Original Article

Role of Pyrrolidine dithiocarbamate in inducing odontoblastic differentiation of dental pulp stem cells

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Abstract

Background: Pyrrolidine dithiocarbamate (PDTC) is an efficient, reproducible, and biological antioxidant of clinical utility, which may also be preferred for obtaining human dental pulp stem cells (hDPSCs) for the purpose of tissue engineering and regenerative medicine.

Aim and Objectives: The study was conducted to evaluate the effects of PDTC on the propagation and differentiation of hDPSCs.

Materials and Methods: hDPSCs were isolated by explant culture method and characterized for stem cell properties using flow cytometry method. The effects of PDTC-induced odontoblastic differentiation of hDPSC at different concentrations (0.5, 1.0, and 5.0 mM) were determined by staining for mineralization.

Results: Mineralization was more prominent and significantly higher in the induced hDPSCs treated with 1.0 mM concentration of PDTC.

Conclusion: PDTC at 1.0 mM concentration could have a significant pharmacological role in activating and enhancing odontogenic differentiation of dental stem cells and make it an important step in regenerative dentistry.

Keywords: Differentiation; human dental pulp stem cells; isolation; odontoblast; pyrrolidine dithiocarbamate

INTRODUCTION

Human dental pulp stem cells (hDPSCs) are a heterogeneous population of cells isolated from the tooth pulp.[1] Pulp tissue contains many undifferentiated mesenchymal cells, which retain the ability to differentiate into mature cells. Induced pluripotent stem cells have been developed from various cell sources, including dental pulp-derived stem cells, and evaluated for potential application to regenerative therapy.[2] hDPSCs have been isolated as adherent mononucleated cells with *in vitro* differentiation capacity toward several lineages, including osteoblasts, adipocytes, and neural cells. Differentiated odontoblasts establish a structure known as the dentin-pulp complex; therefore, these cells have been proposed as tools in dental regeneration and repair.[2]

Among various sources of mesenchymal stem cells (MSCs), deciduous teeth dental pulp stem cells (DTSCs) are a potential source as they are easily obtained, can be cryopreserved and have higher expansion rate, and wider *in vitro* and *in vivo* differentiation potential than those isolated from bone marrow and adipose tissue. Although their differentiation potential is wide, their present clinical use is limited to case reports in craniofacial bone regeneration.[3] Wider clinical use of DTSCs is limited with

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small number of cells obtained after extraction of one tooth, ranging from 0.03 to 0.3 × 10^6 cells after 10–15 days of cultivation of pulp explants.[4] Majority of studies report the local use of 0.2–5 × 10^6 cells that should be available in a short time.[5] the microenvironment that stimulates cell proliferation would have clinical usefulness.

Types of stem cells in regenerative dentistry can be stem cells from exfoliated deciduous teeth (SHED) or MSCs. DPSCs and SHED express MSC markers.[1] Although both cell types had calcium deposits, DPSCs presented a higher alkaline phosphatase (ALP) activity level, seen by measuring amount of ALP in the blood.[2] In addition, DPSCs showed higher levels of osteogenic and odontogenic differentiation. These results suggest that DPSCs are closer to the phenotype of odontoblasts than SHED and may improve the efficacy of human dental tissue-derived MSCs therapeutic protocols.[6]

The aim of this study is to evaluate isolation, characterization, and differentiation of hDPSCs into odontoblasts using pyrrolidine dithiocarbamate (PDTC).

MATERIALS AND METHODS

The collection of samples started after obtaining the ethical clearance from the institutional committee with reference number IC-SCR/RM08/18. Before starting the procedure, informed consent was taken from all the participants to collect their extracted teeth. After collection of the teeth, they were sectioned under sterilized conditions using carborundum disc under saline irrigation to extract the pulp from them. After sectioning, the pulp tissue was removed carefully using tweezers and transferred to the regenerative laboratory in vials containing phosphate-buffered saline.[7]

Explant culture method was used for the isolation of hDPSCs.[7] Smaller fragments of the tissue were cut following which these pieces were put in the culture flask which contained complete culture media consisting of Dulbecco's Modified Eagle Medium (DMEM) along with a supplementation of 20% fetal bovine serum (FBS). The dishes were then incubated at 37°C in a humid incubator containing 5% CO_2. The nutritive medium was replaced with fresh media every 2 days. The cells were checked for outgrowth under a microscope. The cells which showed the differentiation were further used for the study.[7]

Colony-forming unit assay was then performed for checking the clonogenic ability of hDPSCs. Culture plates well in culture media (DMEM with 10% FBS) were used to inoculate the cells. Post 7 days wait during incubation, crystal violet was used to stain the wells.

After these steps, to determine the proliferative capability of hDPSCs, cells from individual samples were then inoculated into well cell culture dishes. Cell counting was done for 14 days. The growth of cells was checked by plotting growth curve using the cell numbers.[7]

In the next step, characterization of hDPSCs was checked using flow cytometry. On incubation for 30 min with CD106 and CD34 antibodies, the cells were washed with phosphate-buffered saline and later were analyzed on the flow cytometer. The isotype control was correlated with the degree of positive staining. For each lineage of cells, the cell differentiation potential was evaluated in three replicates.[7]

Odontogenic differentiation

PDTC at different concentrations (0.5, 1.0, and 5.0 mM) was introduced to an osteogenic induction medium composed of α-MEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL L-ascorbic acid, 10 mM β-glycerophosphate, and 10 nM calcitriol (1α,25-dihydroxyvitamin D3), along with 10 nM dexamethasone. The incubation of cells was done for 14 days, and new media change was done for every alternate day, till the end. Post 14 days, the cells were fixed using 4% paraformaldehyde which was followed by staining for mineralization with 2% alizarin red S (pH, 4.1–4.3). Then, the quantification of alizarin red S-stained odontoblasts was performed by dissolving stained cells in 4% NaOH. The dissolved stain was again read spectrophotometrically at 450 nm [Figure 1] using phase-contrast microscope (Olympus CKX53, Japan).[8]

Statistical analysis

The values of all the data were shown by the way of mean ± standard deviation. The experimental groups were then compared with each other using one-way analysis of variance test.

RESULTS

MSC markers expressed CD106, but the expression of CD34 was observed to be negative in cultured

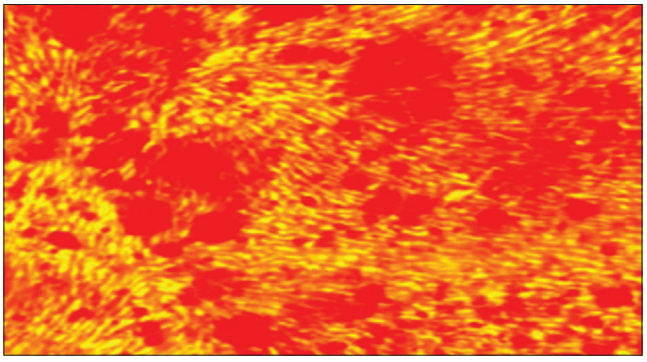

Figure 1: Odontogenic differentiation seen under phase-contrast microscope

hDPSCs. The hDPSCs from the samples showed the ability to differentiate into adipocytes, osteoblasts, and chondrocytes. Treatment of hDPSCs with different concentrations of PDTC showed a decrease in the cell metabolites. However, a slightly higher dose of 1.0 mM showed a significant increase in the cell metabolism. The cell proliferation was found to be slower at a higher concentration of PDTC (5 mM). It was observed that the mineralization was more prominent and significantly higher in the induced HDPSCs treated with 1.0 mM concentration of PDTC [Figure 2].

DISCUSSION

Stem cells originating from tooth pulp have proven their importance for utilization in a variety of clinical uses.[6,8] It is extremely useful to investigate the factors that can regulate the biological behavior of hDPSCs. For clinical usage, there should be a source of MSCs which should be easily available and can be accessed with high content of totipotent stem cells.[9] Dental pulp is one unique tissue that fulfills all these criteria, and therefore, it is preferred for stem cell regenerative therapy.[7,9,10]

The source of cell therapies is from biological sources, such as MSCs. These cells can then be manipulated in a laboratory to develop the required cell therapy product, for example, dentin-like tissues.[10-12] Even with their clinical and commercial promise, novel cellular therapies are recent technologies that have not attained widespread clinical utility or commercial viability.[7]

Stem cells show lesser cell survival and increased rate of cellular death either during differentiation in a laboratory or after transplantation of cells at the ischemic areas. The production of reactive oxygen species (ROS) because of continuous oxidative stress throughout the differentiation process and in injured tissues is one of the main hurdles for the success of this cell therapy.[13,14] PDTC is a precursor of biological antioxidants whereas GSH (Glutathione) functions as a strong inhibitor of ROS.[15,16] The effects of these on the differentiation of HDPSCs still remain unidentified.

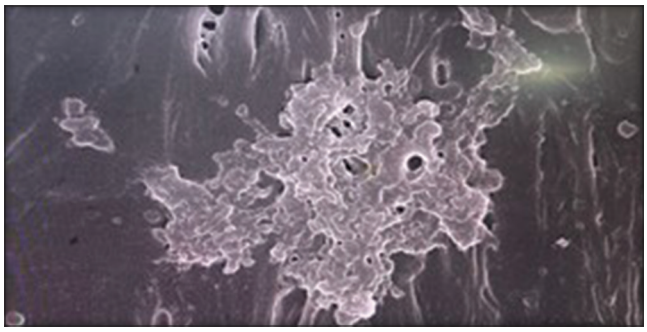

Figure 2: Odontoblastic differentiation at 14 days – SEM view

The explant culture method was used for the cell isolation from HDPSCs.[7] This method was preferred over the enzymatic digestion method to avoid any form of chemical or mechanical stress.[7] hDPSCs can differentiate into odontoblast lineages when a suitable induction medium is provided as they are totipotent cells.[10,17] After isolation in the explant culture method, the cells showed characteristics as well as MSC-specific cell surface markers, which can be an ideal source for clinical cellular therapeutic applications.[18] CD34 is a marker for identification of primitive pluripotent stem cells. However, it is assumed to be a negative marker for MSCs.[19]

PDTC exhibits a variety of therapeutic effects over a range of disorders such as chronic obstructive pulmonary disease, chronic bronchitis, cystic fibrosis, heavy metal toxicity, and also the psychiatric/neurological disorders. The cells were treated with various concentrations of PDTC with the aim of thwarting cellular death caused by oxidative stress during cellular differentiation.[18] With the introduction of PDTC, the cells demonstrated enhanced proliferation rate and reserved their stemness (stemness combines the ability of a cell to perpetuate its lineage) properties and the differentiation potential.[19]

Odontoblasts are the cells responsible for dentinogenesis which result in the formation of dentin in response to the synthesis of organic matrix and the deposition of subsequent mineralized crystals.[20,21] The arrangement of dentin formation which occurs is similar to that of the bone, which is also a mineralized connective tissue.[10,17]

PDTC-induced osteogenic differentiation in hDPSCs showed enhanced mineralization. RUNX2, which is an important transcription factor and master regulator, also controls the formation and development of the bone and teeth by the regulation of totipotent MSC differentiation into the lineages of odontoblast.[15,22] The upregulation of RUNX2 confirmed the odontoblastic differentiation process when treated with PDTC. Dentin sialophosphoprotein (DSPP), which is synthesized by the odontoblastic cells in high amounts, is vital for the development and calcification of the dentin matrix.[23,24]

Research conducted over the years has revealed that DSPP is associated with the mineralization of reparative dentin as well as bone.[22,23,25] Therefore, DSPP remains as one of the first characteristic markers for odontogenic differentiation. The studies have also shown the expression of DMP-1 during earlier osteogenic differentiation.[10,17] Hence, DSPP and DMP-1 are typically designated as definite protein markers of odontogenic differentiation which are used to identify the odontogenic differentiation capability of hDPSCs.[22]

hDPSCs induced with different concentrations of PDTC had different types of effects on cultured cells. It can therefore

be stated that the effect of PDTC on hDPSCs is dependent on the dose. The concentration of 1.0 mM PDTC showed better cell proliferation and increased osteogenic potential of cultured cells.

CONCLUSION

PDTC is an efficient, reproducible, and biological antioxidant of clinical utility, which may also be preferred for obtaining hDPSCs for the purpose of tissue engineering and regenerative medicine. The effect of PDTC on hDPSCs is dose dependent. The results propose that PDTC could have a significant pharmacological role in activating and enhancing odontogenic differentiation of dental stem cells and possibly a prospect in regenerative dentistry.

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Conflicts of interest
There are no conflicts of interest.

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