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Creatine and Retinoic Acid Effects on the Induction of Autophagy and Differentiation of Adipose Tissue-Derived Stem Cells into GABAergic-like Neurons

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ABSTRACT

BACKGROUND AND OBJECTIVE: Deficit of inhibitory GABAergic neurons as a part of central nervous system (CNS) pathogenesis was reported in neurodegenerative disorders; and adipose-derived stem cells (ADSCs) were shown to be a feasible option for cell transdifferentiation in neuronal disorders therapy. In this study, the role of autophagy in differentiation was considered by evaluating the expression of the autologous genes of LC3, P62 and GABARAP in fatty stem cells and after the pre-induction stage.

METHODS: In this experimental study, under sterile conditions ADSCs were obtained from pararenal fat of two male adult rats. The cells were divided into three groups of fatty stem cells, pre-induction and induction. Following third passages of cell culture, ADSCs were preinduced to neural-like cells (NLCs) using 1mM β-mercaptoethanol (βME) and 10µM retinoic acid (RA), and then NLCs were induced by creatine(Cr) in 1, 5, 10, 20 millimolar for 5 days. In induction stage, the effects of creatine on differentiation were studied by anti nestin and GABA antibody immunostainig. The roles of GABARAP, LC3 and p62 autophagy genes in transdifferentiation were assessed by RT-PCR.

FINDINGS: Immunocytochemical studies on ADSCs using CD49d indicated that cultured cells were ADSCs. In the immunochemical studies of the induction stage, at a dose of 10 mM creatinine for 5 days, the expression of the GABA neurons and the nestin-like neuronal cell marker were 58±2% and 56±5%, respectively which had a significant difference with other doses and control group ($p<0.05$). RT-PCR results indicated that in pre-induced cells autophagy genes of GABARAP, LC3 and p62 were expressed but only P62 gene was expressed in fatty stem cells.

CONCLUSION: This study demonstrated that fatty stem cells after induction are able to express nestin and GABA neuronal markers. GABARAP, LC3 and p62 autophagy genes were expressed in pre-induced cells, which indicates the potential role of autophagy in the differentiation of fatty stem cells into nerve-like cells.

KEY WORDS: *GABAergic-like neurons, Adipose derived stem cells, Creatine, Autophagy.*

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Introduction

GABAergic cells are inhibitory cells in the nervous system that cause many neuronal degenerative diseases, such as Huntington's (1), alzheimer's disease (2), epilepsy (3), schizophrenia (4), autism (5), parkinson's disease, and brain and spinal cord injury (7). Therefore, the creation of GABAergic cells (8, 9) and their transplantation at the site of the lesion may be effective in the treatment of these diseases. Today, adipose-derived stem cells (ADSCs) have been shown to produce a range of fully differentiated connective tissue such as tendon (10) cartilage (11), bone (12), and nerve cells (13).

In cell therapy and gene therapy, high amount of cellular resources, easily extracted and controllable are used (14, 15). Fatty stem cells are easily differentiated into nerve cells and can be used as substitutes for damaged cells of the nervous system (16, 17).

Autophagy is a process in which cells, organelles, proteins, and degraded cells and cytoplasm components are incorporated into a two-layer membrane and lysosomes decomposes them, resulting in products derived from cellular degradation are used in energy production, synthesis of proteins and new cell structures. Defects in pathways associated with autonomic nerve cells may cause parkinson's disease, alzheimer's disease, huntingtin's disease, and other neurological diseases (18).

The LC3, P62 and GABARAP genes play a role in the formation of autophagosome membranes and autophagy (19). The induction of autophagy by using retinoic acid induces cell differentiation (20). Retinoic acid is a metabolic product of vitamin A (retinol), which plays a role in induction, neuronal differentiation, axonal growth, and neurogenesis (21). Simultaneous combination of retinoic acid by using various growth factors leads to the creation of various types of neuronal cells, such as GABAergic cells from embryonic stem cells (22), mesenchymal stem cells(8, 9) and neuronal stem cells (23).

Creatine Cr/is an organic nitrogenous acid naturally existing in the body of vertebrates and its primary role is energy production in the nervous and muscular system (24). Creatine is substrate of creatine kinase enzyme, which regulates the amount of ATP in the cell (25). The results of the studies showed that creatine increased the differentiation of stem cells into GABAergic-like cells, as well as GABAergic neurons from different sources of cells (26, 27). In previous studies, fat stem cells as a cell source were used to differentiate into GABAergic-like neurons. In this study, the role of autophagy in differentiation was considered by evaluating the expression of the autophagy genes of LC3, P62 and GABARAP in fatty stem cells and after the pre-induction stage.

Methods

Isolation and culture of fatty stem cells: This study was conducted using laboratory animals of Qazvin University of Medical Sciences with confirmation in the Ethics Committee with code 131.94IR.QUMS.REC. Two male Sprague-Dawley rats (Razi Institute, Tehran, Iran) and 200 g weight were randomly selected. Animals were anesthetized by intraperitoneal injection of 50 mg/kg ketamine and 5 mg/kg xylasein. The abdominal area of the animal was opened after sterilization by using scissors and a surgical blade. The fat was extracted from the around of kidneys.

During the removal of the fat, the destruction of the vessels and the destruction of the samples were prevented. Separated adipose tissues were divided into very small pieces with sterile scalp in a sterile plate containing 1 cc PBS (phosphate buffered saline) and antibiotics. In all stages of isolation and cell culture, penicillin 100 U/ml and streptomycin 100 µg/ml were used. Fragmented pieces of the adipose tissue contained in a large falcon tube containing 20 ml DMEM (Dulbecco's Modified Eagle's Medium), with antibiotics and type 1 collagenase enzyme (0.075%), were shaken for about half an hour to obtain a milky solution. The solution was mixed with FBS (fetal bovine serum) to neutralize the enzyme and then centrifuged for 20 minutes in 1000 rounds and the supernatant of the cells was discarded.

The deposited cells were pipetted with 2 mL of non-serum medium (preventing foaming during pipetting), and then the cell suspension was passed to remove additional components from nylon filter mesh with a pore diameter of 100 micrometers. Cell suspensions were centrifuged again for 5 minutes at 3000 rpm and stored in an incubator at 37 ºC and 95% relative humidity in a flask containing DMEM and FBS 10% and antibiotics (28).

Identification of fatty stem cells: To investigate the nature of stem cells adhering to the flask and confirm their mesenchymal origin with CD49D antibody (Fatty cell marker), CD34 (hematopoietic cell marker), CD31 (vascular endothelial cells marker) were evaluated by immunocytochemistry.

Cell groups: The cells were divided into three groups: adipose stem cells (ADSCs), pre-induction group

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(adipose stem cells exposed to ME β 1 mM for one hour and then RA 10 µM for two days) and the induction group (pre-induced cells subjected to creatine (Cr) for 5 days at doses of 1, 5, 10, 20 mM).

Viability Test: The study of the percentage of cell survival in adipose cells of the 3rd passage (before the onset of the pre-induction phase) and in the stages of pre-induction and induction was performed. A volume of cell suspension and an equal volume of trypan blue were mixed and cell count was performed using a neobar lamella under a microscope. In this method, the color penetrates into the dead cells and comes in blue, and non-stained cells represent living cells, which resulted in the count of whole cells and stained cells of the percentage of live cells. The differentiation of adipose stem cells into GABAergic-like cells was performed in two stages of pre-induction and induction. In these two stages, nestin and GABA antibodies were used to assess the differentiation.

Pre-induction stage: After trispinning the adipose stem cells of the third passage, 10,000 cells were equally plated in each of the wells of the 24-well plate. In the first stage of pre-induction, betammercaptoethanol with a dose of 1 mM was used on adipose tissue stem cells containing serum free medium (29). After an hour from the first pre-induction stage, washing was performed by PBS. The second stage of pre-induction was induced by adding the medium containing 15% FBS and 10 µM retinoic acid (9) for 2 days on the cells. The stages of immunocytochemistry examination and counting and determination of the percentage of alive cells were performed.

Induction stage: In the induction stage, after removing the culture medium containing retinoic acid and washing the cells with PBS, neural-like cells were placed in DMEM / F12 medium containing 15% FBS and 1, 5, 10, and 20 mM creatinine for 5 days. Initially, using the cell survival test, days and doses in which the mortality rate of the cells was increased, were determined. Then the cells were exposed to the non-toxic dose of creatine in the appropriate days.

At the end of this step, the evaluation of primary antibodies including nestin and GABA antibodies was performed by immunocytochemistry on the cells. All steps, as well as how to count and determine the percentage of cells were done as before. Thus, the highest percentage of differentiation to GABAergic-like cells in the induction group was determined.

Immunocytochemistry study: At each stage, after trispinning the cells, 10,000 cells were cast evenly in each of the six wells. After adhesion of the cells to plate floor, Immunocytochemistry tests were carried out. Cells were placed in a 4% paraphormaldehyde solution for 20 minutes, after washing with PBS, cells were placed in 0.3 Triton X for 15 minutes. The cells were then washed with PBS. Cells were placed in primary antibody for 24 hours at 4ºC. Initial antibodies were nestin (;Abcam 1: 300) and GABA (;Abcam 1: 500). The cells were washed with PBS and subjected to a FITC conjugated secondary antibody which is seen in green color (1: 100; Chemicon) and placed at room temperature for 2 hours. For cell count, the propidium iodide color was used in which the nucleus of the cells is red. Cells with positive immune response were counted with fluorescence microscope.

RT-PCR: To perform RT-PCR for LC3, p62, GAPDH and GABARAP genes, the required primers were firstly designed by Gene Runner software (Table 1). In this technique, the total RNA was extracted from the cells of each group by extraction kit (RNA extraction kit). In order to remove the unwanted DNA, the DNase I amplification (grade kit) was used. Then, the RNA was converted to a complementary DNA (cDNA) using the cDNA production kit (synthesis kit) and the reverse transcriptase enzyme. The resulting cDNA was amplified using RT-PCR. The GAPDH gene was used as an internal control.

Statistical analysis: The data obtained from cell viability and cell count were analyzed by Student T-test and one way ANOVA and TUKEY tests. $P < 0.05$ was considered significant.

Table 1. Primers

Gene	Primer(5'--> 3')	Sequence size
GAPDH	F: GTTGTCTCCTGCGACTTCA R: GGTGGTCCAGGGTTTCTTA	190 bp
LC3	F: TGTTAGGCTTGCTCTTTTGG R:GCAGAGGAAATGACCACAGAT	219 bp
p62	F:TCCTACAGACCAAGAATTATGAC R: TTCTCATGCACTTTCCTACTG	232bp
GABARAP	F: TTGATGTGCCTCCTACCTCC R: TGTTTACCCTCCATTCCCAC	200bp

Results

Separation and culture of adipose stem cells: In study with optical microscopy of the adipose stem cells in culture medium, appearance of the cells after three passages was relatively uniform morphologically. The cells in the culture medium were mostly observed in

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two shapes. There were a few rounded, spherical, and small apparent replicating cells, and the rest, which were dominated by the majority of the cells, were characterized by broad, spindle, and pseudo-fibroblasts (Fig. 1A).

Confirmation of the nature of adipose stem cells: Fatty cell stem cells were positive for CD49d (adipose stem cells marker), and cell cytoplasm became green as a result of the use of conjugation of secondary antibodies to the FITC, the nucleus of the cells were also red using propidium iodide (Fig. D1). However, adipose stem cells were negative for CD34 (hematopoietic stem cell markers) and CD31 (endothelial cell markers) (Fig. B, C1).

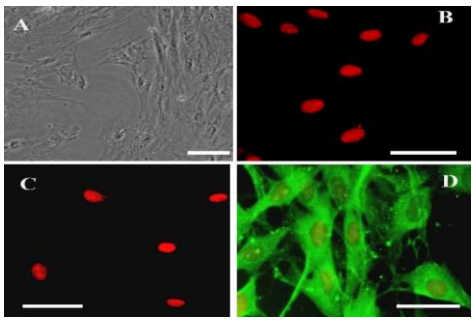

Figure 1. Figure A shows the adipose stem cells in the third passage, B and C are images of adipose stem cells which were negative for CD34 (hematopoietic stem cell markers), CD31 (endothelial cell markers). Image D indicates the adipose stem cells were positive for CD49d (adipose stem cell markers) and cellular cytoplasm became green due to the use of FITC conjugated secondary antibodies, and the nucleus of the cells was red by using Propidium iodide (50 µm scale line).

Determination of cell viability during pre-Induction and induction stages: The survival rate of pre-induced and induced adipose stem cells indicated that the survival rate of non-induced adipose stem cells or the control group was 95.8±2.58% which was significantly ($p<0.05$) more than induced cells in other groups, but was more than compared to βME in the pre-induced group with no significant difference (Fig 2). The survival rate of pre-induction group received beta mercaptoethanol 1mM for 1 hour was 90.2±0.83, but after using retinoic acid 10 µM for 2 days, survival rate was 82.4±3.43% and the difference between them was not significant, but the mortality rate was higher than that of the adipose stem cells. In the induction group on day 5, the mortality rates at doses of 1, 5 and 10 mM were 78.4%±2.88%, 75%±54.2% and 71%±1/22, respectively which have the lowest mortality rate ($p<0.05$) than the 20 mM creatine group (46.6±3.04%) and the difference was significant ($p<0.05$). Meanwhile, this study shows that the highest mortality rate was observed in the 20mM creatine dose on the fifth day with significant difference ($p <0.05$) than other groups that the cell survival rate becomes less than 50%. Therefore, this dose is eliminated in studies (Fig 2).

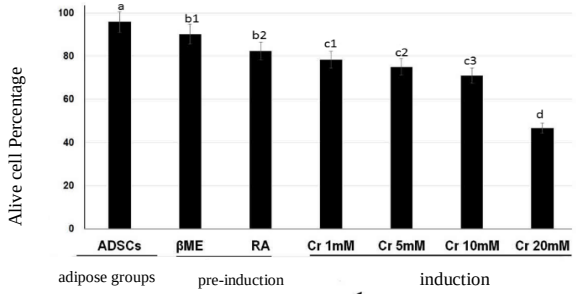

Figure 2. Percentage of cell survival in adipose groups, pre-induction, induction

ADSCs: adipose stem cells as control, βME: A group receiving betamercaptoethanol at a dose of 1 mM for one hour, a group of RA receiving 10-µmA acid retinoic for 2 days. Cr: Groups receiving creatine with doses of 1, 5, 10 and 20 mM for 5 days. Groups were identified with Latin letters and group a except b1 had a significant difference with the rest of the groups. Also, the group D had a significant difference compared woth all groups, and also, c3 with b1 had a significant difference ($p<0.05$). The rest of the groups did not show any significant difference ($p> 0.05$).

Determination of the degree of differentiation by immunocytochemistry: In immunocytochemistry of adipose stem cells after pre-induction and induction stages with nestin antibodies (neuronal like marker) and GABA (GABAergic like marker), different percentages of cells positivity was detected by fluorescence microscopy. In order to determine the percentage of neuronal-like immunopositive cells and also GABAergic like immunopositive cells, cell counting was performed.

On the fifth day of induction, the expression of the nestin antibody in the group of adipose stem cells was 1%, in the group MEβ 3±2%, in the RA group was 5±2% and in the groups of creatine 1 mM 2±8%, 5 mM 47±4%, 10 mM 56±5% and 20 mM 49±2 %(Fig 3). On the fifth day of induction, the amount of GABA antibody expression was 2±1% in the adipose stem cell group, 4±1 % in the MEβ group, 6±1% in the RA group and in the creatine groups, 1 mM 12%±1, 5 mM 50±2%, 10 mM 58±2% and 20 mM 40±3%, respectively (Fig 3). On the fifth day of induction by creatine at a dose of 10 mM, the expression of nestin markers with mean 56±5% and GABA with 58±2% were significantly higher than the other doses (Fig 3).

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Compared to other doses, between induction groups of 1 and 20 mM, the difference in expression between nestin antibody and GABA was significant, but at 5 and 10 mM, the difference in expression between the nestin and GABA antibodies was not significant. The differentiation to neuronal like cells and GABAergic like cells in the pre-induction stage was less than 6%, indicating that the pre-induction stage lonely could not produce neuronal and GABAergic cells (Fig 3).

Cell examination by RT-PCR: autophagy genes of LC3 and GABARAP was not expressed in adipose stem cells, but the p62 gene was expressed. In neuronal like cells after pre-induction stage and use of 10 µm retinoic acid for 2 days, LC3, p62 and GABARAP genes were expressed. GAPDH gene was expressed as internal control in all groups (Fig 4).

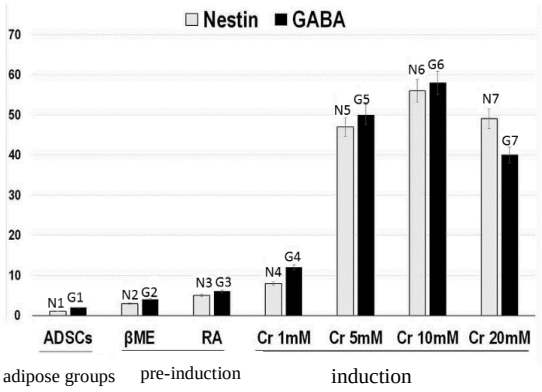

Figure 3. Expression ratio of Nestin and GABA in different cell groups using specific antibodies

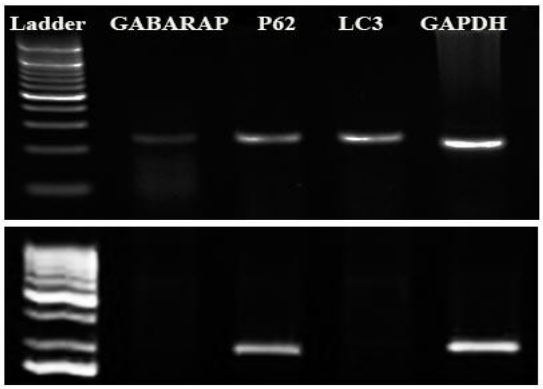

Figure 4. The above image indicates RT-PCR of nerve-like cells, after the pre-induction stage and the use of 10 µm retinoic acid for 2 days, LC3, p62 and GABARAP genes were expressed.

The lower image indicates RT-PCR in adipose stem cells, the autophagy genes of LC3 and GABARAP was not expressed, but the p62 gene was expressed. GAPDH gene was expressed in two cell groups.

In nestin, expression increased from N1 to N6 continuously, but in the N7 group, the expression reaches a level similar to N5. There is a significant difference between all groups except N5 with N7 ($p<0.05$). In GABA, expression increased from G1 to G6 continuously, but in the G7 group, the expression reaches a level similar to G5. There is a significant difference between all groups except G5 with G7 ($p<0.05$). The highest expression of nestin and GABA was observed in the group receiving 5mg of creatine 10mM in the induction stage, which had a significant difference in expression of nerve-like antigens in other groups.

Discussion

In this study, the direct method was used for the creation of nerve-like cells. After the third passage, adipose stem cells were converted into nerve-like cells by 1 mM βME for one hour, and 10 µM retinoic acid for two days, followed by 10 mM creatine (30) for five days, the highest levels of neuronal like cells were created.

Today, cell therapy and patients' body cells are used for treatment of nervous system diseases, because the patient's body cells do not have a problem with the immune system. Nervous stem cells are located in the hippocampus and around the ventricles (31). Extracting these cells and using them to treat patients' illness is almost impossible.

The easiest idea in treating these patients is to extract mesenchymal stem cells, such as fat from the person itself, and differentiate them into neural stem cells. The creation of neuronal like cells from adipose stem cells by retinoic acid is probably through activation of autophagy. In addition to the classical mechanism of retinoic acid through nuclear receptors, its other mechanism of differentiation has been described recently by activating autophagy (20).

The study of biochemical, cellular and molecular, genetic and epigenetic pathways are widely used to study various neurological diseases (30, 32-34). One of the targeted uses of these studies is the use of cell therapy and gene therapy in therapeutic studies (38-35). One of important biochemical pathways in neurological diseases is autophagy. Many proteins are involved in the onset and completion of autophagy (39, 40). The LC3 protein is needed to start autophagy and to form autophagosomes. In studies, the expression of LC3 and p62 are indicator of autophagy activity, and a

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higher rate of LC3-II suggests an increase in the formation of autophagy vacuoles in the cell (19). GABARAP also plays a role like the LC3. Both are only one family and improve the binding capacity of autophagosomes (19).

P62 is an intracellular protein that is induced by cellular stress and contributes to the regulation of the signaling pathways of life and cell death. P62 function is in signal transduction, proliferation, cell survival, death, swelling, tumor formation, and in response to oxidative stress, and this protein is expressed by induction of autophagy (41). In this study, LC3 gene was not expressed in adipose stem cells, but in neuronal like cells after pre-induction stage and use of 10 µm retinoic acid for 2 days, the P62, LC3 and GABARAP genes were expressed, indicating activation of autophagy in these cells. In the induction step, 10-mM creatine was used to differentiate nerve-like cells to the GABAergic like cells, and the highest levels of GABAergic-like neurons were created. Creatine has neuronal protection properties in nerve cells against ischemic and hypoxic injuries (42). In cerebral and spinal cord injury models, creatine prevents increased damage.

Creatine significantly prevents necrosis and apoptosis in the neuronal cells by maintaining the mitochondrial membrane potential constant, decreasing mitochondrial calcium levels, and maintaining the balance of ATP (42). Creatine is supplied by food and also as endogenous in the body. Creatine has direct anti-apoptotic effects (43). Lack of creatine has been observed in patients with damage to the nervous system due to injuries (43).

In previous studies, creatine has been shown to increase the differentiation of stem cells into GABAergic-like cells (26, 27). Creatine has also been reported to have neuroprotective effects in mice of spinal cord injury (44). After spinal cord injury, Creatine prevents excessive destruction of neurons by improving the function of energy metabolism (45), as well as creatine induces GABAergic cells from the striatum (26) and the spinal cord of the mouse embryo (46). Gabaergic neurons are the most important source of GABA neurotransmitter production. In addition, the neurons and glial cells produce and release GABA in the developing brain.

GABA is produced from glutamate by the glutamate acid decarboxylase enzyme. GABA, in addition to being an inhibitory and stimulating neurotransmitter, is also a signaling molecule that has the effects of autocrine and paracrine, and plays an important role in the formation of the nervous system in the fetal period. GABA, like factors of neuronal growth, causes the growth of the axon (47).

GABA is a factor contributing to the release of growth factors by depolarizing the cell membrane and increasing the free calcium inside the cell (48). GABAergic cells play a role in reducing pain, spinal cord injury activates apoptotic agents, followed by increased oxidation, inflammation, and ultimately leads to the death of GABAergic cells in the spinal cord, and pain is no longer inhibited (49). In the embryonic period, if GABAergic cells are damaged, increased glutamatergic stimulation cells increase cortical catalytic activity and ultimately lead to epilepsy (50).

The adipose stem cells in the pre-induction stage by βME and retinoic acid in the culture medium were differentiated into neuronal-like cells, while the degree of differentiation to GABAergic cells was very low. These cells, after a period of 10 mM induction by creatine for 5 days, a large percentage of GABAergic neurons were created. Autophagy genes were expressed in pre-induced cells, and possibly autophagy plays a role in cell induction.

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