ORIGINAL ARTICLE

New method to induce neurotrophin gene expression in human adipose-derived stem cells *in vitro*

Dhiya Altememy, Maryam Haji Ghasem Kashani[1], Amirahmadi Fateme[1], Pegah Khosravian[2]

Department of Pharmaceutics, College of Pharmacy, Al-Zahraa University for Women, Karbala, Iraq, [1]Department of Cellular and Molecular Biology, School of Biology, Institute of Biological Sciences, Damghan University, Damghan, [2]Medical Plants Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Rosemary leaf extract, a well-known medicinal plant, can induce neurotrophin gene expression and proliferation in stem cells. Human adipose-derived stem cells (hASCs) with high proliferation and differentiation capacity are easily accessible and can be extracted with the least damage. This study evaluated the effect of rosemary extract (RE) on neurotrophin gene expression at 48 h postinduction in hASCs. hASCs were isolated from healthy female donors, aged 28–35 years, who had undergone abdominal liposuction. Passage-4 stem cells were cultured and treated with different doses of RE (from 30 to 70 μg/ml) containing 40% carnosic acid for 48 h. Reverse transcription-polymerase chain reaction was used to check the expression of neurotrophin genes. The expression of *NTF3*, *NTF4*, and nerve growth factor genes in cells treated with 40–60 μg/ml and the expression of *GDNF* in cells treated with 50–70 μg/ml of RE for 48 h showed a significant increase compared to cells cultured in serum-containing medium. However, different doses of RE showed no effect on brain-derived neurotrophic factor gene expression in the treated cells. RE (50, 60 μg/ml) leads to an increase of neurotrophin gene expression in hASCs as compared to routine cell culture. Hence, this protocol can be used to prepare ideal cell sources for cell therapy.

Key words: *In vitro* culture, neurotrophins, rosemary extract, reverse transcription-polymerase chain reaction

INTRODUCTION

In the past, adipose tissue was only considered to be a tissue that stores fat. In 1964, Rodbell first extracted stem cells from subcutaneous adipose tissue in rats.[1] In 2002, Zuk *et al*. extracted multipotent stem cells from human adipose tissue, which are called adipose-derived stem cells (ASCs) and introduced them as adult stem cells.[2] Friedenstein first researched mesenchymal stem cells (MSCs) in 1970.[3] MSCs have been widely used in regenerative medicine and tissue engineering due to their self-renewal characteristic and generated different types of mature cells in specific conditions.[4-6]

Nowadays, obesity has become one of the most common public health problems. Therefore, many people undergo liposuction. Researchers have extracted stem cells from discarded adipose tissues.[7] One gram of fatty tissue can extract many stem cells to repair damaged and aged tissues,

Address for correspondence:
Dr. Maryam Haji Ghasem Kashani,
Department of Cellular and Molecular Biology, School of Biology, Institute of Biological Sciences, Damghan University, Damghan, Iran.
E-mail: kashani@du.ac.ir

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tissue regeneration, and trauma.[8-10] ASCs are differentiated into different mesodermal cells, such as adipocytes, chondrocytes, osteoblasts, and skeletal muscle fibers, as well as nonmesodermal cells, such as pancreas, hepatocytes, endocrine cells, neurons, myocardial, and endothelial cells.

ASCs, like bone marrow-derived MSCs (BMSCs), can differentiate into different ectodermal lineages, such as neurons and glial cells, by neural induction.[9,11] Nowadays, one of the world's major transplantation concerns is the need for more tissue and organ donors. Therefore, scientists tried to find a method or technique to induce regeneration in damaged tissues. As a result, tissue engineering in association with stem cells is suggested to solve this issue.[12,13]

The notable characteristics of stem cells regarding accessibility, easy extraction with minimal tissue damage and pain, high differentiation and proliferation, and the possibility of autogenous or allogeneic transplantation without worrying about transplant rejection make stem cells a befitting option for cell therapy.[14,15] BMSCs are like ASCs in all aspects except their proliferation rate. In the past few years, BMSCs were the most important source for obtaining MSCs, but recently, ASCs have been used worldwide.[16]

ASCs can be obtained more easily, compared with BMSCs with less complication. Recent studies indicated that BMSCs from passage five onward undergo spontaneous differentiation and become more inclined to differentiate into osteoblasts.[9] Huang *et al*. have demonstrated that ASCs did not spontaneously differentiate beyond 15 and higher passages and showed fewer changes in phenotypic variation with age.[17,18]

Other studies have also shown that in the long-term and late passages, a tiny percentage of ASCs express astrocytic glial fibrillary acidic protein and neural nestin markers and can differentiate into neuronal cell lines.[19] ASCs and BMSCs are similar in phenotype, surface markers expression, and differentiation, while ASCs have higher self-renewal capacity than BMSCs. The proliferation ratio of ASCs began to slow earlier, perhaps due to collagenase digestion and spending more time from extraction to culture. The survival rate, differentiation capacity, and the number of stem cells in BMSCs decreased with increasing age.[20] While ASCs are more resistant than BMSCs, there is no limit on the number and proliferation when extracted from older donors.[21] Fraser *et al*. demonstrated that ASCs are about 500 times higher than BMSCs within an equivalent volume of fat and bone marrow samples.[22,23]

In addition to the features mentioned above, the availability of ASCs, the relatively easy extraction ability, and the lack of host immune response after transplantation make these cells a good candidate in regenerative medicine and tissue engineering.[24] ASCs can secrete epidermal growth factor, basic fibroblast growth factor, vascular endothelial growth factor, platelet-derived growth factor, transforming growth factor-β1, hepatocyte growth factor, nerve growth factor (NGF), GDNF, and brain-derived neurotrophic factor (BDNF).[25] Neurotrophins have been identified as important regulators of neuronal proliferation, survival, improving function, and differentiation capacity, so be considered promising new therapeutic agents for treating various neurodegenerative diseases. Rosemary extract (RE) is a rich source of phenolic acids, and the most important ones are carnosic acid, carnosol, and rosmarinic acid. The first two compounds have about 90% antioxidant activity.[26,27] Carnosic acid is the primary rosemary-derived phenolic diterpene compound and has been reported to have antioxidant, antimicrobial, anti-obesity, anti-tumor, neuroprotective, anti-cancer, and anti-depressant activities.[28,29] In addition to antioxidant activity, this extract has been reported to increase NGF synthesis in human glioblastoma cells *in vitro*.[30,31]

Today, due to the carcinogenic effects of synthetic antioxidants, the tendency to replace these antioxidants with natural ones has been increasing. Incubation conditions, type of culture medium, and antioxidant supplements in the culture medium can affect cells. Studies have shown that adding antioxidants to culture media can increase the proliferation and expression of some neurotrophin genes in cultured cells and make cells more effective for transplantation.

In this study, we investigated the effect of RE at different concentrations on the expression of some neurotrophic genes in human ASCs (hASCs). Then, it is possible to use this antioxidant to produce a suitable cell bank *in vitro* to speed healing in damaged tissue after transplantation.

MATERIALS AND METHODS

Isolation and culture of human adipose-derived stem cells

The subcutaneous adipose tissue female samples were collected (28–35 years old) undergoing liposuction. The principle of informed assent was obtained from the participants. This study is approved under the ethical approval code of IR.DU. REC.1401.002. One gram of adipose tissue was digested mechanically using a scalpel blade and then divided into small pieces under sterile conditions. Then, 0.2% collagenase enzyme with 1.5 ml/g of adipose tissue was used at 37°C for 60 min. Collagenase was inactivated in an equal volume of α-minimum essential medium (MEM) medium enriched with 10% bovine fetal serum (FBS). After centrifugation (1200 rpm, 5 min, and 37°C), the cells were incubated in α-MEM enriched with 10% FBS and 1% penicillin-streptomycin. After 72 h with the replaced culture medium, the floating cells were removed, In addition to attaching MSCs to the flask's bottom. The cells were passaged upon reaching 80% confluence. According to the method described in our previous study, isolated

cells were characterized.[32] The experimental groups were as follows: control (cultured cells at passage 4 in medium containing 10% FBS), RE30–RE70 groups were cultured and treated with various concentrations of RE (30, 40, 50, 60, and 70 μg/ml) containing 40% carnosic acid (purchased from Hunan Geneham Biomedical Technology) for 48 h, as described by Izadi *et al*.[33]

RNA extraction from human adipose-derived stem cells and reverse transcription-polymerase chain reaction analysis

Reverse transcription-polymerase chain reaction (PCR) technique was used to evaluate the expression of *BDNF*, *GDNF*, *NGF*, neurotrophin-3 (*NTF3*), and neurotrophin-4 (*NTF4*) genes in hASC cells. RNA extraction was performed based on the RNX Plus kit. RNA concentration was measured with a spectrophotometer at 260 nm. Agarose gel electrophoresis was also used to check the quality of the RNA. The cDNA was fabricated according to the instructions of the Fermentas-K1622 kit. First, 1 μg of RNA (equivalent to 10 μl) and 1 μl of oligo primer were poured into the microtube and placed at 70°C for 5 min. Then 5× buffer, deoxynucleotide triphosphates (dNTP), and ribonuclease inhibiter were added to the above solution, and the solution was maintained at 37°C for 5 min. After that, reverse transcriptase enzyme was added to the solution, incubated for 60 min at 42°C, and finally exposed to 70°C for 10 min. PCR reaction (Cambridge, UK) was performed in 25 μl. To ensure the equal distribution of materials, the master mixture was prepared and then transferred to the microtube with oligo primer and cDNA. The program of the thermal cycler device was set as follows: 2 min for 94°C and 30 s for each 94°C, 58°C–58°C, and 72°C. At the end of 30 cycles, the mixture was exposed to 72°C for 10 min. The annealing temperature (Tm) was determined based on primers Tm. The primer Tm of *NTF3*, *NGF*, *BDNF*, and *NTF4* genes was 57°C, and the primer Tm of *GDNF* and *GAPDH* genes was determined to be 55°C. The *GAPDH* gene was used as the internal control gene. Gene expression and the PCR product were examined using electrophoresis with a 1.5% agarose gel to determine how much the targeted fragment was amplified. The gels were imaged with Image J software. The sequence of primers is shown in Table 1.

Statistical analysis

IBM SPSS V16 software (Chicago, IL, USA) was used for data analysis. The significance of differences between the groups was examined using the Kolmogorov and independent-sample *t*-tests, once the normal distribution of the data had been established. A statistical significance level of $P \leq 0.05$ was considered.

RESULTS

Cells morphology

Adherent hASCs at passage 4 with spindle or fibroblast-like shapes were observed [Figure 1a]. Images of hASCs postinduction with different concentrations of RE are shown in Figure 1b-f. Furthermore, the cell colonies formed after induction with doses of 50 and 60 RE can be seen in Figure 1d and e.

Evaluation of neurotrophin gene expression by reverse transcription-polymerase chain reaction

The agarose gel electrophoresis was used to examine the PCR results, as shown in Figure 2.

Neurotrophin genes were examined in cells treated with different amounts of RE for 48 h [Figure 3]. The intensity of *BDNF* gene expression was compared in the studied groups. None of the doses of RE affected the expression of the relevant gene, and no significant difference was observed between the groups. No significant difference was observed between the 40 and 70 μg/ml of RE. The expression intensity of the *NTF4* gene in cells induced with 40, 50, 60, and 70 μg/ml of RE showed a significant increase compared to control and RE30.

A significant difference was not observed in hASCS treated with 30 μg/ml of RE concentration compared to a control group. All the groups treated with RE showed a significant increase in *NTF3* gene expression compared to the control

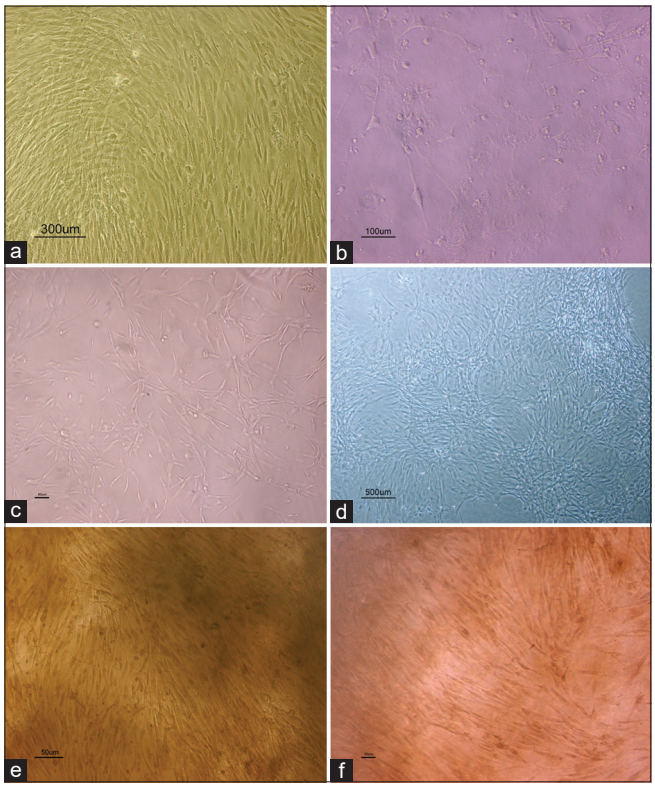

Figure 1: Morphology of human adipose-derived stem cells after induction with different doses of rosemary extract. (a) Cells cultured in medium containing bovine fetal serum. The cells treated with rosemary extract at doses of 30, (b) 40, (c) 50, (d) 60, (e) and 70 μg/μl (f) in a serum-free medium, respectively

Table 1: The primers used for reverse transcription polymerase chain reaction

Primer	Sequence	Primer size (bp)	Product size (bp)
NGF - F	5′-ATACAGGCGGAACCACACTCAG-3′	22	174
NGF - R	5′-GTCCACAGTAATGTTGCGGGTC-3′	22	
BDNF - F	5′-AGAGGCTTGACATCATTGGCTG-3′	22	167
BDNF - R	5′-CAAAGGCACTTGACTACTGAGCATC-3′	25	
NT-3 - F	5′-GGGAGATCAAAACGGGCAAC-3′	20	222
NT-3 - R	5′-ACAAGGCACACACACAGGAC-3′	20	
GAPDH - F	5′-GCTGGGGCTCATTTGCAGG-3′	19	258
GAPDH - R	5′-CGGAGGGGCCATCCACAGT-3′	19	
GDNF - F	5′-CACCAGATAAACAAATGGCAGTGC-3′	24	365
GDNF - R	5′-CGACAGGTCATCATCAAAGGCG-3′	22	
NT-4 - F	5′-CTGTGTGCGATGCAGTCAGT-3′	20	141
NT-4 - R	5′-TGCAGCGGGTTTCAAAGAAGT-3′	21	

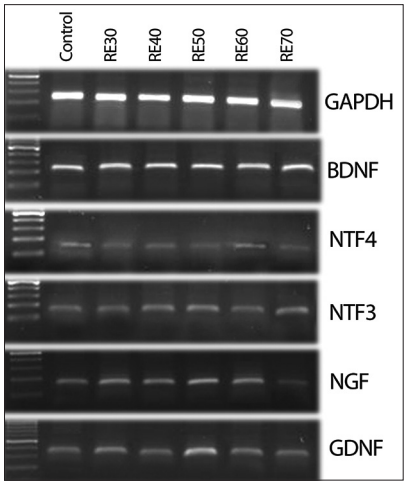

Figure 2: Agarose gel electrophoresis of polymerase chain reaction (PCR) products: Agarose gel electrophoresis image shown the PCR products analysis of brain-derived neurotrophic factor, NTF4, NTF3, nerve growth factor and *GDNF* genes. BDNF: Brain-derived neurotrophic factor, NGF: Nerve growth factor, RE: Rosemary extract

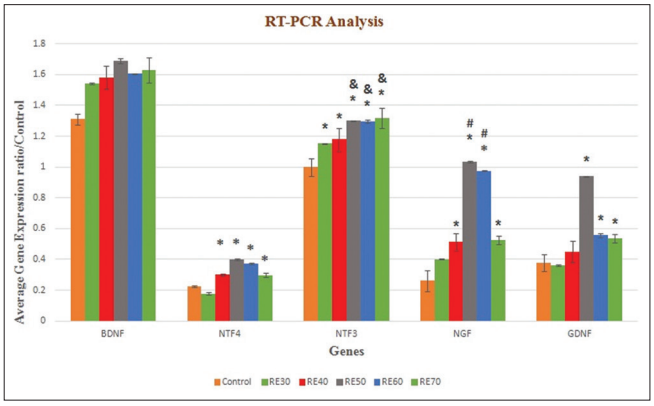

Figure 3: Comparison of brain-derived neurotrophic factor, *NTF4, NTF3, NGF,* and GDNF genes expression in human adipose-derived stem cells treated with different doses of rosemary extract (RE) for 48 h. Control: Cells cultured in medium with 10% fetal bovine serum; RE 30–70 groups: Cells treated with 30–70 μg/ml of RE respectively. RT-PCR: Reverse transcription-polymerase chain reaction, BDNF: Brain-derived neurotrophic factor, NGF: Nerve growth factor, RE: Rosemary extract, *: $P<0.05$ versus control, &: $P<0.05$ versus RE (30, 40), #: $P<0.05$ versus RE (30, 40, 70)

group. However, no significant difference was observed between the different concentrations of RE, including 50, 60, and 70 μg/ml; these groups showed a significant increase compared to 30 and 40 μg/ml of RE. RE with 40–70 μg/ml concentrations significantly increased *NGF* gene expression compared to control and RE30 groups. However, there was no significant difference in the expression of this gene between the control and RE30. The expression intensity of this gene in the cells exposed to RE at concentrations of 50 and 60 μg/ml showed a significant increase compared to other doses of RE. *GDNF* gene expression in hASCs exposed to RE at concentrations of 50–70 μg/ml showed a significant increase compared to the control group. Furthermore, no significant difference was observed between the RE30 and RE40 groups compared to a control group.

DISCUSSION

Recently, human adipose-derived adult stem cells due to their ease of extraction with minimal time, tissue damage and pain, high differentiation, and the possibility of autologous or allogeneic transplantation without fear of rejection,[9,16,17] as well as their ability to proliferate rapidly *in vitro* have considered being ideal for application in regenerative medicine. The effect of different concentrations of RE at 24 and 48 h on the proliferation rate and viability of hASCs was studied 48 h was reported as the most appropriate time by Izadi, *et al.*[33]

The average of living cells among the group for 48 h of cell exposure to RE at a concentration of 50 g/ml showed a significant increase compared to cells cultured in a medium containing serum. It can be reported that RE exhibited a significant trophic effect on hASCs compared to serum. In addition, the cells exposed to RE at concentrations of 50 and 60 μg/ml showed a significant increase in neurotrophic genes compared to cells cultured in a medium containing

serum. Because RE is a potent antioxidant from the mint genus, it has attracted the attention of researchers. Carnosic acid is abundant in rosemary leaves.[34] The antioxidant activity of RE is related to its carnosic acid, carnosol, and rosmarinic acid compounds.[35] In 2003, Kosaka and Yokoi added carnosic acid to the culture medium of human glioblastoma cells, resulting in increased synthesis of NGF in these cells. He used the extract to improve memory loss in Alzheimer's disease so that the extract was able to cross the blood–brain barrier, enhance NGF synthesis, and have a trophic effect on cholinergic neurons in the brain.[31] This is because NGF is a large protein that cannot cross the blood-brain barrier. In another study by the same researcher in 2010, the tumor cells in the adrenal medulla were induced by carnosic acid *in vitro,* and reported that carnosic acid stimulates these cells to secrete NGF.[30] In 2008, Ito *et al*. indicated that rosmarinic acid has similar effects as antidepressants. Both carnosic and rosmarinic acid could stimulate neuronal proliferation in the dentate gyrus of the hippocampus.[36] Carnosic acid was shown to have potent antioxidant activity with anti-mutagenic properties of bacteria and anti-cancer activity in various cellular and animal models. Some laboratory studies have shown that carnosic acid has an antiproliferative effect on leukemia cells and seems to be an effective treatment for cancer with other anti-cancer drugs.[37,38] In this study, the effect of different doses of RE on the expression of neurotrophin genes was investigated, and the results showed that none of the doses affected *BDNF* gene expression, and there was not a significant difference between treated and control groups. In 2008, Park reported that carnosic acid protects dopaminergic neurons exposed to the neurotoxin dieldrin (used to model Parkinson's disease) by stimulating BDNF secretion.[39,40] Another study revealed that the administration of carnosic acid intranasally enhanced BDNF and NGF secretion in the brain.[41] In this study, the NTF3 in treated groups with 30–70 μg/ml of RE significantly increased compared to cells cultured in the serum media. The expression of this gene in cells exposed to 50, 60, and 70 μg/ml concentrations of RE showed a significant increase compared to the other doses of RE.

In contrast, no significant difference was observed between the other treated groups. Therefore, the administration of RE at 50–70 μg/ml concentrations increased the gene expression in hASCs. RE with 40–70 μg/ml concentrations significantly increased the expression of NGF, NTF4, and *NTF3* genes compared to the control. In contrast, there was no significant difference between RE30 and control groups. Therefore, doses of 50–70 μg/ml of RE had a more influential role in neurotrophin gene expression than serum. The administration of RE at 50 μg/ml instead of serum can stimulate the expression of neurotrophin genes in hASCs so that it can provide a suitable cell source for cell therapy.

CONCLUSION

Because RE can cross the blood-brain barrier and stimulate the secretion of neurotrophins and it is well-known that neurotrophins are not able to cross the blood–brain barrier, so neurotrophins are available to damage neural cells and accelerate the healing rate in the brain in this treatment. Therefore, due to the properties of the extract, it can be used as a complementary method for treating neurodegenerative diseases. Using herbal medicines as stimulants of stem cell proliferation and differentiation in treatment may be cost-effective, widely available, and properly replace chemical drugs. There are many plant extracts used in traditional medicine worldwide. Compatible and qualified standard herbal medicines with apt effects and cell therapy can be used in most diseases. The administration of the extract (oral or injectable) will have a more significant therapeutic effect if combined with cell therapy. The extract can cross the blood–brain barrier, reach the site of injury, and prevent oxidative neuronal death by its antioxidant effect or by stimulating transplanted cells to secrete neurotrophic factors at the injury site. It may have a neuroprotective effect and provide an appropriate microenvironment for the neurons remaining at the injury site.

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Conflicts of interest

There are no conflicts of interest.

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