmid) increased compared with the MSC-V group (transfected with empty plasmid) (*P*<0.05, figure 3A). As shown in figure 3B, the expression of GCLC up regulated consequently after increased Nrf2 expression in the cells which receive recombinant plasmid, and this gene was expressed more in the MSC-Nrf2 group than the MSC-V group (*P*<0.01, figure 2B).

Increasing of Nrf2 expression had no effect on HO-1 and NQO1 expression

After up regulation of Nrf2, expression of HO-1 and NQO1 was considered as well. RT-PCR results

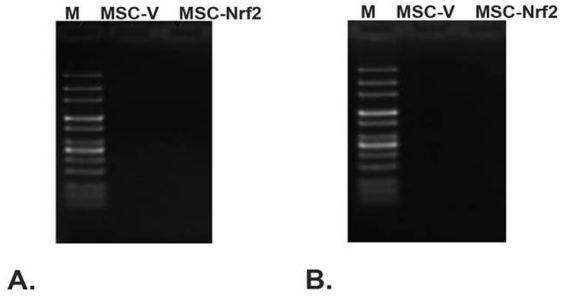

Figure 4: Evaluation of HO-1 and NQO1 expression by RT-PCR. A: HO-1 expression was assayed after transfection with empty and recombinant vectors. MSC-Nrf2 and MSC-V did not express this mentioned gene. B: There was no difference between the expression level of NQO1 in MSC-Nrf2 and that of in MSC-V (P> 0.05). M: DNA marker (100 bp), MSC-V: MSCs transfected with empty plasmid, MSC-Nrf2: MSCs transfected with recombinant pcDNA3.1-Nrf2 plasmid.

indicated that HO-1 was not express in both groups (P>0.05, figure 4A).

As shown in figure 4B, there was no relative band indicating NQO1 expression in UC-MSCs transfected with recombinant plasmid and empty plasmid. In other words, expression of NQO1 in both groups did not show any significant difference (P>0.05).

Considering the results, it was totally determined that after over-expressing the Nrf2 in UC-MSCs, some of its downstream genes such as TXNRD1 and GCLC were more expressed but the expression of NQO1 and HO-1 did not alter in these cells.

Discussion

MSCs have different applications in medicine because of their easy isolation and ability to differentiate to various cell types [6,7]. However, they are exposed to several damages during collection, culture and processing steps which decreases their viability and potentialities *in vivo* [8]. Therefore, several studies have been conducted to improve the survival of MSCs in cell therapy applications [30-32].

In this study Nrf2 gene was over expressed in UC-MSCs. Nrf2 is the essential gene involved in protection of cells against stressful conditions. Nrf2 induces expression of cytoprotective genes that are important to vital cell processes including oxidoreduction, apoptosis, and proliferation [10,12,14,18].

The umbilical cord is an available sample which can be obtained with easy non-invasive sampling and does not have any ethical problem [33]. Moreover, UC-MSCs are primitive stem cells with a faster proliferation rate and more colony forming capacity [34,35]. Non-viral vector, pcDNA3.1 plasmid, and transfection reagent FuGENE HD were used to increase Nrf2 expression in UC-MSCs. PcDNA3.1 is a shuttle vector that could be cloned in prokaryotic system and expressed in eukaryotic system [36].

Using the plasmid vectors (instead of virus vectors) addresses the concerns about non-specific immune reactions and mutagenesis [37]. The FuGENE reagent increases the rate of DNA transition into the cells and it has no negative effect on cell proliferation and differentiation [38].

The expression of TXNRD1 and CGLC genes (some of the Nrf2 downstream genes) up-regulated in UC-MSCs following by over expression of Nrf2. But the increasing of the Nrf2 expression, did not have any effect on the expression of HO-1 and NQ01 genes. In this study, any alteration in gene expression level was analyzed by RT-PCR method only and results were quantified eventually using Image J2x software. It is better to use quantitative techniques like RT-PCR with cyber green florescent dye or other materials to quantify the rate of gene expression. However, time and financial limitations contributed to the lack of using more confirmatory tests to evaluate gene expression in our study.

Mohammadzadeh and colleagues reported that increasing Nrf2 gene expression in MSCs, will lead to increasing the expression of SOD1&2 (Superoxide dismutase 1 &2), increasing cell

resistance and decreasing apoptosis in these cells [26]. They used a viral vector for transduction of bone marrow derived-MSCs. The use of viral vectors has been accompanied by concerns about gene entrance into the genome, immune response to the viral vector, and cancer [38-40].

Another study on increased Nrf2 expression in vascular smooth muscle using a viral vector showed that these cells can increase cell resistance and decrease tissue injuries in vascular diseases with high oxidative stress and inflammation [27]. Cao and co-workers applied over expression of Nrf2 to strength cells against oxidative stress [28]. In their study neuroblastoma cell line had been applied. In our study, UC-MSCs were used as multi-potent primary cells which have more applications. Cell lines, are not primary cells and they are produced by some manipulations.

In other studies it has been reported that Nrf2 can increase the expression of some antioxidant enzymes including HO-1, SOD 1&2, GCLC, GPX, and NQO1 [41-43]. In contrast, the results of the current study show that increased Nrf2 expression, did not have any effect on NQO1 expression. Using different kinds of stem cells, different methods to increase Nrf2 expression, or applying different methods and materials with different sensitivity and specificity may be of the probable reasons of these discrepancies.

Conclusion

Increased Nrf2 expression in MSCs leads to more expression in GCLC and TXNRD1 genes in these cells. It seems that the cytoprotective role of Nrf2 might be influenced by these genes which requires more ongoing complementary studies.

Conflict of Interest

The authors declare that there is no conflict of interest.

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