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Estrogen treatment enhances neurogenic differentiation of human adipose derived stem cells *in vitro*

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ABSTRACT

Objective(s): Estrogen is a sexual hormone that has prominent effects on reproductive and non-reproductive tissues. The aim of this study is to evaluate the effects of estrogen on the proliferation and neural differentiation of human adipose derived stem cells (ADSCs) during neurogenic differentiation.

Materials and Methods: Isolated human ADSCs were trans-differentiated in neural induction medium containing neurobasal medium, N2 and B27 with or without 17β-estradiol (E2) treatment. Proliferation rate and neural differentiation of human ADSCs were assessed using MTT assay, immunostaining and real time RT- PCR analysis, respectively.

***Results*:** Analysis of data show that estradiol treatment can significantly increase proliferation rate of differentiated cells (P<0.05). Immunocytochemical and real time RT-PCR analysis revealed that the expression of precursor and mature neuronal markers (nestin and MAP2) was significantly higher in the E2 treated cell cultures when compared to the untreated cell cultures (P<0.05).

Conclusion: According to our findings, estrogen can promote proliferation and neuronal differentiation of human ADSCs.

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Introduction

Mesenchymal stem cells can be a safe stem cell source, and their therapeutic effects have been shown in the treatment of neurodegenerative diseases (1, 2). Previous studies demonstrated that stem and progenitor cells from adult adipose stromal tissue retain the capacity to differentiate toward mesodermal and non-mesodermal lineages (3-5).

Estrogen as a female sexual steroid hormone also is important in modifying the activities of non-reproductive tissues. One of the most important targets for estrogen is brain. It has neurotrophic and neuroprotective effects on brain (6-8). Estradiol (E2), one of the major estrogens in the central nervous system, can influence neurogenesis by increasing the ratio of neurons to glial cells during differentiation of neural stem cells (NSCs) (9-11). Effect of E2 on neural differentiation can be exerted through neurotrophic factor secretion. E2 enhances the expression of brain derived neurotrophic factor (BDNF) as well as nerve growth factor (NGF) in the frontal cortex and hippocampus of rats (12, 13). Furthermore, it can increase the BDNF, neurotrophin-3 (NT-3), and NGF production in the olfactory cortex of ovariectomized rats (14). In addition, E2 stimulates the level of glial derived neurotrophic factor expression in developing hypothalamic neurons and peripheral organs (15). Therefore, E2 treatment can increase elongation and branching of neurites in cortical culture (16).

E2 influences the neuronal differentiation in human umbilical cord blood mesenchymal stem cells as well as in embryonic stem cells and NSCs (17, 18). Kang *et al,* believed that it might be due to neurotrophic factor expression (17).

It has been reported that ADSCs secret some growth factors such as BDNF, insulin-like growth factor, and fibroblast growth factor (FGF), which are important for nerve regeneration during peripheral nerve injury (19, 20). In addition, previous study indicated that ADSCs-induced repair may act through cell differentiation as well as secreting trophic factors (21). These results suggest that E2 via neurotrophic factor modulation might participate proliferation and neurogenic differentiation of human ADSCs.

We focused our attention on human adipose derived

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stem cells (ADSCs) because these cells can be obtained by less invasive method and cultured with a greater proliferation rate than other mesenchymal stem cells (5). However, ADSCs can be obtained easily and expanded *in vitro* for autologous transplantation. Therefore, ADSCs appear to be an appropriate source for cell-based therapy.

On the other hand, the effect of E2 on the proliferation and neural differentiation of ADSCs are unclear. Therefore, we examined the effects of E2 on the proliferation and neural differentiation of human ADSCs *in vitro.*

Materials and Methods

Isolation and culture of human ADSCs

All procedures were approved by the Ethics Committee of Isfahan University of Medical Sciences. Human adipose tissue was obtained from three elective lipoaspirate samples of abdominal fat from female donors (age range: 20–45 years old), after receiving informed consent and cultured as previously described (22). Briefly, samples were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed aspirates were treated with 0.075% collagenase type I in PBS for 30 min at 37°C with gentle agitation. Later, the collagenase I was inactivated with an equal volume of Dulbecco's Modified Eagle's Medium (DMEM)/10% fetal bovine serum (FBS) and then, the supernatant was centrifuged for 10 min at 800 rpm. The cellular pellet was resuspended in DMEM/10% FBS and plated in DMEM: F12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. After 24 hr, the non-adherent cells were removed and expansion of adherent ADSCs was acquired by serial passage for accessing the pure population of ADSCs. The primary cells were cultured in 25 cm^2 flasks for 4–5 days until they reached a confluency of approximately 80% in a 37°C humidified incubator with a 5% carbon dioxide (CO_2) environment. They were defined as passage 0. At confluence, the cells were detached with 0.25% trypsin/0.02% ethylene diamine tetraacetic acid (Gibco, BRL, Paisley, UK) at a ratio of 1:3 in each passage. The cells used in the present study were from passages 3 to 5. All chemicals, except where specified otherwise, were purchased from Sigma-Aldrich, St. Louis, MO, USA.

In order to determine stemness of isolated cells, human ADSCs within 3–5 passages were harvested by trypsinization, and then the cells were washed twice with 1% bovine serum albumin/PBS and incubated with antibodies against CD90, CD44, CD105, CD34, CD14, and CD45 (Chemicon, Temecula, CA, USA) for 30 min. Primary antibodies were directly conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin. For isotype control, nonspecific FITC-conjugated IgG was substituted for the primary antibodies. Flowcytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Induction of neurogenic differentiation

The transdifferentiation procedure was carried out according to previous study (23). Human ADSCs within 3–5 passages were induced into neurospheres. Briefly, we dissociated human ADSCs (80–90% confluence) with 0.25% trypsin (Gibco, BRL, Paisley, UK) and then plated them on plastic dish at a concentration of 1-2 × $10^5/cm^2$ in DMEM/F12 supplemented with 20 ng/ml human epidermal growth factor (EGF), 20 ng/ml basic FGF and 2% B27 (1:50, Gibco) at 37 °C in 5% CO_2. Fresh medium was added every 3–4 days. After 7 days, neurospheres were triturated using a fire-polished Pasteur pipette and re-plated in fresh medium. The triturated neurospheres and 2×10^4 singled cells re-plated in 24 well plate in neurobasal medium supplemented with 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 1% N2, 1% none essential amino acids, 2% B27, for 7 days. To determine the effect of E2 on neural differentiation, according to previous study we added E2 10 nM to the neural induction medium approximately every day in treated culture, whereas in control culture E2 was absent (17).

MTT assay

To examine the viability of differentiated cells (3×10^3 cells/well) in the E2 presence 7 days post induction , 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was dissolved in 1 ml of PBS. The stock solution was added to the culture medium at a dilution of 1:10. The plates were incubated at 37°C for 4 hr. The medium was then aspirated and 200 µl of dimethyl sulfoxide was added to extract the MTT formazan. The absorbance of each well was detected by a microplate reader (Hiperion MPR 4+, Germany) at the wavelength of 540 nm.

The percentage of cell viability was obtained using the following equation:

% Cell Viability= (treated cell OD-treated blank OD)/ (untreated cell OD-untreated blank OD) × 100

Immunocytochemistry

After fixation with 4% paraformaldehyde (PFA)/PBS, cells were treated with blocking solution (PBS containing 4% goat serum and 0.1% triton X-100) for 45 min at room temperature. Then, cells were incubated in primary antibodies in PBS/0.1% triton X-100 and 1% goat serum overnight at 4°C. Anti-nestin (1:300, Abcam, UK), anti-microtubule-associated protein 2 (MAP-2) (1:300, Abcam, UK), and anti-glial fibrillary acidic protein (GFAP) (1:300, Abcam, UK) were used. After washing with PBS, the slides were exposed to secondary antibodies and

Table 1. The primer sequences (forward, reverse) which were used in the real-time RT-PCR technique

Gene	Forward (top) Reverse (bottom)	Size (bp)	Real time RT-PCR program
Nestin	*5'-AACAGCGACGGAGGTCTCTA-3'* *5'-TTCTCTTGTCCCGCAGACTT-3'*	220	94 ˚C – 20 sec, 59 ˚C – 30 sec , 72 ˚C – 30 sec 35 cycles
MAP2	5'-TCAGAGGCAATGACCTTACC-3' 5'-GTGGTAGGCTCTTGGTCTTT-3'	321	94 ˚C – 20 sec , 57 ˚C – 30 sec , 72 ˚C – 30 sec 45 cycles
GFAP	*5'-CCTCTCCCTGGCTCGAATG-3'* *5'-GGAAGCGAACCTTCTCGATGTA-3'*	161	94 ˚C – 20 sec , 59 ˚C – 30 sec, 72 ˚C – 30 sec 40 cycles
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'	452	94 ˚C – 20 sec , 60 ˚C – 30 sec , 72 ◦C – 30 sec 25 cycles

rabbit anti-mouse FITC-conjugated (1:500; Abcam, UK). They were incubated at room temperature for 1 hr. Diamidino-2-phenylindole (DAPI, 1:1000) was used for nuclear counterstaining. For negative controls, primary antibody was omitted from the reaction series in each experiment. Cells were observed using a fluorescence microscope (Olympus BX51, Japan). To perform quantitative analysis, the number of positive cells was counted on each slide image acquired by ImageJ 1.42 (NIH), and the ratio to the number of nuclei was analyzed for each antigen. The number of immunopositive cells was counted in a minimum of 200 cells per slide. All immunocytochemical experiments were repeated twice.

Real time RT-PCR

Total ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Equal amounts of total RNA were reverse transcribed into complementary deoxyribonucleic acid (cDNA) using oligo-dT and RevertAid First Strand cDNA Synthesis Kit (Fermentas). Real-time polymerase chain reaction (PCR) was performed using a thermal cycler (Rotor-Gene 6000, QIAGEN), with 12.5 µl SYBR Green PCR Master Mix (QIAGEN), 5 pM of each of forward and reverse primers, and 1.5-2 µl cDNA (50 ng/µl cDNA) for each reaction in final volume of 20 µl. Cycle conditions were carried out according to the manufacturer's instructions (QIAGEN). Relative gene expression was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). All samples were normalized to levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which was used as the internal control. All measurements were done in triplicates. The list of primers used in this study are depicted in Table 1.

Statistical analysis

Data are presented as mean ± standard error from 4 to 5 independent cell cultures. Kruskal-Wallis one-way analysis of variance and Dunn's multiple-comparison tests were used to determine the statistical significance between data. Statistical significance was considered when $P<0.05$.

Results

Morphological features and characterization of isolated human ADSCs

After initial plating of human ADSCs, the primary culture appeared to be a mono-layer of flat cells. As the cells approached confluency, they assumed a more spindle-shaped, fibroblastic morphology (Figure 1A). To further characterize these cells, cell surface markers were examined by flow cytometry. Flowcytometry analysis of human ADSCs within 3–5 passages showed that ADSCs CD90, CD105, and CD44 were positive, but CD45, CD34, and CD14 were negative, as previously described (22).

Viability of differentiated ADSCs

We examined the viability of differentiated ADSCs using MTT assay. The mean percentage of cell viability in the E2 treated cell culture (186.7 ± 21) was significantly increased as compared to the untreated group (124.1 ± 25) ($P< 0.05$).

Differentiation to neuron-like cells phenotype

In preinduction medium human ADSCs were spheres of floating cells (Figure 1B). Whereas, in terminal induction medium differentiated human ADSCs showed cytoplasmic retraction and ramified shapes (Figure 1C).

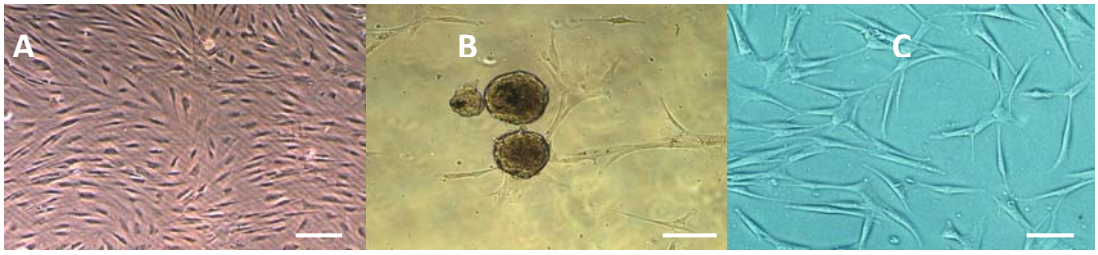

Figure 1. Phase contrast image of (A) stem cells derived from human adipose tissue, (B) neurospheres formation, (C) differentiated cells derived from human adipose derived stem cells 14 days after neural induction in untreated cell cultures. It shows bipolar and multipolar cells with elongated processes, Scale bars denote in A= 150 µm, B= 200 µm and in C= 100 µm

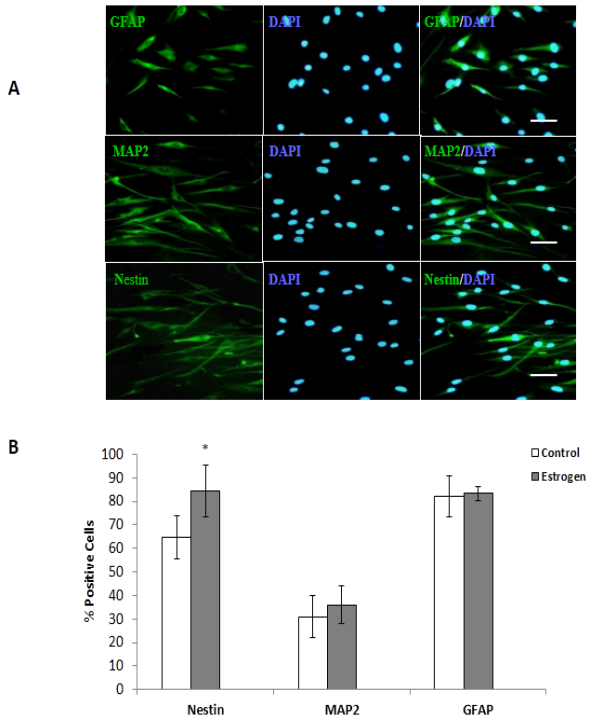

Figure 2. Immunocytochemistry of neural differentiated cells from human adipose derived stem cells (ADSCs) in the presence or without estradiol (E2). Cells were positive for glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP-2), and nestin markers. Cell nuclei were counterstained with diamidino-2-phenylindole (blue). Scale bars: nestin, MAP-2, and GFAP= 50 µm. Comparative analysis between the mean percentages of immunoreactive positive cells for nestin, MAP-2, and GFAP markers in differentiated cells derived from human ADSCs with or without E2 treatment. A significant mean difference in the expression of nestin positive cells was observed (**P*<0.05)

To characterize neural differentiation, induced cells (in the presence or absence of E2) were stained with the markers against nestin (neuronal progenitor cell), GFAP (astrocyte), and MAP-2 (mature neuronal cell) and cell nuclei were counterstained with DAPI. The mean percentage of positive cells for neural markers of nestin, GFAP, and MAP-2 were evaluated 7 days after terminal differentiation. As shown by immunostaining, the mean percentage of nestin positive cells of differentiated cells was significantly increased in treated cell cultures (84.3% ± 11.0) when compared with untreated cultures (64.7% ± 9.2) (*P*<0.05). The mean percentage of MAP-2 positive cells was increased in the presence of E2 (36.0%±5.5) compared with the cultures without E2 treatment (31.0% ± 6.8). Moreover, the mean percentage of GFAP positive cells was increased in E2 treated cultures (83.25 ± 2.80) when compared to untreated cultures (82% ± 8.6) (Figure 2).

Real-time reverse transcriptase (RT)-PCR provided further evidence for neurogenic differentiation of human ADSCs. Our results show that E2 significantly up-regulated the expression level of MAP-2 compared

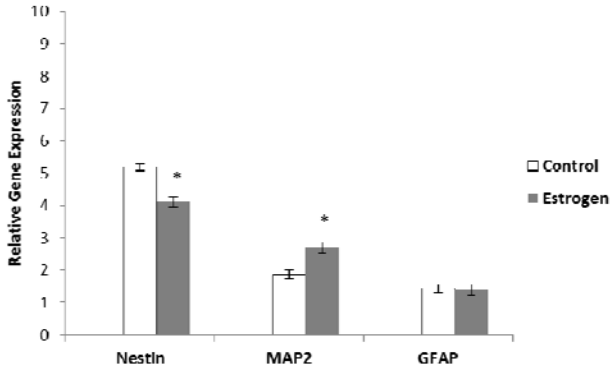

Figure 3. Comparative analysis of some neural markers in treated and untreated cultures examined by real-time reverse transcriptase polymerase chain reaction. The expression of nestin gene, marker of neural precursor cells was significantly down-regulated in estradiol (E2) treated cell cultures compared to untreated cell cultures (**P*<0.05), but the expression of microtubule-associated protein 2 (MAP-2) was significantly up-regulated in E2 treated cell cultures relative to untreated cell cultures (**P*<0.05). While, the level expression of glial fibrillary acidic protein gene was equal in both of differentiated cell cultures

to the untreated cultures (*P*<0.05). While, the expression of nestin gene, marker of neural precursor cells was significantly down-regulated in E2 treated cell cultures as compared to untreated cell cultures (*P*<0.05). While, the level of expression of glial fibrillary acidic protein gene was similar in both of differentiated cell cultures (Figure 3).

Discussion

In the current study, we assessed the potential of E2 to assume selective typical features of neuronal cells during neurogenic differentiation of hADSCs. Using neurosphere formation protocol, human ADSCs induced to neuron-like cells and expressed some neural markers 7 days post-induction. We added E2 with 10 nM concentration, in neurogenic induction medium every day in the treated culture consistent with previous study (17), but some studies have used a different concentration (10^{-11}–10^{-3} M) of E2 (18, 24).

Immunocytochemical analysis of the present study revealed that in the E2 treated cell culture, the differentiated ADSCs had higher expression of the neuronal specific markers (nestin and MAP-2 markers) following neural differentiation. In addition, real-time RT-PCR results show that in the presence of E2, the differentiated ADSCs expressed more MAP-2 markers than the control group, whereas, the expression of nestin was down regulated in the E2 treated cell cultures as compared to untreated cell cultures.

Moreover, the results of MTT assay show that the mean percentage of cell viability and proliferation rate in the E2 treated cell cultures are higher than others. It may be due to presence of EGF and b-FGF

as mitogenic factors in the preinduction medium. Also, addition of E2 to cell cultures could lead to rise in the number of neuronal differentiated cells in treated cell cultures when compared to non-treated cell cultures.

Some evidences show that estrogen acts not only on reproductive organs, but also on brain function by affecting mood, cognition and emotional behavior (25, 26). Hormone therapy via estrogen can improve learning, memory, and protect against cerebral stroke (27, 28).

Specific effects of estrogen are mediated by estrogen receptors (ERs) and act as ligand-activated transcription factors to regulate the expression of estrogen-responsive genes (29, 30). Estrogen has two types of receptors including ERα and ERβ. They are differentially expressed in various tissues with special function (31, 32). Expression of both of these receptors in the brain was known (33). Moreover, expression of ERs in embryonic and mesenchymal stem cells was reported, and it suggests that estrogen may modify the function of stem cells (34, 35).

Wong *et al* (2003) found that chronic exposure to 17β-E_2 had anti-mitotic, but neuroprotective effects; although, pulsed 17β-E_2 treatments significantly increased mitogenesis of granular cells. They reported that neurotoxicity effect of pulsed 17β-E3 was mediated through mitogen-activated protein kinase (MAPK) pathway, but the neuroprotective effects of chronic E2 exposure were MAPK-independent. Therefore, the same concentration of E2 can induce either neuroprotection or neurotoxicity, depending on the time of the exposure. Thus, duration and concentration of E_2, were two important factors in the mitogenesis effect and viability of cells (36).

Previous studies demonstrated that estrogen treatment can increase expression of some neurotrophic factors in the central nervous system (12-15). On the other hand, human ADSCs act as a source of variety of neurotrophic factors with neuroprotective activity, which may prevent neuronal degeneration and are involved in neuronal development (23, 37). Therefore, estrogen can promote proliferation and neuronal differentiation of human ADSCs during neural induction through production of neurotrophic factors.

Conclusion

It could be concluded that estrogen may improve proliferation rate and neuronal differentiation of human ADSCs by production of neurotrophic factors and supports the survival and regeneration of nervous tissue in the neurodegenerative diseases. However, further studies are required to confirm this potential.

Conflict of interests

The authors declare that they have no conflict of interests.

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