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In vitro Transdifferentiation of Bone Marrow Stromal Cells into GABAergic-Like Neurons

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ABSTRACT

Background: Cell therapy of many neurodegenerative diseases using bone marrow stromal cells (BMSC) requires the differentiation of BMSC into neuronal subtype. However, the transdifferentiation of BMSC into GABAergic phenotype requires more investigation. **Methods:** In this study, BMSC of adult female rats were pre-induced into neuroblast-like cells using 1 mM β-mercaptoethanol (βME) and 10 μM retinoic acid (RA), followed by 40 mM potassium chloride as inducer. The BMSC were evaluated by fibronectin as well as Oct-4. The percentage of nestin, neurofilaments (NF 68, NF 160, and NF 200) and GABA immuno-reactive cells was used to evaluate the GABAergic differentiation at the pre-induction and induction stages. The statistical analysis was carried out using unpaired student's *t*-test and ANOVA with Tukey's multiple comparison. **Results:** The BMSC in the fourth passage expressed fibronectin up to 91.24 ± 0.82%. The pre-induced cells after 2 days of RA exposure showed the expression of neuroblastic markers of nestin and NF68 (81.56 ± 2.64% and 82.12 ± 2.65%, respectively). The yield of GABAergic neurons with β-ME for 1 h and RA as pre-inducer for 2 days followed by potassium chloride as inducer (40 mM for 3 days) was 60.64% ± 1.97%. In addition, NF160 and NF200 were detected in the transdifferentiated cells. RT-PCR showed no expression of Oct-4 after the induction and pre-induction stages. **Conclusion:** GABAergic-like neurons obtained from BMSC can be potentially used in cell transplanting for some neurodegenerative disorders. *Iran. Biomed. J. 13 (3): 137-143, 2009*

Keywords: Bone marrow stromal cells (BMSC), GABAergic-like neurons, Transdifferentiation, Cell therapy

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INTRODUCTION

GABAergic neurons, which play fundamental roles in neural development and function, are the pre-dominant inhibitory neurons in the mammalian central nervous system [1]. It has been shown that GABAergic neurons, as interneurons in the spinal cord, effectively attend the neural circuit while some of them surround the motoneurons [1, 2]. GABAergic neurons are important in several diseases such as epilepsy, Alzheimer and Huntington [3, 4] and lead to disregulation of cortical neuronal circuit function. In adult mammalian brain tissue, damaged by diseases such as neurodegeneration disorders, the regeneration of these neurons may not occur spontaneously [5]. Thus, transplantation of GABAergic neurons may provide a therapeutic approach to repair the damaged nervous tissues. Rodent and human bone marrow stromal cells (BMSC) can, under certain conditions, differentiate into neurons, cardiac muscle and other types of cells [6]. It has been shown that BMSC can be induced to express a neuronal phenotype *in vitro* under specific experimental conditions. For example, Woodbury *et al.* [7] observed that in the presence of β-mercaptoethanol (βME) and dimethylsulfoxide, BMSC might differentiate into the cells that express neuron specific enolase and neurofilaments (NF).

Retinoic acid (RA) is well known as the biologically active form of vitamin A that plays an important role during embryogenesis [8]. It has often been observed that treatment with high concentrations of RA promotes neural gene

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expression and represses mesodermal gene expression [9]. In addition, it has been thought to be one of the most important extrinsic inductive signals that can be used for neural differentiation of olfactory neuronal cells *in vitro* [8]. It has been reported that sequential exposure of neural stem cells (NSC) culture to RA and KCl leads to GABAergic neurons [5]. Studies have shown that adult human BMSC grown in suspension culture give rise to neural spheres progenitor cells capable of expressing both dopaminergic and GABAergic phenotypes [10]. There has been no report published so far on the differentiation of BMSC into GABAergic neurons. Therefore, in the present work, we induced BMSC into GABAergic-like neurons *in vitro* by sequential exposure to βME and RA followed by KCl in two stages.

MATERIALS AND METHODS

BMSC preparation. The protocols used were approved by Animal Studies Ethical Committee of Tarbiat Modares University, Tehran, Iran. The bone marrow was extruded from 250-300 g Sprague-Dawely rats (Razi Vaccine and Serum Research Institute, Tehran, Iran) tibias and femurs using an 18 G needle. The bone marrow cells were cultured in α-MEM (Gibco, UK) supplemented with 10% FBS, penicillin (100 U/ml) and L-glutamine (2 mM/ml) on a 75-cm² flask (Nunc, Denmark) at 37ºC by a 5% CO_2 incubator. After 24 h, the hematopoietic stem cells and the non-adherent cells were removed by medium changing and then a fresh medium was added to the flask. The medium was changed every other day after washing the cells by PBS. The bone marrow cells were harvested by trypsin/EDTA (Gibco, UK) before being confluent in order to obtain a single cell suspension. The BMSC of the fourth passage were plated in gelatin-coated flasks, or on 24-well plates containing gelatin-coated glass cover slips (nearly 5,000 cell/cm²). The cells were checked for purity by fibronectin immuno-staining and stemness using Oct-4 RT-PCR on mRNA extraction of the fourth passage of the cultured cells. The experiments were carried out in two stages: stage 1, pre-induction and stage 2, induction). At the stage 1, the cells were pre-induced 24 h after plating with βME (1 mM), for 1 h in α-MEM medium without FBS. After washing the cells with PBS, the medium was changed with α-MEM medium and 10% FBS containing all trans-RA (10 μM) and evaluated the cells after 1, 2 and 3 days. The results of the antibody staining for nestin and NF during 3 days of RA exposure were compared and the cells in the day with the highest percentage of positive immuno-reactions were induced by KCL in the stage 2. We considered two groups as control parallel to the experiment. In the first control group, treatment was continued with βME and RA during the induction stage. The second control group was treated only with α-MEM containing 10% FBS during the pre-induction and induction stages. KCl with different doses (0, 20, 40 and 80 mM/ml) was added to α-MEM containing 10% FBS on days 1, 3 and 5 for the experimental group during the induction stage. Then, the cells were harvested for evaluation by the viability test. Immuno-cytochemical analyses were performed on the adherent cells on the cover slips.

Antibodies and Immuno-cytochemistry. The BMSC at the fourth passage and the cells from stages 1 and 2, which were plated on gelatin-coated glass cover slips, were washed with PBS and fixed by acetone for 5 min. Then, the fixed cells were washed twice with PBS before staining. Blocking of non-specific antigen reaction and permeabilization were carried out in a blocking buffer consisting of 0.1% Triton X-100 and 10% normal goat serum in PBS for 1 h. The primary antibodies (mouse anti-fibronectin monoclonal antibody 1:300, mouse anti-nestin polyclonal antibody 1:300, mouse anti-NF68 monoclonal antibody 1:300, mouse anti-NF160 monoclonal antibody 1:300, mouse anti-NF200 polyclonal antibody 1:400 and mouse anti-GABA monoclonal antibody 1:500) were incubated at 4ºC overnight and washed three times in PBS. Then, the cells were incubated with the secondary antibodies (anti-mouse FITC-conjugated and anti-rabbit FITC-conjugated, Chemicon, 1:100) at room temperature for 2 h. The cells were washed twice in PBS for 15 min and counter-stained with ethidium bromide for 1 min in order to demonstrate the nuclei. Then they were washed again in PBS and examined with a fluorescence microscope at 200× magnification (Zeiss, Axiophot, Germany). The number of immuno-reactive cells was divided by the total cell number in order to estimate the percentage of immuno-reactive cells. Each experiment was replicated at least 5 times so that reproducibility could be ensured.

Expression of Oct-4 gene. Expression of Oct-4 gene was done using forward primer:

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5´AAGCTGCTGA AACAGAAGAGG 3´, and backward primer: 5´ACACGGTTCTCAATGC TA GTC 3´, (215 bp, accession number: NM-001009178, annealing at 57ºC). The internal control was β2 microglobulin with 5´CCGTGATCTTTCTG GTGCTT 3´and 5´TT TTGGGCTCCTTC AGAGTG 3´as forward and backward primers, respectively (300 bp, accession number: NM-012512, annealing at 58ºC). The pre-induced and induced BMSC were evaluated for the expression of Oct-4. RNA was extracted from each cell population (1-2 million cells) by using 1 ml RNX plus (RNX plus™ Kit Cinnagen, Tehran, Iran). Then, the cells were treated with DNase for RT-PCR. Total RNA (1 µg) was used as template in 20-µl cDNA synthesis reaction containing 0.5 µg oligodT. Both RNA and primers were denaturated at 70°C for 5 min and chilled on ice immediately. A mixture of 20 U ribonuclease inhibitor, 1 mM dNTP and the 5× buffer supplied by the manufacturer was added into deionized water (nuclease free) up to 19 µl and the obtained solution was incubated at 37°C for 5 min. Then, 200 U RevertAid^{TM} M- MuLV Reverse Transcriptase (Fermentas, Canada) was added to the reaction and the tube was incubated in a thermocycler (BIO RAD, USA) at 42ºC for 60 min, and at 70ºC for 10 min afterwards. Two negative control reactions (without RNA and without M-MuLV, and with RNA and without M-MuLV, respectively) accompanied each reaction. PCR was performed using 2 µl of the synthesized cDNA with 1.25 U Taq polymerase (Cinnagen, Tehran, Iran), 1.5 mM of $MgCl_2$, 200 µM dNTP, 1 µM of each primer, 10× buffer supplied by the company, and deionized distilled water in a 50 µl total reaction volume. All the common components were added into the master mix and then aliquoted in tubes. The cycling conditions were as follows: initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC for 30 s, 57-58ºC (depending on the primers annealing temperatures) for 30 s, 72ºC for 45 s, and a final extension of 72ºC for 5 min. The product size was checked on 1.5% agarose gel electrophoresis. Each experiment was repeated at least three times in order to ensure its reproducibility.

Statistical analyses. The statistical analyses were carried out using unpaired student's *t*-test and ANOVA with Tukey's multiple comparison. For each parameter, the significance level was determined using SPSS 10 (SPSS Inc., Chicago, IL, USA).

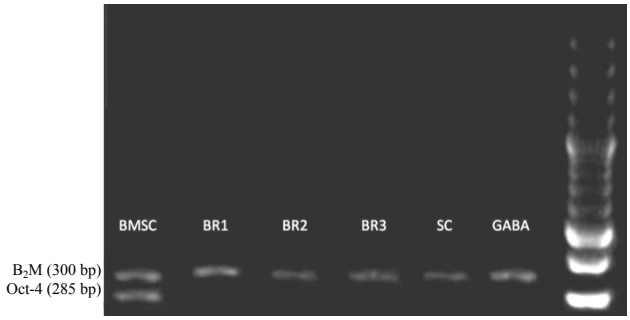

Fig. 1. Detection of β_2M and Oct-4 mRNA from rat bone marrow stromal cells by RT-PCR. BMSC, bone marrow stromal cell; BR1, βME + RA for 1 day; BR2, βME + RA for 2 days; BR3, βME + RA for 3 days; SC, spinal cord and GABA, GABAergic-like cells.

RESULTS

RT-PCR results. The RT-PCR results (Fig. 1) showed that mRNA of Oct-4, stemness marker, was expressed in the BMSC, while treatment of the BMSC with βME (1h) and RA (on the days 1, 2 and 3) during the pre-induction stage, followed by KCL during induction stage, caused the disappearance of Oct-4 expression in both stage as compared to the BMSC. This gene was not expressed in the adult spinal cord.

Immuno-cytochemistry results. To further define the cellular phenotype in the forth passage of the BMSC in the culture, we used anti-fibronectin antibody, a stromal marker of BMSC, and observed that more than 91.24 ± 0.82% (mean ± SEM) of the cells were fibronectin immuno-positive. Also, very few number of the cells in this stage expressed detectable neural markers such as Nestin, NF68, NF160, NF200 and GABA (Fig. 2).

Pre-induction stage. A time course evaluation of RA effect (on the 1st, 2nd and 3rd days) following βME (1 h) was done using immuno-reactive cells for different antibodies. Figure 2 represents the means and the standard errors of the means of the percentages of cell immuno-reactivity to fibronectin, nestin, NF68, NF160, NF200 and GABA. The expression of fibronectin decreased during the pre-induction stage to 4.12 ± 0.59% until the days 3, whereas there was an increase in the expression of nestin and NF68 two days after RA exposure (81.56 ± 2.64% and 82.12 ± 2.65%, respectively) and their expression level decreased on the day 3 (72.84 ± 2.66% and 62.44 ± 2.31%,

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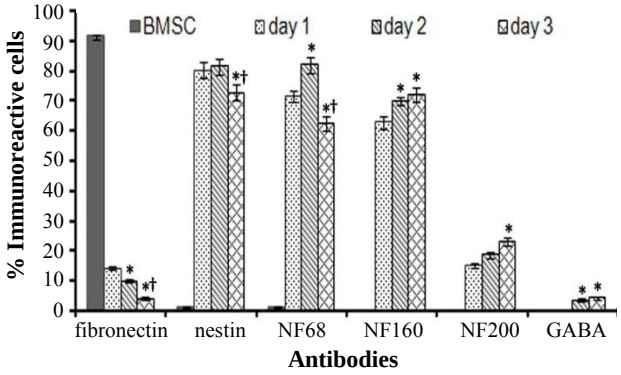

Fig. 2. A quantitative analysis of different antibodies in the treated and the untreated bone marrow stromal cells during 3 days of pre-induction (mean ± SEM), represented by the percentage of immuno-reactive cells. A significant difference with fibronectin is seen in each group. Asterisks indicate statistical significance between 1 day RA and the other experimental groups. † shows the statistical significance between the 2 day RA and the other experimental groups (significancy level $P<0.05$).

respectively). Therefore, the day 2 was chosen for the next step to carry out the induction with KCL. Although the level of NF160, NF200 expression increased constantly during the pre-induction time, the increase in the expression level of GABA was not considerable (4.4 ± 0.64% on the day 3).

Induction stage. During the induction stage, the cells were evaluated by GABA antibody. Figure 3 shows the means and the standard errors of the means of the percentages of the immuno-reactive

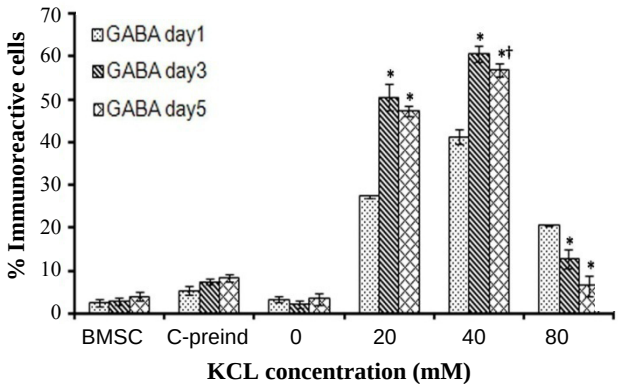

Fig. 3. The means and the standard errors of the percentage of immuno-reactive cells according to the time course and dose response studies with KCl at days 1, 3 and 5 after pre-induction with βME (1 h) and RA (2 days) with different doses of KCl (with the doses of 0, 20, 40 and 80 mM) . Asterisks indicate the statistical significance between the GABA day 1 group and the other experimental in each groups. † shows the statistical significance between the GABA day 5 and the GABA day 1. C-preind, continue pre-induction (continue with RA) (significancy level $P<0.05$).

cells. All doses showed, to some extent, an increase in the expression of GABA except the dose 80 mM. The immuno-cytochemical results of GABA antibody showed that treatment with KCl (40 mM) on the day 3 had the highest differentiation result (60.64 ± 1.97%) compared with the other dose and day groups. The expression of GABA in the control groups continued with RA and in the group, deprived of RA, during the induction stage, did not exceed more than 8.32 ± 0.89% and 3.42 ± 1.3%, respectively. The comparison of the day 2 of the pre-induction stage and the day 3 of KCL 40 mM of the induction stage showed a significant decrease in fibronectin, nestin and NF68 expression and a significant increase in NF200 and GABA expression (Fig. 4). Figure 5 shows the fluorescence microscopic images with relevant phase contrasts of each antibody.

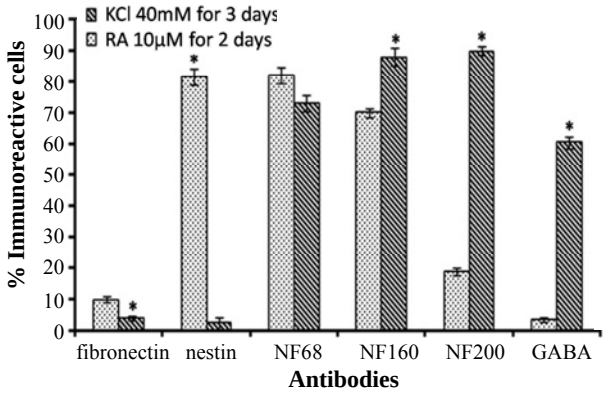

Fig. 4. Comparison of the means and the standard errors of the percentage of immuno-reactive cells induction with 40 mM KCl on the day 3 and pre-induction with βME (1 h) and RA10 μM on the day 2. Asterisks indicate statistical significance between RA on the day 2 and the 40 mM KCl on the day 3 (significancy level $P<0.05$).

DISCUSSION

This study proposes a protocol for step by step neural differentiation. The BMSC were evaluated for fibronectin and Oct-4 expression. The results showed that the BMSC expressed few fibronectins in the fourth passage.

Progressive downregulation of fibronectin, a classical stromal cell marker, was noticed during the pre-induction stage, which is consistent with other findings [11]. Expression of Oct-4 gene, a stem cell marker, was reported in the undifferentiated BMSC [12]. In this study, Oct-4 was detected in undifferentiated BMSC, but not in the BMSC after pre-induction and induction stages. Other investigators confirmed this finding by reporting that

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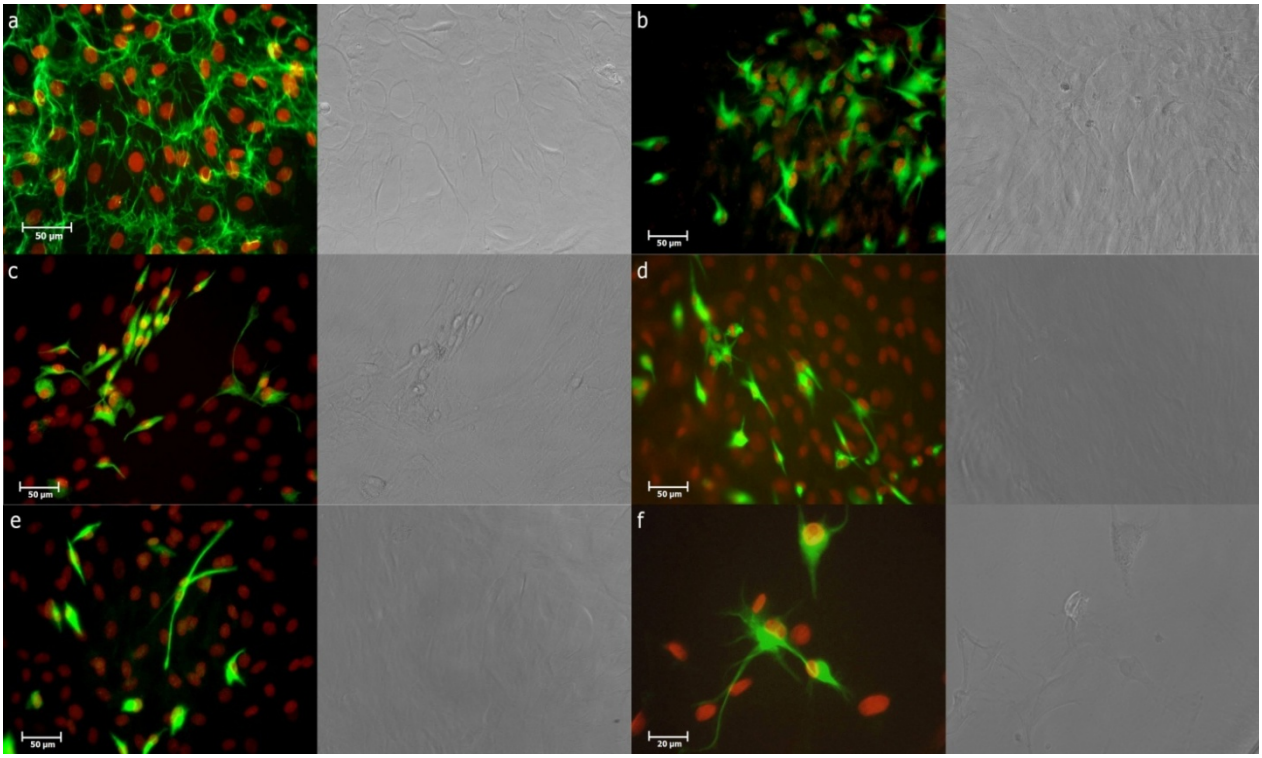

Fig. 5. Photomicrographs of immuno-histochemistry for antibodies used to identify the transdifferentiated bone marrow stromal cells. The right panels represent the phase contrast of the immuno-stained cells. All the pictures were taken on the day 3 of the induction stage. **(a)** represents the immuno-stained cells with anti-fibronectin antibody; **(b)** represents the immuno-stained cells with anti-nestin antibody; **(c)** represents the immuno-stained cells with anti-neurofilament 68 KDa antibody; **(d)** represents the immuno-stained cells with anti-neurofilament 160 KDa antibody; **(e)** represents the immuno-stained cells with anti-neurofilament 200 KDa antibody and **(f)** represents the immuno-stained cells with anti-GABA antibody. Followed by treatment with the primary antibodies, the cells were exposed to the FITC conjugated secondary antibodies and were counter stained with ethidium bromide.

Oct-4 expression is suppressed in the differentiating embryonic stem cells, NSC [13] and umbilical cord blood stem cells [14, 15]. Although a high expression of Oct-4, a marker for cell stemness, was reported in the undifferentiated BMSC, such an expression was not detected in the pre-induced BMSC, which is consistent with the other investigator's findings [10, 15]. In order to identify factors able to lead differentiation of BMSC towards the cells of different neural lineages, treatments by many inducers were examined. Expression of early neuronal markers, NF-160 and nestin [7, 16], was reported by two single inducers, βME and isobutylmethylxanthine, respectively.

The use of βME as a pre-inducer for transdifferentiation of BMSC into neurons was first reported by Woodbury *et al.* [7] and the results showed anti-oxidant and thiol reduction effects of βME [16], inducing of the BMSC and expression of the neuroblastic markers, like nestin and NF160, in these cells [7]. Though the results of a study revealed the transdifferentiation of BMSC by βME is a artifact [17], we reexamined the βME with RA as a pre-inducer and the obtained results confirmed the differentiation of BMSC by βME and RA into neuronal phenotypes as reported by Lu *et al.* [17].

RA is present in various tissues of both embryonic and adult animals, in particular in the nervous system [18-20], where it promotes neuronal differentiation [21]. Previous studies have demonstrated that RA induces both a greater number of neuritis and increases neurite length in the cultured neurons [22]. RA has been used in combination with other factors to induce differentiation of BMSC into neural cells [6, 23]. Since it has been suggested that βME is capable of supporting the viability and differentiation of fetal mouse brain neurons [24], we used this factor in combination with RA. The results showed that the BMSC were slowly differentiated into neuron-like cells and during the 3 days of treatment, they expressed nestin, NF68 and NF160.

The pre-induced cells showed a high percentage of immuno-reactivity to NF68 and nestin markers for neuroblasts [23] and NF160, a marker for neuroblasts and neurons [23, 25].

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The percentage of NF200 immuno-reactive, a marker for differentiated neurons, was low, which indicates the majority of the cells transdifferentiated into neuroblasts [23, 25]. On the day 2, a high rate was noticed for the expression of NF68, NF160 and nestin (82%, 70% and 81%, respectively). This justifies the selection of the day 2 for the induction stage with KCl using 20, 40 and 80 mM doses. During the induction stage, the viability test was used as a parameter to decide on the best doses for induction. The result of viability test showed that the best doses for induction were 20 and 40 mM during the days 1, 3 and 5. Other investigators have reported that KCl had a direct effect on the differentiation of NSC into GABAergic neurons [5]. Miquel [5] has reported that the best dose of KCl for the induction of NSC into GABAergic neurons was 40 mM on the fourth day. Although other investigators used RA or neurotrophins as GABAergic inducer, the result of induction of GABAergic phenotype was not high [26]. There is evidence that KCl, as a depolarizer agent, could downregulate the immature neuronal markers such as nestin and increase the number of post mitotic neurons [5]. Moreover, some reports showed that depolarization (caused by KCl or glutamate) decreased the mitotic activity of neuronal precursors [27].

Our results indicated that BMSC could be induced into 60% GABAergic-like neurons with 40 mM-KCl on the third day after pre-induction. Comparing to the Miquel's report [5], this finding is not too high, maybe due to the heterogeneous population of BMSC against NSC. While BMSC are able to differentiate into multiple mesenchymal and ectodermal derivatives *in vitro* and *in vivo* [7, 28], several studies have shown that the exposure of many different components to NSC leads to different neural lineages such as GABAergic neurons [5, 29, 30]. The pre-induction stage with βME for 1 h and RA for 2 days was followed by the induction stage using 40 mM KCL for 3 days, which resulted in 60% increase in production of GABAergic-like neurons, a high *in vitro* yield by this method. The potential therapeutic applications of BMSC or differentiated NSC have become a major focus of research on various diseases, especially neurologic disorders such as Alzheimer's, Parkinson's and Huntington's diseases [4, 5, 10].

In vitro differentiated GABAergic-like neurons in our study can be a potential source for cell therapy in GABAergic deficiency disorders and offer hopes for more *in vivo* investigation. To our knowledge, this is the first report for *in vitro* transdifferentiation of BMSC into GABAergic-like neurons.

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