International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (1): 834-841
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Comparative Analysis of Extracted and Exfoliated Deciduous Tooth for Mesenchymal Progenitor Cell Banking

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Received: 9 Oct 2018 / Accepted: 8 Nov 2018 / Published online: 1 Jan 2019
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Abstract
Human deciduous teeth have the ability to produce multipotent mesenchymal progenitor cells. Mesenchymal Progenitor cell (MPC's) applications are increasing ever in regenerative medicine & tissue engineering. In this present study focused on comparative analysis of the banking feasibility between mesenchymal progenitors cells cultured from extracted versus exfoliated deciduous tooth. Mesenchymal progenitor cells harvested from both extracted and exfoliated tooth samples were analyzed for their proliferation rate, progenitor cell surface markers expression, multilineage trans-differentiation potential and colony forming unit assay. Progenitor cells from the extracted tooth and exfoliated tooth sample have shown similar characteristics in morphology, cell surface marker expression and differentiation potentials. Exfoliation while is a natural relatively painless process, extraction involves sedating the person and extracting the tooth via invasive procedures, thereby causing discomfort to the patient. Results obtained by this study recommend that isolation and harvesting of MPC's from dental source by exfoliated method is more feasible than extracted method.

Keywords
Mesenchymal progenitor cells extracted/ exfoliated teeth, dental pulp progenitor cells, stem cell storage.

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INTRODUCTION:
Over the last 20 years mesenchymal stem cells (MSCs) have been isolated from several sources from the body (1). One of the major source and most accessible source is the dentine tissue (2). MSCs are potential capability to develop growth and differentiation into several distinct cell types of the body. MSC's has been explored along with their possible role in regenerative medicine for tissues destroyed or tissue affected by disease and physical injury. Grontho's were the 1st group in the year 2000, identify a clonogenic and rapidly proliferative group of cells called adult dental pulp stem cells (DPSC). This had the ability to regenerate into dental tissue such as odontoblast and pulp like tissue cells. These stem cells had the capability to differentiate into the dentin, dental pulp tissue and periodontal ligament (3). These groups of stem cells were termed as dental

pulp stem cells (DPSC). These cells have been determined as self-renewal capabilities and multipotency of the DPSC, and differentiate into adipocytes, neural cells and osteoblast (4). In recent years, scientist have been able to isolate stem cells from several different dentine tissues, they are: (i) dental pulp stem cells (DPSCs) (3) (ii) periodontal ligament stem cells (PDLSCs) (5) (iii) stem cells from human exfoliated deciduous teeth (SHEDs) (6) stem cells from apical papilla (SCAPs) (7) and (v) dental follicle progenitor cells (DFPCs) (8).

In 1985, McCulloch et al isolated a population of cells from the mouse molar periodontal ligament known as periodontal ligament stem cells (9) (PDLSCs). Following which PDLSCs were derived from different animal sources (10, 11 and 12) and humans (5). These CD166, CD105, and STRO- 1 positive cells are capability of self-renew and possess the tendency to differentiate into osteoblast, cementoblast, chondrocytes and adipocytes (13, 14). In comparison with bone marrow mesenchymal stem cells (BMMSC), DPSCs & PDLSC they have a higher ability to inhibit the activity of peripheral blood mononuclear cell (PBMNC) thus harboring strong immunomodulatory properties (15). Another interesting feature of PDLSCs is that when derived from a diseased sources, they don't seem to lose their stem cell like properties, it possess the same proliferative capacity compared to normal PDLSCs, proving to be an ideal source for stem cell derivation (16) . Park et al, was confirmed that PDLSCs can assist in regenerating sections of the swine periodontal tissues, making it a potential source for therapy for periodontitis (17). Kim SH et al., (2009) reports have also confirmed the ability of PDLSCs of aiding in alveolar bone regeneration (12).

Stem cells from apical papilla (SCAPs) is the population of stem cells which is isolated from the apical papilla region, these cells can help in the installation of the root pulp and dentine tissue (18 & 19). These cells primarily trans-differentiated into osteogenic lineages, by regulating the IGF-1 factor 1 one can regulate the osteogenic potential of the SCAP cells (20). In a comparative study with DPSCs, SCAPs had higher proliferation capacity, cellular migration, and mineralization capacity and were capable of creating 3D mineralized units (21). Combination of apical papilla stem cells along with other groups of stem cells have helped in forming several regenerative therapy models: for example SCAPS along with human umbilical endothelial vein cells together showed the potential to regenerate and form a 3D blood vessel (21). SCAP and PDLSCs when transplanted together along with an implant were capable of generating a root-periodontal complex; enhancing tooth function (22). SCAP along with PDLSC and DPSC loaded onto a carrier which when transferred into patient's oral cavity regenerated the periodontal tissue and stabilized the bio root (23).

Another new population of stem cell isolated are dental follicle progenitor cells (DFPCs), these cells differentiate into the dentine tissue such as cementoblast, periodontal ligament cells and osteoblast cells. Since this tissue is in the proximity of the tooth germ layer, it has been proposed that these cells emerge from neural crest cells. These cells are found positive for NESTIN and notch like neural stem cell markers, and even showed a higher presence of IGF-1(osteogenic potential regulator) then human MSCs (8). DFPCs are capable of differentiate into osteoblast/cementoblast, adipocytes and neurons. Their multi-potentiality is promising a great deal of application in regenerative medicine (24, 25). DFPCs have shown equivalent potentiality to form new bone tissue as PDLCs and BMMSC and when transplanted along with DPSCs and primary enamel organ epithelial cells into the omentum of an immune compromised rat are capable of forming a dentin pulp complex along with traces of a nascent periodontal ligament (26).

Out of all the types of dental stem cells, SHED (Stem cells from human exfoliated deciduous teeth) along with DPSCs is the populations on which extensive research has been carried out (29). SHED a highly proliferative population of cells, which can differentiate into adipo, osteo and neural lineages (28, 29). Each section of stem cells derived from the dentine tissue seems to possess slightly different characteristics and thus can have different potential uses (27). Wang et al., (2012) reported that SHED cells have the ability to differentiate into neural lineages and form dopaminergic neurons therefore possessing a promising therapeutic potential to alleviate Parkinson's disease (28). SHED cells are capable of surviving in vivo transplantation and inducing dentine and bone formation in the dentine tissue (6). In another study SHED cells interaction with the neighboring epithelial cells along with cells influence over ECM complexes facilitated and amplified wound healing process (30). Several studies have been carried out to show how stem cells can be used to improve the efficiency of the current technology play by SHED cells. SHED cells seeded in human tissue derived biodegradable scaffolds and transplanted in immune-deficient mice were capable of integrating with the micro-environment and forming cells of the endothelial lineage and could

regenerate the dental pulp tissue (31). Similarly SHED cells when seeded onto a synthetic scaffold without the addition of any growth factors, they were able to differentiate into cells of the pulp tissue forming pulp tissue constructs, these constructs could be implanted into human teeth in order to facilitate regeneration of the dental pulp tissue (32).

The extensive progress of research in therapeutic potential of progenitor cell derived from dentine tissue is absolutely essential to encourage and standardize the practice of dental stem cell banking. The main goal of this study is to successfully isolate dental pulp stem cells from extracted and exfoliated teeth and perform a comparative analysis of potentiality of these stem/progenitor cells in terms of proliferation, trans-differentiation, CFU and gave clarity in standardizing techniques for dental stem cell banking.

MATERIALS & METHODS:

Isolation of Mesenchymal progenitor cells from extracted & exfoliated tooth sample:

The surface of the tooth was first cleaned with sterile PBS and a groove of 0.5–1.0 mm deep was cut around the circumference of the tooth using a sterile hand-held hard wire cutter. The dental pulp was exposed by splitting the teeth with a chisel along the groove. The pulp tissue of the tooth was then extracted with endodontic files. The extracted pulp tissue was subsequently digested with 0.25% trypsin (1X, Invitrogen) for 30 minutes at 37^0C with 5 % CO_2. The resulting pulp was neutralized with the same volume of complete cell culture media. The digested pulp was then cultured in complete medium containing a Dulbecco's modified eagle's medium (DMEM / F12, GIBCO, USA), 10% FBS (GIBCO), 10,000 units penicillin and 10mg Streptomycin (HIMEDIA), EGF and VEGF (GIBCO).Dental pulp progenitor cells were cultured at 37^0C with 5% CO_2. Fresh medium was replaced every 2 days. The cells that were adherent were passaged for 3 cycles and cryopreserved for further experiments.

Phenotypic determination of both extracted & exfoliated tooth derived progenitor cells:

Cell surface markers:

Cell surface markers are proteins that serve as markers for specific cell types and are generally present on cell surfaces. For example B-cell and T-cell markers identify their lineage and the differentiation stages. These lymphocytes differentiate in to various subtypes of cells, required for a particular biological process. During a variety of biological processes, lymphocytes express a number of cell surface receptors, which can be used to identify cellular subtypes. Testing the expression of a specific cell surface marker is important in confirming whether experimental drugs or small molecule are identified by the desired cell type. Mesenchymal progenitor cells are adherent to plastic surfaces. They must express CD105, CD90 and CD73 but not express CD34 and CD45, according to the international society for cellular therapy.

Progenitor surface marking:

Progenitor cells at passage 3 (P3) were used for carrying out immunophenotyping experiments. Tali (image based Cytometer) was used to determine the surface markers. The progenitor cells were dissociated with 0.05% trypsin and cells were re suspended in phosphate buffered saline at 1 million cells/ ml. For 100µl of cell suspension, 5µl of primary antibody was added and incubated for 5 minutes in the dark at room temperature. From that 25µl sample was loaded on the analysis slide and analyzed for positive and negative markers like CD73, CD90, CD 34, CD45, CD105, HLA ABC and HLA DR.

Colony forming assay (CFU):

Progenitor cells in passage 4 (P4) of both exfoliated and extracted tooth samples were seeded in 90 mm tissue culture treated dishes. Freshly prepared growth medium was added to both the dishes. These plates were incubated for 14 days at 37^0 C and 5 % CO_2. After 14 days, growth medium was removed and the plate was washed with PBS. Now 5% crystal violet solution was added to the dishes and incubated at room temperature for 10 minutes. Post incubation, sample dishes were washed with running tap water. Now the images were captured under a phase contract microscope. Colony forming unit (CFU) efficiency was calculated based on the initial seeding density of 100cells/10cm^{2}dish in the complete growth media. Cloning efficiency was estimated as a percentage of cells that generated clones from total cell number/dish.

Trans-differentiation of mesenchymal progenitor cells:

Adipogenic trans-differentiation:

Adipocyte differentiation medium (Himedia HiAdipoXLTM) was used for both types of progenitor cell differentiation. The differentiation was carried out as per the manufacturer's protocol. Briefly, 0.3x 10^6 cells were seeded in a 35 mm plate. The differentiation medium was changed every alternate day for about 14 days. The differentiation was confirmed by Oil Red O staining. Phase contrast images were captured using a motic inverted microscope.

Osteocyte trans-differentiation:

For the Osteocyte trans-differentiation, $0.3*10^6$ cells were seeded on 35 mm tissue culture coated dish. As per manufacturer's instructions, passage 3 (P3) cells were used for the experiment. After mesenchymal progenitor cells attaining 70 % confluency, Osteocyte differentiation medium (Himedia HiOsteoXLTM) was added to the culture dish. Differentiation medium was changed on every alternative day for 28 days. Osteocyte differentiation was confirmed by Vonkossa staining.

Neurogenic trans-differentiation:

Extracted and exfoliated tooth derived mesenchymal progenitor cells from P3 were seeded on 35 mm tissue culture coated dish. As per the manufacturer's protocol after attaining 60-80% confluency, growth medium was replaced with neurogenic differentiation medium (Promo cell C-28015) and subsequent medium changes were carried out for every 48 hours. Differentiation process occurred in 3days. Neuronal differentiation was confirmed by the microscopic images.

Myogenic trans-differentiation:

Harvested progenitor cells P3 from the both extracted and exfoliated tooth samples were seeded on 35 mm tissue culture coated dish. After reaching 70% confluency growth medium supplemented with 5% horse serum, dexamethazone (0.1µm) and hydrocortisone (50 µm). The medium was changed every alternative day for a period of 45 days. Myogenic trans-differentiation was quantified by microscopic images and positive green immune florescence staining.

RESULTS AND DISCUSSION:

The integrity of Mesenchymal progenitor cells:

Mesenchymal progenitor cells harvested from extracted and exfoliated dental pulp tissue have shown similar morphological characteristics. Mesenchymal progenitors were fibroblastic with long morphology and spindle shaped. They were adhering to tissue culture coated plastic dish. The cell average size was 14-16 µm in diameter. Population doubling time for both sources was 48 hours. (Figure 1)

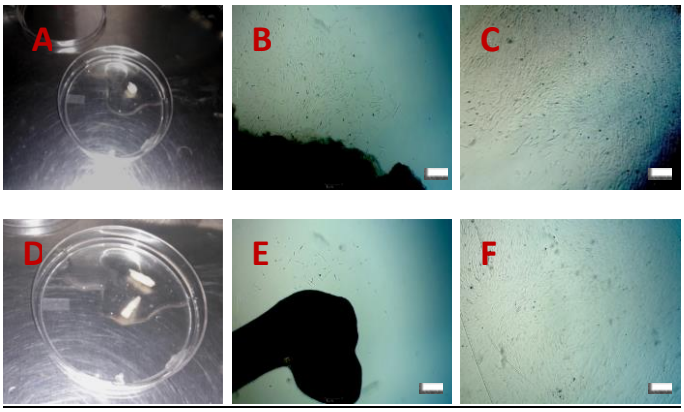

Figure 1. Mesenchymal progenitor cells on day 10 & day 15 in culture dish extracted vs. exfoliated at 10X Microscopic images

A: Extracted tooth
B: Mesenchymal progenitor cells on day 10 at 10 X magnification from extracted tooth
C: Mesenchymal progenitor cells on day 15 at 10 X magnification from extracted tooth
D: Exfoliated tooth
E: Mesenchymal progenitor cells on day 10 at 10 X magnification from exfoliated tooth
F: Mesenchymal progenitor cells on day 15 at 10 X magnification from exfoliated tooth

Mesenchymal progenitor cell specific phenotype:

Mesenchymal progenitor cells were checked for the absence of HLA DR, CD45, CD34 surface antigens. Both sources were positive for HLA ABC, CD105, CD73 and CD90. The expression percentage of both types was quantified in the table. (Figure 2 and Table No. 1)

Table 1: 1 The surface antigen expression percentage of extracted exfoliated dental pulp cells

Source	CD34%	CD45%	CD73%	CD90%	CD105%	HLAABC%	HLADR%
Extracted dental pulp derived Progenitor cells	3	2	85	97	99	89	3
Exfoliated dental pulp derived Progenitor cells	3	2	86	97	99	89	3

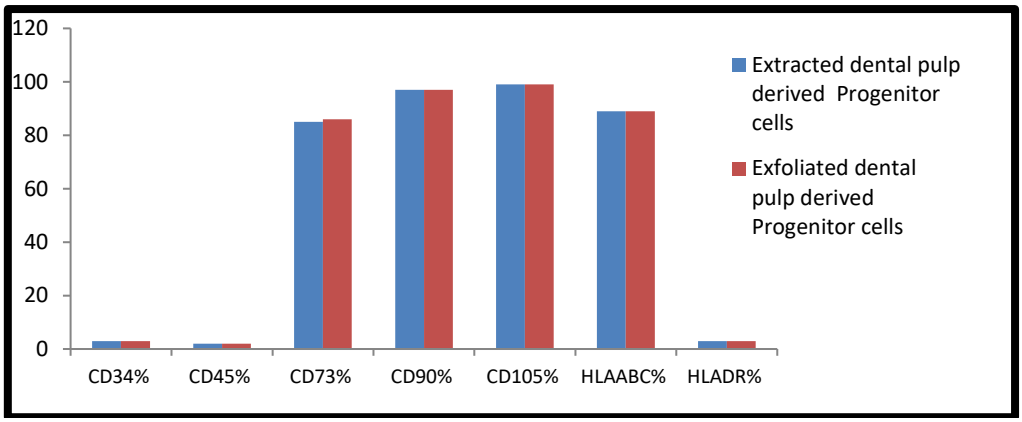

Figure 2. Mesenchymal Progenitor cell surface markers of extracted vs. exfoliated

Colony forming assay (CFU):

To assess clonogenicity, we performed colony forming unit assays at passage 2 for both extracted and exfoliated teeth, which demonstrated that exfoliated teeth have a cloning efficiency of 64.75% (SD 7.65%) versus 65.75% (SD 4.64%) for extracted teeth. (Figure 3).

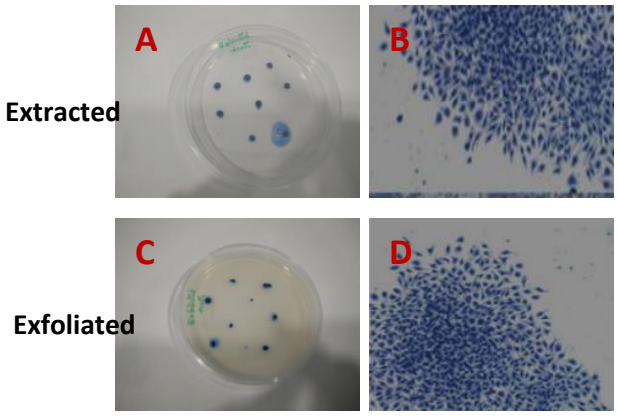

Figure 3. Mesenchymal progenitor cell colony forming unit assay of extracted vs. exfoliated

A & B: Extracted tooth derived mesenchymal progenitor colonies

C & D: Exfoliated tooth derived mesenchymal progenitor colonies

Trans-differentiation of mesenchymal progenitors to specific lineages:

Extracted and exfoliated tooth pulp derived progenitors differentiated into adipocyte, osteoblast, myocytes and neurons upon induction. Lipid-rich vacuoles are stained with oil-o-red stain after 28 days of adipogenic trans-differentiation. Post osteo trans-differentiation by Vonkossa stain calcium deposits were detected. Post myogenic differentiation myocytes were formed, which were confirmed by immunoforosence staining. Neuronal differentiation was confirmed with microscopic images (Figure 4).

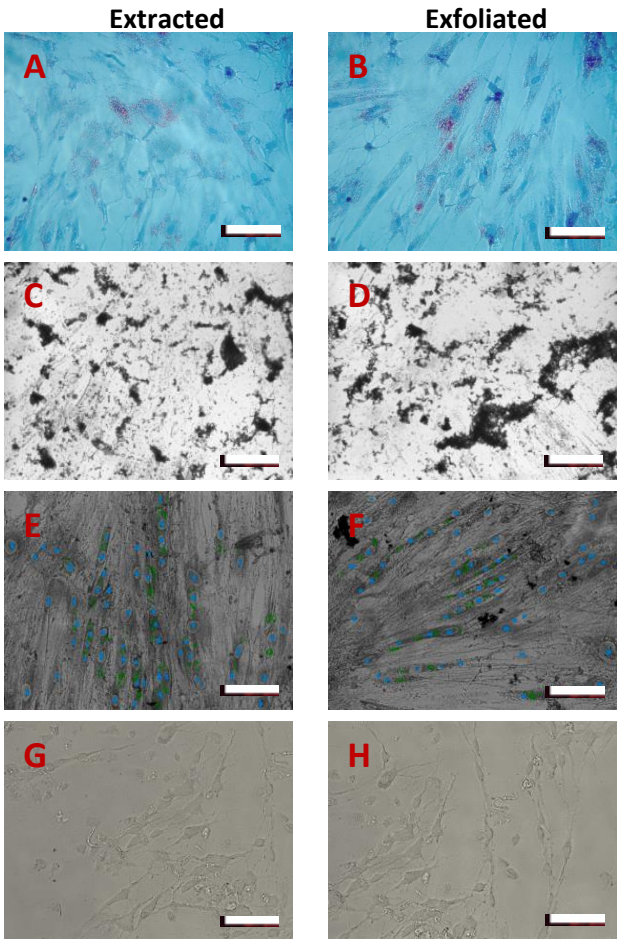

Figure 4. Trans-differentiation of Mesenchymal Progenitor cells

A : Adipogenic differentiation of extracted tooth derived mesenchymal progenitor cells . oil –o-red stained images showing oil droplets
B : Adipogenic differentiation of exfoliated tooth derived mesenchymal progenitor cells. oil –o-red stained images showing oil droplets
C : Osteogenic differentiation of extracted tooth derived mesenchymal progenitor cells. Vonkossa stained images showing calcium deposits
D : Osteogenic differentiation of exfoliated tooth derived mesenchymal progenitor cells showing calcium deposits
E : Myogenic differentiation of extracted tooth derived mesenchymal progenitor cells Immunocyto nuclear staining of MyoD of myocytes
F : Myogenic differentiation of exfoliated tooth derived mesenchymal progenitor cells Immunocyto nuclear staining of MyoD of myocytes.
G : Neurogenic differentiation of extracted tooth derived mesenchymal progenitor cells microscopic images
H : Neurogenic differentiation of exfoliated tooth derived mesenchymal progenitor cells microscopic images

From our above studies we have definitely realized that, several regions in the dentine tissue, offer various population of stem cells which have several beneficial characteristics. Some of the characteristics

all of the dental tissue derived stem cells have in common are their self-renewability, high proliferation rates, immunomodulatory and immune-regulatory properties, they are known to regulate the amount of Treg cells along with suppressing activity of the T cells (33) and their multipotent lineage, Innate ability to differentiate into myogenic, adipogenic, osteogenic and neurogenic lineages (34, 28).

Both the populations of stem cells obtained from the extracted and exfoliated dental pulp, showed similar characteristics, they were mesenchymal in nature, spindle shaped, easily adhered to the culture dish, with a high proliferative rate. This report further confirms previous findings which indicate the high proliferative rate and high population doubling time, suggesting a high number of stem/ progenitor cells can obtain from a single source, within a single extraction (35).

Stem cells derived from both the sources, were found positive for mesenchymal stem cell markers such CD73, CD90, CD105, HLA ABC and negative for CD 34, CD45 and HLA DR. The percentage amount of these markers in the cells was compared between both the populations. Similar results indicate that the stem cells obtained from both extracted and exfoliated teeth show the same degree of stemness, therefore both the populations' stem cells will play an identical role when used for therapeutic purposes.

We also checked the multiline age potential of these cells, populations of both the cells showed the ability to differentiate into adipogenic, myogenic, osteogenic and chrondogenic lineages. Cells obtained from both the sources exfoliated and extracted tooth showed almost the same potential for lineage differentiation.

Out of the several sources for mesenchymal stem/ progenitor cells, dental tissue derived is considered to be an ideal source because the source is easily accessible, the derivation of the stem cells or progenitor cells from the either the pulp or the periodontal ligament, is also a very simple process and the amount of stem/progenitor obtained is high in number and these cells have high proliferative rates. The derivation of these stem cells along with being non-invasive can be a painless and less hassle one too. One can just derive the stem cells from exfoliated teeth as oppose to deriving it from extracted teeth, as both the populations show similar characteristics and therapeutic potential.

CONCLUSION:

Progenitor cells isolated from the deciduous tooth are pluripotent in nature. They exhibit a high proliferating capacity. Dental pulp derived progenitor cells can trans-differentiate into a variety of cell types like osteocytes, neurons like cells, adipocyte and chondrocytes. The study has shown that the exfoliated tooth stem cell storage is more feasible for dental stem cell banking compared with extracted.

As dental pulp progenitor cells can be sourced from actual "expendable" tissues without huge dismalness to have and with less restricted moral concern. They display another open door for dentistry to add to the improvement of tissue building. This wide assortment of cell sorts makes a plenty of chances for the utilization of dental progenitors in tissue recovery. A lot of work has to do before dental progenitor cell-based treatments can turn into a clinical reality.

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