Int. J. Dev. Biol. 53: 579-584 (2009)
doi: 10.1387/ijdb.082623eh

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Neurogenic and mitotic effects of dehydroepiandrosterone on neuronal-competent marrow mesenchymal stem cells

ESMAEIL H. SHIRI[1,2], NARGES-ZARE MEHRJARDI[1], MAHMOOD TAVALLAEI[2], SAEID K. ASHTIANI[1] and HOSSEIN BAHARVAND*[,1,3]

[1]Department of Stem Cells, Cell Science Research Center, ACECR, Royan Institute, [2]Department of Biology, Imam Hossein University and [3]Department of Developmental Biology, University of Science and Culture, ACECR, Tehran, Iran

ABSTRACT To establish whether dehydroepiandrosterone (DHEA) as a neurosteroid could enhance the rate of neuronal differentiation in neuronal-competent bone marrow mesenchymal stem cells (BM-MSCs), we added DHEA before and after plating the neurosphere-like aggregates. Flow cytometric analysis of Tubulin-III and Tau positive cells revealed that the percentages of these cells were increased significantly for the two markers following DHEA treatment at both stages. Moreover, Western blot analysis revealed that Tubulin-III protein was strongly induced by DHEA. The expression of neuronal specific genes such as *Isl-1, Tubulin III, Pax6* and *Nestin* was also detected by RT-PCR analysis as well as BrdU incorporation and found to have increased significantly after DHEA induction. In conclusion, these results provide evidence that DHEA can affect neuronal-competent MSCs in inducing the expression of a comprehensive set of genes and proteins that define neuronal cells. DHEA was also able to induce the division of neuronal-competent MSCs, thereby increasing the number of cells with major neuronal characteristics. To our knowledge, this is the first report which shows that DHEA can induce the division and differentiation of MSCs into neurons *in vitro* and should provide an improved basis for new treatments using MSCs of a wide variety of neurological diseases.

KEY WORDS: *dehydroepiandrosterone, mesenchymal stem cell, neuronal differentiation*

Introduction

Dehydroepiandrosterone (DHEA), its sulfate ester (DHEAS) and other related steroids as well as allopregnanolone (Allo) represent the most abundant steroid products of the adrenal cortex. They are found to be synthesized de novo in brain glial cells and their concentrations are particularly high in the brain and are therefore considered to be neurosteroids (Kaasik *et al*., 2001, Lapchak and Araujo, 2001, Marx *et al*., 2000). Moreover, DHEA levels have been found to decline in mental illnesses such as major depressive disorder or in systematic diseases that respond to DHEA supplementation (Bloch *et al*., 1999, Roshan *et al*., 1999). The decline of neurosteroid levels during aging may also leave the brain unprotected against neurotoxic challenges (Charalampopoulos *et al*., 2006b). DHEA has further been shown to be neuroprotective after oxidative stress in rat hippocampal neuronal cultures (Bastianetto *et al*., 1999) and hippocampal damage induced by N-methyl-D-aspartate (NMDA) (Kimonides *et al*., 1998) or chromaffin cells and the sympathoadrenal PC12 cells (an established model for the study of neuronal cell apoptosis and survival) against serum deprivation-induced apoptosis (Charalampopoulos *et al*., 2006a). Furthermore, it can directly stimulate biosynthesis and release of neuroprotective catecholamines, exerting a direct transcriptional effect on tyrosine hydroxylase and regulate actin depolymerization and submembrane actin filament disassembly, a fast-response cellular system regulating trafficking of catecholamine vesicles (Charalampopoulos *et al*., 2006b). DHEA can also affect the endocrine, immune, and metabolic systems (Bellino *et al*., 1995).

Recent studies have shown that mesenchymal stem cells (MSCs) derived from bone marrow cell suspensions can be

Abbreviations used in this paper: BM, bone marrow; DHEA, dehydroepiandrosterone; MSC, mesenchymal stem cell; NSC, neuronal stem cell.

***Address correspondence to:** Dr. Hossein Baharvand. Department of Stem Cells, Cell Science Research Center, Royan Institute, P.O. Box: 19395-4644, Tehran, Iran. Fax: +98-21-2231-0406. e-mail: Baharvand@RoyanInstitute.org

Accepted: 07 March 2008. *Published online:* 2 April 2009.

ISSN: Online 1696-3547, Print 0214-6282

expanded by their selective attachment to plastic tissue culture dishes (Friedenstein *et al.*, 1976, Reyes *et al.*, 2001, Sekiya *et al.*, 2002).

By far the most prominent advantage of using MSCs over other cell types in cell-replacement therapy is their autologous characteristic. In addition, these cells were reported to differentiate *in vitro* and *in vivo* into cells expressing neuronal and glial markers (Sanchez-Ramos *et al.*, 2000, Woodbury *et al.*, 2000, Zhao *et al.*, 2002). It has also been shown that like embryonic stem cells (ESCs), adult human MSCs in the presence of epidermal growth factor (EGF) and basic-fibroblast growth factor (bFGF) can become converted into a clonogenic neuronal stem cell-like population growing in neurosphere-like structures also called marrow-derived neuronal stem cells (mNSC). These can then be differentiated into cells with morphological and functional characteristics of neuronal, and glial cells *in vitro* (Hermann *et al.*, 2004). Therefore MSCs provide an innovative model to understand developmental and molecular mechanisms of neurosteroid actions. Moreover, the use of MSCs in auto-graft protocols in neurological diseases necessitates the identification of the molecular events that are important for the induction of neuronal differentiation of MSCs. We have shown here that DHEA can induce the differentiation of neuronal cells from MSCs and increase their cell proliferation *in vitro*. These results have important implications for MSCs and are important in the understanding of DHEA actions on the process of neuronal differentiation from MSCs.

Results and Discussion

MSC characterization

MSCs were isolated from the femurs of adult mice and propagated *in vitro* as established previously in our laboratory (Eslaminejad *et al.*, 2006). At low plating densities, MSCs grew as a monolayer of large, flat cells and made colony forming unit-fibroblast (CFU-F, Fig. 1A). As the cells approached confluency, they assumed a more spindle-shaped and a fibroblastic morphology (Fig. 1B). Flow cytometric analysis at the passages (4-6) demonstrated that the cells were negative for CD11b (Fig. 1C), CD45 (Fig. 1D), CD 34 (Fig. 1E), Oct-4 (Fig. 1F), cell surface markers associated with lymphohematopoietic cells and cell nuclear marker associated with multipotential adult progenitor cells (MAPCs). The MSCs did express CD44 (Fig. 1G) and Sca-1 (Fig. 1H), consistent with their undifferentiated state.

Neuronal Differentiation

To convert MSCs into cells with characteristics of NSCs, we detached the MSCs after 4-6 passages and cultured them in 1% agarose-coated dishes in serum-free neurobasal medium supplemented with bFGF+EGF. The cells did not adhere to the surface of culture dishes and formed small spheres of floating cells (Fig. 2 A,B) and finally made mNSCs *in vitro*. Immunocytochemistry showed that mNSCs in the presence of only bFGF+EGF expressed high levels of Nestin (Fig. 2C). The flow cytometric analysis showed that Nestin expression in MSCs increased from 27.5±2.1 to 77.5±0.6 ($P<0.001$, Fig. 2D) in day 7 and decreased in day 11 to 62.5±1.5 ($P<0.001$, Fig. 2D). Therefore in order to confirm the differentiation potential of these cells, neurospheres-like aggregates were dissociated and plated to poly-L-lysine coated plates for 12 days under differentiating conditions. We first asked what role DHEA may play in the neuronal differentiation of MSCs in culture. To establish whether DHEA could enhance the differentiation rate, we added different concentration of DHEA (0.1, 1.0 and 10 μM) with and without RA (1 μM) at the plating stage. Within 4 to 12 days after plating, changes in the morphology of responsive cells assuming neuronal identity became apparent. These morphological changes were similar to those reported previously (Hermann *et al.*, 2004), in which the cytoplasm in the flat cells was initially retracted towards the nucleus, forming a contracted multipolar cell body leaving membranous processes like peripheral extensions. Over the subsequent days, the cell bodies became increasingly spherical and retractile, exhibiting a typical neuronal perikaryal appearance. Gradually, MSCs-derived neurons displayed distinct neuronal morphologies ranging from simple bipolar to large, extensively branched multipolar cells (Fig. 2E), making conncetions via their processes (Fig. 2F). Some of the differentiated cells also acquired morphological and phenotypical characteristics of astrocytes (Fig. 2G). Following the fixation process, the cells were immunostained for the neuronal markers; Tubulin-III (Fig. 2H) and Tau (Fig. 2I), as well as the astrocyte marker; GFAP (Fig. 2J). The results further verified the presence of many well differentiated neurons and astrocytes. Moreover, the expression of neuronal specific genes such as Isl-1, Tubulin III, Pax6, and Nestin were also studied by RT-PCR analysis (Fig. 2K) in which they were found to be weakly expressed in MSCs. Westren blot analysis using anti-Tubulin III antibody revealed that Tubulin-III protein was only induced strongly following the DHEA addition (Fig. 2L). The expression of neuronal markers in mouse bone marrow-derived MSCs has been reported in the past where the MSCs were found to express certain neuronal phenotype markers spontaneously in the absence of specialized induction reagents in culture. These cells were believed to be probably «primed» toward a neuronal fate by the constitutive expression of neuronal antigens and seemed to

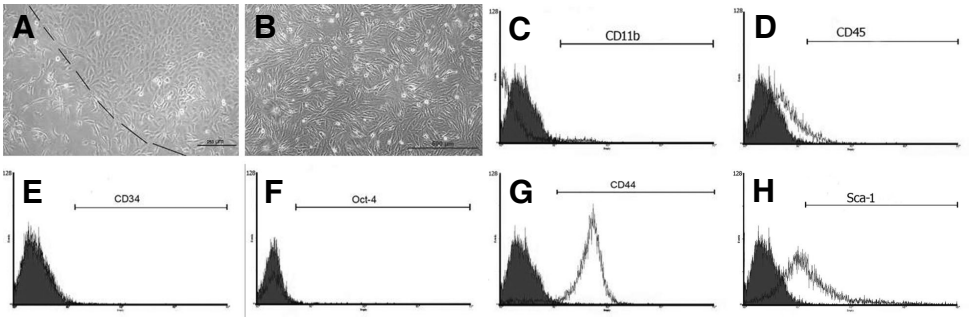

Fig. 1. Characterization of cultured mouse MSCs. *At low plating densities, MSCs grew as a monolayer of large, flat cells and made colony forming unit-fibroblasts (CFU-F, lines)* **(A)**. *As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology* **(B)**. *Flow cytometric analysis at passage number 4-6 demonstrated that the cells were negative for CD11b* **(C)**, *and CD45* **(D)**, *CD 34* **(E)**, *and Oct-4* **(F)**, *while CD44* **(G)** *and Sca-1 were still expressed* **(H)**.

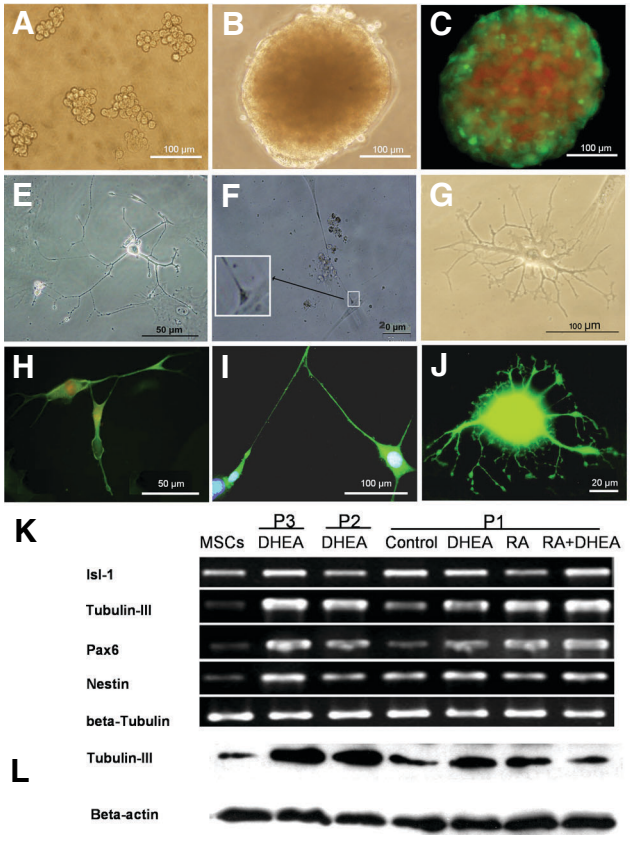

Fig. 2. Characterization of mouse MSC-derived neuronal-like cells. *To convert MSCs into cells with characteristics of NSCs, MSCs were cultured in agarose-coated dishes in serum-free neurobasal medium supplemented with bFGF+EGF to form small floating spheres* **(A)** *and finally formed neurosphere-like aggregates containing mNSCs* **(B)**. *Immunocytochemistry showed that mNSCs expressed high levels of nestin* **(C)** *and the flow cytometric analysis showed that nestin expression increased by differentiation of MSCs up to day 7 and decreased on day 11* **(D)**. *The plated neurospheres-like aggregates, displayed gradually distinct neuronal morphologies ranging from simple bipolar, to large extensively branched multipolar cells* **(E)** *and making connections via their processes* **(F)**. *Some of the differentiated cells however acquired morphological and phenotypical characteristics of astrocytes* **(G)**. *Immunocytochemistry of the differentiated cells showed the expression of Tubulin-III* **(H)** *and Tau* **(I)**, *mature neuron markers and GFAP* **(J)**, *astrocyte marker. The nuclei were conterstained with propidium (H, J) and DAPI (I). Moreover, the expression of neuronal specific genes such as Isl-1, Tubulin-III, Pax6, and Nestin were determined by RT-PCR analysis* **(K)**. *Western blot analysis using anti-Tubulin III antibody revealed that Tubulin-III protein was strongly induced by only DHEA* **(L)**. *The analysis of Tubulin-III positive cells by flow cytometry of the cells after DHEA treatment in plating stage revealed that the percentage of these cells was increased significantly following the DHEA or RA treatment (at least P<0.01),* **(M)** *and this difference was not significant between different concentrations of DHEA (0.1, 1.0 and 10 μM). However, only high concentration of RA+DHEA improved neuronal-like cells differentiation in comparison with RA+DHEA (0.1 or 1 μM).*

respond with an appropriate neuronal pattern of differentiation, when exposed to the environment of the developing brain (Deng *et al.*, 2006).

The analysis of Tubulin-III positive cells by flow cytometry revealed that the percentage of these cells was increased significantly following DHEA or RA treatment (at least P<0.01, Fig. 2M) and that there was no significant differences between different concentrations of DHEA (0.1, 1.0 and 10 μM). We therefore continued our experiments with 1 μM DHEA but found that only the highest concentration of DHEA (10 μM) when used with RA treatment improved the neuronal differentiation and not the DHEA (0.1 or 1 μM) or RA alone (Fig. 2M).

To assess the DHEA influence in the stage of neurospheres-like aggregate formation and the effect of bFGF+EGF, DHEA was added in either the presence or absence of bFGF+EGF in culture. Flow cytometric analysis of Tubulin-III and Tau positive cells after plating, revealed that the percentage of these cells was increased significantly for the two markers following DHEA treatment at both the neurosphere-like aggregate formation stage (protocol 2 and 3) and plating stage (protocol 1) (at least P<0.01, Fig. 3). The percentage increase in these markers following DHEA treatment and bFGF+EGF however, was not found to be significant. Moreover, Western blot analysis revealed that Tubulin-III protein was strongly induced by only DHEA in the neurosphere-like aggregate formation stage with or without bFGF+EGF in comparison with addition of DHEA in the plating stage (Fig. 2L).

DHEA has also been shown to be a potential signaling molecule in neuronal differentiation during development (Compagnone and Mellon, 1998) and has recently been shown to increase neurogenesis in the adult rodent hippocampus (Karishma and Herbert, 2002). DHEA is also found to be involved in the maintenance and division of human NSC cultures derived from the fetal cortex (Suzuki *et al.*, 2004). Moreover, it is reported that DHEA enhances the differentiating effect of RA on neuroblastoma cells via a signalling pathway that is not RA receptor-mediated (Silvagno *et al.*, 2002).

To evaluate the possible mechanism of DHEA function, it was administrated in the neurosphere-like aggregate formation stage with and without bFGF+EGF (protocol 3 and 2, respectively). The diameter of neurosphere-like aggregates was found to increase significantly when DHEA used at this stage (P<0.001) in either the presence or absence of bFGF+EGF (Fig. 4A). To evaluate this enhancement, BrdU incorporation assay were performed with mNSCs after DHEA induction at day 7 in the presence or absence of bFGF+EGF (Fig. 4B1, 4B2, 4C1, 4C2, 4D1, 4D2). Approximately 87.9±2.4% of DHEA stimulated mNSCs exhibited BrdU-positive cellular nuclei with bFGF+EGF in comparison with the control (without DHEA, 75.8±5.0%, P<0.001). However, only

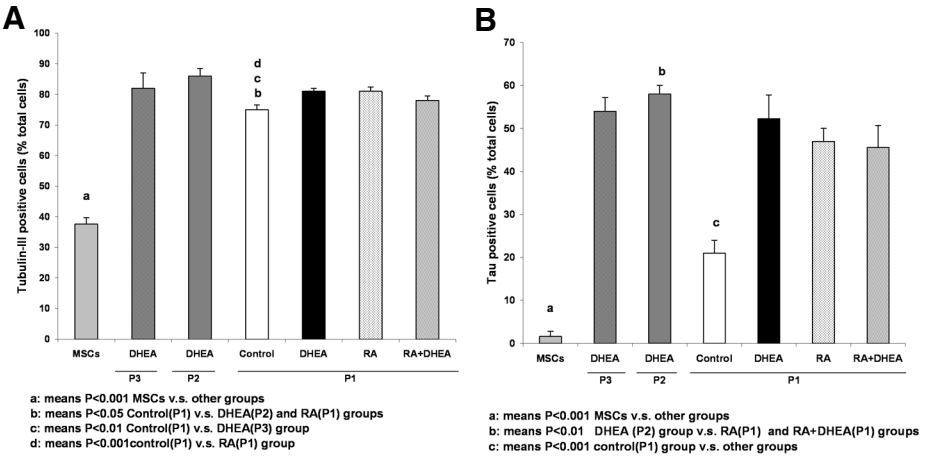

Fig. 3. Flow cytometric analysis of MSC-derived neuronal-like cells under different treatments. *Flow cytometric analysis of Tubulin-III* **(A)** *and Tau* **(B)** *positive cells after plating revealed that the percentages of these cells were increased significantly for the two markers following DHEA treatment in both stages at the neurosphere-like aggregate formation stage (protocol 2 and 3) or plating stage (protocol 1) (at least P<0.01). P: protocol.*

72.4±3.3% of mNSCs without bFGF+EGF and in the presence of DHEA (protocol 3) were positive for BrdU staining, (P<0.001 vs other groups, Fig. 4E). Therefore the BrdU incorporation has increased significantly in the presence of bFGF+EGF by DHEA and these data also show that DHEA has selectively increased the division of the cells responsive to bFGF+EGF within the neurosphere-like aggregates.

In general neurosteroids possess the ability to effect neurons through activation of γ-aminobutyric acid (GABA), NMDA, and sigma receptors (Baulieu, 1997, Baulieu, 1998, Suzuki *et al.*, 2004, Xilouri *et al.*, 2007, Xilouri and Papazafiri, 2006), however the exact role of each factor with regard to DHEA is not well established. Moreover, the prosurvival effect of DHEA appears to be mediated by G-protein-coupled-specific membrane binding sites (Charalampopoulos *et al.*, 2006a). This involves the antiapoptotic Bcl-2 proteins, the activation of prosurvival transcription factors; CREB and NF-kappaB and the expression of upstream effectors of the antiapoptotic Bcl-2 protein, as well as a posttranslational activator of Bcl-2; the prosurvival kinase PKCalpha/beta, (Charalampopoulos *et al.*, 2006b). The neuroprotection also involves phosphorylation of the prosurvival factor Src and the induction of the anti-apoptotic protein Bcl-2 (Charalampopoulos *et al.*, 2006a). DHEA is found to inhibit excitotoxic cell death of P19-N neurons, by directly maintaining the activation of PKB/Akt kinase and interfering with the intrinsic mitochondrial apoptotic pathway, preserving cytochrome C in the mitochondria and Bax in the cytoplasm (Xilouri *et al.*, 2007).

Taken together, these results have shown that DHEA can

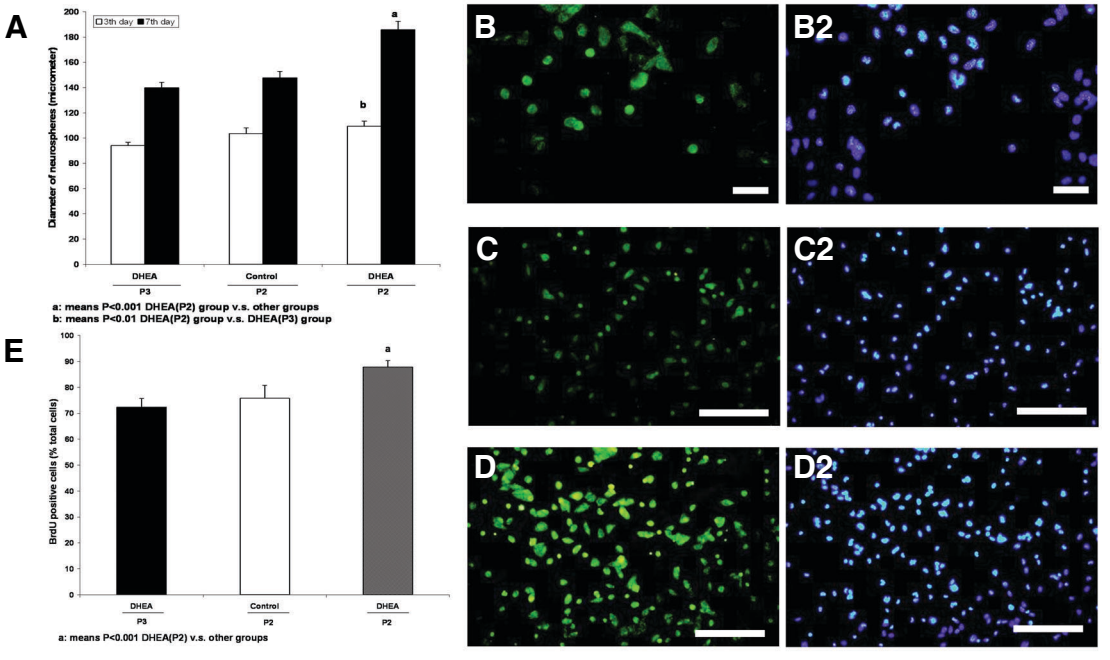

Fig. 4. Cell proliferation after DHEA treatment. *To evaluate the possible mechanism of DHEA, the diameter of neurosphere-like aggregates increased significantly when DHEA administrated at the neurospheres-like aggregate formation stage with and without bFGF+EGF (protocol 3 and 2, respectively) (P<0.001) in presence or absence of bFGF+EGF* **(A)**. *To evaluate this enhancement, BrdU incorporation assays were performed with mNSCs after DHEA induction at day 7 in the presence or absence of bFGF+EGF* **(B - E)**. *Immunostaining (B, C, D) and flow cytometry analysis (E) of the differentiated cells with anti-BrdU showed in the presence of bFGF+EGF, DHEA significantly increased BrdU incorporation (protocol 2, B: DHEA+bFGF+EGF, C: +bFGF+EGF). However, in absence of bFGF+EGF, DHEA did not increase BrdU-positive cellular nuclei (protocol 3, D: only DHEA). These data show DHEA selectively increases the division of the bFGF+EGF -responsive cells within the neurosphere-like aggregates (E). The nuclei were counterstained with DAPI* **(B2, C2, D2)**.

induce the expression of a comprehensive set of genes and proteins that define neuronal cell identity in neuronal-competent MSCs forming mNSCs. Moreover, our results suggest that DHEA can induce the division of mNSCs and so increase the number of cells with major neuronal-like characteristics. To our knowledge, this is the first report that DHEA has been shown to induce the division and differentiation of MSCs into neuron-like cells *in vitro*, when added at either the neurosphere-like aggregate formation stage and/or the plating stage. MSCs may therefore be useful in the treatment of a wide variety of neurological diseases, offering significant advantages over other "stem" cells. The marrow cells are readily accessible, overcoming the risks of obtaining either NSCs from the brain or ESCs from preimplantation embryos while also provide a renewable population of cells.

Materials and Methods

Culture and expansion of MSCs

Mouse MSCs were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 2 mM L-glutamine. They were grown to confluency before being detached by Trypsin/EDTA treatment and were seeded at $1◊10^4$ cell/cm^2 in culture flasks before being evaluated for their multipotency while differentiating into chondrocytes, adipocytes, and osteoblasts (Eslaminejad *et al.*, 2006). All aforementioned materials were purchased from Invitrogen-Gibco.

Neuronal Induction

MSCs were dissociated with 0.05% trypsin/0.04% EDTA and cultured on agarose (Sigma-Aldrich)-coated dishes (60 mm, Nunc) at a concentration of $1x10^5$ cells/ml in neurobasal medium containing B27 (2%, Invitrogen-Gibco), 1% insulin-transferrin-selenite (ITS, Invitrogen-Gibco), L-glutamine, 1% penicillin/streptomycin supplemented with 20 ng/ml of both EGF and bFGF (both from Sigma-Aldrich) at 5% CO2.

Induction of terminal neuronal differentiation was initiated by plating the cells on poly-L-lysine- (Sigma-Aldrich) coated plates at a concentration of 3 x 10^4 cells/cm^2 in the same medium without bFGF+EGF and in presence of 10% FCS and 1 μM all-trans-retinoic acid (RA, Sigma-Aldrich). Cells were differentiated for 12 day and the medium was changed every four days. DHEA was added in plating stage (protocol 1) or in neurospheres-like stage in presence (protocol 2) and/or absence (protocol 3) of bFGF+EGF (Table 1).

Immunofluorescence staining

Cultured cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) and rinsed with PBS (Invitrogen-Gibco)-Tween20 (Sigma-Aldrich). The cells were permeabilized by 0.5% Triton X-100 and blocked with 10% normal serum produced from the same species as the secondary antibody. This was followed by an overnight incubation with primary antibodies including anti-Nestin (Chemicon, MAB353, 1:100), anti-β-Tubulin-III (Sigma-Aldrich, T5293, 1:200), anti-Tau (Millipore, MAB5270, 1:400), anti-BrdU (Roche, 11296736001, 1:200), and anti-glial fibrillary acidic protein (GFAP, Chemicon, MAB3402, 1:400). After rinsing, samples were incubated with secondary antibody: fluorescein isothiocyanate (FITC)-conjugated anti-mouse (Chemicon, AP308, 1:250) and the cells which were labeled with FITC were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D-8417, 0.02 μg/ml) and/or 1 μg/ml propidium iodide (PI, Sigma-Aldrich, P4864) for 3 min at room temperature. Omission of the primary antibody in the sample was used as a control for all markers. Labeled cells were examined with a fluorescence microscope (Olympus, BX51, Japan) and images were acquired with an Olympus D70 camera.

TABLE 1

DIFFERENTIATION INDUCTION PROTOCOL OF NEURONAL-LIKE CELLS FROM MSC

MSCs	Neurosphere formation (7d)	Plating and Maturation (5d)
Protocol 1 →	+(bFGF+EGF) →	+/-DHEA (0.1, 1, 10 μM) +RA
Protocol 2 →	+(bFGF+EGF) +/-DHEA (1 μM) →	
Protocol 3 →	+DHEA (1 μM) →	

MSCs were cultured in 1% agarose-coated dishes in serum-free neurobasal medium supplemented with (protocol 1 and 2) or without (protocol 3) bFGF+EGF to form small spheres of floating cells and finally made marrow-derived NSC-like cells *in vitro*. To establish whether DHEA could enhance differentiation rates, we added different concentration of DHEA (0.1, 1.0 and 10 μM) (protocol 1). To assess the stage of DHEA influence or its effect along with bFGF+EGF, DHEA was added in the presence (protocol 2) and absence (protocol 3) of bFGF+EGF during the neurospheres-like aggregate formation stage. In all protocols, cells were differentiated for 12 days.

Flow cytometric analysis

All staining reactions were performed in staining buffer consisting of PBS supplemented with 2% FCS. After determination of the viability of the cells by trypan blue exclusion, cells were washed two times in staining buffer and fixed in 4% paraformaldehyde and triton X-100 0.5% was used for permeabiliztion. Non-specific antibody binding was blocked with a combination of 10% heat-inactivated goat serum in staining buffer and 1-1.5 x 10^5 cells were used per sample. Cells were incubated with the appropriate primary antibodies or appropriate isotype matched controls (Dako, X0927, 1:100). Primary antibodies used here were: anti-Nestin (1:20), anti-β-Tubulin III (1:200), anti-Tau (1:400), and anti-BrdU (1:100). The cells were washed two times in staining buffer and incubated for 30 min at 4 °C with FITC-conjugated secondary antibody. After washing, flow cytometric analysis was performed with a BD-FACS Calibur Flow Cytometer. The experiments were replicated three times and the acquired data was analyzed by using the WinMDI (2.9) software.

RNA extraction and RT-PCR analysis

Total cellular RNA was extracted from the cells in different stages using total RNA (Nucleospin®) purification kit followed by treatment with RNase-free DNase (Qiagen, Hilden, Germany). Standard RT was performed using 2 μg total RNA, oligo (dT), and the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using mouse specific primers. Amplification conditions were as follows: Initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55-70 °C for 45 sec (see Table 2 for temperatures used) and extension at 72 °C for 45 sec, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis on a 1.7% agarose gel stained with ethidium bromide (1μg/ml) after which they were visualized and photographed on

TABLE 2

PRIMERS AND THE REACTION CONDITIONS OF RT-PCR

Genes	Primer sequences (5'-3')	Size (bp)	Annealing Temperature	Accession No.
Isl-1	F: GACTTTGAGCAAGGGGTTACG R: ACATGAAAAGTGGCAAGTCTCC	439	60	NM-021459
β-Tubulin III	F: GTTCCCACGTCTCCACTTCTTC R: CCAGGTCATTCATGTTGCTCTC	479	63	NM-023279
Pax6	F: GAGAGGACCCATTATCCAGATG R: GCTGACTGTTCATGTGTGTTTG	467	61	NM-013627
Nestin	F: TCGAGCAGGAAGTGGTAGG R: TTGGGACCAGGGACTGTTA	352	58	NM-016701
β-Tubulin	F: TCACTGTGCCTGAACTTACC R: GGAACATAGCCGTAAACTGC	317	58	NM-011655

gel documentation system (UVidoc, UK). A description of primers and the sizes of their final products are described in Table 2.

Western blot Analysis

Twenty micrograms of protein extracted from the cells at different stages was separated by 10% SDS-PAGE electrophoresis using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) by semidry blotting (Bio-Rad) using Dunn carbonate transfer buffer (10 mM NaCHO3, 3 mM Na2CO3, and 20% methanol) and membranes were blocked for 1.5 h using Western blocker solution (Sigma-Aldrich, W0138) before being incubated with an antibody against β-Tubulin III (1:200) or anti-actin (Sigma-Aldrich, A5441, 1:5000). After washing, the membranes were incubated with the peroxidase-conjugated secondary antibody goat anti mouse horse reddish peroxidase (Sigma-Aldrich, A9044, 1:80).

Diameter of neurosphere-like aggregates

The diameter of neurosphere-like aggregates was measured under phase contrast inverted microscope (Olympus, Japan) using an Olysia Bioreport software in days 3 and 7.

Statistical analysis

The experiments were replicated at least three times. The data were expressed as mean ± SD (standard deviation). One-way ANOVA followed by the Tukey post *hoc* test multiple group comparison was used to analyze group differences of the data collected from flow cytometric analysis. The difference between groups was then considered to be statistically reliable if the value of $P< 0.05$ was obtained.

Acknowledgement

This study was funded by the grant provided from the Royan Institute and the Iran National Science Foundation. We gratefully thank Dr. N.F. Dolatshad for her manuscript proofreading.

References

BASTIANETTO, S., RAMASSAMY, C., POIRIER, J. and QUIRION, R. (1999). Dehydroepiandrosterone (DHEA) protects hippocampal cells from oxidative stress-induced damage. *Brain Res Mol Brain Res* 66: 35-41.

BAULIEU, E.E. (1997). Neurosteroids: of the nervous system, by the nervous system, for the nervous system. *Recent Prog Horm Res* 52: 1-32.

BAULIEU, E.E. (1998). Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* 23: 963-987.

BELLINO, F.L., DAYNES, R.A., HORNSBY, P.J., LAVRIN, D.H. and NESTLER, J.E. (1995). *Dehydroepiandrosterone (DHEA) and Aging*. N.Y. Acad. Sci., New York.

BLOCH, M., SCHMIDT, P.J., DANACEAU, M.A., ADAMS, L.F. and RUBINOW, D.R. (1999). Dehydroepiandrosterone treatment of midlife dysthymia. *Biol Psychiatry* 45: 1533-1541.

CHARALAMPOPOULOS, I., ALEXAKI, V.I., LAZARIDIS, I., DERMITZAKI, E., AVLONITIS, N., TSATSANIS, C., CALOGEROPOULOU, T., MARGIORIS, A.N., CASTANAS, E. and GRAVANIS, A. (2006a). G protein-associated, specific membrane binding sites mediate the neuroprotective effect of dehydroepiandrosterone. *Faseb J* 20: 577-579.

CHARALAMPOPOULOS, I., ALEXAKI, V.I., TSATSANIS, C., MINAS, V., DERMITZAKI, E., LASARIDIS, I., VARDOULI, L., STOURNARAS, C., MARGIORIS, A.N., CASTANAS, E. *et al.* (2006b). Neurosteroids as endogenous inhibitors of neuronal cell apoptosis in aging. *Ann N Y Acad Sci* 1088: 139-152.

COMPAGNONE, N.A. and MELLON, S.H. (1998). Dehydroepiandrosterone: a potential signalling molecule for neocortical organization during development. *Proc Natl Acad Sci U S A* 95: 4678-4683.

DENG, J., PETERSEN, B.E., STEINDLER, D.A., JORGENSEN, M.L. and LAYWELL, E.D. (2006). Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. *Stem Cells* 24: 1054-1064.

ESLAMINEJAD, M.B., NIKMAHZAR, A., TAGHIYAR, L., NADRI, S. and MASSUMI, M. (2006). Murine mesenchymal stem cells isolated by low density primary culture system. *Dev Growth Differ* 48: 361-370.

FRIEDENSTEIN, A.J., GORSKAJA, J.F. and KULAGINA, N.N. (1976). Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 4: 267-274.

HERMANN, A., GASTL, R., LIEBAU, S., POPA, M.O., FIEDLER, J., BOEHM, B.O., MAISEL, M., LERCHE, H., SCHWARZ, J., BRENNER, R. *et al.* (2004). Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells. *J Cell Sci* 117: 4411-4422.

KAASIK, A., KALDA, A., JAAKO, K. and ZHARKOVSKY, A. (2001). Dehydroepiandrosterone sulphate prevents oxygen-glucose deprivation-induced injury in cerebellar granule cell culture. *Neuroscience* 102: 427-432.

KARISHMA, K.K. and HERBERT, J. (2002). Dehydroepiandrosterone (DHEA) stimulates neurogenesis in the hippocampus of the rat, promotes survival of newly formed neurons and prevents corticosterone-induced suppression. *Eur J Neurosci* 16: 445-453.

KIMONIDES, V.G., KHATIBI, N.H., SVENDSEN, C.N., SOFRONIEW, M.V. and HERBERT, J. (1998). Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci U S A* 95: 1852-1857.

LAPCHAK, P.A. and ARAUJO, D.M. (2001). Preclinical development of neurosteroids as neuroprotective agents for the treatment of neurodegenerative diseases. *Int Rev Neurobiol* 46: 379-397.

MARX, C.E., JARSKOG, L.F., LAUDER, J.M., GILMORE, J.H., LIEBERMAN, J.A. and MORROW, A.L. (2000). Neurosteroid modulation of embryonic neuronal survival *in vitro* following anoxia. *Brain Res* 871: 104-112.

REYES, M., LUND, T., LENVIK, T., AGUIAR, D., KOODIE, L. and VERFAILLIE, C.M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* 98: 2615-2625.

ROSHAN, S., NADER, S. and ORLANDER, P. (1999). Review: Ageing and hormones. *Eur J Clin Invest* 29: 210-213.

SANCHEZ-RAMOS, J., SONG, S., CARDOZO-PELAEZ, F., HAZZI, C., STEDEFORD, T., WILLING, A., FREEMAN, T.B., SAPORTA, S., JANSSEN, W., PATEL, N. *et al.* (2000). Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp Neurol* 164: 247-256.

SEKIYA, I., LARSON, B.L., SMITH, J.R., POCHAMPALLY, R., CUI, J.G. and PROCKOP, D.J. (2002). Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 20: 530-541.

SILVAGNO, F., GUARNIERI, V., CAPIZZI, A. and PESCARMONA, G.P. (2002). Synergistic effect of retinoic acid and dehydroepiandrosterone on differentiation of human neuroblastoma cells. *FEBS Lett* 532: 153-158.

SUZUKI, M., WRIGHT, L.S., MARWAH, P., LARDY, H.A. and SVENDSEN, C.N. (2004). Mitotic and neurogenic effects of dehydroepiandrosterone (DHEA) on human neural stem cell cultures derived from the fetal cortex. *Proc Natl Acad Sci U S A* 101: 3202-3207.

WOODBURY, D., SCHWARZ, E.J., PROCKOP, D.J. and BLACK, I.B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 61: 364-370.

XILOURI, M., AVLONITIS, N., CALOGEROPOULOU, T. and PAPAZAFIRI, P. (2007). Neuroprotective effects of steroid analogues on P19-N neurons. *Neurochem Int* 50: 660-670.

XILOURI, M. and PAPAZAFIRI, P. (2006). Anti-apoptotic effects of allopregnanolone on P19 neurons. *Eur J Neurosci* 23: 43-54.

ZHAO, L.R., DUAN, W.M., REYES, M., KEENE, C.D., VERFAILLIE, C.M. and LOW, W.C. (2002). Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 174: 11-20.