Dental Pulp Stem Cells from Primary and PermanentTeeth: Quality Analysis

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Objective: *The objective of the study was to identify and isolate stem cells from healthy and inflamed dental pulp and characterize their differentiation potential into multiple lineages.* ***Study design:*** *Study was conducted in dental pulp tissues obtained from the children in the age range of 5-14 years. Tissue samples were collected from teeth indicated for pulp therapy and extractions for orthodontic purpose. Samples were processed in the laboratory including cell culture, isolation and differentiation into multiple lineages. The results for the analysis of various cell surface markers used for dental pulp were compared with bone marrow which is considered as a gold standard.* ***Results:*** *There was no statistically significant difference found in the expression of various surface markers between inflamed dental pulp and bone marrow. Healthy pulp from the primary teeth was not sufficient to use as a source for harvesting stem cells moreover the healthy tissue obtained from permanent teeth failed to show any results at all.* ***Conclusion:*** *Inflamed pulp discarded during pulp therapy procedures is a potential source for harvesting adult stem cells.*

Keywords*: Stem cells, SHED, primary teeth, Permanent teeth*
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Stem cell research is one of the most fascinating areas of biology today because of the optimism it provides to the clinician in terms of its therapeutic implications. Stem cell as defined by Rao, 2004[15] is a cell that has the ability to continuously divide and produce progeny cells that differentiate into various other types of cells or tissues.

Various researchers have been able to successfully expand stem cells *in vitro* on a reproducible basis without changing the inherent properties of the stem cells.[8] Bone marrow is the most utilized and well-studied source of stem cells, but the procurement is extremely painful, expensive for patients and yields low numbers of harvested cells. The need of the day however, is to find alternate sources of adult stem cells which should have a similar potential as that of bone marrow mesenchymal stem cells.

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.

On the basis of recent advances in postnatal stem cell biology and knowing the multilineage differentiation potential of adult stem cells, we can contemplate the use of osteoprogenitor cells derived from sources other than bone marrow as a practical cellular based approach for regenerative therapy in dentistry.

This study attempted to isolate mesenchymal stem cells from inflamed pulp tissue of primary teeth. This idea was conceived based on the fact that inflammation appears to increase homing of bone marrow-derived stem cells, macrophages, or myofibroblasts within the peripheral sites and may actually be required for successful engraftment. Similarly in inflamed dental pulp tissue initially, neutrophils and tissue mast cells are thought to be recruited as part of body's defense mechanism which is a multifactorial system that coordinates inflammatory cell involvement. Not many studies have been conducted to explain the molecular mechanisms that might affect the expression of stem cells in such tissues, although studies have been conducted to show the beneficial effect of stem cells therapy on inflammatory and exudative disorders (Lee et al, 2010)[7]. Another major aspect of utilizing inflamed pulp tissue is to find out if the discarded tissue waste during the therapy can be used as reported by Jazedje et al, 2009[6] in their study on fallopian tubes.

In view of above the present study is undertaken with the aims and objective to identify and isolate stem cells from healthy and inflamed dental pulp.

The aims of the study were:

1) To isolate, identify and characterize stem cells from

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both the inflamed and healthy dental pulp;
2) To see the difference in the tissue potency of dental pulp of primary and permanent teeth as a source of harvesting stem cells;
3) To compare the stem cells obtained from dental pulp with those obtained from bone marrow;
4) To identify various lineage forms that can be derived from the stem cells of deciduous tooth pulp.

MATERIALS & METHODS

The study was conducted in the Department of Pedodontics and Preventive Dentistry, Manipal; in collaboration with Stempeutics, Manipal.

The objective of the study was to identify and isolate stem cells from healthy and inflamed dental pulp and characterize their differentiation potential into multiple lineages.

The study was based on demonstrating 5% difference in the marker positivity which was thought to be clinically significant.

To give 80% power and a 5% significance level using a two-sided independent sample T test, a sample of 8 was calculated to be sufficient to demonstrate a significant difference per group. To generate minimum 8 samples per group a convenient sample size of 50 teeth was selected including 25 primary incisors and molars and 25 permanent premolars according to the inclusion criteria, obtained from the outpatient children.

The children included in the study were in an age range of 5-14 years and in a sex ratio of 1:1.

Subjects included in the study were healthy individuals with no history of illness in the last one year; patients with primary teeth indicated for single visit pulp therapy or extraction of primary and permanent teeth indicated for orthodontic treatment. Patients having long term systemic illness, undergoing antibiotic therapy for past one month and those with primary teeth indicated for multiple visit pulp therapy were excluded from the study.

Institutional ethical committee approval was obtained from the Ethical Committee of Kasturba Hospital, Manipal. A written informed consent based on the ethical committee guideline was obtained from the parents of the children included in the study.

A convenient sample of 50 teeth (25 deciduous and 25 permanent teeth) was divided into three groups as follows:

Group A: Included inflamed dental pulp samples from deciduous teeth indicated for single visit pulp therapy or orthodontic extraction = 13 teeth

Group B: Included healthy pulp samples from deciduous teeth extracted with physiologic mobility = 12 teeth

Group C: Included dental pulp samples from permanent teeth extracted for orthodontic purpose = 25 teeth

Sample collection

After a rinse with 0.2% chlorhexidine for 60 seconds a topical anesthetic gel was applied and lignocaine with 1:200000 adrenaline was administered to achieve anesthesia. Under rubber dam isolation access cavity was prepared using a round bur, barbed broach was used to remove the pulp tissue from the root canals. Similarly teeth indicated for orthodontic treatment were extracted following pulp extirpation.

Transportation

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

Processing

In the laboratory the sample was processed inside the culture room in a laminar flow chamber. In the beginning 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) at 37ºC for 10-20 minutes after mincing. After incubation the culture medium i.e. 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, (DMEM-F12). 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.

Lab procedures

In the laboratory cells were passaged up to the 8th stage and 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). (Table 1)

Table 1: Type of markers studied	
Mesenchymal markers	CD 90, CD 73, CD166
Hematopoietic markers	CD34, CD45

Cells Lineage induction was performed when cultures had reached 60-70% confluences. 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 to do Von Kossa staining for osteoblasts, oil red O for

adipocytes and safronin for chondrocytes.

Percentage positivity for both mesenchymal and hematopoietic markers was analyzed for the dental pulp and bone marrow stem cells from the samples available at Stempeutics, Manipal using fluorescence assisted cell sorting. The data was fed into the SPSS (Version 15.0) software for statistical analysis. Independent sample-t test was used for the comparative evaluation of the markers in the dental pulp stem cells and bone marrow cells.

RESULTS

Table 2: Age and sex distribution of sample

Sample	Sex	Number of teeth	Age range
Primary dental pulp	13 male 12 female	13 12	5-14 years 5-14 years
Permanent dental pulp	13 male 12 female	13 12	11-14 years 11-14 years
Bone marrow	5 male 5 female	- -	20-35 years 20-35 years

Table 3. Results with respect to group distribution

Group	Number of teeth	Number of tissues grown in culture	Positive for stem cell growth
A	13	13	7
B	12	12	3
C	25	00	0
A: Deciduous inflamed B: Deciduous healthy C: Permanent healthy			

Since there were no positive results from the healthy permanent group, this was not further investigated.

Table 4: Sample distribution

Source of sample	Number	Positive
Dental pulp Primary	25	10
Inflamed *Healthy*	*13* *12*	*07(53.8%)* *03(25%)*
Permanent	25	00
Inflamed *Healthy*	*00* *25*	*00* *00*
Bone marrow	10	10*(100%)*

Table 5. Comparison of percentage expression of cells positive for mesenchymal markers in dental pulp (Group I) and bone marrow (Group II)

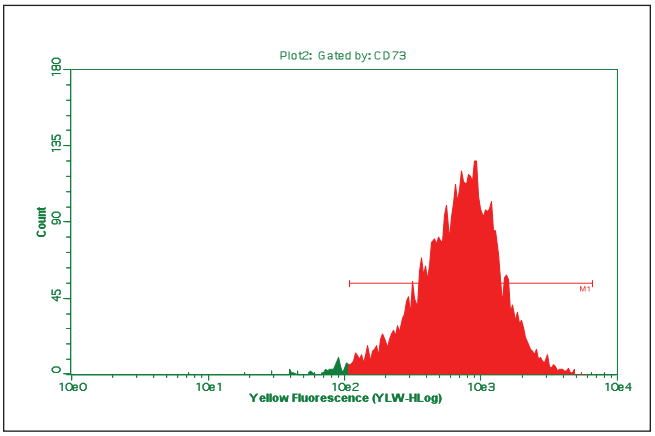
Group		N	Mean%	Standard Deviation	p
CD 73	I II	10 10	94.18 94.18	3.25 3.41	0.8 ns
CD 90	I II	10 10	93.97 91.05	6.01 4.60	0.2 ns
CD 133	I II	10 10	6.56 6.70	14.9 15.20	0.9 ns
CD 166	I II	10 10	90.25 89.15	6.23 6.09	0.6 ns
ns: non significant					

Table 6. Comparison of percentage expression of cells positive for hematopoietic markers in dental pulp (Group I) and bone marrow (Group II)

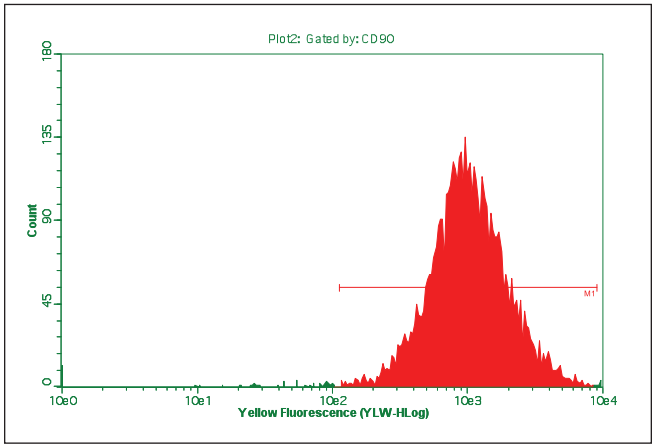
Group		N	Mean%	Standard Deviation	p
CD 34	I II	10 10	1.39 1.58	0.92 0.93	0.6 ns
CD 45	I II	10 10	1.23 1.26	0.96 0.83	0.9 ns
ns: non significant					

Table 7. Differentiation of dental pulp stem cells into multiple lineages

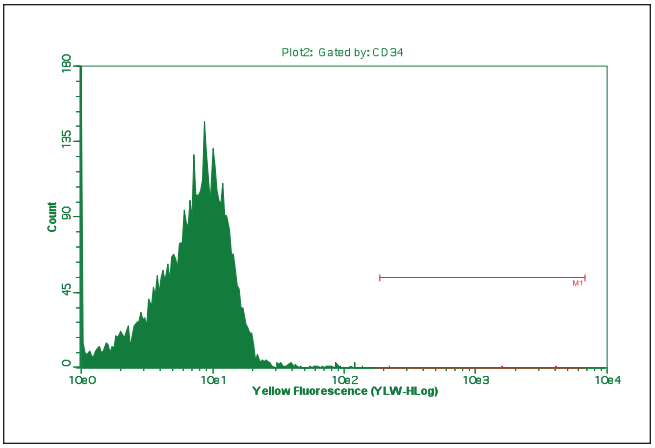
Sample	Chondrocyte	Osteoblast	Adipocyte
Dental pulp (n=10)			
Inflamed (n=7)	6 (85.7%)	6 (85.7%)	4 (57.1%)
Healthy (n=3)	3 (100%)	3 (100%)	1 (33.3%)
Bone marrow(n=10)	10 (100%)	10 (100%)	10 (100%)



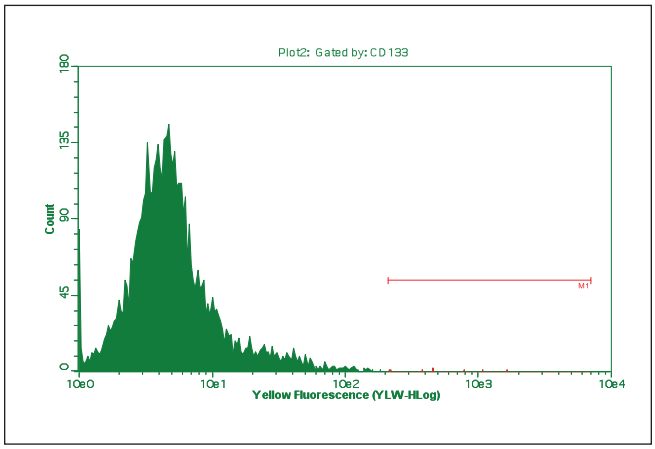
Graph 1. Phycoerythrin antibody conjugated expression of CD 73 in dental pulp stem cells



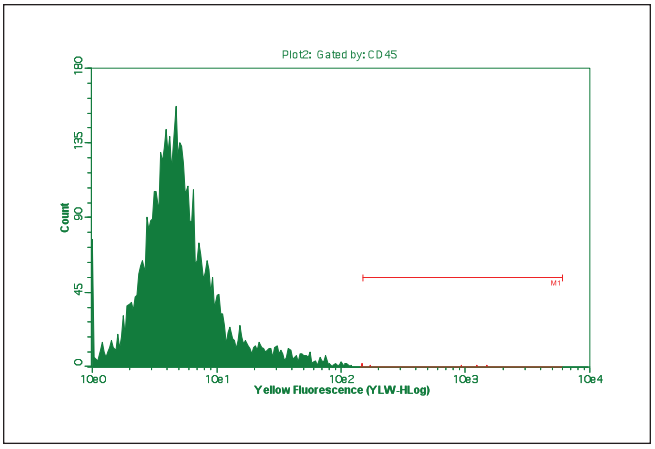
Graph 2. Phycoerythrin antibody conjugated expression of CD 90 in dental pulp stem cells



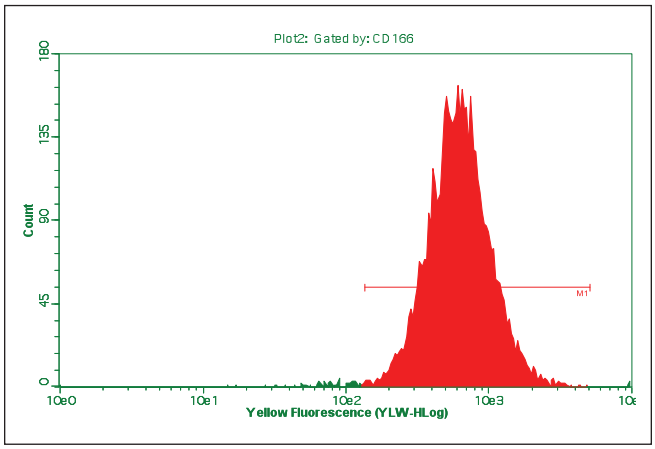
Graph 5. Fluorescence isothiocynate antibody conjugated expression of CD 34 in dental pulp stem cells



Graph 3. Fluorescence isothiocynate antibody conjugated expression of CD 133 in dental pulp stem cells



Graph 6. Fluorescence isothiocynate antibody conjugated expression of CD 45 in dental pulp stem cells



Graph 4. Phycoerythrin antibody conjugated expression of CD 166 in dental pulp stem cells

DISCUSSION

The present pilot work was undertaken with the conceived idea of utilizing discarded inflamed tissue during pulp therapy procedures as a potential source of harvesting stem cells and comparing the tissue potency with both permanent and primary healthy pulp. For this the tissue was collected under complete isolation and transported to the lab for further processing within 30 minutes. In the lab the tissue was cultured and analyzed by using various markers. The markers used in the present study namely CD73, CD90, CD34, CD45 were in accordance with the guidelines of the International Society for Cellular Therapy. Various studies have also been conducted [4,5,13,16] where the expression of these markers has been seen for mesenchymal stem cells.

A variety of dental mesenchymal stem cells have been isolated including stem cells from human exfoliated deciduous teeth (SHED),[9] periodontal ligament stem cells (PDLSC),[17] and stem cells from apical papilla (SCAP).[18] This study was the first attempt to isolate mesenchymal stem

cells from inflamed pulp tissue of primary teeth. In the present study, to minimize chances of contamination the pulp tissue was removed with a barbed broach after preparing an access cavity under complete isolation with rubber dam, prior to extraction. 10% fetal calf serum with 100μM ascorbic acid was used for culture of the pulp tissue as recommended by Suchanek *et al*.[19] In the present study progenitor cell from the inflamed pulp tissue of primary teeth was used where due to initial inflammation, neutrophils and tissue mast cells are recruited as part of body's defense mechanism which is a multifactorial system that coordinates inflammatory cell involvement. Inflammation increases the recruitment of bone marrow-derived stem cells, macrophages, or myofibroblasts at the peripheral sites which is essential for the successful engaftment of such tissues. Stromal cells are found in an increased number in inflamed tissues contributing to the local production of growth factors and chemokines.[14] This gives an explanation to the increased positivity of stem cells derived from the inflamed tissue as compared to healthy, in the present study. Inflammation leads to recruitment of the undifferentiated cells at the inflammatory site as body's defense mechanism and hence the sampling of such tissue could probably result in an increased positivity for isolation of undifferentiated cells. This seems to be a breakthrough in clinical research as it raises the possibility of utilizing the inflamed discarded dental pulp tissue during pulp therapy procedures as potential and non-invasive source of harvesting undifferentiated mesenchymal stem cells. In the present study 53.8% positive results were shown with the inflamed pulp tissues (Table 4). The remaining 46.2% negative results from the inflamed tissue can be probably explained by the difference in the stages of inflammation. During the initial stage, the undifferentiated cells are not yet recruited to the site of injury whereas during the advanced stage the cells are already destroyed by the foreign organisms or undergo necrosis. Sampling of such tissue will not yield a healthy population of undifferentiated mesenchymal cells. Another explanation for less number of positive results can be due to the inadequate amount of tissue present for growth of cells due to the resorption of roots and nearing physiologic exfoliation.

Permanent dental pulp tissue studies[3,4,19] have used human impacted third molars for the isolation of dental pulp stem cells. Human third molars due to incomplete root formation and presence of follicle may harbor a good number of undifferentiated mesenchymal cells. However in the present study for the isolation of permanent dental pulp premolars extracted for orthodontic treatment were used; these teeth had a complete root formation with closure of apical foramen and also there were no signs of any inflammation which could possibly be the reason for non-recruitment of stem cells from permanent teeth. Since in this study the permanent teeth did not yield positive result, they were not further investigated.

Bruder *et al* 1997[1] 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 and bone marrow derived stem cells was 90.25% and 89.15% respectively and the difference was statistically non-significant 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 *et al* [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, CD90 and CD166 were strongly positive (varying from 90 to 94%) in the dental pulp cells suggesting they are a population of mesenchymal stem cells (Graph 1, 2, 3). Additionally, the expression of CD73, CD90 and CD166 in dental pulp cells was comparable to the bone marrow derived cells,[4] and the difference was found to be statistically non significant. Pittenger *et al* [13] stated that criteria for undifferentiated mesenchymal stem cells are that the cells must be negative for blood cell or hematopoietic progenitor cell markers like 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. The values were in agreement with those obtained from bone marrow and the difference was statistically not significant (Graph 5, 6). Gronthos *et al*,[4] compared dental pulp stem cells from impacted third molars from 19-29 year old patients with the marrow aspirates of normal human adult volunteers 20–35 years of age. Similar to the present study immunoreactivity profiles of CD 34 and CD 45 hematopoietic markers were negative for primary cultures of both dental pulp and bone marrow derived stem cells. 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.[10] They bind to the regulatory regions of targeted gene and finally determine the cells destiny, keeping pluripotency or turning to differentiation.[20] 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. In the present study we could differentiate the stem cells from extracted primary teeth into osteoblasts, adipocytes and chondrocytes by processing the tissue samples immediately after extraction to minimize chances of contamination. However, Perry *et al* [11] 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. Gronthos *et al* [4] 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 *et al* [3] have stated that the adipogenic potential of dental pulp can be increased 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). 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.[2] The cells cultured in the present study strongly identified 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 indicating that the inflamed pulp tissue has an equal potency as a source of harvesting stem cells.

CONCLUSIONS

In this preliminary study conducted with a conceived idea, inflamed tissue of dental pulp has shown excellent potency in harvesting the stem cells. However, there is a need to carry out further elaborate study with a larger sample size and comparing the difference at the molecular level.

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