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ORIGINAL ARTICLE

Inhibitory Effect of Gamma-Secretase on the Survival Rate of Dental Pulp Stem Cells: An in Vitro Study

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Abstract

Objective: To evaluate in vitro the effect of gamma-secretase inhibition on the survival of dental pulp stem cells. **Material and Methods:** Sound teeth have been used. Dental pulp stem cells were isolated by enzymatic digestion. An appropriate number of cells were treated with different concentrations of gamma secretase enzyme (DAPT) (1, 3, 6.25, 12.5, 25.5, 37.5, 50 and 100 µM). The metabolic activity of cells and the distribution of cells in different phages of cell cycle was evaluated by MTT assay and flow cytometry, respectively. Statistical analysis was made one-way ANOVA. Comparison was made between the groups on the level of $p<0.05$. **Results:** In low concentration of DAPT (1, 3, 6.25, 12.5) the growth rate of the cells increases, whereas in high concentration of DAPT (25.5, 37.5, 50, 100) can significantly reduce the viability of the treated cells. The results also indicate that DAPT can interrupt the cell cycle in G1 phase. **Conclusion:** The DAPT for dose-dependent survival rate of dental pulp stem cells and affect cell population increase in the G1 phase of the cell cycle.

Keywords: Stem Cells; Dental Pulp; Receptors, Notch.

Introduction

Mesenchymal stem cells are primary, unspecialized cells extractable from various tissues and cells. They have the potential to differentiate and proliferate into different cell layers. The fundamental principles of reviving medical science are based upon stem cells [1].

The key to successful tissue engineering is to identify the appropriate source of stem cells. Due to high availability, less-invasiveness and interaction with biomaterials, dental pulp stem cells (DPSC) are the most effective cells for healing and regeneration of tissues, specifically dental defects [2]. These cells were first differentiated and identified in vitro by the enzyme digestion method [3]. DPSCs are multipotent stem cells of ectomesenchymal and neural crest nature with the phenotype of mesenchymal stem cells and may be differentiated into neuron, cartilage, osteoblast, heart cells and Islets of Langerhans β cells [4]. Notch signaling pathway play key roles in protection and function of stem cells and may inhibit or stimulate them [5]. Asymmetric proliferation of stem cells will assure differentiation and self-renewal of these cells and will be used in healing and regeneration of tissues and organs. In non-symmetrical division of stem cells, the Notch signaling pathway inhibits one of the daughter cells and activates the other. So, two types of cells are created with different functions [6].

Notch signaling pathway is an evolving protected ligand-receptor pathway, which plays an important role in completion and homeostasis of tissues [5]. In mammals, four Notch receptors (Notch 1- Notch 4) consisting of intracellular domains (NICD) and extracellular domains (NECD) and five ligands (Jagged1, Jagged2, [Delta-like] DLL1, DLL3 and DLL4) were identified. The mechanism of ligand-receptor occurs due to the activation of two types of enzymes, TACE/ADAM10 and γ-secretase, by which, the intracellular domain differentiates from the receptor and transfers into the nucleus and links to the protein that binds to DNA (RBP-Jκ / CBF1) and activates and transcripts the target genes [7,8].

The jagged ligand is expressed in the mesenchymal epithelial interactions with Notch 1-3 receptors in the epithelial and mesenchymal cells during the growth of teeth and differentiates between the DPSCs into cementoblast, periodontal ligaments and odontoblast [9]. The gamma secretase enzyme is a key enzyme in the Notch signaling pathway. The enzyme inhibition due to inhibitors distorts the function of the stem cells. DAPT is a beta-amyloid compound, which inhibits the gamma secretase enzyme [10]. Since this pathway affects physiological and pathological conditions of dental tissue significantly, in this paper the inhibitory effect of the gamma secretase enzyme (DAPT) on the vital activity of cells and cell cycles was analyzed.

Material and Methods

Extraction and Culturing the DPSCs

This empirical study was made in the embryology and stem cells laboratory of the Anatomical Sciences Faculty of Ardebil University of Medical Sciences [11]. Dental samples were sliced after washing by phosphate buffer saline (PBS) and ethylic alcohol 70%. Thereafter, the slices

were incubated in a temperature of 37°C and 5% CO2 exposed to collagenase enzyme type 4 (Invitrogen).

After digestion of pulp tissue, to inactivate the collagenase enzyme, the culture medium of αMEM (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), two times the enzyme volume, was added, consisting of fetal bovine serum (FBS)(Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), 100 u/ml penicillin (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), 2.5µg/ml amphotericin (Sigma-Aldrich, Darmstadt, Germany), l00 u/ml Streptomycin (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), Glutamax 1% (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) and nonessential amino acid 1% (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). The liquid was accumulated in a 15mm falcon tube to be centrifuged for 10 min with 1800 rpm. The obtained cells were plate in 25cm2 flasks containing full culture medium, and then incubated by an incubator. The culture medium was changed once/48hr. Afterwards, flask floors were filled and the cells were passaged in a ratio of 1 to 2. Fourth-passaged cells were used in the following stages.

Cell Treatment and Metabolic Activity Evaluation

Cells were treated by DAPT concentrations of 1, 3, 6.25, 12.5, 25, 37.5, 50 and 100 µmolar and were studied 48 hr later. Metabolic activity of cells was measured by MTT method. In this method, first 500 cells were cultured in 96-well plates and incubated for 24 hr. After incubation, the cells were treated by aforementioned concentrations of DAPT. 48 hr later, the medium of wells was replaced with 200µl culture medium containing Methyl Thiazol Tetra Zolium- MTT (Sigma-Aldrich, Darmstadt, Germany) with the concentration of 5mg/l of PBS and incubated for 4 hr. After incubation, to dissolve formazan crystals and create colors, 200 µl solution of disufoxid methyl (DMSO) (Sigma-Aldrich, Darmstadt, Germany) was added and the photoabsorption was read by a Synergy HT (BioTek Instruments Inc., Winooski, VT, USA) ELISA-reader in the wavelength of 540 nm.

Cell Cycle Evaluation by Flow Cytometry

To make flow cytometry, 2x104 cells with a concentration of 25.5 µmolar DAPT were treated in 25T flasks. After 48 hr, in order to remove cells from the container's floor, trypsin enzyme was added (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA). The obtained liquid was centrifuged two times and the supernatant was removed. Four milliliters of ethanol 4% was added gradually to fix the cells and stored for 4 hr in 4°C. Cells were centrifuged by 3000 rpm for 5min and washed by PBS. Thereafter, DAPI (4',6-diamino-2phenylindole) solution was added to cells by nylon mesh filter and the cell distribution trend was analyzed by a flow cytometer (Sysmex Partec GmbH, Görlitz, Germany).

Data Analysis

The FloMax software was used to investigate the percentage of G1, S and G2/M-phased cells. Statistical analysis was made by SPSS 16 and one-way ANOVA. Comparison was made between the groups on the level of $P<0.05$. The results were reported in mean ± SD.

Results

Extraction and Culturing of DPSCs

Differentiation of cells was performed by enzyme digestion method. Cells had fibroblast-like and needle-like morphologies, illustrated in Figure 1.

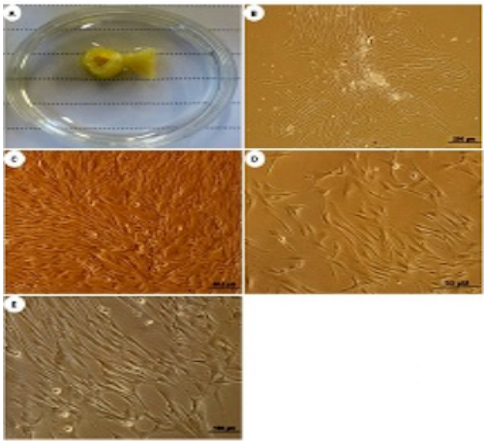


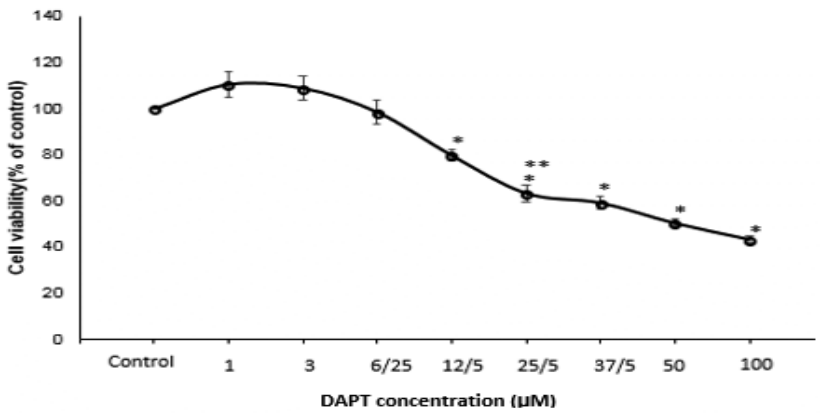
Figure 1. Stem Cells extracted from the DPSCs. Cells grow as fibroblast-like colonies.

The Effects of DAPT on DPSCs by MTT Assay

This test relies on conversion of Tetra Zolium dissolved salt into un-dissolved crystals of formazan by mitochondrial dehydrogenase succinate enzyme. The precipitation that is obtained by dissolving these crystals with DMSO is purple. Color intensity is related to viable cells. The results showed that when the concentration of DAPT was 1 and 3 µmolar, cell growth rate was 110±5.4 and 108±5.2, respectively, compared to the control group, which was not statistically significant. With a higher DAPT concentration, cell growth rate diminished; so, with the concentration of 6.25 and 12.5, the cell growth rate decrease was not statistically significant compared to the control group. With higher concentrations (25, 37.5, 50 and 100 µmolar), the cell growth rate decrease was, respectively, 64± 3.7, 59±2.6, 50±1.5, and 43±1.5, which was statistically significant on the level of $p<0.05$ and $p<0.001$. Cell growth curve is shown in Figure 2.

Flow Cytometry

Flow cytometry is an appropriate method for determination of percentage of G1, S, and G2/M-phased cells. In this regard, use is made of flow-chromes like DAPI, which are able to penetrate the nucleus and bind to the DNA. The results showed that the cell diffusion in phase G1 was 50±2.7 for the treatment group with a DAPT of 25.5µmolar, which is higher than the control group (44±0.6). However, cell population distribution in phase S was significantly decreased in the treatment group on the level of $p<0.05$. The results are illustrated in Figures 3 and 4.



*Statistically significant difference compared to the control group (p<0.001); **Statistically significant difference compared to the concentration of previous group (p<0.05).

Figure 2. Effects of various DAPT concentrations on cell viability of DPSCs by MTT. The results for treatment and control groups are shown as curves.

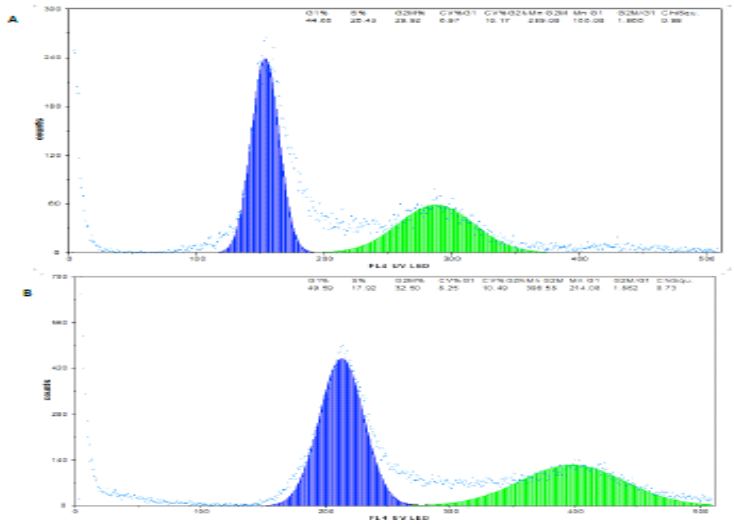
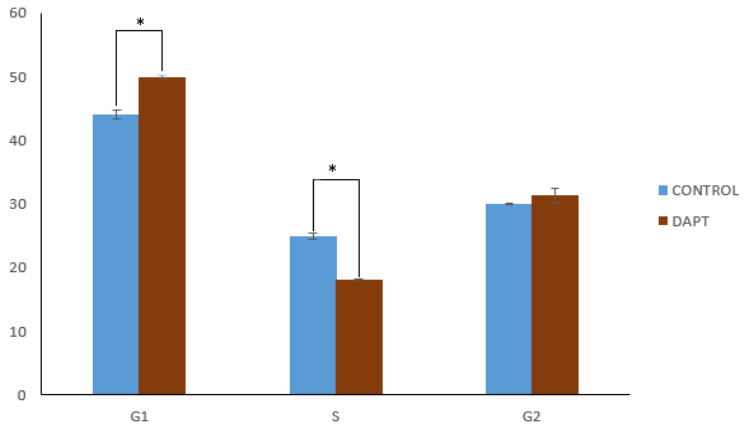


Figure 3. Effects of DAPT on DPSCs by flow cytometry. The peak areas show, respectively, the percentage of G1, S and G2/M-phased cells. The control and treated groups are shown in A and B, respectively.



*Statistically significant difference compared to the control group (p<0.05).

Figure 4. Cell distribution percentage in the cell cycle by flow cytometery. In this graph the cell population percentage is shown along with DAPT in control and treatment groups.

Discussion

In recent years, there has been a significant increase in the stem cell studies. From among the tissues that are rich in stem cells with high availability, dental tissues can be mentioned which are suitable for tissue engineering. Mesenchymal stem cells, found in the dental tissue, include DPSCs, dental papillae, dental follicle and periodontal ligament. These cells can be differentiated and purified and grow under specific culturing conditions and be used in tissue engineering, especially, for regeneration of teeth, nerves and bones [12].

Mechanism like induction of apoptosis, stimulation of immune responses and dental physiological transformations, which cause dental damages, would induce differentiation of DPSCs to odontoblasts and periodontal ligaments [9]. A major factor for function adjustment and differentiation and proliferation of stem cells is Notch signaling pathway. Any changes in the Notch signaling pathway will distort the functions of stem cells. An important enzyme in this pathway is gamma secretase which may be affected by inhibitors and induce changes in the Notch signaling pathway. So, the function, growth and differentiation of the cells will be affected by these inhibitors [13]. Therefore, a better understanding of the adjustment method of pulp stem cells in pathological situations will be definitely helpful for dental treatment, restoration and regeneration.

In this study, the inhibitory effects of secretase enzyme (DAPT) on DPSCs were investigated in vitro. In order to assess the cell growth and the metabolic activities of DPSCs, MTT was used. After treatment with low concentrations (1, 3, 6.25, 12.5μmolar), cells showed 10% growth, which was not statistically significant. With high concentrations (25.5, 37.5, 50 and 100μmolar DAPT), the growth and metabolic activity of the cells reduced to 50% of control group. Therefore, DAPT affects cell growth and proliferation in a dose-dependent manner. In a study on Notch pathway inhibition for DPSCs, it was shown that stimulation of Notch signaling pathway with jagged-1 ligand would inhibit cell differentiation into odontoblast [9]. In the same light, in their study, a researcher showed that over-expression of NICD in the pre-osteoblastic cells of MC3T3 and mesenchymal cells of ST-2prevented from osteoblastic differentiation [14].

Flow cytometry not only is able to determine normal, apoptotic and necrotic cells, it is also an appropriate instrument for determination of the cell distribution percentage in various cell phases [15]. Graphs and calculations showed that by treatment with DAPT, cell distribution will increase 50% in G1 phase and reduce 18% in S phase. Results comparison showed that DAPT's effects on cell cycle and cell inhibition in a certain phase will inhibit growth and proliferation of DPSCs. In their study on the effects of ligand Delta1-RNA interference on DPSCs by flow cytometry method, showed that cell proliferation decreases in S phase [16]. Damages to cell DNA inhibit the cell cycle and affect its function. These will often harm the DNA telomerase in replication. Shortening of telomerase will decrease the viability of the cell and it is aged prematurely; so, the stem cells would not function appropriately in restoration and regeneration [17]. Quantitative analysis of genes involved in this pathway remains for future studies.

Conclusion

The results showed that gamma secretase enzyme (DAPT) affects the viability of dental pulp stem cells in a dose-dependent manner and causes increase in cell population in phase G1 of the cell cycle.

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Conflict of Interest: The authors declare no conflicts of interest.

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