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Research Article

Isolation and Characterization of Human Dental Pulp Stem Cells and Their *In Vitro* Differentiation into Odontoblast and Nerve-Like Cells

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Abstract

Objective: The aim of this study was to isolate and characterize human dental pulp stem cells and to induce their *in-vitro* differentiation into odontoblast and nerve-like cells.

Materials and methods: Human dental pulp tissues were collected from impacted third molars immediately after extraction. The collected pulp tissues were then enzymatically digested using 0.2% collagenase type II to release the primary total pulp cells. The obtained cells were cultured in DMEM supplemented with 15% FBS and antibiotic mixture. Isolation of STRO-1+ cells was done using immuno-magnetic separation. STRO-1+ HDPSCs were induced to differentiate into odontoblast-like cells using Dexamethasone/ vitamin D3 and into nerve-like cells using a neurogenic induction cocktail.

Results: STRO-1+ cells were successfully isolated and were induced to differentiate into odontoblast and nerve-like cells, confirmed by immuno-staining.

Conclusions: It was found That STRO-1+ HDPSCs are capable of extensive proliferation and multi-lineage differentiation into more specialized cell types.

Keywords: *Dental pulp; Dentin-pulp complex; Dental pulp stem cells; Characterization; Odontoblasts; Odontogenic differentiation; Nerve-like cells*

Abbreviations: DMEM: Dulbecco's modified eagle's medium; FBS: Fetal bovine serum; STRO-1: Stromal cell marker; HDPSCs: human dental pulp stem cells' DPSCs: dental pulp stem cells; MSCs: mesenchymal stem cells; CD-146: cluster of differentiation-146; PBS: Phosphate buffered saline; CO_2: Carbon dioxide; EDTA: Ethylene Diamine Tetra-acetic acid; DAPI: 4,6- diamidino-2-phenylindole dihydrochloride; MACS: magnetic-activated cell sorting/selection

Introduction

Human organs and tissues are made up of about 200 kinds of cells that develop from stem cells. Stem cells are undifferentiated cells with the ability to differentiate into any tissue type in order to carry out specific tasks. Currently, two approaches are being investigated for reconstructing teeth using cell culture procedures. One approach is to seed tooth germ cells into tooth-shaped scaffolds made of biodegradable materials. The other approach is to reconstitute teeth by re-aggregating tooth germ from dissociated single epithelial cells and mesenchymal cells [1]. Human dental pulp tissues contain stem cells called "dental pulp stem cells" (DPSCs). DPSCs are quiescent and reside in a specific peri-vascular microenvironment where they maintain their stem cell characteristics. DPSCs show multi potential differentiation ability, which is similar to that of mesenchymal stem cells (MSCs). These DPSCs express MSC markers, including Stro-1 and CD-146, and undergo colony forming *in vitro* and can regenerate the dentin/pulp complex *in vivo*. Moreover, cultured primary pulp cells

can be induced to differentiate into odontoblast-like cells and generate dentin-like mineral structure in culture dishes [2]. Therefore, the aim of this research was to investigate the *in-vitro* regeneration potential of the cellular elements of the dentin pulp complex to regenerate hard dental tissues and neural elements.

Materials and Methods

Isolation of human dental pulp stem cells (HDPSCs) from impacted human third molars

Collection of teeth

All the methods of this study has been granted by the ethical committee of the faculty of oral and dental medicine, Cairo university as well as the review board of the department of endodontics in the faculty. All patients signed an informed consent by which they agree to give away their impacted third molars immediately after extraction, to contribute to the research.

Selection criteria of teeth (Sample inclusion criteria)

Human impacted third molars [n=10] were collected from patients [n=8], age ranged (19-26), the inclusion criteria for the teeth were as follows;

Fully impacted third molars, with no communication to the oral cavity.

The teeth show no signs of cavitation (caries-free).

The teeth shouldn't be included in pathologic lesions.

The collected teeth were kept immediately after extraction in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) supplemented with Penicillin/streptomycin (Gibco BRL) mixture and 15% fetal bovine serum (FBS). The teeth were then split open by creating vertical grooves using a high speed hand piece with generous cooling, followed by hammering the tooth at the grooves using a chisel and mallet. The pulp was carefully removed and then kept in DMEM [3,4].

Preparation of collagenase digesting solution

0.2% type II collagenase (Worthington) was adjusted in Phosphate Buffered Saline (PBS, Sigma-Aldrich) [4].

Isolation of primary total pulp cells

All cell culture procedures were done in a sterile environment, in a biological safety cabinet. The Collected pulp tissue was washed in PBS. Tissues were then moved to a dish and carefully dissected into small pieces using sterile blade. The dissected pulp tissue was then moved to a polypropylene tube and washed again with PBS. It was then centrifuged in a cooling centrifuge at 500 rpm for 2 minutes at 4°C.

The supernatant was discarded and the previously prepared 0.2% collagenase solution was then added to the pulp tissue. The tube was then put in a 37°C water bath with shaking in the water bath shaker for 30 minutes. After 30 minutes the tube was centrifuged at 500 rpm for two minutes at 4°C. The supernatant -now containing the digested pulp cells- was then moved to another polypropylene tube and then centrifuged at 1500 rpm for five minutes at 4°C. The supernatant was discarded and fresh DMEM media supplemented with 15% FBS, was added to the cells. The tube, now containing the primary total pulp cells was then centrifuged at 1500 rpm for 5 minutes. Cells were transferred into a 3.5 inch culture dish and incubated at 37°C in 5% CO_2. Cells were monitored everyday using inverted phase contrast light microscope with digital camera [4].

Detachment

On time of passaging, the cells in each culture dish were treated with 0.5% Trypsin/EDTA for few minutes with gentle tapping [4].

Characterization of the isolated human dental pulp stem cells (STRO-1+ cells)

To obtain STRO-1+ stem cells, DPSCs were indirectly sorted using immuno magnetic beads [5]. Cells were incubated with mouse anti-human STRO-1 supernatant (R&D systems, Minneapolis, MN) at 4°C for 30 minutes, washed with PBS/5% FBS, and re-suspended with rat anti-mouse IgM-conjugated Dynabeads (Invitrogen technologies) on a rotary mixer for 60 minutes at room temperature.

Differentiation of human dental pulp stem cells, *In-vitro*, into odontoblast-like cells, using dexamethasone and 1,25- dihydroxy vitamin D3

The characterized human dental pulp stromal cells (STRO-1 + cells) were grown to 70%-80% confluence, passaged and seeded into well plates in the previously mentioned culture medium (DMEM) supplemented with 10% FBS and Penicillin streptomycin mixture. The media was changed three times a week.

Odontogenic differentiation were induced by 100 nM dexamethasone (Sigma), 10mM β-glycerophosphate, 50 mg/mL ascorbate phosphate, 5x10-8 M 1, 25- dihydroxy vitamin D3 (1, 25 $(OH)_2$ D3) (Sigma) into the culture medium for 7 days. At the end of the induction period, cells were washed three times with PBS and fixed in 70% ethanol for 45 minutes at 4°C followed by washing with water [3].

The cultures were then stained with 2% Alizarin red S (Sigma-Aldrich) (pH 4.0) for 5 minutes at room temperature to stain the extracellular matrix [3], and then washed. Evidence of mineralization was examined using phase contrast inverted microscope.

Differentiation of human dental pulp stem cells, *In-vitro*, into nerve-like cells, using neurogenic induction cocktail

Cells were incubated in the neurogenic induction cocktail composed of Neurobasal A (Invitrogen technologies), B27 supplement (GIBCO-BRL), 20 ng/mL epidermal growth factor (Lonza, USA), and 40 ng/mL fibroblast growth factor (Lonza, USA). After 4 weeks, cells were analyzed by immunofluorescence for the expression of the neural cell marker, βIII –tubulin by the addition of monoclonal mouse anti-human βIII-tubulin antibodies (Promega, Madison, W.I) for 1 hour at room temperature [6].

Cultures were incubated with anti-mouse Alexa fluor 594 for 1 hour at room temperature and the cell nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain, and examined under a fluorescence microscope.

Results and Discussion

Isolation of the human dental pulp stem cells (HDPSCs)

Following the isolation and culturing procedures, Cells initially appear having a rounded floating appearance (Figure 1, A and B).

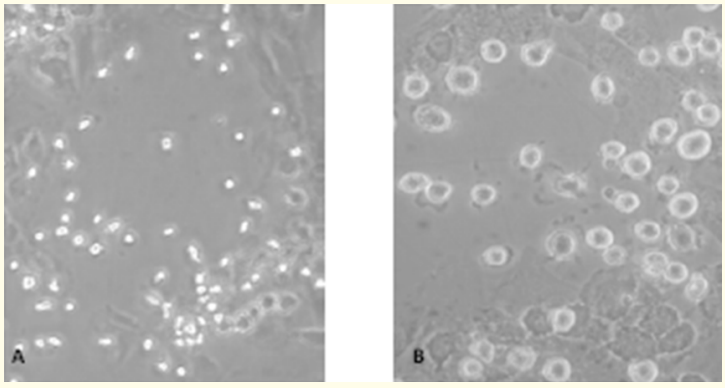

Figure 1: *(A) Isolated primary total pulp cells. [Original magnification 20x].*
(B) Isolated primary total pulp cells. [Original magnification 40x].

After 7 days, the cells appeared with various morphologies, some appeared spindle like and others with fibroblastic like appearance thus reflecting the morphological diversity of the pulp cells (Figure 2).

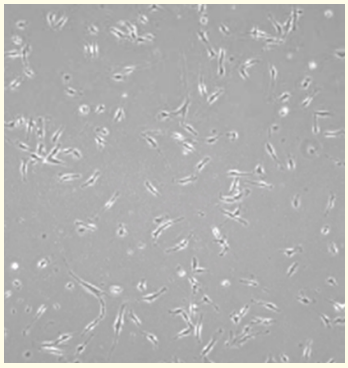

Figure 2: *Isolated human dental pulp stem cells (HDPSCs) after incubation in CO_2 incubator for 7days, observed under phase contrast inverted microscope.*

The cells were successfully passaged up till 9th passage. Some displayed enlarged cell bodies and elongated appearance with one or two long cellular processes maintaining their fibroblastic and spindle like appearance up till 90 days (Figure 3).

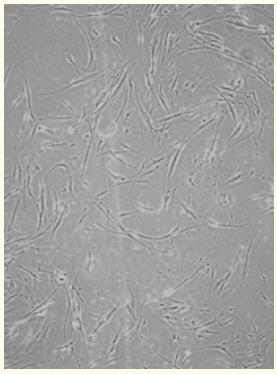

Figure 3: *Isolated human dental pulp stem cells (HDPSCs) after incubation in CO_2 incubator for 21 days, observed under phase contrast inverted microscope.*

Characterization of the isolated human dental pulp stem cells (STRO-1+ cells)

To characterize the human dental pulp stem cells, Immuno-magnetic cell separation (magnetic activated cell selection - MACS) using STRO-1 marker is used to separate the HDPSCs from the primary total pulp cells.

In the 1st passage, there are multiple, positively isolated STRO-1+ cells, having small cell bodies and multiple cellular processes (Figure 4-A), while in the 9th passage, there are multiple positively isolated STRO-1+ cells having small cell bodies and multiple long processes anastomosing with one another (Figure 4-B).

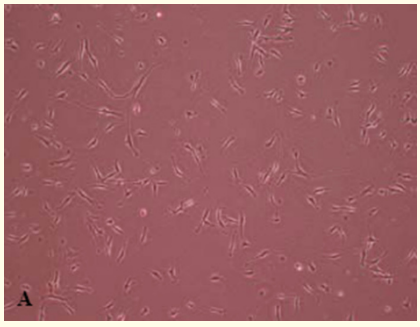

Figure 4A: *Multiple positively isolated STRO-1+ HDPSCs at passage 1 (p1) having small cell bodies and multiple short processes, as examined under phase contrast inverted microscope (original magnification X20).*

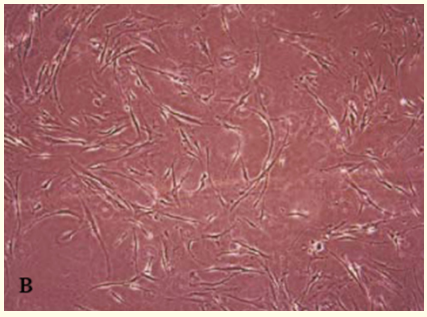

Figure 4B: *Multiple positively isolated STRO-1+ HDPSCs at passage 9 (P9) having small cell bodies and multiple long processes anatomizing with one another, as examined under phase contrast inverted microscope (original magnification X20).*

Differentiation of human dental pulp stem cells into odontoblast-like cells, *in-vitro*, using dexamethasone and vitamin D3

Alizarin red stain was used to detect odontogenic differentiation of the isolated STRO-1+ human dental pulp stem cells into odontoblast-like cells and displayed calcified nodules (Figure 5).

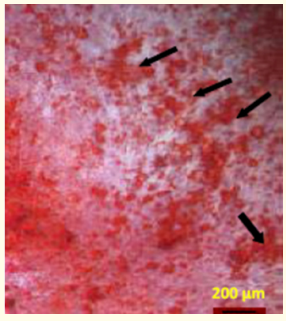

Figure 5: *HDPSCs were stimulated to differentiate into odontoblast like cells, and lay down calcium deposits (black arrows) stained red, using alizarin red stain.*

Differentiation of human dental pulp stem cells into nerve-like cells

Cells under neurogenic induction underwent morphological changes, where the DPSCs possessed spindle-shaped fibroblastic cell bodies with mainly long cellular processes. A subpopulation of these cells expressed the neurogenic marker βIII tubulin.

After washing, cultures were incubated with anti-mouse Alexa Fluor 488 for 1 hour at room temperature and the cell nuclei stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI stain).

Images analyzed under fluorescence microscope, revealed multiple bipolar and multipolar cells, having multiple long processes. The cellular extensions appeared red-stained (due to fixation of anti- βIII tubulin antibodies) while the nuclei appeared blue-stained (positive chromatin staining) (Figure 6).

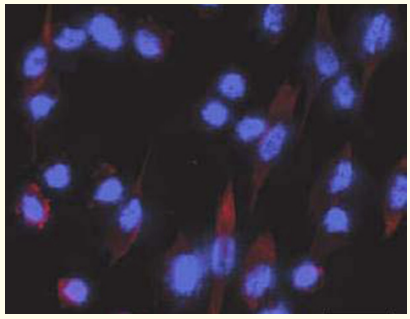

Figure 6: *Multiple bipolar and multi polar nerve-like cells with ling anastomosing cellular processes. Red: β III- tubulin, Blue: DAPI [Scale bar: 30 μm].*

Discussion

In the present study, dental pulp was used as a source to obtain stem cell, where healthy human third molars indicated for extraction due to impaction were chosen. These sources are easily available from discarded teeth after extraction [7].

Other studies have used deciduous teeth as a source of stem cells identification. However, in the present study we intended to use permanent teeth as a source for mature stem cells, since deciduous teeth are only present only till 12 years of age, whereas, most dental conditions requiring interference occur during adulthood when deciduous teeth are no longer present [8-10].

Another reason that hindered our use of the deciduous teeth is that by the age of shedding, most of the deciduous teeth are either, pulp treated or badly decayed, due to reduced level of dental care in Egyptian population [11].

As dental caries are a source of pulpal contamination leading to damage of the culture medium, the teeth with caries were excluded from the study. In this study, we used cow horn forceps for pulp removal, which caused minimal trauma; as the forceps' edges do not touch each other; the pulp does not crushed when the tooth breaks into pieces. We tried to get undamaged pulp tissues by making a vertical groove on the tooth to guide the forceps to form a clear cut fracture without damaging the pulp tissues [12].

This study was conducted using fetal bovine serum (FBS) as a supplement to culture media since cells are unable to proliferate without essential growth factors, hormones and nutrients that are present in the serum [13,14].

Enzyme digestion method was applied to isolate and characterize pulp cells grown in vitro. The use of Trypsin: EDTA dissociation combined with subsequent culture in Dulbecco's modified eagle's medium (DMEM) provided a better approach to obtain viable cells which exhibit a relatively high proliferative capacity.

In the present study, fully spindle-shaped cells were observed 2 weeks after cellular culturing. Also, spindle-shaped fibroblasts were present in the culture medium that attached to the flask floor, which were eliminated during passage or washing procedures as they came to the medium surface because of weight differences. This was in accordance with the findings of [15].

Certain aspects of our observation on the morphological characteristics of HDPSCs grown in culture dishes were consistent with those reported by other investigators [16,17] in that, after isolation and incubation in CO_2 incubator, cells began to attach to the bottom of dishes, form colonies and they show stellate or spindle shape morphology on microscopic examination.

Characterization of the isolated HDPSCs via separation of STRO-1+ cells from the isolated human dental pulp specimens by means of indirect magnetic activated cell separation (MACS).

STRO-1 recognizes a trypsin-resistant cell-surface antigen present on stromal progenitor cells. Previous studies reported that a proportion of 9.98% to 20% for STRO-1-positive cells was present in the enzymatically released cell culture of human dental pulp, using flow cytometric analysis [18,19]. Our data indicated that the isolated population of HDPSCs expressed MSC marker (STRO-1)

In our study, investigations involved mesodermal as well as ectodermal differentiation capacity of HDPSCs, in the form of differentiation into odontoblast like cells and nerve-like cells respectively. The ability of stem cells to differentiate into various cell types was previously investigated, where they were differentiated into adipocytes, muscle cells and osteoblasts [20-23].

The potential of HDPSCs to differentiate into odontoblast-like cells was done by testing and confirming their ability to form mineral nodules in cultures following stimulation with hard-tissue promoting agents; dexamethasone and 1, 25 $(OH)_2$ vitamin D3.

Dexamethasone promotes recruitment, proliferation and differentiation of mesenchymal stem cells into odontoblasts while 1, 25 $(OH)_2$ vitamin D3 mediates the deposition of minerals to form mineralized nodules *in vitro*, or hard tissues *in vivo* [24].

Alizarin red stain [19] used to confirm successful formation of calcified nodules represented by the positive red stained areas. This agrees with the previous work done by other authors who used Alizarin red stain to confirm the effect of dexamethasone and vitamin D3 in odontogenic differentiation of dental pulp stem cells [24].

As regards the differentiation of HDPSCs into nerve-like cells, the isolated HDPSCs were differentiated into cells expressing antigens that are indicators of glial and neuronal differentiation (βIII tubulin) as demonstrated by immuno histochemical staining.

With regards to the contents of dental pulp, it is obvious that odontoblasts and nerve cells are the most commonly differentiated cell types, therefore, when comparing our study to recent literature, testing the multipotent ability of dental pulp stem/progenitor cells was in accordance with many recent studies [12,26,27].

Conclusion

Human dental pulp stem cells [HDPSCs] have peculiar multi-lineage differentiation ability. They can differentiate into hard tissue-forming cells [odontoblast-like cells] and nerve-like cells, thus confirming their ability to regenerate two of the major components of the dental tissue.

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Conflict of Interest

The authors deny any financial or administrative conflict of interests.

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