

# Assessment of the Neural-Like Cells Differentiation from Endometrial Stem Cells following Fluoxetine Treatment

Morteza Motaghedifard<sup>1,\*</sup>, Javad Verdi<sup>1</sup>, Ali-Reza Shoaee-Hassani<sup>1</sup>,  
Hamid-Reza Banafsheh<sup>2</sup>, Esmail Fakharian<sup>3</sup>

<sup>1</sup>Research Center for Science and Technology in Medicine,  
Imam Khomeini Hospital, Tehran University of Medical Sciences, Tehran, Iran

<sup>2</sup>Department of Pharmacology, Kashan University of Medical Sciences, Kashan, Iran

<sup>3</sup>Department of Neurosurgery, Kashan University of Medical Sciences, Kashan, Iran

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## Abstract

\*Corresponding author:  
Morteza Motaghedifard  
Research Center for Science  
and Technology in Medicine,  
Imam Khomeini Hospital,  
Tehran, Iran  
motaghedi.morteza@gmail.com

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Fluoxetine is a common antidepressant which selectively inhibits serotonin reuptake at synaptic level. Some research findings have proposed the effect this drug on neurogenesis, neuronal survival as well as proliferation of the neural progenitor cells. Endometrium is a part of uterus which harbors mesenchymal stem cells. This source of stem cells can be differentiated into neural cells which may potentially be used in treating many diseases. Given the above, this study was designed to assess the effect of fluoxetine on neural cells differentiation from endometrial stem cells. Endometrial stem cells obtained from stem cell banking were cultured in Dulbecco's modified eagle's medium (DMEM) containing fetal bovine serum (FBS) in the presence of Retinoic Acid, fluoxetine and Retinoic Acid+fluoxetine for 10 days. To assess the differentiation of endometrial stem cells into neural-like cells, we used immunocytochemistry and RT-PCR. The viability of cells was assessed using the trypan blue test. Data analysis revealed that 61% of endometrial stem cells differentiated to neural-like cells. Moreover, the biopotency of neural-like cells on fluoxetine treatment was more pronounced across differentiation days. Based on our findings, fluoxetine was shown to be a suitable inducer for the differentiation of neural-like cells from endometrial stem cells.

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## Introduction

Many disorders of central nervous system including stroke, Parkinson's disease, Alzheimer's disease and spinal cord injury are characterized by loss of neural cells and their physiological function (1). After the successful work of J. Kessler at Northwestern University in the repair of spinal cord injury in rats (2), new hopes for cell therapy in the treatment of CNS lesions started to emerge.

The human uterine endometrium has a very vascular stroma which regenerates more than any other tissue in normal physiological conditions.

Its upper functional layer sheds during menstruation, and restores by the proliferation of the basal layers. The presence of stem cells in different layers of the uterus has been demonstrated by immunohistochemistry and

identification of different cell markers especially CD-146 and CD-105 (3-8). The ability of these cells to differentiate into various cells including neurons has also been demonstrated over recent years (3, 9-12).

Many different materials including antioxidants and growth factors have already been used as inducer of stem cells through different mechanisms. So far, although it has been proven that some antidepressant drugs increase nerve cells' differentiation, the effects of serotonin reuptake inhibitor antidepressants (SSRIs) effects on these cells has remained less understood (13-17). The possibility that the cAMP signal transduction cascade contributes to the regulation of neurogenesis by antidepressants is supported by several studies (13). Some studies show that antidepressants (especially fluoxetine) promote the hippocampal neurogenesis. As such, a causal relation between the stimulation of neurogenesis and the effect of antidepressants has started to establish (15).

In this study the effects of fluoxetine, as an SSRI, on the endometrial stem cells differentiation into neurons, as well as the extent of differentiation is scrutinized.

## **Method**

The lyophilized, freeze-dried endometrial stem cells prepared in National Center for Genetic Resources of school of New Medical Technologies of Tehran University were transferred to tissue engineering and stem cell laboratories of Science and Technology in Medicine Research Center (Imam Khomeini Hospital, Tehran).

### **Differentiation of endometrial stem cells**

Differentiation of endometrial stem cells into neuron-like cells was identified by the morphologic changes and formation of cell body. Suspension of cells were treated with Retinoic acid (4  $\mu\text{g}$ ) alone, Fluoxetine (10 and 100 ng, 1 and 10  $\mu\text{g}$ ) alone or the combination of Retinoic acid (4  $\mu\text{g}$ ) and Fluoxetine (1  $\mu\text{g}$ ) for 10 days. The Control group consisted of cells without treatment.  $1.5 \times 10^5$  cell/ml was used in each group.

The human endometrial stem cells were placed on collagen precoated plates with DMEM low glucose medium. The cultures were kept in a humidified 10% CO<sub>2</sub> atmosphere at 37°C and 95% humidity for 10 days. The media were replaced with fresh media every 3 day and the cells were examined under the microscope on daily basis. Stem cells which grew to 70% confluence were pretreated with 1  $\mu\text{M}$  dimethyl-sulfoxide (DMSO,

Sigma, USA) and then treated with Fluoxetine (Sigma, USA) and Retinoic acid (RA, Sigma, USA).

After a week, phenotypic changes of neuronal differentiation appeared in the cells as dendritic processes and axons. Approximately 10 days following the treatment, cells were examined through reverse transcriptase PCR and immunocytochemistry assays.

### **Reverse Transcriptase PCR Analysis**

To collect total RNA from treated endometrial stem cells, we used an Isogen kit according to the manufacturer's instructions (Nippon Gene, Tokyo, Japan). RNA quantity and purity were determined by spectrophotometry (Beckman DU-65). Standard reverse transcription was performed using the AMV kit (Takara Biomedical, Ohtsu, Japan) with 1  $\mu\text{g}$  RNA and 0.5  $\mu\text{g}$  oligo-dT per reaction, according to the manufacturer's instructions. Reaction mixtures included 2.5  $\mu\text{L}$  cDNA, 1x PCR buffer (AMS TM, Cinnagen, Iran), 200  $\mu\text{M}$  dNTPs, 0.5  $\mu\text{M}$  of each of forward and reverse primers (Table 1), and 1U Taq DNA polymerase. Polymerase chain reactions were performed at 94 °C for 1-min, 30 cycles 94 °C for 30 s, 55–63 °C for 30s, and 72 °C for 30s, and 72 °C for 10 min. Amplified DNA fragments were electrophoresed on 1.5% agarose gel. The gels were stained with ethidiumbromide (10  $\mu\text{g}/\text{mL}$ ) and photographed on a UV trans illuminator (Uvidoc, UK).

### **Immunocytochemistry**

The inductive effect of fluoxetine on endometrial stem cells was assessed by the appearance of Nestin and MAP-2 protein in immunocytochemical studies.

The treated cell groups were:

-Fixed by incubation in 9% paraformaldehyde for 20min and permeabilized with 0.5% Triton X-100 for 10min (18),

-Added primary antibody against Nestin (Sigma, USA) and MAP-2 (Sigma, USA) incubated for 2 h at 37 °C,

-Washed with PBS with adding fluorescent isothiocyanate conjugated secondary antibody (Sigma, USA) for 1h at room temperature,

-Washed with PBS for three times and each for 5 minutes,

-Examined cells with fluorescent microscope.

### **Cell viability assay**

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is a useful colorimetric test for measuring the activity of

cellular enzymes that reduce the yellow tetrazolium dye, to its insoluble formazan, giving a purple color. Differentiated mesenchymal stem cells were tested for their survival time in the presence or absence of Fluoxetine. The EnSCs were plated into 96 well enzyme linked immunosorbent assay (ELISA) plates. After the differentiation for replacing the culture media, Fluoxetine was added to three wells and PBS was used as a negative control in three other wells. Culture plates were incubated for one week. On incubation (7d), culture media were replaced with medium, containing Fluoxetine and PBS. Cell viability was assessed by MTT assay kit (Biotium, Hayward, USA). The MTT reagent (10  $\mu$ L) was added to the wells and incubated for 3 h. At the end of the incubation period, the medium was removed and 100  $\mu$ L DMSO was added into each well. To dissolve the formazan crystals, the supernatant was pipetted several times. Absorbance was measured on an ELISA plate reader at a wavelength of 540 nm.

### 5-Bromo-20-deoxyuridine

One  $\mu$ M BrdU was added to the cultures for 6 h on the 8th day of Fluoxetine induction. The cells were observed on the 9th day of BrdU labeling and then fixed on the 10th day of development and stained for BrdU and Nestin with anti-BrdU monoclonal antibody (Sigma, St. Louis) overnight at 4°C and labeled with secondary antibody conjugated with Rhodamine for 120 minutes at room temperature. BrdU- and Nestin-positive cells were counted in 10 microscopic fields. The percentage and standard deviation of double stained cells among all Nestin-positive cells were calculated.

### Statistical Analysis

This is a parallel experimental study on endometrial stem cells in duplex group. ANOVA test was used to determine the P value between the groups, and Tukey's test was employed to examine the mean differences between them. Differences at  $P \leq 0.05$  were considered as statistically significant.

### Results

After cell culture and differentiation, cells were examined under phase contrast invert microscope. A group of cells which were detached from the plate were kept in -70 °C refrigerator for molecular recognition. Another batch of cells was used for immunocytochemistry.

Differentiation and viability of cells after induction

The best concentration of Fluoxetine found for induction of neural cells from endometrial stem

cells was 1  $\mu$ g/ml of cell culture medium. Concentrations above 10  $\mu$ g/ml of Fluoxetine had cytotoxic effects, and concentrations of 100 ng/ml or less had no effect on the differentiation of stem cells into neural cells (Table 2 and Figure 1).

Cell differentiation by 4  $\mu$ g/ml of retinoic acid, in a ten-day period was about 91%. The effect of retinoic acid and fluoxetine on the differentiation of stem cells was up to 74% after 10 day; while it was less than 61% for Fluoxetine alone. However, the rate of spontaneous differentiation of endometrial stem cells into neurons was less than 5% (Table 2 and Figure 1).

**Table 1.** Primers used for specific neuronal genes expression

<b>Mash 1</b>	Forward	5'- AAGTCAGCGGCCAAGCAGGATAAG-3'
	Reverse	5'- CGCAGCGTCTCCACCTTGCTCATCT-3'
<b>Pax 6</b>	Forward	5'- CTTATAATCCAAGCATCAGT -3'
	Reverse	5'- CCAGCCAGAGCCAGCATGCAGAACA -3'

Cells culture with the aforementioned drugs showed the appearance of neuronal phenotype with dendritic processes and axons in all cells after one week (Figure 2).

Results of the viability test using Trypan blue test between the groups showed that the number of surviving cells in the control group (without inducer) on all days of the experiment was higher than the experiment groups. In the group treated with Fluoxetine and the combination of Fluoxetine and Retinoic acid, cell viability was significantly higher than other groups. The toxic effect of Retinoic acid turned to be higher than other groups (Table 3 and Figure 3).

**Table 2.** Study results in different groups of cells after induction

Group	Differentiation
Control	4
Retinoic Acid 4 $\mu$ g	91
Fluoxetine 10 ng	49
Fluoxetine 100 ng	54
Fluoxetine 1 $\mu$ g	61
Fluoxetine 10 $\mu$ g	65
R.A. 4 $\mu$ g + FLX 1 $\mu$ g	74
P. Value	<0.0001

Neuronal cell markers after induction

Nestin immunocytochemistry study showed that retinoic acid was the strongest neuron inducer agent of our experiment, followed by combination of

Retinoic acid and Fluoxetine and Fluoxetine alone (Figure 4).

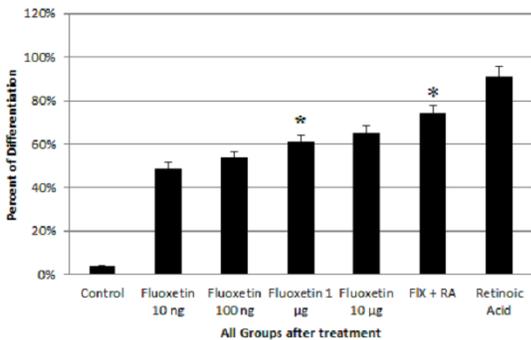
Immunocytochemical expression of Map-2 marker in stimulated cells showed that although Retinoic acid had slightly higher effects, the difference between treatment groups was insignificant (Figure 5 and 6).

**Table 3.** Percentage of viability in differentiated endometrial stem cells throughout the study and across groups

	Day 1	Day 3	Day 5	Day 7	Day 10
Control	100	103	110	116	119
R.A.	96	91	88	83	77
Fluoxetine	98	95	92	90	83
R.A. + FIX	97	93	91	87	80
P. value	0.709	0.541	0.609	0.344	0.570

**Table 4.** Results of neural cell markers after induction (Immunocytochemistry and RT-PCR)

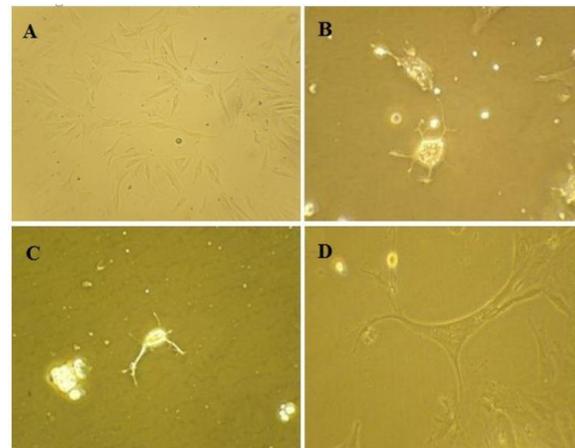
	MAP-2	Nestin	Mash-1	Pax-6
Control	8	5	11	10
R.A.	92	95	89	98
Fluoxetine	58	53	60	61
R.A.+ Fluoxetine	69	70	72	61
P. Value	<0.001	<0.0001	<0.001	<0.001



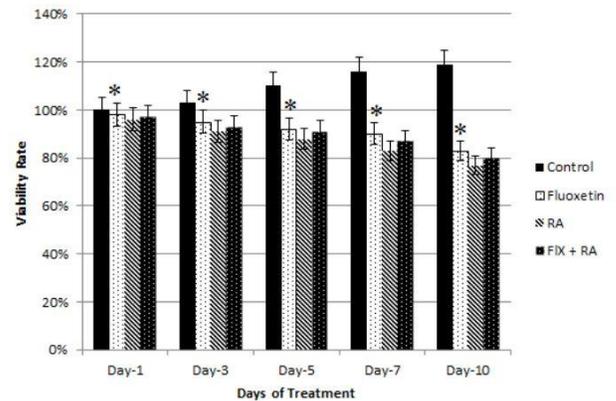
**Figure 1.** Differentiation of endometrial stem cells after 10 days of treatment across groups

RT-PCR to detect the expression of Mash1 and Pax-6

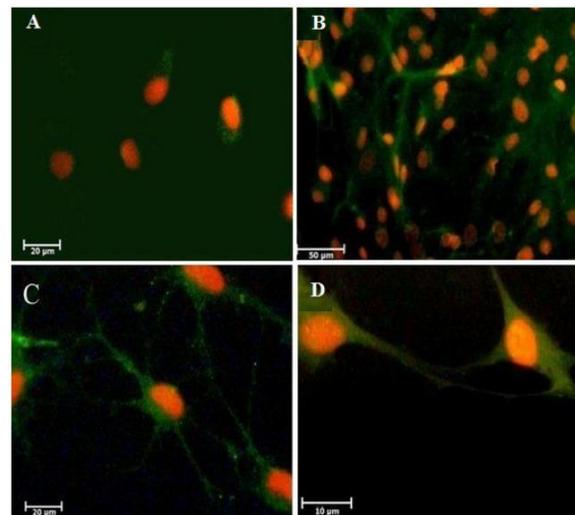
Using RT-PCR by specific primers for genes Mash1 and Pax-6 resulted in higher expression of Mash1 in the group induced by Retinoic acid. This effect was even more significant for Pax-6 (Figure 7 and 8).



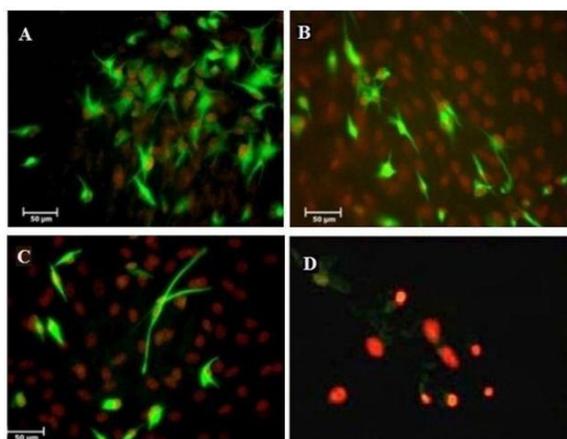
**Figure 2.** All study cell groups after 10 day treatment. A-Control group without any treatment, B-Cells that treated with Retinoic acid, C-Cells treated with Retinoic acid and Fluoxetine and D-Cells treated with Fluoxetine alone



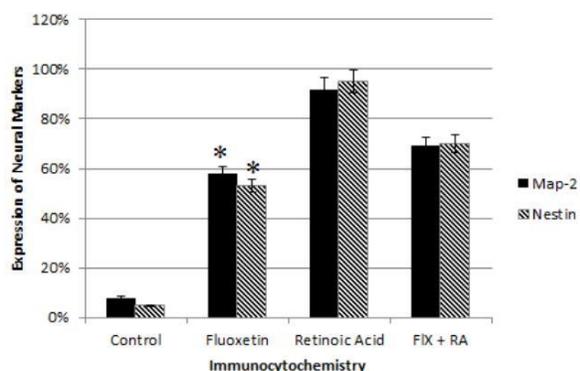
**Figure 3.** Viability of differentiated endometrial stem cells throughout the study and across groups



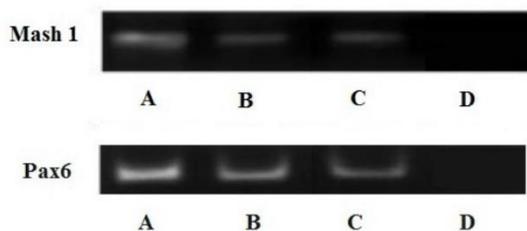
**Figure 4.** Immunocytochemistry for Nestin marker in all cell groups. A-Control group without any treatment, B-Cells treated with Retinoic acid, C-Cells treated with Retinoic acid and Fluoxetine and D-Cells treated with Fluoxetine alone



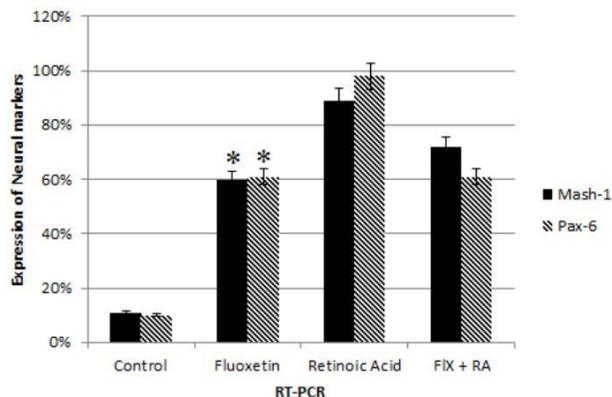
**Figure 5.** Immunocytochemistry for Map-2 marker in all cell groups. A- Control group without any treatment, B- Cells treated with Retinoic acid, C-Cells that treated with Retinoic acid and Fluoxetine and D-Cells treated with Fluoxetine alone.



**Figure 6.** Immunocytochemistry results for neural cell markers after induction



**Figure 7.** Expression of differential genes. A-Cells treated with Retinoic acid, B-Cells treated with Retinoic acid and Fluoxetine, C-Cells that treated with Fluoxetine and D-Control group without any treatment



**Figure 8.** RT-PCR results for neural cell markers after induction

## Discussion

The induction was up to 91% by Retinoic acid, 61% by Fluoxetine, and 74% by Fluoxetine and Retinoic acid combination. However, Retinoic acid had a higher rate of cell death compared to 1 µg/ml Fluoxetine.

Rahmani and his colleagues have done similar research indicating differentiation and survival of endometrial stem cells by phosphorylation Akt1, following treatment with fluoxetine (17). In their study, the cells were divided into two groups, separately treated with Fluoxetine and Retinoic acid. Duration of treatment was 10 days and the applied dose was different to our project.

Mash1 and Pax-6 expression level of genes in a concentration of 1 µg on the cells was shown to be satisfactory, thereby the differentiation of the cells was not reversible even after removal of the inducer.

The inducer material itself is very effective in the pathway of induction of cell. Keilhoff and colleagues demonstrated that Retinoic acid induces stromal stem cells to Schwann cells, while glial cell growth factor induces them to astrocytes. The combination of Retinoic acid and fibroblast growth factor has been shown to induce cell differentiation into neuroglial category.

Nestin, a marker of neural precursor cells has been seen in induced cells after 10 days. On the other hand, Sanchez and colleagues showed that epidermal growth factor and brain-derived neurotrophic factor result in expression of Nestin in bone marrow stromal cells.

The presence of synaptic marker Map-2 in a high percentage of cells in this study might suggest the differentiation of study cells into cholinergic neurons.

Cholinergic neurons are found in many parts of the nervous system including the midbrain, thalamus, cerebral cortex, and the cranial nerves.

Amacrine cells in the retina, as well as many of the neurons in the auditory pathway, the autonomic system, pre- and post-ganglionic parasympathetic ganglion fibers and sympathetic ganglion cells are examples of cholinergic neurons (19). Gray matter of the brain and pericentral gray matter of the spinal cord contain collection of cholinergic cells. There exist cholinergic medium in the the intermediate layer of gray matter which are referred to as the partitioning cholinergic motor neurons (20).

In 1991, Bakhit and colleagues demonstrated that after spinal cord injury, the level of acetyl choline transferase is decreased in the cord, especially the injured parts. This is notably seen during the first week after injury and continues until about four weeks, and then returns to the initial state all over except in the injured region (21).

Studies on stem cell transplantation in neurological damage have shown better results when performed in the early stages of differentiation (22). In addition, neural stem cells better match the new environment in neuroblastic stage (23). Axon regeneration by neuroblasts has been reported in spinal cord injury (24). Ability of undifferentiated stem cells to live in brain without induction and secrete some cytokines after exposure to microenvironment has similarly been reported (25). Cholinergic neurons appear to be more important across these processes.

In our present investigation, by the end of first week, about 75% of the study cells induced with the combination of Fluoxetine and Retinoic acid differentiated into cholinergic neuron-like cells and approximately 70% of such cells expressed Map-2

marker, while the percentages for Fluoxetine alone was 61% and 58%, respectively. Cholinergic-like cells in culture have long processes subjected to damage upon transportation between different media; therefore, the induced cells must be separated from culture within maximum of four days to avoid damage as much as possible.

## Conclusion

It can be concluded that SSRI drugs (Fluoxetine in particular) are potentially able to induce differentiation of stem cells into neural-like cells and neurons. This ability for Fluoxetine, especially in combination with Retinoic acid, which has been shown in our study may be further examined in future studies hoping to offer a novel chance for repairing neurological damages.

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