Dental Pulp Stem Cells from Primary Teeth Quality Analysis: Laboratory Procedures

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Objectives: *To present details of isolation, processing and differentiation of stem cells from inflamed dental pulp of primary teeth.* ***Materials and methods:*** *Tissue sample was collected from teeth indicated for a single visit pulp therapy. Samples were transported and processed in the laboratory which included culturing of cells, isolation and in vitro differentiation into multiple lineages. The results for the analysis of various cell surface markers used for dental pulp were compared with bone marrow.* ***Results:*** *There was no statistically significant difference found in the expression of various surface markers between dental pulp and bone marrow. The stem cells from dental pulp were differentiated into multiple lineages.* ***Conclusion:*** *Isolation of cells from oral tissues is technique sensitive.*

Keywords: *Stem cell, inflamed pulp, deciduous teeth, stem cell marker, in vitro differentiation,*
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INTRODUCTION

The discovery of stem cell based therapy has proven to be a boon to regenerative medicine because of the potential of a stem cell to differentiate into various cell types and thus help in tissue regeneration following injury or disease.

Development of stem cells begins with the most primitive totipotent stem cell, the zygote, which is the result of the fusion of two germ cells (oocyte and sperm) during the process of fertilization. A stem cell is a cell from the embryo, fetus, or adult that has, under certain conditions, the ability to reproduce itself for long periods or, in the case of adult stem cells, throughout the life of the organism. It also can give rise to specialized cells that make up the tissues and organs of the body. A breakthrough in human embryonic stem cell research came in 1998 when a group led by Thomson[1] at the University of Wisconsin-Madison first developed a technique to isolate and grow the cells when derived from human blastocysts donated by couples undergoing treatment for infertility. The cells were sub cultured successively, yet they retained their normal karyotypes, which is a characteristic chromosome complement of a eukaryote species. The dental pulp contains undifferentiated mesenchymal cells that constitute a reservoir of cells on which the body can call to assume functions that are not ordinarily needed. Dental pulp stem cells are an elusive population of self -renewing cells with potential utility in generating biologic replacement teeth in humans.

For the first time, Gronthos, *et al* in 2002[2] isolated human postnatal dental pulp stem cells from normal molar teeth of adult patients. These cells were isolated using enzymatic digestion and colony-forming-unit-fibroblast assay, when each postnatal dental pulp stem cell colony was originated from a single progenitor cell. The cells within each colony presented a typical fibroblast-like morphology resembling those of bone marrow derived mesenchymal stem cells. They showed higher colony-forming efficiency and cell proliferation *in vitro* when compared to bone marrow derived mesenchymal stem cells.

The isolation of post-natal stem cells from an easily accessible source is indispensable for tissue engineering and clinical applications. Dental pulp can provide an alternate source, as the stem cells derived from this tissue have been shown to harbor a similar potency as that of bone marrow derived mesenchymal stem cells.[3]

In our previous paper[4] we have already concluded that inflamed pulp discarded during pulp therapy procedures is a potential source for harvesting adult stem cells. The present paper provides the details of the laboratory procedures involved in the isolation, processing and differentiation of stem cells from inflamed dental pulp of deciduous teeth.

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MATERIALS AND METHOD

This study retrieved stem cells from a discarded tissue waste.

The samples indicated for a single visit pulp therapy were chosen for the study. A tooth was indicated for single sitting pulpal treatment when there was an accidental iatrogenic injury to the pulp or due to a carious lesion that was either approaching or approximating the pulp without involving the tissue. This situation has been chosen to minimize the risk of infection of the sample tissue and collect it in its various stages of inflammation before it gets infected.

Isolation of Human dental pulp

Patient was seated comfortably on the dental chair and advised to rinse with 0.2% chlorhexidine mouth rinse for 60 seconds prior to the sample collection to minimize the bacterial contamination. A topical anesthetic gel was applied and lignocaine local anesthetic with 1:200000 adrenaline was administered. Rubber dam was applied for adequate isolation and to minimize contamination with oral microflora. Access cavity was prepared using a round bur, to obtain access to the pulp chamber and root canals. After gaining access to the pulp chamber, barbed broach was used to remove the pulp tissue from the root canals.

The tissue was collected in 15 ml conical base centrifuge tubes (BD Falcon), containing Dulbeco's phosphate buffered saline (DPBS) solution (Invitrogen) containing no calcium or magnesium ions and was transported to Stempeutics, Manipal within 30 minutes of collection.

Culturing of cells

In the laboratory the tissue sample was washed thrice with Dulbeco's phosphate buffered saline solution (Invitrogen) with no calcium and magnesium. After this, 0.05% trypsin (Invitrogen) was added and tissue minced to smaller pieces to increase the surface area for action of trypsin, using number 21 surgical scalpel. The tissue was incubated in the incubator (Heracel Thermo) for 10-20 minutes after mincing. After incubation the culture medium; Dulbeco's modified Eagle's Medium- F12 (DMEM-F12) (Invirtogen) with 10% Fetal Bovine Serum (Hyclone), 100µM ascorbic acid and 2mM L-Glutamax supplemented with 100U/ml penicillin and 100µ/ml streptomycin, was added to the incubated sample to minimize trypsin action. The sample was now centrifuged (Eppendorf Centrifuge Machine 5415 R, Germany) at 1200 rpm for 7 min. The supernatant was discarded and the tissue pellet collected was plated in 35 mm^2 tissue culture flasks (BD Falcon) marked appropriately containing 2 ml of the culture medium**.** The cells were incubated (Heracel Thermo) in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC. The plates were reviewed after 48 hours to check for growth and attachment of cells**.**

Cells were washed with Dulbeco's phosphate buffered saline (DPBS) solution followed by addition of 0.05% Trypsin and EDTA for detachment. After incubation at 37ºC for 5 minutes, complete media was added to neutralize trypsin action. Culture was centrifuged at 1200 rpm for 5 minutes. 0.1 ml of 0.4% Trypan blue solution was mixed with 0.1 ml of cell suspension in media (dilution 1:1) in an Eppendorf tube. 10µl of this mix was pippetted into coverslip chambers and observed at 10X magnification under the microscope to check if there were more than 50 cells/ square mm, which indicates 80% confluence. Thereafter the cells were transferred to another culture dish, indicating one passage. In the similar manner stem cells obtained from dental pulp were cultured up to passage number 8.

Flow Cytometric Analysis

Analysis of cell surface molecules on dental pulp was performed using a panel of fluorochrome-labelled monoclonal antibodies diluted according to the manufacturer's instructions (Pharmingen). Once the cells at the fifth passage were found confluent they were trypsinised (detached from surface). The pellet thus obtained was resuspended in appropriate amount of phosphate buffered saline and then aliquoted such that each tube contained 0.5 x 10^6 cells. The

Table 1. Significance, location, type of markers and source of antibodies

Marker name	Cell type	Significance	Antibody type and source
Cluster differentiation (CD) 90 or Thy-1	Mesenchymal and hematopoietic stem cells	Cell surface protein, used as a marker for variety of stem cells	Phycoerythrin conjugated (PE) 555596 (BD Pharmingen)
CD 73 Ecto 5'-nucleotidase	Mesenchymal stem cells, lymphocytes	During development of both T & B cells its expression is increased and has been described as lymphocyte maturation marker	PE, 550257 (BD Pharmingen)
CD 166 Activated Leucocyte Cell Adhesion Molecule (ALCAM)	Expressed on human bone marrow derived mesenchymal stem cells	Promotes adhesion of leucocytes, expression lost during development of cells into differentiated phenotypes	PE, 559263 (BD Pharmingen)
CD 133 Prominin1 (PROM1)	Expressed on glioblastomas, neuronal and glial stem cells	Localizes to cellular protrusions	PE, 559263 (BD Pharmingen)
CD 34 & CD 45	Hematopoietic stem cell or Endothelial progenitors	Cell surface proteins in bone marrow cell, indicative of hematopoietic and endothelial progenitor	CD 34 PE, 550761; CD 45 FITC, 347463 (BD Pharmingen)

optimum titer of primary and secondary antibodies were standardized and were added to the cell suspension and incubated in dark for an hour at 4°C. After the incubation period, 1 ml of phosphate buffered saline was added to each tube and centrifuged (Eppendorf Centrifuge Machine 5415 R, Gennany) at 1800 rpm for 5 minutes at 4°C. To the cells stained with unconjugated primary antibodies, the respective secondary antibodies (1 µl) were added for half an hour in dark at 4°C. 1 ml of phosphate buffered saline was added and centrifuged at 1800 rpm for 5 minutes at 4°C. The pellets thus obtained were suspended in 300 µl of 1% paraformaldehyde for fixation of the cells and then were transferred into flow tubes and analyzed for the cell surface markers in the flow cytometer. (FACS; LSR 2, BD)

Surface markers

The various surface and intracellular markers used in the study are described in table 1. All the optimal dilutions of the antibodies to these markers were empirically determined with relevant isotypes phycoerythrin and fluorescence isothyocynate conjugated controls.

Differentiation

Before initiation of differentiation, all samples were cultured up to passage number 8.

Lineage induction of cells was performed when cultures had reached 60-70% confluences.

OSTEOBLAST

The mesenchymal stem cells were cultured in complete media supplemented with induction media (1µl/ml Dexamethasone, 6µl/ml Ascorbic acid, 20□l/ml B- glycerophosphate) in 35 mm^2 dish. Media was replenished every 3 days. After 7 - 21 days, cells were fixed to do Von Kossa staining.

Media was aspirated and each well was rinsed twice with Dulbeco's phosphate buffered saline solution. 1ml of 4% formalin was added and kept at room temperature for 30 minutes. Thereafter formalin was discarded and again the fixed cells were incubated with 5% $AgNO_3$ for 60 minutes under UV light. The 5% $AgNO_3$ was aspirated and 1ml of 2.5% sodium thio-sulphate added and incubated for 5minutes. The observation and result was recorded in functional assay template.

ADIPOCYTE

The cells were cultured for up to 3 weeks in complete media supplemented with induction media (1µM Dexamethasone, 0.5mM isobutylmethylxanthine, 1µgm/ml Insulin, 100 µl Indomethacin) in 35mm^2 dish (BD Falcon). The media was replenished every 3 days. After 7 days cells were fixed for staining with oil red for adipocytes.

Media was aspirated and each well rinsed twice with Dulbeco's phosphate buffered saline solution. 4% formalin was added and kept at room temperature for 5 minutes. The formalin was discarded and the same volume of fresh formalin was again added into the wells. They were incubated for 1 hour and washed with 60% isopropanol. Oil Red O was then added to the working solution (200µl) for 10 minutes at room temperature. All the Oil Red O was removed and immediately rinsed with distilled water, at least five times. It was then examined under inverted microscope.

CHONDROCYTE

The mesenchymal stem cells were cultured in complete media supplemented with induction media (1µl/ml Dexamethasone, 6µl/ml Ascorbic acid, 20µl/ml B- glycerophosphate) in 35 mm^2 dish. Media was replenished every 3 days. After 7 - 21 days, cells were fixed for staining with Safronin.

Media was aspirated and each well was rinsed twice with Dulbeco's phosphate buffered saline solution. 1ml of 4% formalin was added and kept at room temperature for 30 minutes. Thereafter formalin was discarded and again the fixed cells were washed with Dulbeco's phosphate buffered saline solution. Then 1% Safronin solution prepared by 0.1N HCl was added to the culture plates for 30 minutes under UV light. The wells were rinsed three times with 0.1 N HCl, and distilled water was added to neutralize the acidity. It was visualized under light microscope. Orange staining indicates synthesis of proteoglycans by chondrocytes. The observation and result was recorded in functional assay template.

RESULTS

Table 1. shows number of samples with positive results and those lost to contamination

Total inflamed pulps	Positive culture	Negative culture
13	7	6

Analysis was done for the following:

A) Growth potential of the dental pulp stem cells
B) Differentiation of the dental pulp stem cells into multiple lineages
C) Characterization of immune phenotype of dental pulp stem cells using flow cytometry

A) Growth potential of the dental pulp stem cells

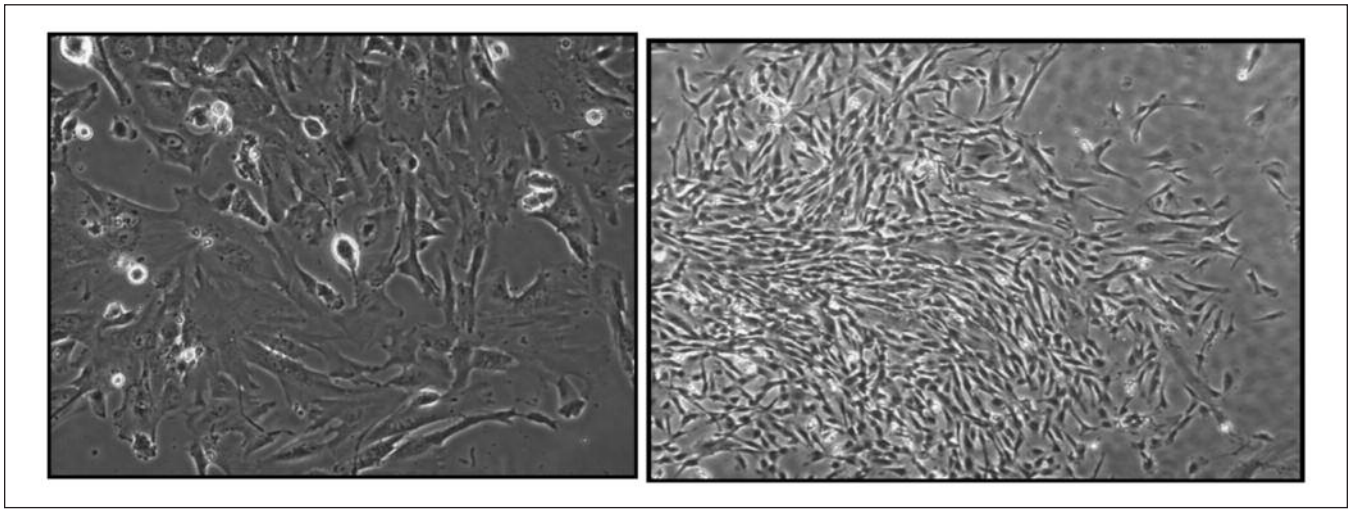
1. Isolation and culture of cells

After incubation for 48 hours, the growth of cells was observed under the phase contrast microscope at day 2 and 4. The cells were again analyzed at day 5 and 6. All the samples positive for stem cells growth reached 80% confluence at day 8; after which it was transferred from passage 0 to passage 1 in another culture plate for further multiplication. The growing dental pulp stem cells were transferred from passage 1 to passage 2 after reaching 80% confluence at day 14 (Figures 1A and B).

2. **Comparison of growth potency of dental pulp stem cells using various markers**

2a). Cells positive for mesenchymal markers

CD 73 shows 94.18% positivity which means that 94.18% of cells counted expressed CD73. CD 90



(A) (B)

Figure 1. (A) shows cells at day 8 and (B) shows cells at day 14 with 80% confluence after which it was transferred from passage 1 to passage 2

was 93.97% positive for dental pulp. CD 133 was 6.56% positive and CD 166 was 90.25% positive for dental pulp.

2b). Cells positive for hematopoietic markers

CD 34 shows 1.39% and CD 45 was 1.23% positive for dental pulp.

2c). Indirect immunoflouresecence of dental pulp stem cells for pluripotency markers

The dental pulp stem cells were analyzed for pluripotent markers such as Oct 4 and Nanog by immune staining with specific antibodies. They were observed under the fluorescent microscope (Nikon Eclipse 80 I Microscope).

Octamer 4 (Oct 4) and Nanog

The dental pulps stem cells demonstrated 4, 6 diamidino – 2- phenyindole (DAPI) fluorescence, which is used to stain the cell nucleus to confirm the presence of the pleuripotent cells. (Figure 2A, 2C). They were then observed

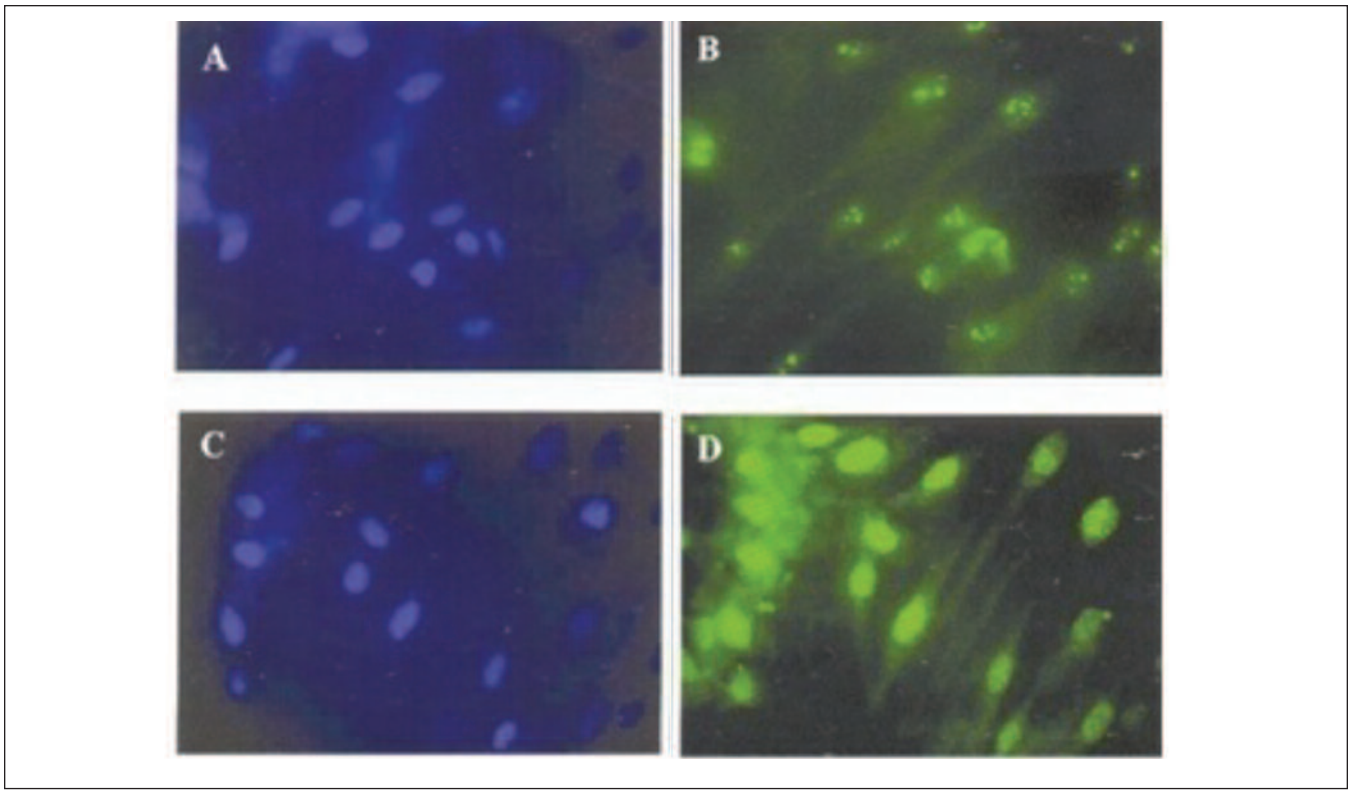


Figure 2. Fluorescent microscopic pictures of Immuno localization of Pluripotency markers Oct 4 and Nanog on Dental Pulp Stem Cells
A, C: Dental pulp stem cells exhibiting 4, 6 diamidino - 2- phenyindole (DAPI) fluorescence, with blue color representing the nuclei
B, D. Dental pulp stem cells depicting positive Oct 4 and Nanog expression with green color representing the fluorescence specific antigens

to be positive for expression of Octamer 4, a transcription factor unique to pluripotent stem cells present intracellularly as observed by a green fluorescence (Figure 2B).

The cells also were found to express Nanog on their surface which is a gene expressed in embryonic stem cells and is thought to be a key factor in maintaining pluripotency (Figure 2D).

B) Differentiation of dental pulp stem cells into multiple lineages (Figure 3, 4, 5; Graph 1)

85.7% of the inflamed dental pulp stem cells differentiated into chondrocytes (6 out of 7 samples differentiated into chondrocytes), 85.7% into osteoblasts (6 out of 7 samples differentiated into osteoblasts) and 57.1% into adipocytes (4 out of 7 samples differentiated into adipocytes). 100% of bone marrow derived stem cells differentiated into chondrocytes, osteoblasts and adipocytes (10 out of 10 samples differentiated into all the three type of cells).

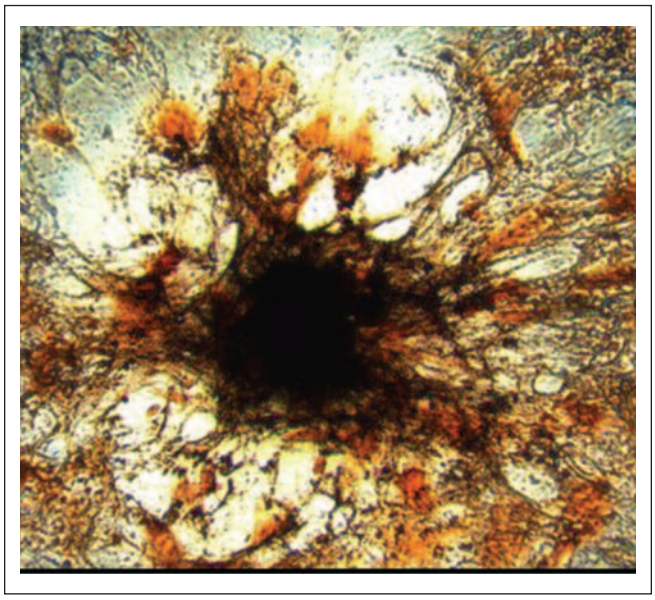


Figure 3. Chondrocyte differentiated from dental pulp stem cells

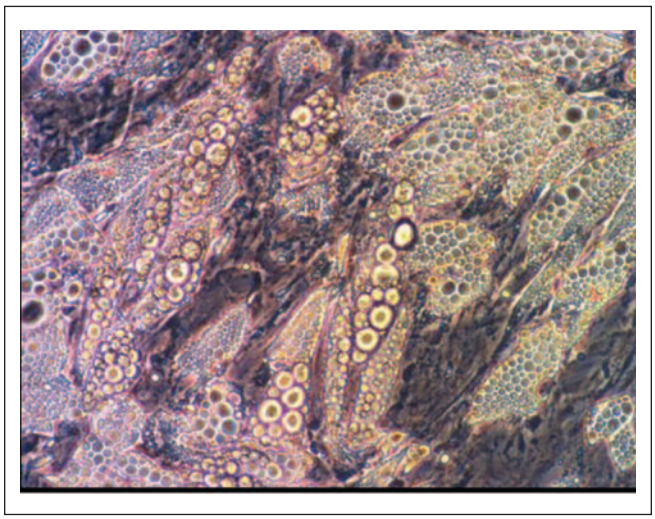


Figure 4. Adipocytes differentiated from dental pulp stem cells

DISCUSSION

The aim was to present a detailed laboratory analysis of isolation and differentiation of stem cells from inflamed deciduous teeth. Ethical committee approval and written informed consent of parents was taken before starting the study. Dental pulp samples were collected from deciduous teeth indicated for single visit pulp therapy. The tissue collected was processed and analyzed using flow cytometry for the presence of stem cells which were compared qualitatively with those obtained from the bone marrow. The differential potential of the dental pulp stem cells into multiple lineages was also evaluated.

The markers used in the present study namely CD73, CD90, CD 34, CD45 were in accordance with the guidelines of the International Society for Cellular Therapy.[31] In addition various studies have been conducted by Pittenger *et al*, 1999[5]; Gronthos *et al* 2000[6]; Reyes *et al*, 2001[7]; Gronthos, Zannettino *et al*, 2003[8] where the expression of these markers has been seen by the mesenchymal stem cells.

Gronthos, Mankani *et al*, 2000[6] isolated pulp tissue from impacted third molars of 19-29 year old individuals. The

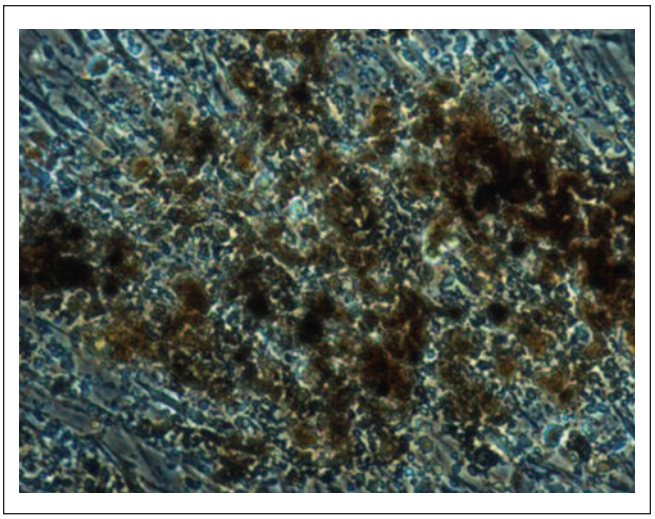
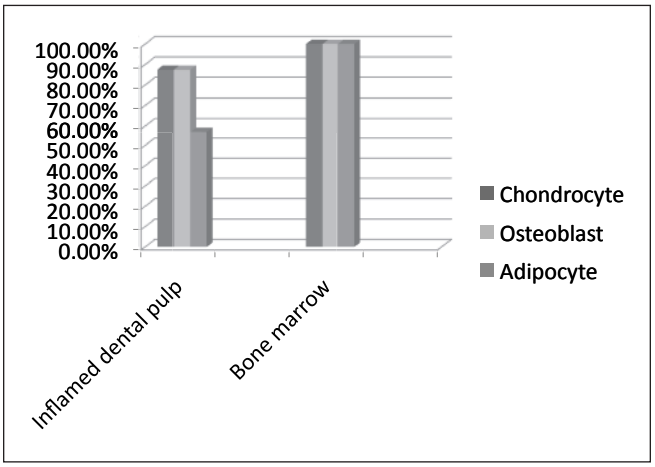


Figure 5. Osteoblasts differentiated from dental pulp stem cells



Graph 1. Percentage of dental pulp and bone marrow samples differentiating into chondrocytes, osteoblasts and adipocytes

teeth were extracted, cleaned and crown structure was removed from root portion by making a groove along the cemento-enamel junction using a sterile fissure bur at high speed. In the present study however, to minimize chances of contamination and also to standardize the procedure as performed in routine clinical practice without sacrificing the tooth, the pulp tissue was removed with a barbed broach after preparing an access cavity under complete isolation with rubber dam, prior to extraction**.**

Suchanek, *et al*, 2009[9] compared efficacy of 2% and 10% fetal calf serum in culture medium for growth of mesenchymal stem cells. They revealed that medium supplemented with 10 % FCS and 100µM ascorbic acid is best for growth of dental pulp derived stem cells. In the present study 10% fetal calf serum with 100µM ascorbic acid was used for culture of the isolated cells as recommended above.

Cell suspensions of dental pulp were seeded plates (BD Falcon) with alpha modification of Eagle's medium (DMEM-F12) supplemented with 100 units/ml penicilliny100 mg/ml streptomycin as reported by (6); to minimize any contamination that occurred while sample collection.

In the present study we could differentiate the stem cells from extracted deciduous teeth into osteoblasts, adipocytes and chondrocytes **(**Graph1**)** by processing the tissue samples immediately after extraction to minimize chances of contamination due to storage and also because of time constraints. However, Perry, Zhou *et al*, 2008[10] isolated dental pulp stem cells from cryopreserved intact teeth and differentiated the same to osteoblasts, adipocytes and chondrocytes. This indicates that dental pulp stem cell isolation is feasible for at least 5 days after tooth extraction, and implies that processing immediately after extraction may not be required for successful banking of the stem cells. Further, the recovery of viable cells after cryopreservation of intact teeth suggests that minimal processing may be needed for the banking of samples with no immediate plans for expansion and use.

Bruder, Horowitz *et al* 1997[11] reported CD 166 expression on human bone marrow derived mesenchymal stem cells that are lost during their development and differentiation into osteocytes. In the present study the positive rate for CD 166 on the dental pulp stem cell surface was 90.25% which indicates a strong capacity to induce cell mediated bone formation. However the study was not conducted up to the differentiation of cells to their mature phenotypes due to time constraints and hence the decrease in the marker expression could not be verified.

In a study by Pierdomenico, Bonsi *et al*, 2005,[12] using flow cytometry showed that 94 ± 4% of dental pulp derived stem cells expressed traditional mesenchymal markers, such as CD73, CD90 and CD166, indicating that this isolated population was highly homogeneous regarding these markers. Similarly in the present study it was also observed using flow cytometry that mesenchymal stem cell markers such as CD73 (94.18%)*,* CD90 (93.97%) and CD166 (90.25%) were strongly positive in the dental pulp cells suggesting they are a population of mesenchymal stem cells.

Pittenger, Mackay and Beck *et al,* 1999[5] stated that criteria for undifferentiated mesenchymal stem cells are that the cells must be negative for blood cell or hematopoietic progenitor cell markers CD34 and CD45. Flow cytometry analysis in the present study demonstrated that the percentage positivity for CD 34, CD45 (hematopoietic stem cell markers) in dental pulp stem cells was 1.39% and 1.23% respectively.

Oct-4 and Nanog are two critical transcriptional factors to keep pluripotency and self-renewal of stem cells in vivo and in vitro, and they usually express only in pluripotent cells and not in differentiated cells.[13] They bind to the regulatory regions of targeted gene and finally determine the cells destiny, keeping pluripotency or turning to differentiation.[14] All the samples tested in the present study were positive for Oct-4 and Nanog using immunostaining methods which depicts the isolated cells to be pluripotent and capable of self-renewal.

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Gronthos, Mankani *et al*, 2000[6] found that there was no evidence of adipogenesis in primary dental pulp stem cell cultures even after 6 weeks of culture whereas clusters of lipid-containing adipocytes were detected in primary cultures of bone marrow stem cells as early as 2 weeks. Similarly in the present study the decreased capacity of dental pulp stem cells was seen to differentiate into adipocytes after 4 weeks, as compared to the bone marrow cells. The reason for such a differential expression can be the absence of adipocytes from normal cellular component in dental pulp.

Gronthos, Brahim *et al*, 2002[2] have stated that the adipogenic potential of dental pulp can be increased by using a more potent adipogenic-induction medium.

The International Society for Cellular Therapy has strongly encouraged the scientific community to adopt a set of criteria that standardizes the identification of mesenchymal stromal cells (MSCs).[31] The first criterion is that MSCs must be adherent to plastic when maintained in culture. Second, the MSCs must be positive for the surface antigens CD105, CD73 and CD90. Additionally, the MSCs must lack markers for monocytes, macrophages and B cells, as well as the expression of the haematopoietic antigens CD34 and

CD45. The final criterion is that the MSCs must have the potential to differentiate into osteoblasts, adipocytes and chondrocytes under standard in vitro differentiating conditions.[15] All the above criteria were met by the cells cultured in the present study strongly identifying the mesenchymal stromal cells from dental pulp.

The major findings of the study were the successful isolation, characterization and differentiation of stem cells from inflamed primary dental pulp into multiple lineages, although a number of samples were lost to contamination with saliva in spite of taking all precautions. Further studies with large sample size are warranted to confirm the same with Reverse Transcriptase – Polymerase Chain Reaction along with differentiation of odontoblast and in vivo regeneration of dentin pulp complex.

CONCLUSION

It can be concluded from the details of isolation procedures that although inflamed pulp can be a potential source of harvesting stem cells the technique is sensitive to contamination in oral cavity. All measures to ensure isolation will minimize loss of sample due to infection.

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