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Original Research Article

An In Vitro - Evalution of Dental Pulp Stem cells by Isolation, Characterization and Differentiation

Rama Brahmam Lanke[1], Arun kumar. S[2], Priya Matani[3]
[1]Masters in Environmental and Occupational Health Sciences, Western Kentucky University
[2] Masters in Conservative Dentistry & Endodontics, Mgs Dental College & Research Centre, Sriganga nagar
[3]Post graduate student in Conservative Dentistry & Endodontics, Mgs Dental College & Research Centre, Sriganga nagar

***Corresponding author**
Arun kumar. S
Email: drarun325@gmail.com

Abstract: Stem cells have been differentiated from variety of tissues. Recently stem cells have been isolated and grown from pulp tissue of permanent teeth (DPSCs), deciduous teeth (SHED), Potential application of stem cells in dentistry as reported in the literature includes endodontic regeneration therapy, periodontal regeneration, whole tooth regeneration, repair and regeneration of craniofacial defects. The purpose of the present study was to isolate, characterize and differentiate the dental pulp stem cells. 20 extracted permanent teeth from humans below age 25 years were collected. Various procedures including Isolation and culturing, expansion and sub culturing, RRNA isolation, Reverse transcription -RT PCR reaction, agarose gel electrophoresis were done to detect the amplicon including surface Characterization of cells. Osteogenic and acidogenic factors were added to the Media. The cells showed positive expression for pluripotent markers, ectodermal markers and mesodermal markers. The cells were found to express oct4 at both the mRNA and protein levels. They expressed ectodermal markers like ncaml, b3 tubulin and nestin. In the present study with the obtained results it can be demonstrated that stem cells are existed in human dental pulp and these cells differentiated showing mineralized tissue.
Keywords: stem cells, dental stem cell, regeneration of dentin, regeneration of pulp, tissue engineering.

INTRODUCTION

Use of the term "Stem cell" dates back at least to William Sedgwick, who used it to describe the regenerative properties of plants in 1886.

The recognition that tissues vary in their capacity to regenerate and the identification of tissues that can self renew over an organism's complete life span are rooted in 19th-century biological and medical science. The existence of a stem cell, viewed as the ultimate origin of self renewal in self-renewing tissues, was perhaps first postulated by Regaud based on his studies of spermatogenesis in starting of nineteenth century [1].

"Stem cells are like little kids who, when they grow up, can enter a variety of professions," Dr. Marc Hedrick of the UCLA School of Medicine says. *"A child might become a fireman, a doctor or a plumber, depending on the influences in their life or environment. In the same way, these stem cells can become many tissues by making certain changes in their environment."*

Stem cells are defined as clonogenic cells capable of both self renewal and multi lineage differentiation [2]. The discovery of stem cells and their potential has led to the development of new cell therapy strategies [3, 4]. Stem cells have been differentiated from variety of tissues such as cord blood, peripheral blood, bone marrow, gut, brain, kidney, liver, heart, testis, skeletal muscle, skin and eye in humans. Stem cell therapy has shown great promise in the management of Variety of chronic/ difficult to treat diseases in medicine like leukemia, breast cancer, inflammatory bowel disease, osteogenesis imperfecta, and cancer, neurodegenerative diseases, spinal cord injuries, heart disease, Parkinson's disease and diabetes.

Recently stem cells have been isolated and grown from pulp tissue of permanent teeth (DPSCs), deciduous teeth (SHED), periodontal ligament, apical papilla from a immature tooth and it has been reported that they generated dentin like tissue both *in vitro* and *in vivo* studies in animals. Transplanted skeletal or dental stem cells may one day be used to repair craniofacial bone or even repair or regenerate teeth [5, 6]. The use of

cryo protectants such as dimethyl sulfoxide (DMSO) is therefore suggested to prevent crystals formation [7].

Regenerative endodontic procedures can be defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex. Regenerative dental procedures have a long history originating around 1952, when Dr. B. W. Hermann reported on the application of Ca $(OH)_2$ in a case report of vital pulp amputation. Subsequent regenerative dental procedures include the development of guided tissue regeneration (GTR) procedures and distraction osteogenesis, application of platelet rich plasma (PRP) for bone augmentation, Emdogain for periodontal tissue regeneration, and recombinant human bone morphogenic protein for bone augmentation; and recombinant human bone morphogenic protein for bone augmentation, and the use of fibroblast growth factor n for periodontal tissue regeneration [8].

Potential application of stem cells in dentistry as reported in the literature includes endodontic regeneration therapy, periodontal regeneration, whole tooth regeneration, repair and regeneration of craniofacial defects. Therefore, regeneration or replacement of oral /dental tissues affected by inherited disorders like trauma, infection or neoplasms expected to solve many dental problems

The objectives of regenerative endodontic procedures are to regenerate pulp like tissue, ideally, the pulp-dentin complex; regenerate damaged coronal dentin, such as following a carious exposure; and regenerate resorbed root, cervical or apical dentin.

Recently it has been suggested that naturally occurring exfoliated teeth would be similar in some way to umbilical cord containing stem cells that may offer a unique stem cell resource for potential clinical applications. Gronthos *et al.;* in his study found that the deciduous tooth had multipotent stem cells which were highly proferalitive and clonogenic capable of differentiating, into variety of cell types including neural cells, adipocytes and odontoblasts. After in vivo transplantation they were able to induce bone, generate dentin and survive in mouse brain along with expression of neural markers.

Given the fact that we have in hand populations of stem cells that reproducibly reform bone and its marrow, cementum, dentin, and perhaps even periodontal ligament, it is possible to envision complete restoration of the hard tissues in the oral cavity using the patient's own cells, thereby avoiding issues of histocompatability This would be a more biological approach rather mere mechanical one [9].

Hence there is a need to gain clarity and further insight into specific properties of dental pulp stem cells, study their proliferation abilities, differentiation potential, and immunoreactivity profiles as these findings are likely to open up new horizons to make this concept a reality. Therefore methods to isolate and characterize the stem cell population are a preliminary step, of any such research and are crucial for the development of novel therapies based on stem cell regeneration

The present study was conducted to evaluate Growth and isolation of pulp stem cells (DPSCs), Dental pulp stem cells characterization (DPSCs), Evaluating the differentiation potential of dental pulp stem cells (DPSCs).

MATERIALS & METHODOLOGY

This study was conducted in Department of Conservative Dentistry and Endodontics, Thai Moogambigai Dental College & Hospital. 20 extracted permanent teeth from humans below age 25 years were collected. Third molars indicated for extraction or premolars indicated for extraction and orthodontic treatment were included in the study. Teeth with dental caries, teeth with pulpal or periapical disease, teeth with periodontal disease were excluded.

Sample collection, storage and handling;

Normal human extracted permanent teeth and extracted/ exfoliated deciduous teeth were collected and surfaces were cleaned. The teeth were cut using sterilized dental burs to reveal the pulp chamber and the pulp was gently separated from the crown and roots using a blunt non cutting forceps and a small spoon excavator.

The pulp was stored in a falcon containing DMEM (Dulbecco's Modified Eagle's Media containing 2x antibiotic and antimycotic) [GIBCO] and taken immediately to Manipal Institute of Regenerative Medicine, Bangalore, where the further experiments were conducted.

Isolation and culturing

Pulp tissue was digested in a solution of 2 mg/ml collagenase for 3 hours till the tissue digested. Then the cells were removed using a micropipette. Cells were incubated for 3 days for cell adhesion to occur. The cells were identified using a Fluorescence Microscope (ZEISS LD PLAN-NEOFLUAR) at 40x magnification.

Characterization of cells

First strand cDNA (complementary DNA) synthesis was perfomed on the cells by using a first strand cDNA synthesis kit (Superscript III, Invitrogen). Polymerize Chain Reaction was performed and later agarose gel electrophoresis was done to detect the

amplicon. After the gel was run, it was then viewed under the UV transilluminator. Surface characterization of cells was done with the help of flow cytometer (BDLSR II) using various surface markers like HLA-DR, CD73, CD44, CD106, CD34, CD10, CD123, CD7, CD31.An Immunocytochemistry analysis was done to see the expression of proteins in the cells. Primary antibodies used in the study were oct4 and nanog specific antibodies (chemicon) which are mouse IgG. Secondary antibodies used were rabbit antimouse IgG (AP160F) conjugated with FITC (chemicon).

Differentiation of cells

Osteogenic and Adipogenic factors were added to the culture Media. The differentiated cells were stained using Alizarin Red and Von Kossa stain for mineralized tissue and Oil Red O stain for Adipocytes.

RESULTS

Isolation and Expansion Of Cells

Identification of cells was done with the help of a phase contrast microscope (ZEISS LD PLAN-NEOFLUAR) at 40x magnification. The figures depict the cells at various stages of confluency. At the end of 5^{th} day the cells reach about 85% confluency. After the cells reach confluence (5 days post plating) the cells are trypsinized at 0.35% concentration and replated. Subsequently immuno flouroscence was performed to check for the expression of pluripotency markers, oct4 and nanog.

Identification and Characterization Of Cells

Examination of cells was done under fluorescence microscope (ZEISS LD PLAN-NEOFLUAR) at 40x magnification. Oct4 and nanog being the stem cell specific markers were used to identify the stem cells within the heterogeneous cell adherent population. Cells were propagated to a minimum of 5 passages and characterized for mesenchymal markers by flow cytometry.

In the present study, the cells were strongly positive for lymphocyte differentiation marker CD73, early adhesion and hyaluronan marker CD 44, and leukocytic cell marker CD 10. Cultures of DPSC and SHED failed to react with endothelial cell marker CD 106, immune cell marker HLA-DR, and were consistently negative for CD34 (marker for early hematopoietic stem cells). They were also negative for T cell marker CD 7 and endothelial cell marker CD 31

The cells were dimly positive for hematopoietic stem cell marker CD 123.

RT-PCR analysis of the expression of various pluripotency markers and derm markers in dental pulp derived stem cells at different passages. The cells shown positive expression for pluripotent markers, ectodermal markers and mesodermal markers. The cells were found to express oct4 at both the mRNA and protein levels. They expressed ectodermal markers like ncaml, b3 tubulin and nestin. They have also expressed mesodermal markers like hand1, bmp4 and gapdh. The cells however did not express endodermal markers.

DISCUSSION

.This study was conducted using the pulp tissue from deciduous and permanent teeth of patients below the age of 23 years. Extracted teeth from young patients were selected because of the presence of more cells and less fiber tissue in the pulp, aiding the motive of our study. The pulp tissue was transported to the laboratory as soon as possible. A flow cytometry analysis of dental pulp derived stem cells using a panel of cell surface markers revealed a similar expression pattern for a variety of markers for both DPSCs and SHED.

This study along with the other previous studies provides evidence that remnant dental pulp derived from DPSCs and SHED contains a multipotent stem-cell population. These stem cells can be isolated and expanded, thereby providing a unique and accessible population of stem cells from an easily available tissue resource

In the present study, the cells were strongly positive for lymphocyte differentiation marker CD73, early adhesion and hyaluronan marker CD 44, and leukocytic cell marker CD 10. vCultures of DPSC and SHED failed to react with endothelial cell marker CD106, immune cell marker HLA-DR, consistently negative for CD34 (marker for early hematopoietic stem cells). They were also negative for T cell marker CD 7 and endothelial cell marker CD 31(PECAM~1) indicating that they don't have the origin from the hematopoietic system. The cells were dimly positive for hematopoietic stem cell marker CD123. This result shows that these cells are not hematopoietic in origin and that they are pure mesenchymal stem cells, A single marker can be expressed by a variety of cells, so always positive and negative expression of multiple markers are sought after, for the characterization of cells.

The present study goes along with the study of Gronthos, who showed that profiles for both cell populations of DPSC and BMSC (bone marrow stem cells) failed to react with the hematopoietic markers CD 14 (monocyte/macrophage), CD45 (common leukocyte antigen), CD34 (hematopoietic stem/progenitor ceUs/endothelium), CD34 (marker for early hematopoietic stem cells) in both the studies showed that these cells are not hematopoietic in origin. In general, DPSCs and BMSCs(Bone marrow stem cells) exhibited a similar expression pattern for a variety of markers associated with endothelium, smooth muscle (a-smooth muscle actin), bone (alkaline phosphatase,

type I collagen, osteonectin, osteopontin, and osteocalcin), and fibroblasts (type III collagen and fibroblast growth factor 2).

Masako Miura in his study showed that ex vivo-expanded SHED were found to express the cell surface molecules STRO-1 and CD 146, two early mesenchymal stem-cell markers previously found to be present in BMSSCs and DPSCs. Laura Pierdomenico demonstrated that the cells expressed CD29, CD166, while CD45, CD34, CD14 proved negative which is again similar to our study [7]. But Irina Kerkis showed in her study that immature dental pulp stem cells are positive for CD13 and CD31. And negative for CD34, CD43, and CD45. This was contradictory to our study which showed the negative expression for CD34 (marker for early hematopoietic stem cells). This variation may be due to the type of media used or the procedure followed. This may also be due to the inherent quality of the cells itself [8].

Pei-Hsun Cheng, did experiments on chimpanzee teeth .There is 98% similarity of their genomes , making the chimpanzee the closest living relative to humans; and found that Both chimpanzee DPSCs and human BMSCs share identical expression profiles on common cell surface antigens. They were all negative for hematopoietic cell surface markers: CD14, CD18, CD24, CD34, and CD45; and positive CD29, CD44, CD59, CD73, CD90, CD105, CD150 and CD166). The present study also got similar results [9].

In the present study, RT-PCR analysis of the expression of various pluripotency markers and derm markers was done at different passages. The cells showed positive expression for pluripotent markers, ectodermal markers and mesodermal markers. The ceils were found to express oct4 at both the mRNA and protein levels. They expressed ectodermal markers like ncaml, b3 tubulin and nestin. They also expressed mesodermal markers like hand1, bmp4 and gapdh. SHED and DPSCs showed similar results. This indicates that these cells might have the capacity to differentiate into ectoderm and endoderm organs. The cells however did not express endodermal markers. Oct-4 and Nanog, markers of cells were expressed which indicates the pluripotent behavior of the cells. However the expression pattern needs to be confirmed at a protein level to emphasize if there is any physical significant.

These results were similar to the results obtained by Gronthos and Masuka Moura showed that SHED expressed a variety of neural cell markers including nestin, Ill-tubulin, as measured by immuno cytochemical staining/. In the present study there was not much difference in the DPSCs and SHED as far as the characterization is concerned. The marker expression for both the samples was similar. However according to Masako Miura deciduous teeth are significantly different from permanent teeth with regards to their developmental processes, tissue structure, and function SHED are distinct from DPSCs with respect to their higher proliferation rate, increased cell-population doublings, sphere-like cell-cluster formation, osteoinductive capacity in vivo, and failure to reconstitute a dentm-pulp-like complex. SHED apparently represent a population of multipotent stem cells that are perhaps more immature than previously examined postnatal stromal stem-cell populations. In his study, SHED demonstrated a strong capacity to induce recipient cell-mediated bone formation in vivo. According to him, SHED could not differentiate directly into osteoblasts but did induce new bone formation by forming an osteoinductive template to recruit murine host osteogenic cells. These data imply that deciduous teeth may not only provide guidance for the eruption of permanent teeth, as generally assumed, but may also be involved in inducing bone formation during the eruption of permanent teeth. It is notable that SHED expressed neuronal and glial cell markers, which may be related to the neural crest-cell origin of the dental pulp [10]. The cells express a particular profile for variety of antigens tested which clearly indicate that they are mesenchymal in origin.

In the present study, the comparison was done between two samples of DPSCs, and one sample of SHED. More number of samples is although required to compare the samples properly, by using more number of cell surface markers. In our study, out of 20 samples used, many of them could not be successfully processed and expanded in vitro suggesting the complexity and technique sensitivity of the procedure involved. Furthermore, the risk of contamination, viability of the cells, addition of ideal factors, inherent nature of the cells and the requirement of a perfect environment cannot be overlooked in performing this study. Identifying and rectifying these limitations is important to complement the technique of isolation, expansion and maintenance of these multilineage cell lines. The cells can be differentiated showing mineralized tissue and also showed the presence of adipocytes. Present study together with the work of other studies indicates the potential for using DPSCs as a source of pluripotent stem cells for future cellular based therapies in medicine and dentistry.

CONCLUSION

Within the limitation of the present study it can be concluded that stem cells are existed in human permanent dental pulp tissue and these stem cells can be isolated, cultivated. The cells are mesenchymal in origin and the cells can be differentiated.

REFERENCES

1. Bianco P, Robey PG. Marrow stromal stem cells. The Journal of clinical investigation. 2000 Jun 15; 105(12):1663-8.
2. Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. Journal of dental research. 2002 Aug; 81(8):531-5.
3. Brar GS, Toor RS. Dental stem cells: dentinogenic, osteogenic, and neurogenic differentiation and its clinical cell based therapies. Indian Journal of Dental Research. 2012 May 1; 23(3):393.
4. Rodríguez Lozano FJ, Insausti CL, Iniesta F, Blanquer M, Ramírez MD, Meseguer L, Meseguer Henarejos AB, Marín Atucha NT, Martínez S, Moraleda Jiménez JM. Mesenchymal dental stem cells in regenerative dentistry.
5. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences. 2000 Dec 5; 97(25):13625-30.
6. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proceedings of the National Academy of Sciences. 2003 May 13; 100(10):5807-12.
7. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue engineering. 2006 Oct 1; 12(10):2813-23.
8. Krebsbach PH, Robey PG. Dental and skeletal stem cells: potential cellular therapeutics for craniofacial regeneration. Journal of dental education. 2002 Jun 1; 66(6):766-73.
9. Shetty SO, Farooq U, Chandra SB, Nanjappa SU. Molecular biology and preservation of tooth vitality current implication. Endodontol. 2006 Jun; 2:12.
10. Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Massironi SM, Pereira LV, Caplan AI, Cerruti HF. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells Tissues Organs. 2006; 184(3-4):105-16.
11. Sonoyama W, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Shi S, Wang S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. PloS one. 2006 Dec 20; 1(1):e79.
12. Cheng PH, Snyder B, Fillos D, Ibegbu CC, Huang AH, Chan AW. Postnatal stem/progenitor cells derived from the dental pulp of adult chimpanzee. BMC cell biology. 2008 Apr 22; 9(1):20.
13. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. Proceedings of the National Academy of Sciences. 2003 May 13; 100(10):5807-12.